ZmbZIP91 regulates expression of starch synthesis-related genes by binding to ACTCAT elements in their promoters

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Abstract

Starch synthesis is a key process that influences crop yield and quality, though little is known about the regulation of this complex metabolic pathway. Here, we present the identification of ZmbZIP91 as a candidate regulator of starch synthesis via co-expression analysis in maize (Zea mays L.). ZmbZIP91 was strongly associated with the expression of starch synthesis genes. Reverse transcription–PCR (RT–PCR) and RNA in situ hybridization indicated that ZmbZIP91 is highly expressed in maize endosperm, with less expression in leaves. Particle bombardment-mediated transient expression in maize endosperm and leaf protoplasts demonstrated that ZmbZIP91 could positively regulate the expression of starch synthesis genes in both leaves and endosperm. Additionally, the Arabidopsis mutant vip1 carried a mutation in a gene (VIP1) that is homologous to ZmbZIP91, displayed altered growth with less starch in leaves, and ZmbZIP91 was able to complement this phenotype, resulting in normal starch synthesis. A yeast one-hybrid experiment and EMSAs showed that ZmbZIP91 could directly bind to ACTCAT elements in the promoters of starch synthesis genes (pAGPS1, pSSI, pSSIIIa, and pISA1). These results demonstrate that ZmbZIP91 acts as a core regulatory factor in starch synthesis by binding to ACTCAT elements in the promoters of starch synthesis genes.

Key words: ACTCAT motif, co-expression, gene transcription, maize, starch synthesis, ZmbZIP91.

Introduction

Maize (Zea mays L.) is an important food and forage crop that is cultivated worldwide. Starch is an essential commodity that is widely used in many industries, such as human food, animal feed, and fuel production (Zeeman et al., 2010), and is the main component of maize kernels, accounting for ~70% of the content. As the quality of maize is directly affected by the type and physicochemical properties of starch (Visser and Jacobsen, 1993; Morley-Smith et al., 2008; Sonnewald and Kossmann, 2013), starch is a key factor influencing maize yield and quality.
The production of starch is mainly catalysed by four enzymes: ADP glucose pyrophosphorylase (AGPase; EC 2.7.7.27), starch synthase (SS; EC 2.4.1.21), starch branching enzyme (SBE; EC 2.4.1.18), and starch debranching enzyme (DBE; EC 3.2.1.68) (Tetlow, 2006). Recent studies have found that starch phosphorylase (PHO) also plays an important role in starch synthesis (Sato et al., 2008; Tetlow, 2011). Mutation of the key genes in the starch synthesis pathway will decrease starch content or alter its structure, thereby affecting maize yield and quality. For example, sh2 and bt2 (mutants of AGPLS1 and AGPS1) (Hannah and Nelson, 1976) exhibit a much lower starch content that the wild type, whereas wx (mutation of GBSSI (Nelson and Rines, 1962) and ae (mutation of SBEIIb) (Fisher et al., 1993) exhibit an altered starch structure. The genes involved in starch synthesis in maize have been cloned, and their functions are relatively clear; in addition, a significant number of authors have reviewed the starch synthesis process (James et al., 2003; Hannah, 2005; Jeon et al., 2010; Keeling and Myers, 2010; Zeeman et al., 2010). Although these studies have allowed for a better understanding of the starch synthesis pathway, little is known about its regulation, especially in maize.

In general, the regulation of gene expression is affected by trans-acting factors and cis-acting regulatory elements: a transcription factor targets a cis-acting element in a gene promoter to activate or inhibit transcription, thus ensuring that the correct gene is expressed (Stower, 2011). Several factors (genes) participating in the regulation of starch synthesis have been reported. SUSIBA2, a WRKY family transcription factor, binds directly to the promoter of pISA1 to regulate its expression, thereby influencing starch synthesis in barley (Sun et al., 2003). FLOURY ENDOSPERM2 positively regulates the expression of starch synthesis genes, and its overexpression could increase the starch content and seed yield in rice (She et al., 2010). Additionally, SERF1, a transcription factor of the AP2 family, negatively regulates rice grain filling, and mutations of the gene could increase the starch content of rice (Schmidt et al., 2013). OsbZIP58 was reported to regulate directly the expression of starch synthesis genes in rice, and mutations of the gene decreased the total starch and amylase contents (Wang et al., 2013). Despite these findings, a comprehensive understanding of the factors that regulate starch synthesis remains elusive, especially in maize. Therefore, the screening and identification of key factors involved in starch synthesis will be of great importance for an understanding of the regulation of starch synthesis.

With the development of high-throughput technology, there has been a massive increase in the amount of gene expression data available, and co-expression analyses based on these large-scale data have been performed for the screening and identification of novel genes that may be involved in metabolic pathways (Saito et al., 2008; Higashi and Saito, 2013). Indeed, many key factors that participate in metabolism have been identified via co-expression analysis, particularly in Arabidopsis (Hirai et al., 2007; Gigolashvili et al., 2009; Sawada et al., 2009). Furthermore, Fu and Xue (2010) screened factors participating in starch synthesis in rice by co-expression analysis and identified RSR1 (Rice Starch Regulator1) as a key factor that negatively regulates the expression of starch synthesis genes; mutation of the gene resulted in increases in both the starch content and yield. Similarly, in our previous research, ZmNAC36 was proven to be involved in starch synthesis (Zhang et al., 2014). Thus, co-expression analysis is a feasible way to screen for key factors involved in the regulation of starch synthesis.

The goal of the present study was to mine the key factors positively regulating several starch synthesis genes, identify their functions, and clarify their regulation mechanisms. Here, we present the identification of ZmbZIP91 as a candidate regulator of starch synthesis via co-expression analysis in maize. The expression pattern and functional properties of ZmbZIP91 were analysed, and its regulation of the expression starch synthesis gene was identified by particle bombardment-mediated transient expression in maize endosperm and leaf protoplasts. In addition, we analysed the phenotype of the Arabidopsis mutant vip1. The vip1 mutant carried a mutation in a gene (VIP1) that is homologous with ZmbZIP91. Complementation experiments with ZmbZIP91 were also performed. In addition, the molecular mechanism of ZmbZIP91 was clarified by a yeast one-hybrid assay and EMSAs.

### Materials and methods

**Screening candidate transcription factors by co-expression analysis**

The data used in this analysis were mainly based on the genome-wide atlas of transcription during maize development, which contains samples from 60 diverse tissues, representing 11 major organ systems and varying developmental stages of maize, GEO number GSE27004 (Sekhon et al., 2011). Co-expression analysis was performed according to Fu and Xue (2010), with minor alterations. Eight key starch synthesis genes were chosen as guide genes, and probes were considered as associated with the guide genes only when Pearson's correlation coefficient (PCC) was >0.60. The genes associated with more than six guide genes were classified as candidate genes, and we then chose transcription factors among the candidate genes. Ultimately, transcription factors highly co-expressed with six guide genes were chosen as candidate factors involved in starch synthesis.

**Plant materials**

Maize inbred line B73 (a gift of Professor Li Yu, Chinese Academy of Agricultural Sciences) was grown in the field under recommended agronomic guidelines, and individuals were self-pollinated. Grains were collected at 7, 12, 17, 22, 27, and 32 days after pollination (DAP) for mRNA extraction. Two-week-old seedlings of B73 cultivated in the lab were used for genomic DNA extraction and protoplast preparation, and 10 DAP endosperm from Chengdan30 (hybrid maize) was used for particle bombardment.

**Gene cloning and plasmid construction**

cDNAs from 7–32 DAP endosperm were pooled at 5 d intervals for cloning of candidate transcription factors. Genomic DNA was used to clone the promoters of starch synthesis genes. All genes were first cloned using KOD enzymes (Toyobo, Osaka, Japan) and then cloned into T vectors for sequencing. 

*pBI221* (Clontech, Takara, Dalian, China) was used for the transient expression assay. We constructed the 35×:Luc reporter as follows. The *Luc* reporter gene was amplified from *pzs53a-Luc* (Hu...
After digestion with BamHI and SacI, the gene was cloned into pBI221 to construct the 35S::Luc vector. pAGPLS1 and pISAI were amplified using primers with HindIII and BamHI sites, and cloned into the 35S::Luc vector. pSBEnH was amplified using primers with HindIII and XhoI sites and cloned into 35S::Luc. As it has been reported that Adh1 intron 1 (Adh1) can enhance promoter activity (Mascarenhas et al., 1990; Cornejo et al., 1993), Adh1 was amplified from p25S3α:Luc with XhoI and BamHI sites and cloned into pSBEnH:Luc to increase the promoter activities of pISAI and pSBEnH and was cloned into ISA1::Luc at BamHI sites using the in-fusion system (Clontech). Ubi::Gus was used as the internal control.

The LacZ system for yeast one-hybrid assays was used. Eight starch synthesis gene promoters (pAGPS1, pAGPS1, pGBSS1, pSSI, pSSIa, pSSIia, pISAI, and pSBEnH) were inserted into the XhoI site of the p178 vector upstream of CYCI. All the promoter–LacZ reporter vectors were constructed using the in-fusion system. ZmbZIP91 was also cloned into the pPC86 vector: ZmbZIP91 was amplified by primers with SacI and SacI sites and cloned into pPC86. The p178 and pPC86 vectors and yeast EGY48 were gifts of Dr. Wang Jiechen (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) (Wang et al., 2013). The vector p2300-GFP (Zhang et al., 2014) was used for subcellular localization analysis. ZmbZIP91 was amplified by primers with Smal and SpeI sites and then cloned into p2300-GFP. The plastid marker was supplied by Dr. Zhang (School of Life Sciences, Sun Yat-sen University, Guangzhou) (Zhang et al., 2011).

pET-32a (Takara, Dalian, China) was used for ZmbZIP1-His fusion protein expression. ZmbZIP1 was amplified by primers with EcoRV and SacI sites and then cloned into pET-32a.

The promoter–LacZ reporter vectors were constructed using the in-fusion system. ZmbZIP91 was amplified by primers with Smal and SpeI sites and then cloned into p2300-GFP. The plastid marker was supplied by Dr. Zhang (School of Life Sciences, Sun Yat-sen University, Guangzhou) (Zhang et al., 2011).

All genes and vectors were sequenced, and the primers used are listed in Supplementary Table S1 available at JXB online.

RT–PCR analyses
Total RNA was isolated using an RNA Extraction Kit (Tiangen, Beijing, China), and reverse transcription was carried out with the Prime Script reagent kit (Takara). The cycling conditions were set according to the SYBR PrimeScript RT-PCR Kit manual (Takara). All primer details are listed in Supplementary Table S2.

Particle bombardment and transient expression assay
The experiment was carried out mainly according to a previous report (Hu et al., 2012). Briefly, maize kernels at 10 DAP were surface-sterilized with 75% (v/v) ethanol, and the developing endosperm was isolated and cultivated on Murashige and Skoog (MS) medium. The tissues wereplasmolyzed on medium for 4 h prior to bombardment. A helium biolistic gun transfection system (Bio-Rad) was used to deliver DNA coated with gold particles. The bombarded tissues were cultivated for 24 h for GUS and LUC activity analyses.

Preparation and transfection of maize leaf protoplasts
Maize leaf protoplasts were prepared according to Yoo et al. (2007), with minor modifications. Young leaves of maize seedlings were cut into strips along the veins. The strips were immediately transferred to 0.6 M mannitol and incubated for 10 min in the dark; the strips were then incubated in an enzyme solution. The protoplasts were collected by horizontal centrifugation (80 g, 3 min; Eppendorf 5810R, Eppendorf, Germany) and then resuspended in MMG solution (containing 0.45 mM mannitol) at a concentration of 2 × 10⁶ cells ml⁻¹. Plasmid DNA (10 μg) was mixed with 100 μl of protoplasts (~2 × 10⁶ cells). A 10 μl aliquot of freshly prepared polyethylene glycol (PEG) solution was added, and the mixture was incubated at room temperature in the dark at 25 °C for 15 min before adding 440 μl of W5 solution. The solution was mixed by gently inverting the tube, which was then centrifuged at 80 g for 3 min. The protoplasts were gently resuspended in MMG solution (containing 0.45 mM mannitol), transferred to multiwell plates, and cultured under light at 25 °C for 9–16 h for the subsequent experiments. When examining the regulatory role of candidate starch synthesis transcription factors, the amount of DNA and solutions used in the transfection system was scaled up five times.

Analysis of ZmbZIP91 functional properties
The subcellular location of ZmbZIP91 was analysed using maize leaf protoplasts. p2300-ZmbZIP91-GFP was transfected into protoplasts, which were incubated at 25 °C for 9–16 h. An AIR-si Laser Scanning Confocal Microscope (Nikon, Kanagawa, Japan) was used for the detection of green fluorescent protein (GFP) fluorescence. Trans-activation of ZmbZIP91 was assessed mainly as previously described (Zhang et al., 2014). The GAL4-ZmbZIP1 fusion protein was expressed from pGBK1T7-ZmbZIP91 in yeast. Transformants were screened by plating on SD/–Trp plates and positive clones were then screened on SD/–Trp plates with X-gal. To test trans-activation, the yeast cells were cultivated at 28 °C for 2 d.

Yeast one-hybrid analysis of interaction between ZmbZIP91 and the promoters of starch synthesis genes
Yeast one-hybrid assays were performed essentially according to Wang et al. (2013). To test the ability of ZmbZIP91 to bind to the promoters of eight starch synthesis genes, the promoters were cloned and inserted into the XhoI site of the p178 vector. Yeast EGY48 was transformed with the vector pPC86-ZmbZIP91 and each of the eight reporter plasmids. To evaluate interaction between ZmbZIP91 and the starch synthesis gene promoters, the transformants were screened by plating on SD/–Ura–Trp+X-gal plates.

RNA in situ hybridization for ZmbZIP91 in maize endosperm
The probes used for in situ hybridization were synthesized by Sangon (Shanghai, China); a gene-specific coding region was chosen for the synthesis of probes (Supplementary Table S3 at JXB online). RNA in situ hybridization was conducted mainly according to Hua et al. (2004), with some modification. Maize seeds from different developmental stages were fixed in formaldehyde solution (4%), dehydrated through an ethanol series, embedded in paraffin (Sigma-Aldrich), and sectioned at 5 μm with a microtome. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulphate, 0.3 M NaCl, 10 mM TRIS-HCl (pH 6.8), 10 mM NaH₂PO₄ (pH 6.8), 5 mM EDTA, 2.2 mM DTT, 0.6 μg μl⁻¹ yeast tRNA, and digoxigenin-labelled probe. Approximately 100 μl of the mixture was mounted onto a slide and covered with a clear coverslip. Hybridization was performed overnight at 65 °C. The slides were then washed twice in 2× SSC at room temperature and then in 1× SSC and 0.1× SSC at 55 °C for 15 min each.

Mutant identification of Arabidopsis
The vip1 T-DNA insertion line (SALK_001014) was obtained from the Arabidopsis Biological Resource Center (ABRC, http://arabidopsis.org). Homozygous plants were identified by PCR. ZmbZIP91 regulates expression of starch synthesis-related genes | 1329
was transformed into mutant seedlings by *Agrobacterium*-mediated transformation. To screen for positive plants, the seedlings were grown on MS plates containing 1% (w/v) sucrose, 0.8% (w/v) agar-agar, and 1 mM kanamycin under long-day conditions (16 h light/8 h dark) in a 22 °C growth chamber. The seedlings were then transferred to soil watered with a nutrient solution (Miracle-Gro, OH, USA) under the same light regime and temperature. Adult plants were imaged using a Stereo Microscope (Olympus, Tokyo, Japan). The primers used in this experiment are listed in Supplementary Table S4 at *JXB* online.

**Morphological analysis of leaf starch granules**

The extraction of leaf starch granules was performed according to *Ritte et al.* (2000) with some modifications. Leaves (~10g) were frozen in liquid nitrogen and homogenized in a mortar. The homogenate was mixed with 50 ml of extraction buffer [100 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 0.05% (v/v) Triton X-100] and further homogenized manually. The homogenate was then passed through a 100 μm nylon mesh, and the filtrate was centrifuged for 5 min at 1000 g. The supernatant was removed, and the precipitate was washed again with 10 ml of extraction buffer and then filtered successively through nylon with mesh sizes of 60, 40, and 20 μm. The filtrate was layered onto a 10 ml cushion [95% (v/v) percoll (Pharmacia, Uppsala, Sweden) and 5% (v/v) 0.5M HEPES-KOH (pH 7.0)] and centrifuged for 15 min at 2000 g. The extract was then dried overnight in an oven at 40 °C. Images of starch granules were observed under a scanning electron microscope (FEI Quanta 450, FEI Company, Hillsboro, OR, USA).

**EMSA for analysis ZmbZIP91-binding elements in starch synthesis gene promoters**

EMSA was performed according to previous research (Zhang et al., 2014). After induced expression of the His-tagged protein, the recombinant cells were disrupted discontinuously by ultrasonic for 10 min at 300 W. The cell debris was pelleted by centrifugation, and the supernatant and pellet were analysed to assess the solubility of the protein. The protein was purified using a protein purification kit (Beyotime, Jiangsu, China). Putative cis-containing elements for ZmbZIP91 binding were synthesized by Songon (Shanghai, China) with a 5' end biotin label. Double-stranded oligonucleotides were prepared by annealing of the complementary single-stranded sequences. Native-PAGE was used for electrophoresis, and the product was then transferred to a polyvinylidene fluoride (PVDF) membrane. A Chemiluminescent EMSA Kit (Beyotime, Jiangsu, China) was used for visualization. The probes are listed in Supplementary Table S3 at *JXB* online.

**Results**

**Identification of ZmbZIP91 and expression pattern analysis**

Nearly 30 genes participate in starch synthesis in maize (Yan et al., 2009). In the present study, eight key starch synthesis genes were selected as guide genes; the details are listed in Supplementary Table S5 at *JXB* online. Mutants of each of these eight genes could seriously affect starch synthesis. Previous expression data (Sekhon et al., 2011) analyses have shown that guide genes are mainly expressed in the endosperm, with lower amounts in leaves and other plant parts (Supplementary Fig. S1). Genome-wide co-expression analysis identified nine candidate factors that have an average co-relationship value of >0.8 with at least six of the eight guide genes (Supplementary Table S6).

We first identified all candidate transcription factors, and our results showed that seven of the nine candidate transcription factors could enhance the activities of the starch synthesis gene promoters. Among the candidate transcription factors, GRMZM2G043600 (bZIP factor family) was found to enhance pISA1 activity significantly (*P* < 0.01) (Supplementary Fig. S2 at *JXB* online). As bZIPS are reported to be participate in the regulation of starch synthesis in rice (Cheng et al., 2002; Wang et al., 2013), GRMZM2G043600 was further analysed. According to previous research (Wei et al., 2012), GRMZM2G043600 is the 91st member of the bZIP family; thus, we designated it ZmbZIP91 (Supplementary Fig. S3). To verify the results of co-expression analysis, the expression of ZmbZIP91 and the eight guide genes was evaluated by RT-PCR; the results showed that the expression of ZmbZIP91 is strongly correlated with the expression of the guide genes, particularly *pSSIIIa, pSSI, ISA1, AGPS1*, and *AGPLSI* (Supplementary Table S7). Moreover, we further measured the expression pattern of ZmbZIP91 by real-time RT-PCR and semi-quantitative RT-PCR, with both demonstrating predominant ZmbZIP91 expression in the endosperm, less expression in leaves, and negligible expression in stems and roots (Fig. 1A, B). RNA *in situ* hybridization also showed that the gene is mainly expressed in the endosperm (Fig. 1C). The functional properties of ZmbZIP91 were also analysed. ZmbZIP91 was found to be localized to the nucleus (Supplementary Fig. 4A), and the protein exhibited transactivation in yeast (Supplementary Fig. 4B). These data suggest that ZmbZIP91, as a typical transcription factor of the bZIP family, might play a role in the regulation of starch synthesis.

**ZmbZIP91 regulates the expression of starch synthesis genes**

To determine the regulatory role of ZmbZIP91 in starch synthesis gene expression, we performed a particle bombardment-mediated transient expression assay in maize endosperm. We initially aimed to determine whether ZmbZIP91 could enhance the expression of all eight guide genes; thus, their promoters were cloned into a region upstream of the *Luc* gene, and the activities of all cloned promoters were measured. Unfortunately, except for that of *AGPLSI*, most promoters showed low levels of activity (Supplementary Fig. S5 at *JXB* online). Previous studies have shown that *Adh1* intron 1 (*Adh*) increases gene expression (Mascarenhas et al., 1990; Cornejo et al., 1993). Therefore, to confirm our result, *Adh* was cloned into the vectors containing starch synthesis gene promoters (*pSSIIIa, pISA1*, and *pSBEIIb*). Along with *pAGPLSI*, four promoters with high promoter activity were used in our experiment. The LUC/GUS assay indicated that ZmbZIP91 could indeed enhance the promoter activity of starch synthesis genes, particularly *pSSIIIa* and *pISA1* (Fig. 2).

Previous studies have shown that starch synthesis occurs in the leaves (Smith, 2012; Stitt and Zeeman, 2012). Although some starch synthesis genes are expressed in a tissue-specific manner, certain starch synthesis genes expressed in maize endosperm also exhibit high expression in leaves, such as *SSI* (Dang and Boyer, 1988), *AGPS1* (Priotul et al., 1994), and *ISA1*.
Fig. 1. Tissue expression analysis of ZmbZIP91. (A) Real-time RT–PCR analysis of ZmbZIP91 expression in different tissues. Two-tailed unpaired t-tests were used to determine significant differences. **P<0.01. (B) Semi-quantitative RT–PCR analysis of ZmbZIP91 expression in different tissues. (C) Detection of ZmbZIP91 mRNA in cross-sections of a maturing maize seeds (13 d after pollination) by in situ hybridization. Pe, pericarp; Em, embryo; En, endosperm; L, leaves; R, root; S, stem. Scale bars=500 μm.

Fig. 2. ZmbZIP91 enhances the activities of starch synthesis gene promoters via particle bombardment of maize endosperm. (A) Diagram of the effector plasmid, reporter plasmid, and internal plasmid. (B) Example of the particle bombardment of maize endosperm; particle bombardment using Ubi::GUS and stained with GUS solution (left); particle bombardment with no construct (right). (C) Response of the pAGPS1 promoter to ZmbZIP91. (D) Response of the pSSIIia promoter to ZmbZIP91. (E) Response of the pSBEIIb promoter to ZmbZIP91. (F) Response of the pISA1 promoter to ZmbZIP91. LUC and GUS activities are shown. The data are provided as the means ±SE of at least five replicates. The significance of the difference between −ZmbZIP91 and +ZmbZIP91 was analysed using a one-sided paired t-test (*P<0.05, **P<0.01). (This figure is available in colour at JXB online.)
We therefore hypothesized that the core regulatory system for starch synthesis is the same between leaves and endosperm. In our experiment, the expression of ZmbZIP91 was also detected in leaves (Figs. 1A, B). Moreover, we examined the influence of ZmbZIP91 on the expression of starch synthesis genes in leaves using the leaf protoplast system, which has been widely used in the characterization of genes and diverse signalling pathways (Sheen, 2001; Yoo et al., 2007). After transfecting ZmbZIP91 into maize leaf protoplasts, we measured the expression of starch synthesis genes by real-time RT–PCR. In each assay, the yellow fluorescent protein (YFP) signal was detect to ensure that ZmbZIP91 was efficiently transfected into maize leaf protoplasts. Five independent experiments showed that ZmbZIP91 increased the expression of starch synthesis genes, particularly AGPS1, SSI, and ISA1 (Fig. 3).

**Mutation of VIP1, a gene homologous to ZmbZIP91, influences starch synthesis in Arabidopsis leaves**

Unlike maize, many mutants of Arabidopsis are available; therefore, this species is widely employed to determine the biological function of genes of interest. By searching MESSA (http://prodata.swmed.edu/MESSA/MESSA.cgi) (Cong and Grishin, 2012), VIP1 was determined to be the closest homologue to ZmbZIP91 in the SWISS-PROT database. Phylogenetic analysis of ZmbZIP91 and all bZIPS in Arabidopsis showed that ZmbZIP91 is most closely related to VIP1 (Supplementary Fig. S6 at JXB online), with 50.64% sequence homology in the core region (Supplementary Fig. S7).

The Arabidopsis vip1 mutant carries a mutation in a gene (VIP1) that is homologous with ZmbZIP91. The vip1 T-DNA insertion line was ordered from the ABRC (SALK_001014) (http://arabidopsis.org); based on sequencing, the T-DNA fragment (http://signal.salk.edu) is inserted in the second intron (Supplementary Fig. S8A at JXB online). Homozygous plants were identified by PCR analysis (Supplementary Fig. S8B), and the expression of VIP1 was measured. No full-length VIP1 RNA was present in the vip1 mutant, suggesting that this gene is most probably mutated (Supplementary Fig. S8C).

We first analysed the phenotype of the vip1 mutant and found that the growth of the mutant was affected, as indicated by its small seeds and leaves (Fig. 4A). The colour of the leaves was also lighter than that of the wild type after KI–I$_2$ staining (Fig. 4B). Starch granules were detected in the wild-type leaves, whereas almost no intact starch granules were observed in the mutant vip1 leaves (Fig. 4C). These results indicate that starch synthesis in vip1 is affected by the mutation.

The starch content of the leaves was measured using the starch (HK) assay kit (Sigma, Shanghai, China). The starch content of the vip1 mutant was decreased by ~5.5 mg starch g FW$^{-1}$ compared with the 8.6 mg starch g FW$^{-1}$ in the wild type (Fig. 5A). In addition, the expression of starch synthesis genes was measured by real-time RT–PCR using At-tubulin as the internal control. The results showed decreased expression of starch synthesis genes in the vip1 mutant, particularly AGPS1, SSI, and ISA1 (Fig. 5B).

**ZmbZIP91 partially complements the phenotype of the vip1 mutant**

To confirm the function of ZmbZIP91 in starch synthesis, ZmbZIP91 was transfected into the vip1 mutant using the

![Fig. 3.](image-url) ZmbZIP91 enhances the expression of starch synthesis genes in maize leaf protoplasts. (A) An intact maize leaf protoplast. (B) The high transfection efficiency of the isolated protoplasts. (C) Expression analysis of starch synthesis genes after ZmbZIP91 transfection. The expression of ZmbZIP91 was promoted by pUbiquition. Untransfected ZmbZIP91 was used as a control (Level 1). The data are expressed as the mean ±SE of five replicates. The significance of the difference between –ZmbZIP91 and +ZmbZIP91 was analysed using a one-sided paired t-test (*P<0.05, **P<0.01). (This figure is available in colour at JXB online.)
**Agrobacterium**-mediated floral dip method to generate the complemented lines. The transgenic vector used was pRI201-AN-GUS (Takara), which contains the *Gus* reporter gene. The transgenic lines were GUS stained, and PCR analysis of each generation was performed using *ZmbZIP91*-specific primers (Supplementary Fig. S9 at *JXB* online). After the
T<sub>1</sub> generation, the phenotypes of vip1/35S-ZmbZIP91 were compared with the wild-type line (Col-0) and vip1 mutant.

The growth of vip1/35S-ZmbZIP91 was similar to that of the wild type, as indicated by normal seeds and leaves (Fig. 4A).KI<sub>1</sub>-I<sub>2</sub> staining also revealed a darker leaf colour of vip1/35S-ZmbZIP91 compared with the vip1 mutant that was almost the same as that of the wild type (Fig. 4B). Starch granules in the leaves were also examined. In general, normal intact starch granules in leaves are oblate in shape. No oblate starch granules were observed in thevip1 mutant line, whereas oblate starch granules were detected in the vip1/35S-ZmbZIP91 line (Fig. 4C). Moreover, using a starch (HK) assay kit (Sigma), the starch content of the leaves was measured: that of the vip1/35S-ZmbZIP91 line was higher compared with that of thevip1 mutant line, which exhibited ~7.5 mg starch g<sup>−1</sup>FW (Fig. 5A). The expression of starch synthesis genes was also measured by RT–PCR, and the expression levels of AGPS1, SSI, and ISA1 were found to have reverted to that of the wild type (Fig. 5B). These findings indicate that ZmbZIP91 partially complemented the vip1 mutant phenotype, with normal starch synthesis.

ZmbZIP91 binds to ACTCAT elements in starch synthesis gene promoters

Transient expression and functional complementation assays demonstrated that ZmbZIP91 regulates the expression of starch synthesis genes. However, we sought to assess whether ZmbZIP91 directly regulates the expression of these genes. Thus, a yeast one-hybrid analysis with the LacZ system (Wang et al., 2013) was used to test the ability of ZmbZIP91 to bind to starch synthesis gene promoters. Eight starch synthesis guide gene promoters were cloned into p178, and ZmbZIP91 was cloned into pPC86. When co-transfected with the promoters of pAGPS1, pISA1, pSSIIIa, and pSSI, only ZmbZIP91 showed positive interactions (Fig. 6). This finding indicated that ZmbZIP91 could directly bind to these four promoters to regulate the expression of the corresponding genes. In addition, the results of our yeast one-hybrid analysis explain why the expression levels of starch synthesis genes, particularly AGPS1, SSI, and ISA1, were strongly influenced in our transient expression experiments.

The potential distribution of binding elements in the promoters of various starch synthesis genes by yeast one-hybrid analysis. pPC86-ZmbZIP91 and p178 with different starch synthesis gene promoters were transformed into EGY48, and colonies were selected on selection medium (SD/−Ura−Trp+X−α-Gal). The blue yeast colonies indicate positive interactions. The different starch synthesis gene promoters are listed next to the corresponding panels.

Discussion

bZIPS are reported to participate in the regulation of starch synthesis, especially in rice (Cheng et al., 2002; Wang et al., 2013). As OsbZIP58 is reported to regulate starch synthesis and can also bind to several starch synthesis gene promoters to regulate the expression of starch synthesis genes in rice (Wang et al., 2013), the ZmbZIP91 binds specifically to the ACTCAT motif, we also compared binding using competition with wild-type and mutant unlabelled probes. The band was unaffected when the mutant unlabelled competitor probe was used, whereas the wild-type (non-mutated) probe did reduce the signal, which is indicative of specific binding (Fig. 7G, H). Some of the examined promoters are predicted to harbour several ACTCAT elements; for example, SSI is predicted to contain two ACTCAT elements. Thus, to determine any differences between the elements among different sites, biotin-labelled ACTCAT elements from different sites of the pSSI promoter were synthesized and used in EMSAs. The results showed differences in the shifted band with ACTCAT elements from various sites, indicating that the sequence flanking the ACTCAT element also affects the binding affinity of ZmbZIP91 (Supplementary Fig. S11 at JXB online).
2013), it is highly likely that \textit{ZmbZIP91} is the orthologous gene to \textit{OsbZIP58}. However, when blast searching using OsbZIP58 for bZIPs in maize, the results of the three closest genes in maize were ZmbZIP60 (GRMZM2G007063), ZmbZIP16 (GRMZM2G019446), and ZmbZIP17 (GRMZM2G016150), and not ZmbZIP91 (Supplementary Fig. S12 at JXB online). The expression patterns of these three genes were analysed in maizeGDB (www.maizegdb.org (last accessed 12.12.2015)), and they were found to be highly expressed in all types of tissues (Supplementary Fig. S13). OsbZIP58 is highly expressed in the endosperm, with low expression in other plant parts such as leaves, indicating that these three genes do not have the same function in starch synthesis regulation as OsbZIP58. In our study, both the instant assay (particle bombardment-mediated transient expression in maize endosperm and leaf protoplasts) and the complementation assay demonstrated that ZmbZIP91 could regulate the expression of starch synthesis genes and influence starch synthesis. Therefore, we propose that sequence analysis cannot always fully explain the function of homologous genes.
It was been previously reported that starch synthesis varies between the leaves and endosperm. The starch in leaves is classified as transient starch, whereas that in the endosperm is categorized as storage starch. However, the pathway of starch synthesis in leaves is largely similar to that in storage organs, which are both orchestrated by AGPase, SS, SBE, and DBE (Santelia and Zeeman, 2011; Smith, 2012; Stitt and Zeeman, 2012). Although the expression of certain starch synthesis genes is tissue specific, some of the starch synthesis genes expressed in the maize endosperm also exhibit high expression in leaves, such as SSI (Dang and Boyer, 1988), AGPS1 (Prioul et al., 1994), and ISA1 (Lin et al., 2013). Because ZmbZIP91 was expressed in both the endosperm and leaves, we used both the former (particle bombardment on maize endosperm) and latter (leaf protoplasts) to determine the function of this protein. The results showed that ZmbZIP91 can positively regulate the expression of starch synthesis genes, especially AGPS1, SSI, and ISA1 (Fig. 3). In addition, analysis of the vip1 mutant phenotype revealed an impact on starch synthesis, with ZmbZIP91 partially complementing the phenotype of the vip1 mutant and resulting in normal starch synthesis. These findings indicated that ZmbZIP91 might be the core factor that regulates starch synthesis in both maize leaves and endosperm. In addition, our yeast one-hybrid analysis also showed that ZmbZIP91 binds exclusively to the pAGPS1, pISA1, pSSI1a, and pSSI promoters, and EMSAs indicated that ZmbZIP91 binds directly to the ACTCAT elements present in the promoter region of starch synthesis genes. Sequence analysis of the other four promoters (pAGP1S, pGBSS1, pSS1Ia, and pSBE1Iib) showed no ACTCAT elements, which can explain why no interactions (no blue spots) were detected in the yeast one-hybrid assay when ZmbZIP91 was co-transfected with these four starch synthesis gene promoters.

Compared with maize, there are numerous mutants available for Arabidopsis, and it was thus easier to generate transgenics in this particular species. Accordingly, to determine the biological function of ZmbZIP91, we utilized the Arabidopsis mutant vip1, which carried a mutation in a gene (VIP1) that is homologous to ZmbZIP91. VIP1 is reportedly involved in the stable genetic transformation of Arabidopsis by Agrobacterium (Li et al., 2005) and plant immunity signalling (Pitzschke et al., 2009); however, another study questioned its function in genetic transformation (Shi et al., 2014). Previous reports also indicate that starch-related phenotypes are not detected in vip1 mutant lines. In the present study, leaf colour was lighter in the vip1 mutant compared with that of the wild-type line after KI–I2 staining (Fig. 4B), and the starch content of the vip1 mutant was lower (Fig. 5A). During growth, the vip1 mutant lines were smaller in size than the wild type (Fig. 4A). As starch present in the leaves during the day is allocated to plant development during the night (Caspar et al., 1991; Schulze et al., 1991), a decrease in starch content might result in altered vip1 mutant growth compared with the wild-type line. It has also been reported that ss1isa1 (SSI and ISA1 mutants) displays a dwarf phenotype and lower starch content; in contrast, ss2isa1 (SS2 and ISA1 mutant) also exhibits a dwarf phenotype but contains almost no starch (Pfister et al., 2014). In our study, the expression of AGPS1, SSI, and ISA1 in the vip1 mutant was remarkably down-regulated, whereas the expression of SSI1 and SSI1I1 changed minimally (Fig. 5B); it is possible that the down-regulation of SSI and ISA1 altered the starch content and phenotype of the vip1 mutant. The expression of some starch synthesis genes returned to normal in vip1/35S-ZmbZIP91. Furthermore, sequence analysis of starch synthesis gene promoters in Arabidopsis indicated that most contain ACTCAT elements, including pSSI, pSSI1, and pISA1. Therefore, ZmbZIP91 in vip1/35S-ZmbZIP91 restored the expression of starch synthesis genes by binding to ACTCAT elements in these promoters.

Recently, VIP1 has been reported to be involved in osmosensory signalling, and its overexpression induces growth retardation under mannilot treatment (Tsugama et al., 2012). This growth inhibition could be largely due to the overexpression of CYP707A13, which is up-regulated during rehydration or under conditions of high humidity (Umezawa et al., 2006; Okamoto et al., 2009). VIP1 also interacts with CYP707A13 promoters. In the present study, we determined whether the overexpression of ZmbZIP91 could induce growth retardation by comparing the growth of the vip1 mutant, vip1/35S-ZmbZIP91-1, and vip1/35S-ZmbZIP91-2 with that of the wild-type line in the presence of 500 mM or 300 mM mannitol. ZmbZIP91 overexpression did in fact result in retarded growth under mannilot treatment (Supplementary Fig. S14A at JXB online), and CYP707A13 expression was up-regulated (Supplementary Fig. S14B). Although the experiments did not prove whether ZmbZIP91 interacts with CYP707A13 promoters, sequence analysis of these promoters indicated that both harbour the ACTCA(T) element. In addition, the ACTCA(T) element was previously determined to respond to osmotic pressure (Oono et al., 2003). Nonetheless, the relationship between starch synthesis and osmosensory signalling remains elusive. It is possible that starch synthesis can lower osmotic pressure by consuming sucrose; however, further studies are necessary to confirm this mechanism.

Microarray analysis has shown that the ACTCAT element is responsive to osmotic pressure (Oono et al., 2003). bZIPs bind to this element in the promoter of ProDH to lower osmotic pressure in Arabidopsis (Satoh et al., 2004). In fact, osmotic pressure is high in the phloem, and sucrose is continuously being imported into the endosperm, resulting in an endosperm with a low osmotic pressure (Patrick and Offler, 2001). We propose that ZmbZIP91 lowers osmotic pressure by consuming sucrose in the maize endosperm, thus increasing sucrose fixation from the source to the sink. Moreover, ZmbZIP91 only binds to the promoters of pAGPS1, pISA1, pSSI1a, and pSSI, though the expression of SEI, AGP1S1, and certain other starch synthesis genes increased via transient expression (Figs 2, 3). Therefore, ZmbZIP91 regulates the expression of other starch genes through direct regulation or interaction with other factors. For instance, it has been reported that SERF1 regulates grain filling and starch synthesis by directly regulating RPBF (directly binding to pGBSS1) (Schmidt et al., 2013).
In conclusion, we present the identification of ZmbZIP91 as a candidate regulator of starch biosynthesis using co-expression analysis in maize. Our transient expression assays demonstrate that ZmbZIP91 can positively regulate the expression of starch synthesis genes in both the leaf and endosperm. The Arabidopsis mutant vip1, which carries a mutation in a gene (VIP1) that is homologous to ZmbZIP91, exhibited altered growth with less starch in the leaves, and ZmbZIP91 was able to complement this phenotype, resulting in normal starch synthesis. Moreover, we demonstrate that ZmbZIP91 can regulate the expression of starch synthesis gene by binding to ACTCAT elements. Taken together, all these data suggest that ZmbZIP91 acts as a core regulatory factor of starch synthesis by binding to ACTCAT elements present in the promoters of starch synthesis genes.

Supplementary data

Supplementary data are available at JXB online.

**Figure S1.** Expression pattern of genes for starch synthesis in 60 samples.

**Fig. S2.** Identification of candidate transcription factors in the regulation of starch synthesis gene expression via particle bombardment of maize endosperm.

**Figure S3.** Evolutionary tree of the bZIP family in maize and their corresponding names.

**Figure S4.** Functional characteristics of ZmbZIP91.

**Figure S5.** Analysis of promoter activities of starch synthesis genes.

**Figure S6.** Evolutionary tree analysis between ZmbZIP91 and bZIPs in Arabidopsis.

**Figure S7.** Sequence homology analysis between ZmbZIP91 and VIP1.

**Figure S8.** Localization of T-DNA insertion and identification of the T-DNA line.

**Figure S9.** Identification of transgenic lines by GUS staining and PCR in each generation.

**Figure S10.** bZIP-binding elements in the promoters of AGPS1, pISA1, pSSI1a, and pSSI.

**Figure S11.** EMSA of ZmbZIP91 with sequences flanking the ACTCAT element in pSSI.

**Figure S12.** Evolutionary tree analysis of OsbZIP58 with bZIPs in maize.

**Figure S13.** Expression pattern analysis of three genes with the closest relationship to OsbZIP58 in maize.

**Figure S14.** ZmbZIP91 overexpression in response to mannitol stress.

**Table S1.** Primers for gene cloning and plasmid construction.

**Table S2.** Primers used for RT-PCR of related genes.

**Table S3.** Sequences for EMSA and RNA *in situ* analyses.

**Table S4.** Primers used for the identification of the Arabidopsis mutant.

**Table S5.** Details of the eight genes chosen as guide genes.

**Table S6.** Details of the nine factors chosen as candidate factors.

**Table S7.** Correlation analysis between ZmbZIP91 and the eight guide genes.

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<th>Table S8. Details of binding sites in the promoters of AGPS1, ISA1, SSSI1a, and SSSI.</th>
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<td><strong>Promoter</strong></td>
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References


