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RESEARCH PAPER

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 tanscription–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 (Satoh *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 *35s::Luc* vector as follows. The *Luc* reporter gene was amplified from *pzsS3a-Luc* (Hu

et al., 2011). After digestion with *Bam*HI and *Sac*I, the gene was cloned into *pBI221* to construct the *35s::Luc* vector. *pAGPLS1* and *pISA1* were amplified using primers with *Hin*dIII and *Bam*HI sites, and cloned into the *35s::Luc* vector. *pSBEIIb* was amplified using primers with *Hin*dIII and *Xba*I sites and cloned into *35s::Luc*. As it has been reported that *Adh1* intron 1 (*Adh*) can enhance promoter activity (Mascarenhas *et al.*, 1990; Cornejo *et al.*, 1993), *Adh* was amplified from *pzsS3a::Luc* with *Xba*I and *Bam*HI sites and cloned into *pSBEIIb::Luc* to increase the promoter activities of *pISA1* and *pSBEIIb* and was cloned into *ISA1:Luc* at *Bam*HI 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*, *pAGPLS1*, *pGBSSI*, *pSSI*, *pSSIIa*, *pSSIIIa*, *pISA1*, and *pSBEIIb*) were inserted into the *Xho*I site of the *p178* vector upstream of *CYC1*. 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 *Sal*I and *Sac*I 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).

p2300-GFP (Zhang *et al.*, 2014) was used for subcellular localization analysis. *ZmbZIP91* was amplified by primers with *Sma*I and *Spe*I 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 ZmbZIP91-His fusion protein expression. *ZmbZIP91* was amplified by primers with *Eco*RV and *Sal*I sites and then cloned into *pET-32a*.

The *pRI201-AN-GUS* (Takara) vector containing the *Gus* (β-glucuronidase) gene served as the transgenic vector. A set of transcription elements containing *35S::ZmbZIP91* and the *Nos* terminator were amplified from *pBI221* and then cloned into *Sma*I sites using the in-fusion system.

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 expression of *ZmbZIP91* in different tissues was analysed by both real-time RT–PCR and semi-quantitative RT–PCR, and the expression patterns of *ZmbZIP91* and starch synthesis genes were analysed by semi-quantitative RT–PCR. *ZmActin* was used as the internal control. The expression of starch synthesis genes in maize leaves and protoplasts as well as Arabidopsis leaves was measured by real-time RT–PCR using the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). 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 were plasmolysed 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 2×10^6 cells ml^{-1}. Plasmid DNA (10 μg) was mixed with 100 μl of protoplasts (~2×10^5 cells). A 110 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 susequent 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 A1R-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–ZmbZIP91 fusion protein was expressed from pGBKT7-ZmbZIP91 in yeast. Transformants were screened by plating on SD/–Trp plates, and positive clones were then screened on SD/–Trp plates with X-α-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 *Xho*I 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-α-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) for 4 d with two changes of paraffin per day (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 NaH_2PO_4 (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*

was transformed into mutant seedlings by *Agrobacterium*-mediated transfection. 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 (~10 g) 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 ultrasound 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 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 *SSIIIa*, *SSI*, *ISA1*, *AGPS1*, and *AGPLS1* (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 *AGPLS1*, 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 *pAGPLS1*, 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* (Prioul *et al.*, 1994), and *ISA1*

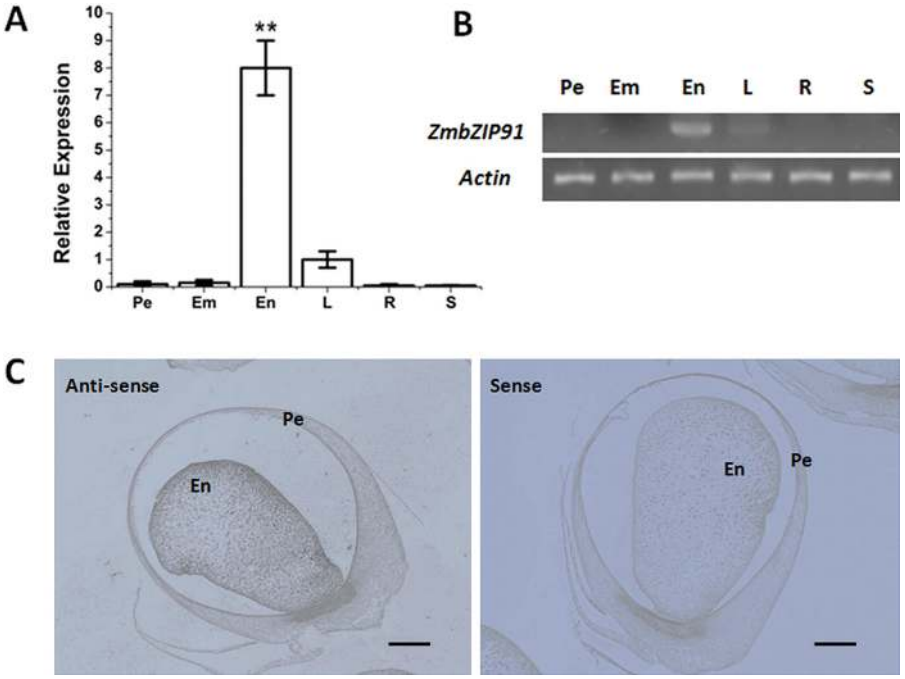

Fig. 1. Tissue expression analysis of *ZmbZIP91*. (A) Real-time RT–PCR analysis of *ZmbZIP91* expression in different tissues. Two-tailed unpaired *t*-tests were used to determine significant differences. ***P* <0.01. (B) Semi-quantitative RT–PCR analysis of *ZmbZIP91* expression in different tissues. (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.

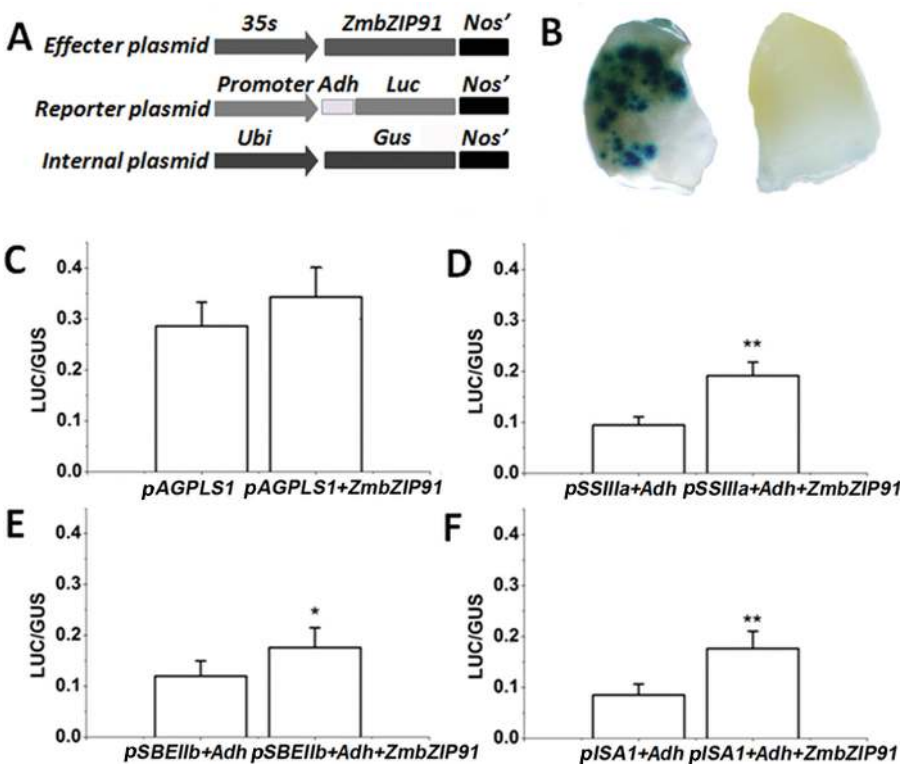

Fig. 2. ZmbZIP91 enhances the activities of starch synthesis gene promoters via particle bombardment of maize endosperm. (A) Diagram of the effector plasmid, reporter plasmid, and internal plasmid. (B) Example of the particle bombardment of maize endosperm: particle bombardment using *Ubi::GUS* and stained 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.)

(Lin *et al.*, 2013). 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 fluorecent 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

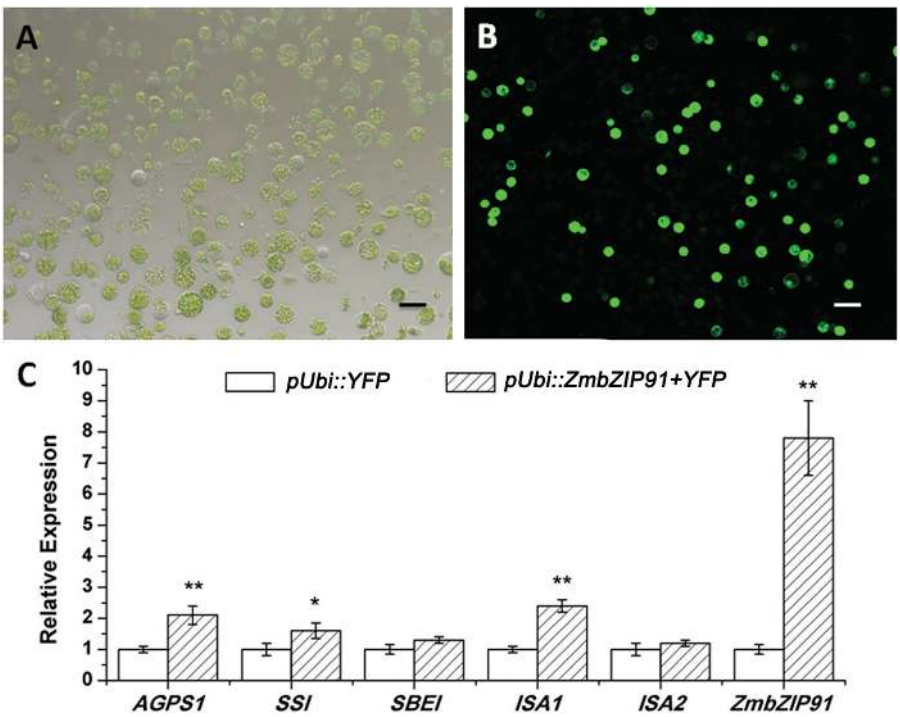

Fig. 3. ZmbZIP91 enhances the expression of starch synthesis genes in maize leaf protoplasts. (A) An intact maize leaf protoplast. (B) The high transfection efficiency of the isolated protoplasts. (C) Expression analysis of starch synthesis genes after *ZmbZIP91* transfection. The expression of *ZmbZIP91* 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.)

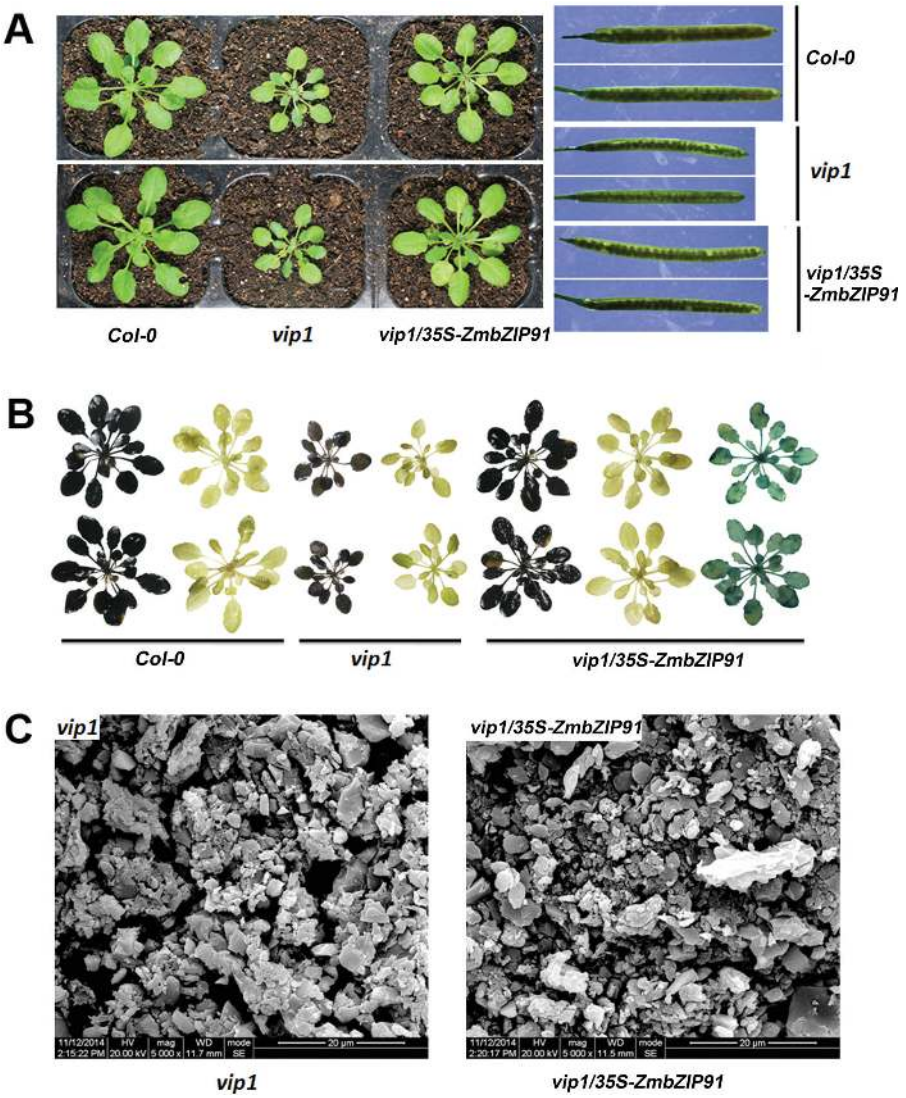

Fig. 4. Phenotypic analysis of the *vip1* mutant and complementation with *ZmbZIP91*. (A) Rosette growth of wild-type (*Col-0*), *vip1* mutant, and transgenic plants constitutively expressing *ZmbZIP91* (*vip1/35S-ZmbZIP91*) (left). Siliques and seeds of the corresponding plants (right). (B) Leaves of the corresponding plants after KI–I_2 staining and destaining with 75% alcohol. GUS staining of transgenic plants constitutively expressing *ZmbZIP91* (*vip1/35S-ZmbZIP91*). (C) Scanning electron microscopy of leaf starch in the *vip1* mutant and the *vip1/35S-ZmbZIP91* line.

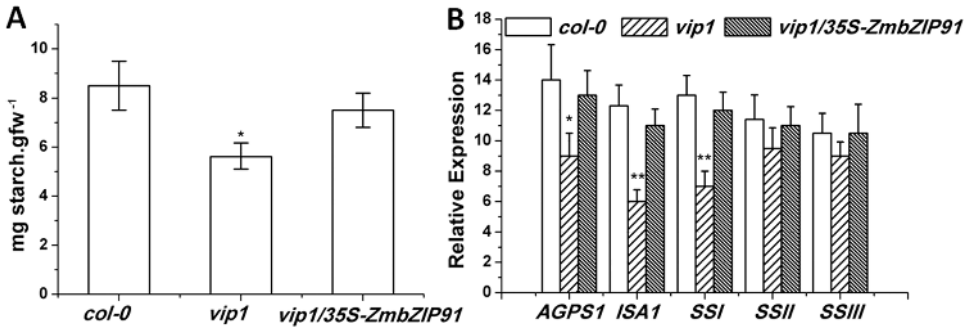

Fig. 5. Starch content and expression levels of starch synthesis genes in Arabidopsis leaves. (A) Starch content in *Col-0*, the *vip1* mutant, and *vip1/35S-ZmbZIP91*. (B) Expression of starch synthesis genes in *Col-0*, the *vip1* mutant, and *vip1/35S-ZmbZIP91*. Total RNA was extracted from leaves at the end of the day. *At-tubulin* was used as the internal control. The experiments were conducted using three replicates. Two-tailed unpaired *t*-tests were used to determine significant differences. **P*<0.05; ***P*<0.01.

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_3 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–I_2 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 the *vip1* 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 the *vip1* mutant line, which exhibited ~7.5 mg starch g FW^{-1} (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 *pAGPS1*, *pISA1*, *pSSIIIa*, and *pSSI* was also examined. The region from −2000 bp upstream of the translation start site (ATG) was used to search for bZIP-binding elements using PlantPAN (Chang *et al.*, 2008), and four fragments were determined to harbour several copies of the bZIP-binding elements (Supplementary Fig. S10 at *JXB* online). The details of the binding sites are listed in Supplementary Table S8. Further analysis of the binding elements indicated the presence of two types of elements: TCATT and ACTCAT motifs. The TCATT element exclusively binds Opaque-2 (bZIP family) to regulate zein protein synthesis in maize (Lohmer *et al.*, 1991). In contrast, the ACTCAT element has been reported to respond to osmotic pressure (Oono *et al.*, 2003), and a bZIP binds to the ACTCAT motif in the promoter of *ProDH* to lower osmotic pressure in Arabidopsis (Satoh *et al.*, 2004).

To identify the specific element to which ZmbZIP91 binds directly, an EMSA was implemented. First, ZmbZIP91-His was expressed and purified (Fig. 7A), and western blotting was performed to confirm expression of the protein (Fig. 7B). Then, biotin-labelled ACTCAT and TCATT elements from the promoters of *pSSIIIa* and *pAGPS1* were randomly synthesized. After ZmbZIP91 was successfully expressed and purified, an EMSA was performed using ZmbZIP91 and each element. A shifted electrophoresis band was only detected when ZmbZIP91 was incubated with the biotin-labelled ACTCAT element, which indicated that ZmbZIP91 is able to bind directly to ACTCAT elements but not TCATT elements (Fig. 7C–F). To determine whether 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).

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.*,

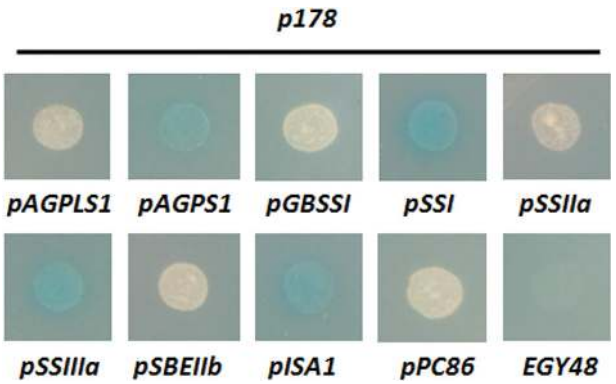

Fig. 6. Detection of the interaction between ZmbZIP91 and the promoters of various starch synthesis genes by yeast one-hybrid analysis. *pPC86-ZmbZIP91* and *p178* with different starch synthesis gene promoters were transformed into *EGY48*, and colonies were selected on selection medium (SD/–Ura–Trp+X-α-Gal). The blue yeast colonies indicate positive interactions. The different starch synthesis gene promoters are listed next to the corresponding panels.

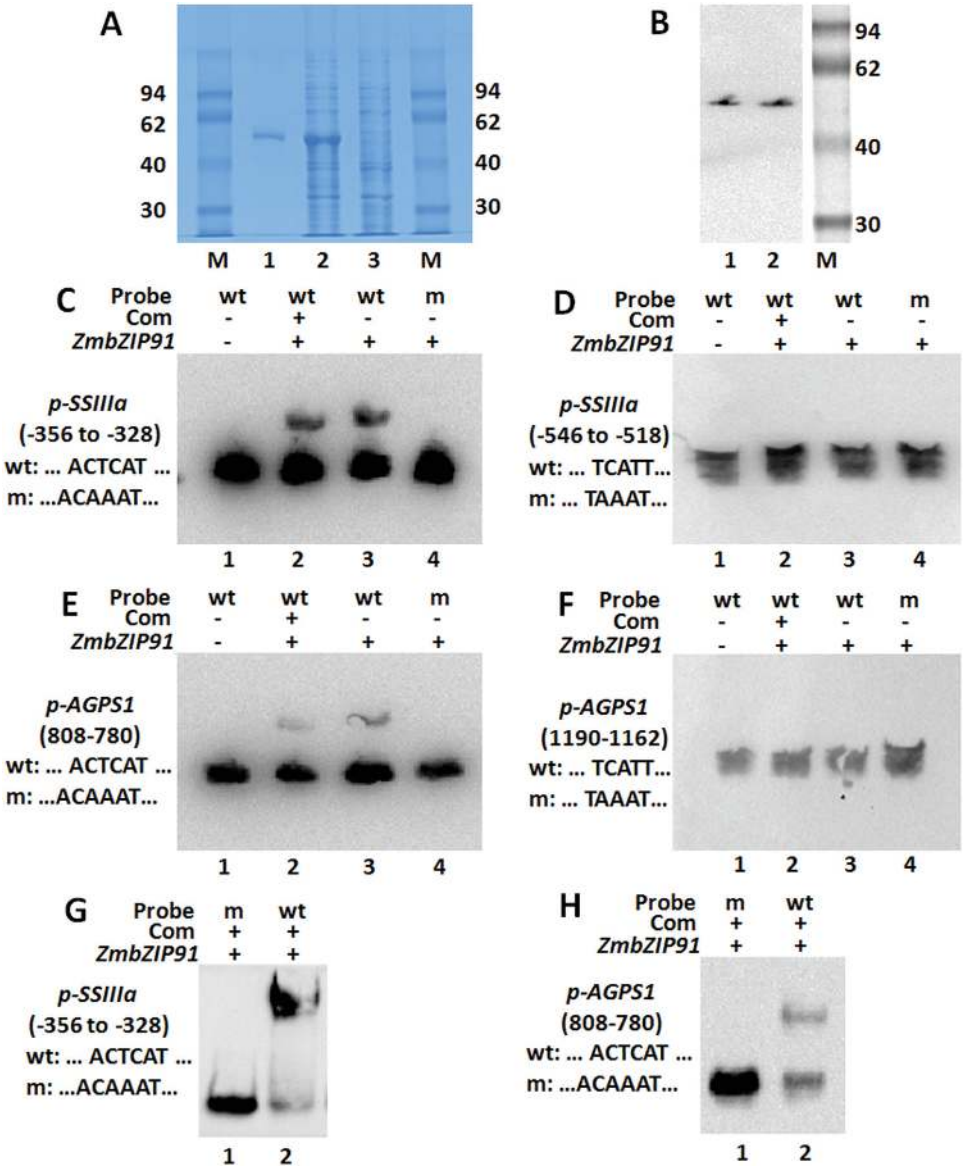

Fig. 7. ZmbZIP91 binds *in vitro* directly to the ACTCAT element but not the TCATT element in the promoters of starch synthesis genes. (A) ZmbZIP91 expression and purification. No treatment (A3), IPTG (isopropyl-β-D-thiogalactopyranoside) treatment (A2), and purified protein (A1) are labelled. (B) Western blot analysis of the induced protein using an anti-His antibody. Lane 1 shows the purified protein; lane 2 shows the total induced protein. (C and D) EMSA performed with the purified ZmbZIP91 protein and ACTCAT (C)/TCATT (D) elements from *pSSIIIa*. (E and F) EMSA performed with the purified ZmbZIP91 protein and ACTCAT (E)/TCATT (F) elements from *pAGPS1*. Lane 1, no protein added. Lanes 2–4, 500 ng of purified ZmbZIP91 protein was added. Lane 2, competition analysis using unlabelled elements; lanes 1–3, the biotin-labelled element was added; lane 4, the biotin-labelled mutant element was added. (G and H) EMSA of ZmbZIP91 performed with the purified ZmbZIP91 protein and the ACTCAT (G)/TCATT (H) elements using competition with wild-type and mutant unlabelled probes; 500 ng of purified ZmbZIP91 protein was used in the EMSA. Lane 1, the biotin-labelled mutant element was added; lane 2, the biotin-labelled element was added. Mutant unlabelled competitor cannot eliminate the band, whereas the wild-type probe can, indicating specific binding.

2013), it is highly likely that *ZmbZIP91* is the orthologous gene to *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*, *pSSIIIa*, 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 (*pAGPLS1*, *pGBSSI*, *pSSIIa*, and *pSBEIIb*) 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–I_2 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 *SSII* and *SSIII* 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*, *pSSII*, 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 mannitol treatment (Tsugama *et al.*, 2012). This growth inhibition could be largely due to the overexpression of *CYP707A1/3*, which is up-regulated during rehydration or under conditions of high humidity (Umezawa *et al.*, 2006; Okamoto *et al.*, 2009). VIP1 also interacts with *CYP707A1/3* 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 100 mM or 300 mM mannitol. *ZmbZIP91* overexpression did in fact result in retarded growth under mannitol treatment (Supplementary Fig. S14A at *JXB* online), and *CYP707A1/3* expression was up-regulated (Supplementary Fig. S14B). Although the experiments did not prove whether ZmbZIP91 interacts with *CYP707A1/3* 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*, *pSSIIIa*, and *pSSI*, though the expression of *SBEI*, *AGPLS1*, 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 *pGBSSI*) (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 *pAGPS1*, *pISA1*, *pSSIIIa*, 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.

Table S8. Details of binding sites in the promoters of *AGPS1*, *ISA1*, *SSIIIa*, and *SSI*.

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