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Novel role of ZmaNAC36 in co-expression of starch synthetic genes in maize endosperm

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Abstract Starch is an essential commodity that is widely used as food, feed, fuel and in industry. However, its mechanism of synthesis is not fully understood, especially in terms of the expression and regulation of the starch synthetic genes. It was reported that the starch synthetic genes were co-expressed during maize endosperm development; however, the mechanism of the co-expression was not reported. In this paper, the *ZmaNAC36* gene was amplified by homology-based cloning, and its expression vector was constructed for transient expression. The nuclear localization, transcriptional activation and target sites of the ZmaNAC36 protein were identified. The expression profile of *ZmaNAC36* showed that it was strongly expressed in the maize endosperm and was co-expressed with most of the starch synthetic genes. Moreover, the expressions of many starch synthesis genes in the endosperm were upregulated when *ZmaNAC36* was transiently overexpressed. All our results indicated that NAC36 might be a transcription factor and play a potential role in the co-expression of starch synthetic genes in the maize endosperm.

Keywords Starch synthesis · Gene regulation · Transcription factor · NAC · Maize endosperm

Abbreviations

AGPase	ADP-glucose pyrophosphorylase
ADPG	Adenosine diphosphate glucose
ATAF1/2	*Arabidopsis thaliana* transcription activation factor
CUC2	Cup-shaped cotyledon
DAP	Day after pollination
DBE	Debranching enzymes
GBSS	Granule-bound starch synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
SBE	Starch-branching enzymes
SSS	Soluble granule-bound starch synthase
SP	Starch phosphorylase

Introduction

Starch is the most abundant storage carbohydrate in many crop seeds, and is closely related to the yield and quality of crops. Starch is also an essential commodity in food, feed, fuel, and industry. Maize starch is one of the best quality starches, with a purity reaching 99.5, and 80 % of the world's starch comes from maize. Thus, it is important to determine the mechanism of maize starch biosynthesis.

The synthesis of starch in higher plants is orchestrated mainly by four major types of enzymes: ADP-glucose

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pyrophosphorylase (EC 2.7.7.27, AGPase), starch synthase (EC 2.4.1.21, SS), starch-branching enzyme (EC 2.4.1.18, SBE), and starch debranching enzyme (EC 3.2.1.68, DBE) (Myers et al. 2000; Hannah 2005). Starch synthesis begins with the synthesis of ADP-glucose (ADPG) from Glu-1-P and ATP by AGPase. SS catalyzes linear chain elongation by the addition of a Glc unit donated from ADPG to the non-reducing end of an acceptor chain. Branch linkage is formed by the action of SBE, which cleaves linear glucan and transfers the released fragment to a C6 hydroxyl group of the same, or neighboring, chain. DBE hydrolyzes the branch linkages, and is involved in amylopectin biosynthesis (Kubo et al. 1999). It was reported recently that starch phosphorylase (EC 2.4.1.1, SP) also takes part in starch synthesis (Zeeman et al. 2004; Tickle et al. 2009; Nakamura et al. 2012). Starch contains both amylose and amylopectin. It is generally thought that granule-bound starch synthase was responsible for amylose synthesis, while other isoforms of SS, together with SBE and DBE, synthesized amylopectin (Ball and Morell 2003).

Many researchers have reported that starch is synthesized by coordinated expression of multiple enzymes. Keeling (1999) reported that the activities of starch biosynthetic enzymes in the seed reached their peaks at the middle of grain filling, and there were significant correlations with each other among the activities of starch synthetic enzymes. Similar results were found in maize inbred lines with different starch contents (Zhang et al. 2008). Recently, studies showed that starch synthetic enzymes constituted a multi-enzyme complex in the storage organs. Tetlow et al. (2004) reported that three forms of SBE and SS enzymes can interact with each other within the amyloplast. Later, he also observed that SSI, SSII and SBEII could constitute a complex in wheat (Tetlow et al. 2008). Hennen-Bierwagen et al. (2008, 2009) showed that starch biosynthetic enzymes formed multisubunit complexes in the developing maize endosperm. Meanwhile, the co-expression of starch biosynthetic genes was observed in maize (Giroux et al. 1994). Ohdan et al. (2005) generated the expression profiles of starch biosynthetic genes in rice using RT-PCR and found that some genes were co-expressed. This co-expression phenomenon was also found in *Arabidopsis thaliana* (Li et al. 2007; Tsai et al. 2009) and potato (Ferreira et al. 2010). In our previous study, 15 out of 24 starch biosynthetic genes were mainly expressed in the maize endosperm, and the 15 genes were co-expressed during maize development (Chen et al. 2011). Though the co-expression of starch synthesis was reported in many species, the mechanism remains unknown.

Gene expression is regulated by trans-acting factors and cis-acting elements. Transcription factors that interact with the upstream gene promoter mediate gene expression (Stower 2011). Multiple transcription factor families in plants, such as no apical meristem (NAM), *Arabidopsis thaliana* transcription activation factor [ATAF1/2] and cup-shaped cotyledon (CUC2) (NAC) (Olsen et al. 2005b), MYB (Du et al. 2009), WRKY(Rushton et al. 2010) and others, play important roles in the regulation of nearly all metabolic processes. Sun et al. (2003) found that transcription factors regulated the expression of starch biosynthetic genes. Fu and Xue (2010), through co-expression analysis, cloned a transcription factor, named *RSR1*, and showed its involvement in negative regulation of starch biosynthetic gene expression. Thus, we speculated that there might also be a common transcription factor that regulates the expressions of many starch biosynthetic genes in the maize endosperm.

NAM, ATAF, and CUC (NAC) transcription factors are one of the largest families of plant-specific transcription factors, and the family is present in a wide range of land plants. NAC transcription factors contain a highly conserved N-terminal DNA-binding domain and a variable C-terminal domain, and are implicated in various aspects of plant development. (Xie et al. 2000; Ren et al. 2000; Olsen et al. 2005a). NACs participate in plant responses to pathogens, viral infections and environmental stimuli (Ren et al. 2000; Kim et al. 2007).

In this paper, we analyzed the co-expression data from Fu and Xue's research (Fu and Xue 2010), and found a gene (LOC_Os01g29840.1) that had a high transcription level in rice seeds, which we thought likely to be involved in starch synthesis. We obtained the gene from maize by homologous cloning according to the LOC_Os01g29840.1 protein sequence and named it *ZmaNAC36* because of its high similarity with the *ZmaNAC36* gene in maize. *ZmaNAC36* probably encodes a transcription factor containing an NAC domain, whose function in starch synthesis has yet to be reported. Nuclear localization, transcriptional activation and target sites of ZmaNAC36 protein were identified. In addition, the expression profile and transient expression in maize endosperm of *ZmaNAC36* were analyzed. All our results indicated that NAC36 might be a transcription factor and play a potential role in the co-expression of starch biosynthetic genes in the maize endosperm.

Materials and methods

Plant materials and growth conditions

Maize seedlings of the 08–641 maize inbred line, provided by Maize Research Institute, Sichuan Agricultural University, were grown in the school farm in summer in 2011, according to the local standard for high yield maize production. When silks emerged, strict self-pollinations were performed every

morning. Developing endosperms were sampled from fresh ears at 5-day intervals during the period from 5 days after pollination (5 DAP) to 35 DAP. Meanwhile, root, stems, embryos and flowers at 10 DAP were also sampled. All samples were immediately frozen in liquid nitrogen, and stored at −70 °C until use. Ten DAP endosperms were chosen as the receptors for transient expression, according to Hu's method (Hu et al. 2011, 2012).

Gene cloning and sequence analysis of ZmaNAC36

In the data of Fu and Xue (2010), there were several genes that correlated with the expressions of starch synthetic genes (Supplement 1. T1). The expression of a gene (LOC_Os01g29840.1) that encoded a NAC protein was co-expressed significantly with 10 starch synthetic genes, and the average PCC value of the encoding genes with co-expressed guide genes was more than 0.8. BLAST searching using the LOC_Os01g29840.1 gene sequence in the maize database (www.maizesequence.org), which identified the corresponding gene sequence (GRMZM2G154182) from the maize endosperm cDNA libraries, named ZmaNAC36. Invitrogen company (Shanghai, China) constructed a cDNA library of the maize endosperm using the CloneMiner II kit. The primers used for the ZmaNAC36 gene amplification were designed by DNAstar software: 5′-AAGAAGAGAGTTTTTGTGCATTTGG-3′ (forward) and 5′-TCAGTACTTCCACACGCCATCC-3′(reverse). The KOD enzyme (TOYOBO, Japan) was used to clone ZmaNAC36, and the PCR product was inserted into pMD19-T (Takara, Dalian, China), and sequenced by Majorbio (www.majorbio.com).

Expression vector construction

A GFP-ZmaNAC36 expression vector was constructed for the subcellular localization of the ZmaNAC36 protein. The full-length coding region of ZmaNAC36 was amplified by PCR using the following primers: 5′-CGCGGATCCATG GCGGCGGACCAGCAG-3′ (forward) and (5′-CTAGACTAGTGTACTTCCACACGCCATCCATC-3′ (reverse) (the underlined sections are *Bam*HI and *Spe*I sites, respectively). The PCR product was inserted into pMD19-T and digested with *Bam*HI and *Spe*I, and the resultant fragment was ligated into pCAMBIA2300-35S-eGFP, which contained a GFP protein driven by the CaMV 35S promoter (plasmid map in Supplement 1. P2). HiFi polymerase (Tiangen, China) was used to check the construct by PCR, and Majorbio (www.majorbio.com) sequenced the PCR product.

The pGBKT7-ZmaNAC36 vector was constructed for transcription activation analysis. ZmaNAC36 was amplified with the following primers: 5′-GGAATTCCATATGATGGCGGCGGACCAGCAG-3′ (forward) and (5′-CGCGGATCCTCAGTACTTCCACACGCCATCC-3′ (reverse) (the underlined sections are *Nde*I and *Bam*HI

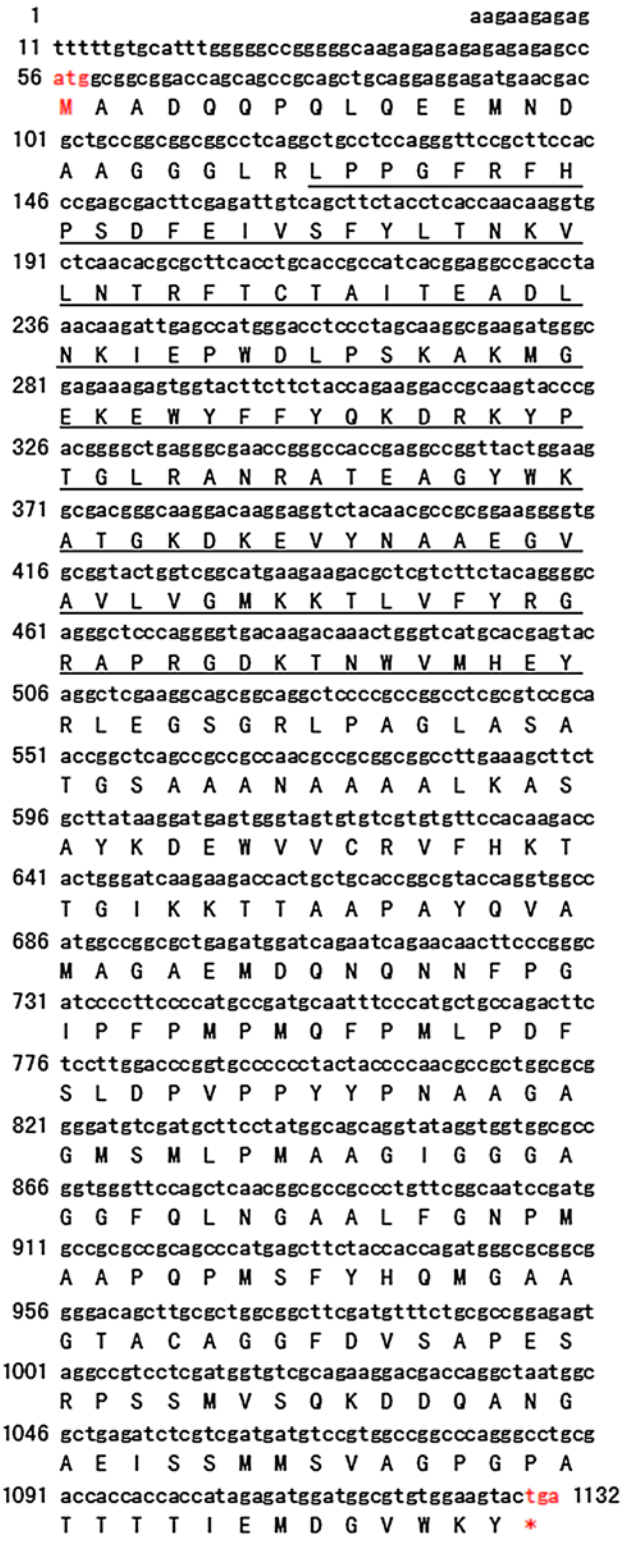

Fig. 1 Nucleotide and deduced amino acid sequences of *ZmaNAC36*. The *underlined* sections indicate conserved tryptophan residues in the NAC domain

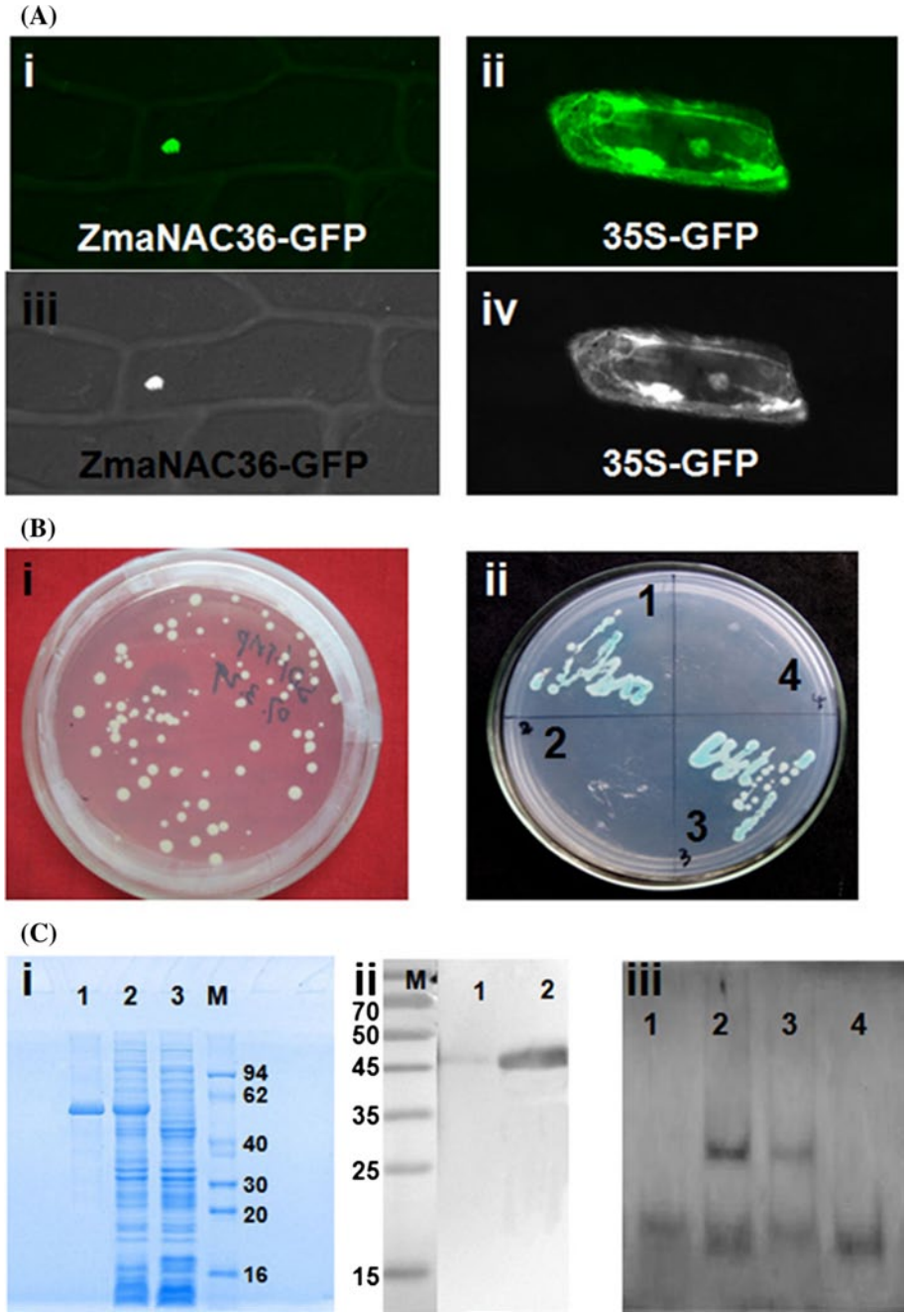

Fig. 2 Nuclear localization, transcriptional activation, Western blot and electrophoretic mobility shift assays of ZmaNAC36 protein. **a** The ZmaNAC36-GFP fusion protein, driven by the 35S promoter, was transiently expressed in onion epidermal cells and analyzed by fluorescent microscopy (**a**, i). GFP alone was used as a control (**a**, ii). Panels (**a**, iii) and (**a**, iv) are images under natural light. **b** Transactivation analysis of ZmaNAC36 in yeast. A fusion protein of the GAL4 DNA-binding domain and ZmaNAC36 was expressed in yeast strain AH109. Co-transformants were screened on SD/-Trp plates (i). (ii) The fusion protein of the GAL4 DNA-binding domain and ZmaNAC36 was screened on SD/-Trp-His-Ade with X-a-gal (1, ii). The co-transformation of GBKT7-lam and PGADT7-T was used as the negative control (2, ii) and the co-transformation of GBKT7-53 and PGADT7-T was used as the positive control (3, ii). The vector pGBKT7 was another negative control (4, ii). The plate was incubated at 28 °C for 3 days. **c** Western blot and Electrophoretic mobility shift assays (EMSA). (i) Prokaryotic expression of ZmaNAC36. ZmaNAC36 can be effectively induced under IPTG treatment (i, 2) compared with the control with no IPTG treatment (i, 3). ZmaNAC36 was successful purified using the His tag (i, 1). (ii) Western blot to verify the specificity of ZmaNAC36 protein. The induced protein (ii, 2) and not induced protein (ii, 1) were probed in immunoblot analysis with the IgG fraction. Anti-His was used as the primary antibody. The anti-mouse IgG antibody was used as secondary antibody. The result further verified ZmaNAC36 was specifically induced. (iii) EMSA of ZmaNAC36. iii,1 to ii4: biotin labeled probe in the absence of protein (iii, 1), with ZmaNAC36 protein (iii, 2), competition experiments with the unlabeled probe (iii, 3) and the biotin labeled mutation probe (iii, 4)

sites, respectively). ZmaNAC36 was cloned into the DNA-binding domain vector pGBKT7 (Clontech, USA).

The pTF101.1-ZmaNAC36 vector was constructed for transient expression of ZmaNAC36 in the maize endosperm. ZmaNAC36 was amplified with the following primers: 5′-CGCGGATCCATGGCGGCGGACCAGCAG-3′ (forward) and 5′-CGAGCTCGGATGGCGTGTGGAAGTACTGA-3′ (reverse) (the underlined section are *Bam*HI and *Sac*I sites, respectively). The map pTF101.1 is presented in the supplementary data (Supplement 1. P3).

Subcellular localization using onion epidermal cells

Onion epidermal cells were bombarded with the GFP-ZmaNAC36 expression vector using a helium biolistic gun transformation system (Bio-Rad, USA), as described previously (Hu et al. 2011), and incubated in light or darkness for 24–48 h at 28 °C. The subcellular localization of GFP fusion proteins was visualized with a fluorescence microscope BX61 (Olympus, Japan).

Transcription activation in yeast

Transcription activation analysis was examined for the presence of a protein activation domain using a yeast assay system. A GAL4 DNA-binding domain-ZmaNAC36 fusion protein would be produced when pGBKT7-ZmaNAC36 vector was transformed into yeast. The yeast strain AH109 was used in our experiment. Transformants were screened by plating them on SD/-Trp plates. The positive clones were confined by PCR, and the colonies were then screened on SD/-Trp-His-Ade plates with X-α-gal. Yeast was cultivated at 28 °C for 3 days to test transcription activation. For details, please refer to Fujita et al. (2004).

Western Blots Analysis

The pET32a (Takara Biotechnology (Dalian) Co., Ltd.) was used for the expression of ZmaNAC36-His fusion protein. Restriction enzyme *BamH*I and *Sal*I were used in our experiment for vector construction. ZmaNAC36 was amplified with the following primers: 5′-CGCGGATCCATGGCGGCGGACCAGCAG-3′ (forward) and 5′-CGCGTCGACTCAGTACTTCCACACGCCATCC-3′ (reverse) (the underlined section are *Bam*HI and *Sal*I sites, respectively). Rosetta (Takara Biotechnology (Dalian) Co., Ltd.) was used as the host cell for protein expression. Strains transformed with ZmaNAC36 plasmid were firstly shaken at 37 °C, 200 rpm until the OD reached about 0.5. And Isopropyl β-D-1-Thiogalactopyranoside(IPTG) (final concentrations was 1 mmol/L) was added, then the strains were continued to shake at 28 °C, 200 rpm for about 4 h. The strains were broken discontinuously by ultrasound under 300 W for 10 min. SDS-PAGE was use to electrophoresis the induced protein. The protein was then transferred to PVDF membrane. After incubation with anti-His and anti-mouse IgG antibody(Abmart (Shanghai) Co., Ltd), the product was lastly chemical self-luminous using chemiluminescent kit (Beyotime Institute of Biotechnology, Jiangsu, China). The details of the western blot analysis were referred to Sambrook and Russell (2001).

EMSA-electrophoretic mobility shift assays (EMSA)

EMSA was used to verify whether the transcription factor could bind with the promoter fragment. The protein expression of ZmaNAC36 was listed as the same as it in Western Blot Analysis. For the protein purification of ZmaNAC36, firstly, the induced strains were broken discontinuously by ultrasound under 300 W for 10 min. The cell debris was pelleted by centrifugation, and the supernatant and pelleted debris were analyzed to confirm if the protein was soluble or not. The protein was purified using a His tag protein purification method (Hengen 1995). In our research, the corresponding protein purification kit was used (Beyotime Institute of Biotechnology, Jiangsu, China). And then,the NAC common binding promoter fragment (NAC binding sequences (NACBSs)) was synthetized according to Olsen et al. (2005a). Single-stranded oligonucleotides 5′-CAGTCTTGCGTGTTGGAACACGCAACAGTC-3′ and its reverse complementary sequence (the underlined sections are the NAC binding sites) was synthetized with a 5′-end biotin label. The substitution of the NACBSs with simple nucleotide

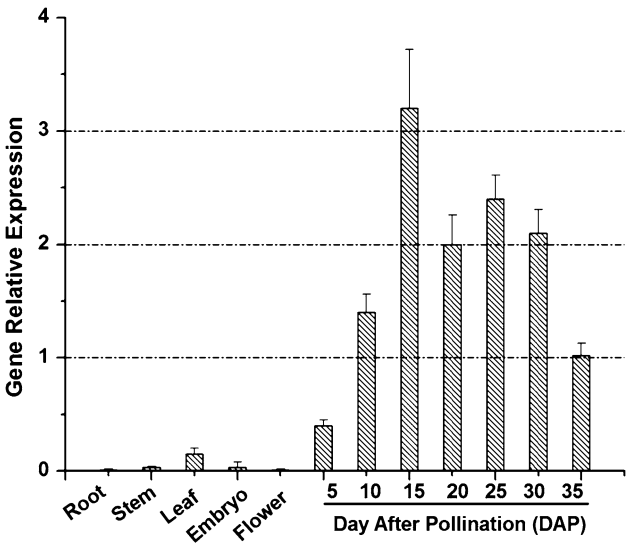

Fig. 3 Expression analysis of *ZmaNAC36* in different organs. The expressions of *ZmaNAC36* in maize endosperm for 5–35 days at 5-day intervals after pollination (DAP) were also measured. The sample of root, stem, leaf, embryo and flower were from maize at 10 DAP

repeats was used to abolish the NAC domain binding (5′-CAGTCAAAAAAATTGGAAGGGGGGGCAGTC-3′ and its reverse complementary sequence; the underlined sections are the mutated NAC binding sites; both were synthetized with a 5′-end biotin label). At the same time, the sequences without a 5′-end biotin labeling, (5′-CAGTCTTGCGTGTTGGAACACGCAACAGTC-3′ and its reverse complementary sequence) were also synthesized. All the sequences were synthesized by Sangon Biotech (http://www.sangon.com/). The double-stranded oligonucleotides used in EMSA were prepared by annealing the complementary single-stranded oligonucleotides. Negative-PAGE was used for the electrophoresis, and the product was then transferred to PVDF. Chemiluminescence was detected using an EMSA kit (Beyotime Institute of Biotechnology, Jiangsu, China) and the major steps for chemiluminescent detection were after Giraud et al. (2009).

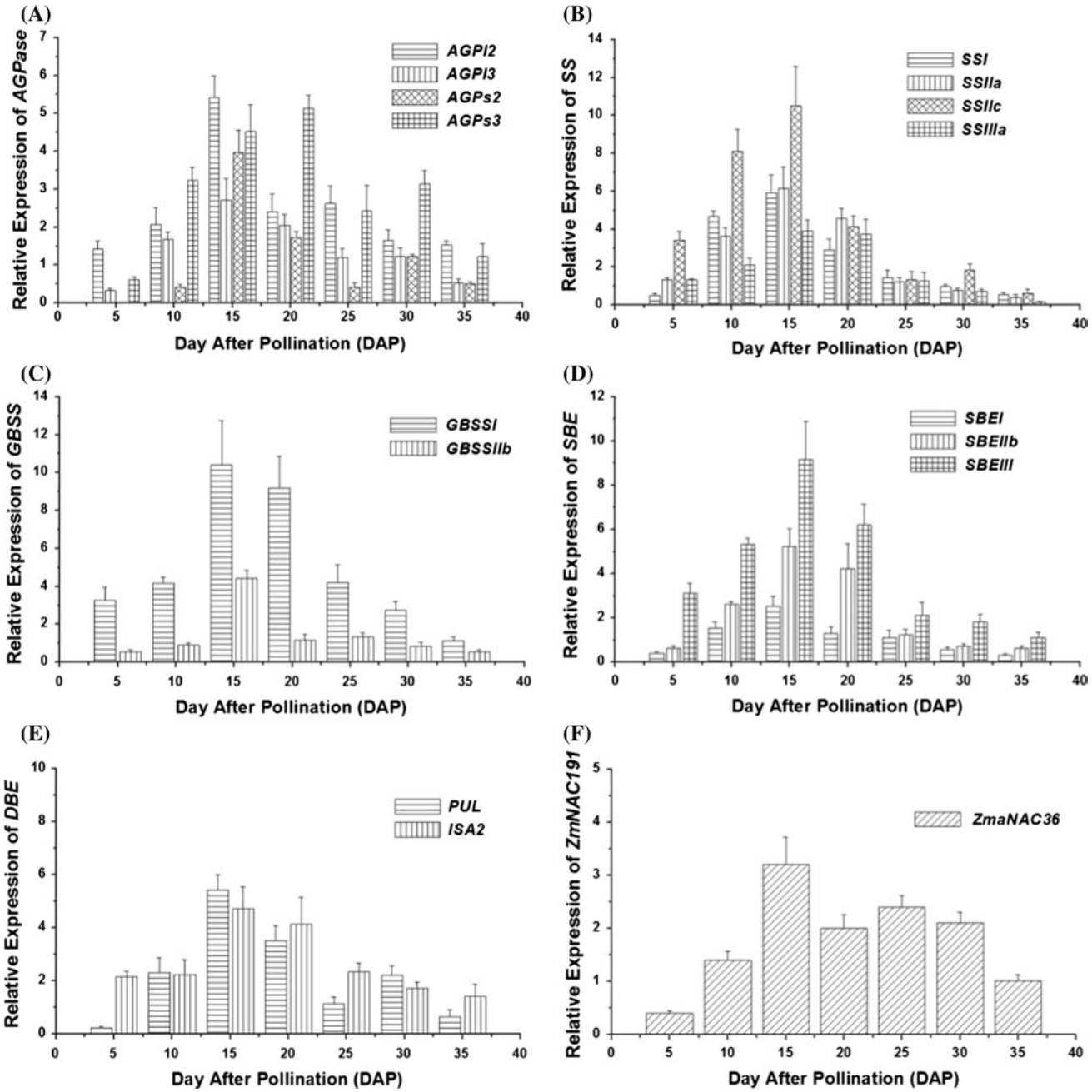

Fig. 4 qPCR analysis of starch synthetic genes in the maize endosperm. The expression of 18S rDNA was used as the internal reference gene. **a** AGPase genes; **b** SS genes; **c** GBSS genes; **d** SBE genes; **e** DBE genes; **f** *ZmaNAC36*

RNA extraction and real-time qPCR analysis

Total RNA was extracted using a Total RNA Extraction Kit (Tiangen Biotech Co., Ltd, Germany). Reverse transcription was carried out using Prime Script™ reagent Kit Perfect Real Time (TaKaRa, Japan), during which the genomic DNA was removed. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) assays were achieved using an iCycler instrument, model 5.0 (Bio-Rad, Hong Kong). The PCR mixture contained (in a total volume of 20 μL), 0.5 μL of forward primer, 0.5 μL of reverse primer, 1 μL of cDNA, 8 μL of doubly distilled H_2O and 10 μL of SYBR Green II (TaKaRa, Japan). The transcription levels of 16 genes (Table 2), including ZmaNAC36 and 15 genes mainly expressed in the maize endosperm, were tested by real-time qPCR. The primers for 16 genes for real-time qPCR were designed to amplify an approximately 200 bp fragment that encompassed an intron–intron junction, and the 18 s rDNA gene was used as a reference gene (Table 2).

Particle bombardment and transient expression assay in the maize endosperm

Maize kernels were surface-sterilized by 75 % (v/v) ethanol. Developing endosperms were isolated from 10 DAP kernels. Endosperms were cultivated on MS medium. The tissues were plasmolyzed on media for 4 h before bombardment. A helium biolistic gun transformation system (Bio-Rad, USA) was used to deliver gold particles coated with DNA. The bombarded tissues were cultivated for 36 h for analysis of the expressions of starch synthetic genes. The experiment was carried out according to our previous report (Hu et al. 2011).

Results

Cloning and sequencing analysis of *ZmaNAC36*

ZmaNAC36 was cloned from maize by homologous cloning using the rice gene LOC_Os01g29840.1. The protein sequence was used in a BLAST search of the maize database (www.maizesequence.org) and GRMZM2G154182 was cloned in our experiment. BLAST searching of the transcription factor database (http://planttfdb.cbi.edu.cn/) revealed that it is similar to the 36th member of the NAC family in maize; thus, we named the gene *ZmaNAC36* (from http://planttfdb.cbi.edu.cn/phylo_tree.php?sp=Zm&fam=NAC). *ZmaNAC36* comprised 1,132 bp with an open reading frame (ORF) of 1,077 bp. The encoded protein was predicted to be 38.40 kDa. *ZmaNAC36* is 53.56 % similar to the corresponding cDNA sequence in rice. Based on bioinformatic prediction (http://prosite.expasy.org/scanprosite/), the predicted protein contained one NAC domain at the amino terminus (Fig. 1), which means that *ZmaNAC36* probably encodes a NAC protein; however, its biological function has not yet been reported.

Characterization of *ZmaNAC36*

Most transcription factors nuclearly localized. Here, the subcellular localization of ZmaNAC36 was measured in onion cells. The results showed that the recombinant fusion protein ZmaNAC36-GFP was located in the nucleus, whereas the control GFP was localized in both cytoplasm and the nucleus (Fig. 2a). We also determined transcription activation activity of NAC36. The construct pGBKT7-ZmaNAC36 was transformed into yeast AH109

Table 1 Correlation analysis between *ZmaNAC36* and starch synthetic genes

	ZmaNAC36		*ZmaNAC36*
AGPl2	0.819*	*GBSSI*	0.68
AGPl3	0.831*	*GBSSIIb*	0.79*
AGPs2	0.798*	*SBEI*	0.75
AGPs3	0.755*	*SBEIIb*	0.65
SSI	0.6	*SBEIII*	0.55
SSIIa	0.57	*ISO2*	0.80*
SSIIc	0.42	*PUL*	0.65
SSIIIa	0.56		

* Correlation was significant at the 0.05 level (2-tailed)

** Correlation was significant at the 0.01 level (2-tailed)

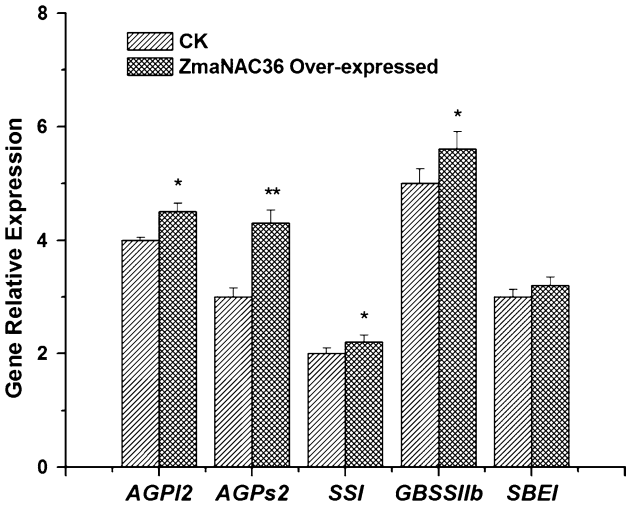

Fig. 5 Expression levels of representative genes in the transient expression experiment. Total RNA extracted from cultivated tissue was used for real-time qPCR analysis.*Significant at 0.05 %; **significant at 0.01 %

Table 2 Characteristics of starch synthetic genes and their RT-PCR primers

Enzymes	Full gene name	Gene name	Acc no.	Primer sequence (5′-3′)
AGPase	*ADP-glucose pyrophosphorylase small subunit2*	*ZmAGPS2*	GU550073.1	F: ATTACCGTTGCTGCCCTACC R: ACTGCTCTCCTTTCGGTTTCTC
	ADP-glucose pyrophosphorylase small subunit3	*ZmAGPS3*	AY032604	F: AACTCTGCTTCGCTCAACC R: TGCTCCTCAAATAGCCACAT
	ADP-glucose pyrophosphorylase large subunit2	*ZmAGPL2*	NM_001112247.1	F: GATGGGTGCGGATTTGTAT R: TTGGAACGCCCTCTTTGT
	ADP-glucose pyrophosphorylase large subunit3	*ZmAGPL3*	NM_001127632.1	F: GGGAGCGGACACCTATGAA R: AGCCTCTTGGATGCCCTTAC
SSS	*Starch synthase I*	*ZmSSI*	AF036891.2	F: CTTTCGGTGAGAATGGAGAGC R: GAGGACTTGTGTTCCCTGTATG
	Starch synthase IIa	*ZmSSIIa*	AF019296.1	F: GGGGAAGTAGGCAGGAAATCAT R: CAGATAAACAGGCAGGAGTGC
	Starch synthase IIc	*ZmSSIIc*	EU284113.1	F: TATCACCAACGAGACCCTTCG R: AGGAACCATCACTCTGACCACT
	Starch synthase IIIa	*ZmSSIIIa*	AF023159.1	F: GCTTCTTCTGTCGTTCTGCTCT R: GCGTAGTTTTCCTTGTGTAGCC
GBSS	*Granule-bound starch synthase I*	*ZmGBSSI*	AY109531.1	F: CATCTACAGGGACGCAAAGAC R: CGAAGGACGACTTGAATCTCTC
	Granule-bound starch synthase IIb	*ZmGBSSIIb*	EF472248.1	F: GTTGAGTTGGATGGTGTCCTTC R: GAGAGCCTTTGCTTCAGTTACC
SBE	*Starch branching enzyme I*	*ZmSBEI*	AY105679.1	F: CCTGTGTGGCTTATTACCGTGT R: GCCTCCTTGTCTTCTTTGCTAC
	Starch branching enzyme IIb	*ZmSBEIIb*	EF433557.1	F: GTGGGGTAGGTTTTGACTATCG R: CCTCCTATTTGTCAGTGTGTGC
	Starch branching enzyme III	*ZmSBEIII*	EU333945.1	F: GTCCACCCAACATTCTTCACTG R: CACACCAACACGATACGACTG
DBE	*Pullulanase*	*ZmPUL*	AF080567.1	F: GAACTGTCAAAACTCCCTCCAG R: CTGGGTTACTTGCATAGCTTCC
	Isoamylase II	*ZmISA2*	AY172633.3	F: GGGGGATGAATGTGGAAACTC R: CTCTGGAAAATGTCTGCTCGTC
ZmaNAC36	NAC factor	*ZmaNAC36*	GRMZM2G154182	F: CCAGGGGTGACAAGACAAACT R: GTGGAACACACGACACACTACC
18 s		*18srRNA*	JF905931.1	F: CTGAGAAACGGCTACCACA R: CCCAAGGTCCAACTACGAG

Acc no.: GenBank accession number in NCBI; primer abbreviations: *F* forward, *R* reverse. GRMZM2G154182; ID in maize sequence database (www.maizesequence.org)

cells and screened on SD/-Trp plates. Positive clones were identified by PCR and then further cultivated on SD/-Trp-His-Ade plates with X-α-gal at 28 °C for 3 days. The yeast cells with pGBKT7-ZmaNAC36 turned the substrate blue, which demonstrated that ZmaNAC36 had transcription activation activity (Fig. 2b). To identify ZmaNAC36 as a member of the NAC transcription factor family, the DNA-binding specificity of ZmaNAC36 was assessed using EMSA. Olsen et al. (2005a) had identified the common binding sites of the NAC transcription factor family. Here, the NAC common binding promoter fragment was synthetized referred to in Olsen's report (Olsen et al. 2005a). ZmaNAC36 was expressed in *E. coli Rosetta*, and Western blot verified ZmaNAC36 was specifically induced. In addition, the purified ZmaNAC36 protein could bind to the common sites of the NAC family, which confirmed that ZmaNAC36 belonged to the NAC transcription factor family (Fig. 2c).

Expression pattern analyses

To determine the expression pattern of *ZmaNAC36*, real-time qRT-PCR was carried out with the *18srDNA* gene as the internal reference gene. The expression of *ZmaNAC36* was extremely low in roots, stems, embryos and flowers when compared with the expression in maize endosperms. Its expression level was the highest during the middle of the maize endosperm development (Fig. 3). This result indicated that ZmaNAC36 may play an important role in the maize endosperm.

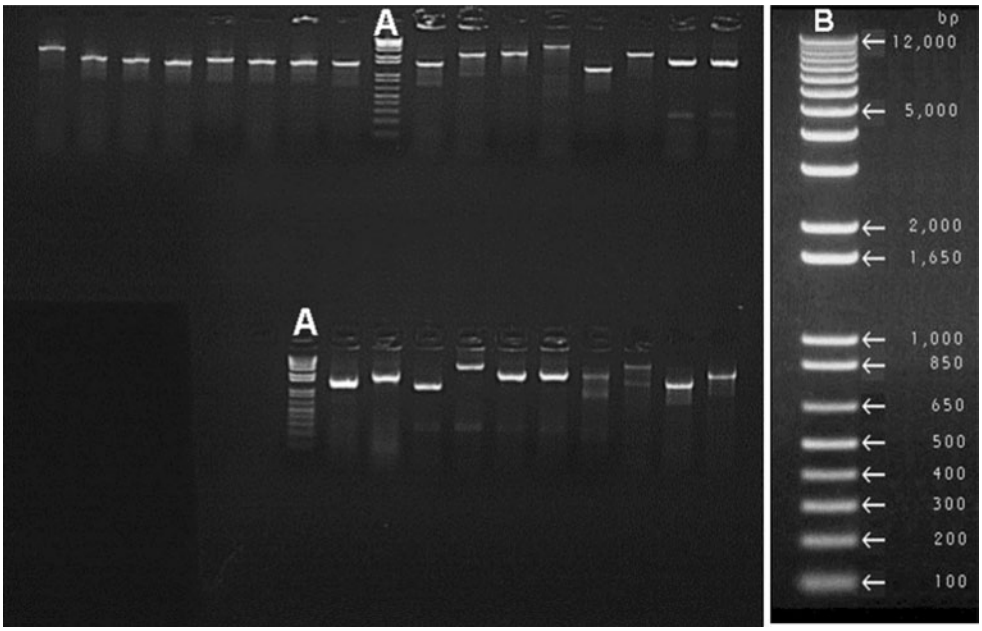

Fig. 6 Testing the quality of the cDNA libraries. **A** The DNA marker; **B** an enlarged picture of the marker

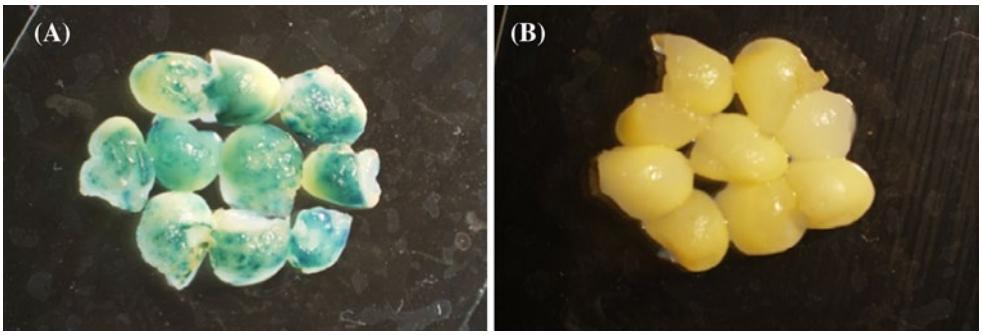

Fig. 7 Detection of GUS activities in maize endosperms after particle bombardment. **a** The endosperm bombarded with the vector with the *GUS* gene; **b** the control

The expression patterns of 15 genes mainly expressed in the maize endosperm were determined by real-time qPCR. The expression profiles of the genes encoding each key enzyme are shown in Fig. 4. Co-expression analysis between *ZmaNAC36* and the 15 starch synthetic genes was conducted. The results showed that *ZmaNAC36* was significant co-expressed with six starch synthetic genes (Table 1).

ZmaNAC36 was transiently overexpressed in the development endosperm. The transgenic endosperms were cultivated for 1 day, and total RNA was extracted for gene expression analysis. The expressions of starch synthetic genes were measured by real-time qPCR. The results showed that *AGPl2*, *AGPs2*, *SSI*, *GBSSIIb* and *SBEI* were upregulated compared with the control. In particular, *AGPs2* expression was significantly increased in the maize endosperm (Fig. 5). While the other starch synthetic genes had little change in our experiment (data not shown). This result indicated that ZmaNAC36 may be involved in the co-expression of many starch synthetic genes (Table 2).

Discussion

It had previously been reported that the starch synthetic genes were co-expressed, but the mechanism was not clear. Fu and Xue (2010) used the co-expression analysis and proved that *RSR1*, encoding an AP2 transcription factor, negatively regulated starch synthetic genes, which provided a guide for our research into gene regulation. In the co-expression analysis of Fu and Xue, in addition to AP2,

there were several other factors that were positively correlated with starch synthetic genes in the rice endosperm, including NAC, bZIP, MYB and GRAS. We chose to clone the highly correlated genes from maize. Unfortunately, we only cloned an NAC (ZmaNAC36) and a bZIP (Opaque-2) gene from the maize endosperm cDNA libraries. One possible reason was that some genes expressed in rice are not expressed in the maize endosperm. The function of Opaque-2 is known (Schmidt et al. 1990, 1992); therefore, *ZmaNAC36* was chosen for further study. It should be mentioned that the maize endosperm cDNA libraries used in our experiment were of high quality. Various genes of different lengths were contained in the libraries (Fig. 6). The characterization and identification of ZmaNAC36 indicated that it is an NAC factor that may play a role in regulating the expression of starch synthetic genes.

Transgenes in maize is very difficult; thus, transient overexpression of *ZmaNAC36* in maize endosperm was used to test its biological function. High efficiency particle bombardment was needed for the transient overexpression. To test the efficiency of particle bombardment, we generated a 35S-GUS vector (Supplement 1 P2). The 35S-GUS vector was bombarded into maize endosperms at about 10 DAP. The bombarded endosperms were stained with the GUS buffer referred to in Jefferson's method (Jefferson et al. 1987). The results indicated that particle bombardment could efficiently transfer the gene to the maize endosperm (Fig. 7).

In our previous report, 15 out of 24 genes involved in starch synthesis were mainly expressed in the maize endosperm, and were induced by sucrose and ABA(Chen et al. 2011). In this paper, ZmaNAC36 also increased the transcription levels of five starch synthetic genes. Thus, we proposed a hypothesis that ZmaNAC36 is first induced by ABA and sucrose, and the higher levels of ZmaNAC36 directly increase the expressions of the genes that were shown to be induced by ABA and sucrose. Subsequent experiments indicated that sucrose and ABA, alone or in combination, could enhance the expression of ZmaNAC36 (data not shown), which supported our hypothesis. Meanwhile, more studies are still required to completely clear the regulatory mechanisms of NAC36 on regulation of starch synthetic genes in maize endosperm.

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