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RESEARCH PAPER

Binding of ABI4 to a CACCG motif mediates the ABA-induced expression of the *ZmSSI* gene in maize (*Zea mays* L.) endosperm

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Abstract

Starch synthase I (SSI) contributes the majority of the starch synthase activity in developing maize endosperm. In this work, the effects of various plant hormones and sugars on the expression of the starch synthase I gene (*ZmSSI*) in developing maize endosperms were examined. The accumulation of *ZmSSI* mRNA was induced using abscisic acid (ABA) but not with glucose, sucrose, or gibberellin treatment. To investigate the molecular mechanism underlying this effect, the *ZmSSI* promoter region (–1537 to +51) was isolated and analysed. A transient expression assay in maize endosperm tissue showed that the full-length *ZmSSI* promoter is activated by ABA. The results of deletion and mutation assays demonstrated that a CACCG motif in the *ZmSSI* promoter is responsible for the ABA inducibility. The results of binding shift assays indicated that this CACCG motif interacts with the maize ABI4 protein *in vitro*. The overexpression of *ABI4* in endosperm tissue enhanced the activity of a promoter containing the CACCG motif in the absence of ABA treatment. Expression pattern analysis indicated that the transcription pattern of *ABI4* in the developing maize endosperm was induced by ABA treatment but was only slightly affected by glucose or sucrose treatment. Taken together, these data indicate that ABI4 binds to the CACCG motif in the *ZmSSI* promoter and mediates its ABA inducibility.

Key words: ABA, ABI4, CACCG motif, expression, maize endosperm, *ZmSSI*.

Introduction

Starch is the end-product of plant photosynthesis and is a major chemical component of crop grains. Starch biosynthesis and accumulation is a critical process that not only determines the final grain yield but also influences grain quality. Starch biosynthesis in higher plants occurs in the plastids through the coordinated activities of four enzymes: ADP glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) (James *et al*., 2003; Tetlow *et al*., 2004; Hennen-Bierwagen *et al*., 2009). It is generally accepted that the activities of these four enzymes in seeds during the grain-filling period affect the starch accumulation rate and the final starch content.

Abscisic acid (ABA) is a key regulator of seed development and plays an important role in regulating grain filling (Kato *et al*., 1993; Yang *et al*., 2001; Linkies *et al*., 2010; Nambara *et al*., 2010). In rice, the ABA content in large grain cultivars was higher than that in small grain cultivars and showed a higher grain-filling rate (Kato *et al*., 1993). It has also been demonstrated that the ABA level is positively correlated with starch content in developing wheat and barley seeds (Bai *et al*., 1989; Seiler *et al*.,

Abbreviations: ABA, abscisic acid; ABI, ABA insensitive; AGPase, ADP glucose pyrophosphorylase; CE1, coupling element1; DAP, days after pollination; DBE, starch debranching enzyme; EMSA, electrophoretic mobility shift assay; IPTG, isopropylthio-β-D-galactoside; qRT-PCR, real-time quantitative RT-PCR; SBE, starch branching enzyme; SON-PCR, single oligonucleotide nested PCR; SS, starch synthase.

2011). Under water stress, the enhanced activities of starch synthetic enzymes in the grains of rice and wheat have been associated with elevated levels of ABA (Yang *et al*., 2001, 2004; Zhang *et al*., 2012). Zhu *et al*. (2011) observed that the ABA concentration in rice superior spikelets is higher than that in inferior spikelets during the early and middle stages of grain filling, resulting in higher transcript levels of starch metabolism-related genes in the superior spikelets of rice than in the inferior spikelets. It has also been reported that some genes involved in starch biosynthetic and metabolic pathways are co-expressed with genes involved in the response to ABA stimulation in rice (Fu *et al*., 2010). However, the molecular mechanisms involved in the ABA-regulated expression of starch synthetic genes remain unclear.

Previous genetic studies have characterized three ABA-insensitive (ABI) transcription factor genes, *ABI3/VP1*, *ABI4*, and *ABI5*, which are expressed in mature seeds to mediate ABA signalling during seed development (reviewed in Linkies *et al*., 2010; Nambara *et al*., 2010). ABI4 is an AP2/ERF domain family transcription factor. Mutant analysis indicates that ABI4 is also crucial for salt or sugar signalling (Laby *et al*., 2000; Quesada *et al*., 2000; Rook *et al*., 2001). In *Arabidopsis* seedlings, ABI4 mediates the effects of sugars on starch metabolism by regulating the expression of starch synthetic genes such as *APL3* and *SBE2.2* (Rook *et al*., 2001; Bossi *et al*., 2009), and starch breakdown genes such as *SEX1* and the β-amylase gene *BMY8*/*BAM3* (Ramon *et al*., 2007). The ABI4 protein binds to *cis*-acting elements mediating the expression of sugar- or ABA-inducible genes, and the *in vitro* selection of DNA binding sites in the maize ABI4 protein identified a coupling element1 (CE1)-like sequence CACCK (Niu *et al*., 2002). However, two base degenerated motifs of this sequence retain some binding capacity: the S box (CACYKSCA), which is similar to the CACCK motif (Acevedo-Hernández *et al*., 2005), and the CCAC motif (Rook *et al*., 2006; Koussevitzky *et al*., 2007). The two degenerated motifs are present in close association, or overlap, with the G box and interact with ABI4 to mediate the ABA and sugar repression of nuclear genes (Bossi *et al*., 2009).

SS is one of the important enzymes involved in starch synthesis. It extends α-glucans by adding glucose residues from ADPG, the product of AGPase, to the glucan chains at non-reduced ends of growing starch molecules through α-1,4 glucosidic linkages. The endosperm is a major organ of the cereal seed in which starch biosynthesis and accumulation occur. In maize, the *SSI*, *SSIIIa*, *SSIV*, *GBSSI*, and *GBSSIIb* genes are highly expressed in developing endosperm tissue (Yan *et al*., 2009). SSI is the most active of these isozymes and accounts for >60% of all SS activity in the soluble fraction of the developing maize endosperm (Cao *et al*., 1999), and similar results have been reported in wheat and rice (Li *et al*., 2000; Fujita *et al*., 2006). To explore the regulatory mechanism of starch synthetic genes in maize endosperm during seed development, the effects of different plant hormones and sugars on the expression of the *ZmSSI* gene in the developing maize endosperm were analysed. It was observed that the exogenous application of ABA enhanced *ZmSSI* expression. The results of deletion and mutation analyses of the *ZmSSI* promoter showed that a CACCG motif in the promoter region is required for ABA inducibility. Furthermore, the results of gel shift assays and transient expression analyses showed that the ABI4 protein interacts with the CACCG motif to mediate the ABA-induced expression of *ZmSSI*.

Materials and methods

Plant materials and growth conditions

The maize inbred line 18R (bred by Sichuan Agricultural University) was grown in the field under recommended agronomic guidelines and self-pollinated. To obtain etiolated seedlings, the maize seeds were imbibed for 48 h and subsequently transferred to dark moist chambers for 10 d after germination.

Real-time quantitative RT-PCR (qRT-PCR) analysis

Five detached maize endosperms were incubated in a Petri plate in 5 ml of buffer (20 mM $CaCl_2$, 20 mM sodium succinate, pH 5.0, and 10 μM chloramphenicol) containing either 200 mM mannitol plus 5 mM glucose, 200 mM glucose, or 200 mM sucrose, or buffer containing 200 mM mannitol plus 5 mM glucose and either 1×10^{-6} M gibberellin (GA) or 1×10^{-4} M ABA (Lu *et al*., 1998; Chen *et al*., 2006). After 36 h of incubation in the dark at 28 °C, total RNA was extracted using Trizol (Invitrogen, CA, USA) and treated with DNase I (TaKaRa, Dalian, China) to remove any genomic DNA. Reverse transcription-PCR (RT-PCR) was performed with 1 μg of total RNA using the PrimeScript™ RT reagent kit (TaKaRa). Subsequently, quantitiative real-time PCR (qRT-PCR) analysis was conducted. The PCR amplification of the 115 bp *ZmSSI* cDNA was performed using ZmSSIF (5'-CTTTCGGTGAGAATGGAGAGC-3') and ZmSSIR (5'-GAGGAC TTGTGTTCCCTGTATG-3') as forward and reverse primers, respectively. The PCR amplification of the 139 bp maize *ABI4* cDNA was performed using ABI4F (5'-CCACACTATACTACGCCAACACG-3') and ABI4R (5'-GTACCTACCTGCGGCTCCTGTA-3') as forward and reverse primers, respectively. The amplification of the 220 bp *18S* rRNA amplicon was performed using 18SF (5'-CTGAGAAACGGCTACCACA-3') and 18SR (5'-CCCAAGGTCCAACTACGAG-3') as forward and reverse primers, respectively. The qRT-PCR analysis was performed using the iQ5 real-time PCR detection system (Bio-Rad, CA, USA) in a total reaction volume of 25 μl of SYBR Green PCR Master Mix (TaKaRa). The Ct value was determined using the instrument's software. The relative quantification of gene expression was monitored after normalization to *18S* rRNA expression as internal control. The relative transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Promoter isolation

Maize genomic DNA was extracted from etiolated seedlings of the maize inbred line 18R. The 5'-flanking region of *ZmSSI* was isolated from genomic DNA using single oligonucleotide nested (SON) PCR, as described previously (Antal *et al*., 2004). The *ZmSSI*-specific primer GSP1 (5'-CACGGCCGAGGGCGTCGCCATTG-3') and the nested *ZmSSI* primer GSP2 (5'-CATTGCGGAGAGGGAGAGCAGACAGGAG-3') were designed from the mRNA sequence of *ZmSSI* (Knight *et al*., 1998) (GenBank accession no. AF036891). The SON-PCRs (50 μl) were performed with 200 μM of each dNTP, 2 μM of primer, and 2 U of LA Taq DNA polymerase (TaKaRa). The DNA template for the primary reaction was 20 ng of maize genomic DNA. The secondary reaction used 2 μl of a 1:50 dilution of the first reaction as a template. The reaction was initiated with a denaturing step at 94 °C for 3 min and was followed by five cycles of amplification (30 s at 94 °C, 1 min at 60 °C, 2.5 min at 72 °C), one ramping step (30 s at 94 °C, 3 min at 29 °C, 3 min ramp to 72 °C, 2.5 min at 72 °C), and 60 (primary reaction) or 30 (secondary reaction) additional amplification cycles (10 s at 94 °C, 1 min at 60 °C, 2.5 min at 72 °C). The reaction ended with a final elongation step of 7 min at 72 °C. The resulting PCR fragment of ~1.6 kb was cloned into the pMD18-T vector (TaKaRa), sequenced, and subsequently referred to as pZmSSI-1.6KT.

Plasmid construction

A series of nested 5' deletions of the *ZmSSI* promoter (1.6, 1.2, 0.8, 0.53, 0.42, 0.36, and 0.25 kb) were amplified using PCR from pZmSSI-1.6KT with the common antisense primer (ASP: 5'-TCG *AGATCT*CGGAGAGGGAGAGCAGACAG-3') and the sense primers SP1 (5'-CCC*CTGCAG*TTGATGTTCTTTTTGTGTCTGTAAC-3'), SP2 (5'-CCC*CTGCAG*GATCTAGACTGGATGCTCTG-3'), SP4 (5'-CCC *CTGCAG*AGGGAGACAGAGGAAGCAC-3'), SP5 (5'-CCC*CTGCAG* CGCACCGCAGAATCTTCCC-3'), SP6 (5'-CCC*CTGCAG*TTCGCTC GCTCGCGGCGAAC-3'), or SP7 (5'-CCC*CTGCAG*ATCCGTTTTTG CTGTGTGCTC-3'), respectively. The underlined sequences in the above primers indicate the *Bgl*II and *Pst*I restriction endonuclease sequences.

To produce the pALuc3 construct, a 1.2 kb fragment was amplified using the SP2 and ASP primers and digested with *Pst*I and *Bgl*II, and the 0.8 kb resulting fragment was gel purified and cloned into the *Pst*I and *Bam*HI sites of pzsS3a-ALuc, upstream of the *ADH1* intron to replace the *ZmSSIIIa* promoter (Hu *et al*., 2011). To generate pALuc1 and pALuc2 constructs, the PCR products containing the 1.6 and 1.2 kb promoter fragments were digested with *Pst*I, and the resulting 0.8 kb and 0.4 kb fragments were gel purified separately. Subsequently, the pALuc3 construct was digested with *Pst*I, dephosphorylated at its 5' ends using BAP (TaKaRa), and ligated with the gel-purified fragments. Clones carrying the pALuc1, pALuc2, and pALuc3 constructs were detected using PCR with the universal primer RV-M and ASP as forward and reverse primers, respectively. The 5'-deletion derivatives were constructed by digesting the corresponding PCR products with *Pst*I and *Bgl*II and cloning these fragments into the *Pst*I and *Bam*HI sites of pzsS3a-ALuc (Hu *et al*., 2011) upstream of the *ADH1* intron to replace the *ZmSSIIIa* promoter, producing the constructs pALuc4, pALuc5, pALuc6, and pALuc7.

The mutagenized reporter constructs were generated using the KOD Plus mutagenesis kit according to the manufacturer's instructions (ToYoBo, Osaka, Japan). To mutate the CACCG motif, the sense primer mCPF (5'-*TGTTA*CAGAATCTTCCCCACGCCAC-3') and antisense primer mCPR (5'-CGGCGGCTGGCTGCCGGATTG-3') were used to generate pmCALuc. For the mutation of motif III, the sense primer mIIIPF (5'-*ATTGTA*CTGCCACTGAAAGCGCTTCGAC-3') and antisense primer mCIIIR (5'-GTGGGGAAGATTCTGCGGTG-3') were used to generate pmIIIALuc. The mutant sites in each primer are underlined. The pALuc5 construct was used as the template for mutagenic PCR. The identity of the promoter region of all constructs was confirmed by sequencing.

To construct the effector vector for the transient gene expression assay, the entire coding region of maize *ABI4* was PCR amplified from 18R genomic DNA using the primers EABI4F (5'-GCG*GGATCC* ATGGAAGCCAGCAACAATG-3') and EABI4R (5'-GCG*GAGCTC* ACTTGAGGAAGACATCAAACC-3') and cloned into the *Bam*HI and *Sac*I sites of the reporter construct pUbi-Gus (Hu *et al*., 2011), replacing the β-glucuronidase (GUS) coding sequence to generate pUbi-ABI4. The underlined sequences in the primers indicate the *Bam*HI and *Sac*I restriction endonuclease sequences.

Particle bombardment and transient expression assays

At 9–11 days after pollination (DAP), the immature maize kernels were surface sterilized with 75% ethanol, and the top 1 mm of the kernel was sliced off. The endosperms were separated under sterile conditions and placed in MSO medium (Murashige and Skoog salts containing 1% agar and 10% sucrose) for 4 h prior to bombardment.

A Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) was used to deliver gold particles (1.6 μm) coated with the DNA constructs using 900 psi He pressure with the sample holder placed at 6 cm from the stopping screen. For the analysis of the 5'-deletion constructs, the molar ratio of the test construct to the internal control plasmid DNA (pUbi-Gus) was 4:1. For the analysis of the effect of ABI4 on the expression of *ZmSSI*, the test construct (pALuc5), effector construct (pUbi-ABI4), and internal control plasmid DNA (pUbi-Gus) were combined at a molar ratio of 4:1:1.

Each independent experiment consisted of four replicates and was repeated 2–3 times with similar results. After bombardment, the Petri dishes containing bombarded endosperms were sealed and incubated at 28 °C in the dark for 24 h. Subsequently, total protein was extracted using 0.2 ml of CCLR buffer [100 mM $KH_2(PO_4)$ pH 7.8, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 7 mM β-mercaptoethanol] as previously described (Chen *et al*., 2006). The fluorogenic assay for GUS activity was performed as previously described (Lu *et al*., 1998). LUC activity was determined using a luciferase assay system (Promega, WI, USA), with the photons emitted integrated over a time period of 10 s. The fluorescence and luminescence were determined using a Luminoskan Ascent (Thermo, IL, USA).

Overexpression of recombinant ABI4 in Escherichia coli

The maize ABI4 coding sequence was excised from pUbi-ABI4 with *Bam*HI and *Sac*I and subcloned into the expression vector pET-28a (Novagen, WI, USA) between the *Bam*HI and *Sac*I sites. The resulting plasmid was transformed into Rosetta™ (DE3) *E. coli*. Overexpression was performed according to the manufacturer's instructions, except that the 1 mM isopropylthio-β-D-galactoside (IPTG) induction was performed at 28 °C for 4 h. The overexpressed protein was purified using Ni–agarose affinity chromatography (Qiagen, MD, USA) under native conditions according to the manufacturer's instructions. SDS–PAGE was conducted as previously described (Zhu *et al*., 2010).

Electrophoretic mobility shift assay (EMSA)

The promoter region (–369 to –301) containing the CACCG element was PCR amplified using RV-M and RP (5'-GGACGGACGAGGTC GAAG-3') as forward and reverse primers, respectively, and the pALuc5 construct was used as template. The amplified fragment was gel extracted and labelled with [γ-^{32}P]dATP using T4 polynucleotide kinase (TaKaRa). Subsequently, EMSA was conducted using an EMSA/ Gel Shift kit according to the manufacturer's instructions (Beyotime, Haimen, China). In parallel, to determine binding specificity, competition experiments were carried out with unlabelled wild-type or mutated DNA amplified from the pmCALuc construct by PCR using the primers RV-M and RP added to the reaction in 20- to 80-fold molar excess over the labelled DNA fragment. After a 15 min incubation at room temperature, the completed reactions were separated using PAGE, and the gel was dried and subjected to autoradiography.

Results

ABA induces ZmSSI expression in maize endosperm

Several research groups have reported that the expression of some starch synthetic genes is induced by plant hormones and sugars (Miyazawa *et al*., 1999; Rook *et al*., 2001; Akihiro *et al*., 2005; Ahn *et al*., 2010). Here, whether the expression of *ZmSSI* in the maize endosperm was also affected by various plant hormones and sugars was investigated. First, developing maize endosperms were detached from 10–12 DAP maize kernels and treated with ABA, GA, glucose, or sucrose for 36 h (mannitol was added to the samples without sugars as an osmotic control). Total RNA was extracted and subjected to qRT-PCR analysis. The results showed that the *ZmSSI* expression was induced (~2.3-fold) with 100 μM ABA but not with glucose, sucrose, or GA (Fig. 1).

ZmSSI promoter activity is induced after ABA treatment

To investigate the mechanism underlying the ABA-mediated transcriptional regulation of the *ZmSSI* gene, the promoter

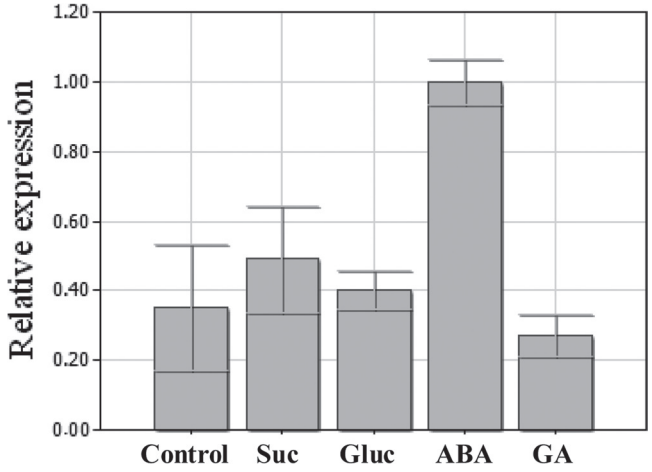

Fig. 1. qRT-PCR analyses of *ZmSSI* expression in maize endosperm in response to various plant hormone and sugar treatments. Twelve DAP maize endosperms were separated and treated with various plant hormones and sugars [200 mM mannitol plus 5 mM glucose (Control), 200 mM glucose (Gluc), 200 mM sucrose (Suc), or 200 mM mannitol plus 5 mM glucose supplemented with 100 μM ABA or 1 μM GA] at 28 °C in the dark for 36 h. Total RNA was isolated and subjected to qRT-PCR. RNA levels were quantified and normalized to *18S* rRNA. Error bars represent the standard deviation of three independent experiments.

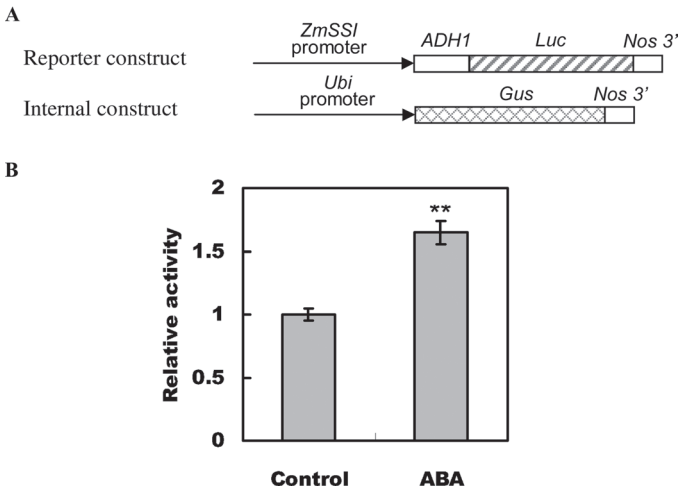

Fig. 2. ABA-induced *ZmSSI* promoter activity. (A). Schematic diagram representing the reporter and internal control constructs used in the experiment. (B) *ZmSSI* promoter response to ABA treatment. The reporter construct, pALuc1, and the internal control construct, pUbi-Gus, were co-bombarded into maize endosperms. LUC activity was normalized to GUS activity after the bombarded endosperms were incubated or not with 100 μM ABA at 28 °C for 24 h. Relative LUC/GUS activity is expressed with control (–ABA)=1. The data are given as the means ±SE of three replicates. The significance of the difference between the –ABA and +ABA conditions was analysed using a one-sided paired *t*-test (**$P < 0.01$).

region of the *ZmSSI* gene was isolated using SON-PCR with gene-specific primers derived from the 5'-terminal regions of its mRNA sequence. After two rounds of high stringency SON-PCR, distinct DNA fragments were generated. An abundant band of ~1.6-kb (indicated by an arrow in Supplementary Fig. S1 available at *JXB* online) was extracted and sequenced. The result of the sequence analysis showed that the fragment was 1637 bp in length (GenBank accession no. EF988335). A comparison with the complete mRNA sequence of *ZmSSI* confirmed the identity of a 51 bp mRNA sequence (see Supplementary Fig. S2).

The 1637 bp region upstream of the ATG start codon was fused to a luciferase cDNA (*Luc*). Because the presence of the maize *ADH1* intron 1 increases gene expression in maize (Lee *et al*., 2007) and other cereals (Schünmann *et al*., 2004) without altering promoter specificity, this intron was inserted between the *ZmSSI* promoter and *Luc* for enhanced expression to generate the pALuc1 construct (Fig. 2A).

The ABA inducibility of each promoter was analysed using a particle bombardment-mediated transient expression assay system. The pALuc1 construct was transfected into maize endosperms along with a reference plasmid, pUbi-Gus, which contains a maize *Ubiquitin* promoter linked to a *Gus* gene (Hu *et al*., 2011), to correct for transfection efficiency. The bombarded endosperms were incubated in the dark with or without 100 μM ABA. The protein extracts were prepared from samples at 24 h post-treatment, and their relative luciferase activities were analysed. As shown in Fig. 2B, an ~60% increase in LUC activity was observed in the bombarded endosperms treated with ABA. The bombarded endosperms were also treated with different concentrations of ABA for different times, and it was observed that the promoter reached a peak in activity after treatment with 100 μM ABA for 24 h (see Supplementary Fig. S3 at *JXB* online). This finding demonstrated that *ZmSSI* promoter activity is induced with ABA, as expected, based on the increased expression observed in qRT-PCR analysis.

A 69 bp promoter region (–369 to –301) is responsible for ABA induction

To localize the ABA response region of the *ZmSSI* promoter, a series of 5'-terminal deletions of the promoter were generated from pALuc1, as shown in Fig. 3A. Each 5'-deletion construct was co-transfected into maize endosperms along with the internal control pUbi-Gus, and the endosperms were incubated in the absence (white bars) or presence (black bars) of 100 μM ABA for 24 h. Protein extracts were generated from the bombarded endosperms, and their relative LUC activities were measured.

In the case of the pALuc1 construct, the LUC activity was significantly induced in the presence of ABA (one-sided paired *t*-test; $P < 0.01$). The ABA-dependent increase in LUC activity was similarly high in the shortened constructs up to –369 (pALuc5) (one-sided paired *t*-test; $P < 0.01$). However, further shortening of the promoter, up to position –301 (pALuc6), not only significantly impaired ABA-induced LUC activity but also resulted in an ~50% decrease in activity upon ABA treatment (one-sided paired *t*-test; $P < 0.01$). A similar loss of ABA

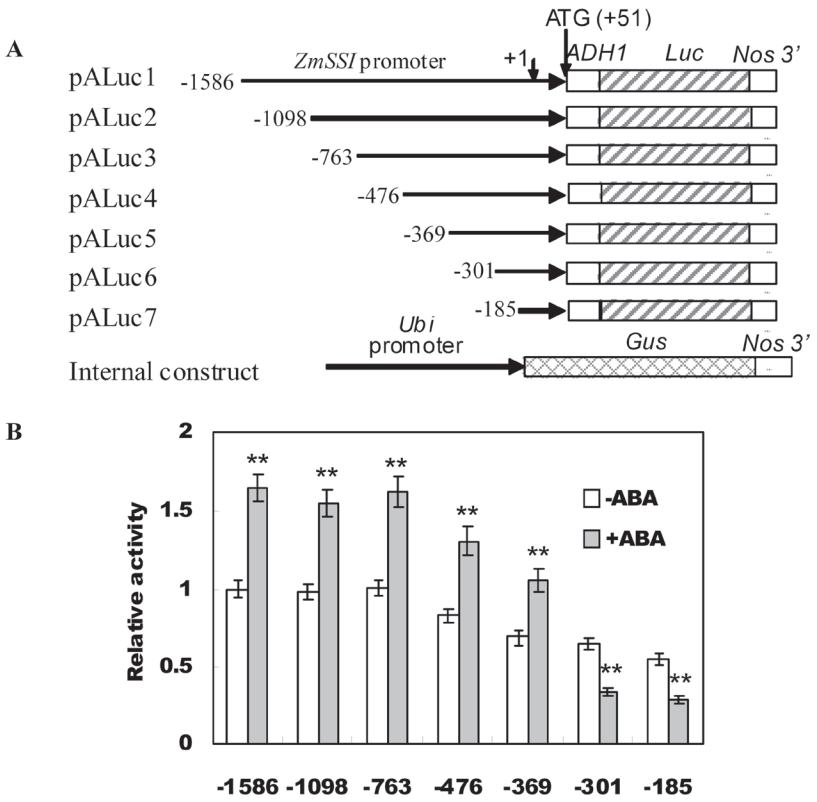

Fig. 3. Identification of the ABA-responsive region by 5'-deletion analysis of the *ZmSSI* promoter. (A) Schematic diagram of the 5'-deletion constructs of the *ZmSSI* promoter fused to the *Luc* reporter gene. The promoter region of *ZmSSI*, the 5'-untranslated region (UTR) of *ZmSSI* from +1 to +51, the first intron of the maize *ADH1* gene, the firefly luciferase (*Luc*) coding region, and the *NOS* 3' end sequences are indicated. The numbers on the left indicate the deletion end-points relative to the transcription initiation site (+1) of the *ZmSSI* gene. (B) Relative LUC activity in deletion constructs of the *ZmSSI* promoter. Each deletion construct shown in A was bombarded into maize endosperms, which were incubated in a buffer containing ABA (+ABA) or lacking ABA (–ABA) at 28 °C for 24 h. Protein extracts were made and LUC activity was determined. The relative activity values are percentages of the pALuc1 level in ABA-lacking buffer (–ABA). The data are given as the means ±SE of three replicates. The significance of the difference between the –ABA and +ABA conditions was analysed using a one-sided paired *t*-test (**$P < 0.01$).

induction was observed for pALuc7 (Fig. 3B). These data indicated that the region between positions –369 and –301 conferred the ABA-specific induced expression of the reporter gene, suggesting that the *cis*-element responsible for the ABA-induced activation of the *ZmSSI* promoter is contained within this 69 bp region. The repression of LUC activity could be caused by the presence of other *cis*-acting regulatory elements downstream of position –185 in the constructs.

Mutational analyses indicate that a CACCG motif is responsible for ABA-inducible expression of the ZmSSI gene

Analysis of the 69 bp (–369 to –301) promoter fragment revealed that the two potential *cis*-acting elements that respond to ABA were present in this region. One element is the CACCG motif (–367 to –363) which is similar to CE1, an element observed in ABA-responsive genes (Busk *et al*., 1997; Niu *et al*., 2002). The other element is a GCCACGCTGC motif (–347 to –338), which is similar to motif III (GCCGCGTGGC) in the ABA response element of the *rab16B* gene in rice, but with an antisense orientation (Yamaguchi-Shinozaki *et al*., 1990) (Fig. 4A).

To investigate whether the putative CACCG motif and the motif III sequence in the 69 bp region (–369 to –301) are associated with the ABA-inducible expression of *ZmSSI*, a mutational analysis of the two sites was performed. The CACCG motif and motif III were mutated using megaprimer mutagenesis with pALuc5 as template (Fig. 4A), generating pmCALuc and pmIIIALuc. Ten DAP maize endosperms were transiently transformed with each mutant construct using particle bombardment. Bombarded samples were treated or not with ABA and assayed for the relative expression of LUC activity. As shown in Fig. 4B, the mutation of motif III had no effect on the ABA inducibility of the promoter; the level of LUC activity generated by pmIIIALuc was enhanced after ABA treatment. However, when the CACCG motif was mutated, the ABA inducibility of the promoter was abrogated; the LUC activity of ABA-treated pmCALuc corresponded to ~50% of the activity in untreated endosperms. Thus, the CACCG motif in this region is responsible for the ABA inducibility of *ZmSSI* gene expression.

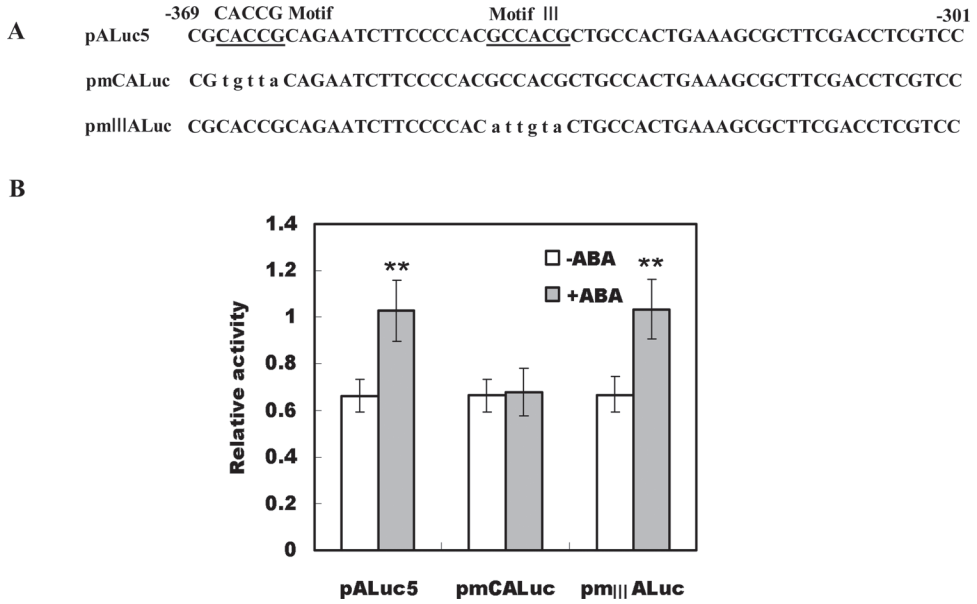

Fig. 4. Effects of mutations to putative *cis*-elements in the truncated *ZmSSI* promoter on induction of expression by ABA. (A) Sequences of the ABA-responsive region (–369 to –301) in the *ZmSSI* promoter and the *cis*-element mutant sequences. The nucleotides in the CACCG motif and in motif III are underlined, and the substituted nucleotides are shown in lowercase letters. (B) Relative LUC activity levels in maize endosperms transformed with the constructs shown and incubated or not with 100 μM ABA at 28 °C for 24 h. Relative LUC activity is expressed with pALuc1 (–ABA)=1. The data are given as the means ±SE of three replicates. The significance of the difference between the –ABA and +ABA conditions was analysed using a one-sided paired *t*-test (**$P < 0.01$)

ABI4 binds to the CACCG motif in the 69 bp (–390 to –301) promoter region in vitro

It has been reported that the CACCK motif interacts with the ABI4 protein, which is involved in ABA-induced gene expression (Niu *et al*., 2002) and sugar response pathways (Bossi *et al*., 2009). Because the CACCG motif in this region is responsible for the ABA inducibility of *ZmSSI*, tests were carried out to determine whether ABI4 binds to the CACCG motif in this promoter region. The coding sequence of maize ABI4 was isolated and cloned into pET-28a, and a recombinant ABI4 protein with a C-terminal His tag was produced in *E. coli* and purified using Ni-NTA (nickel-nitrilotriacetic acid) chromatography. The recombinant protein was separated using SDS–PAGE (see Supplementary Fig. S4 available at *JXB* online) and renatured, then a DNA fragment containing the *ZmSSI* promoter region from –390 to –301, which contains the CACCG motif, was labelled using [γ-^{32}P]dATP and used as a probe. The interaction between the purified renatured recombinant ABI4 and the labelled DNA fragment was assayed using a gel mobility shift assay. The results of this assay are shown in Fig. 5. No binding signal was detected in reactions without recombinant protein (Fig. 5, lane 1), but when incubated with the recombinant protein, a retarded DNA–protein complex was detected (Fig. 5, lane 2). In addition, competition with excess unlabelled DNA fragments reduced the amounts of complexes (Fig. 5, lanes 3–5); however, the mutant competitor DNA lacking a CACCG motif was unable to abolish the binding of the protein to the probe (Fig. 5, lanes 6 and 7). Taken together, these data indicate that ABI4 can indeed recognize the *ZmSSI* promoter *in vitro*, binding specifically to the CACCG motif.

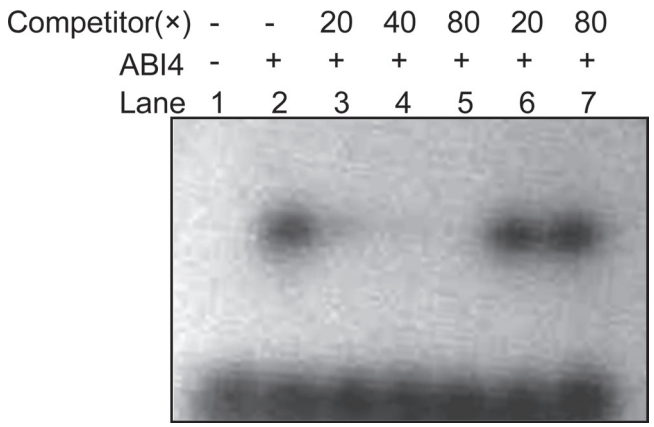

Fig. 5. Electrophoretic mobility shift assays (EMSAs) using a *ZmSSI* promoter fragment containing the CACCG motif and recombinant maize ABI4 protein. Radiolabelled probe was incubated in the absence (lane 1) or presence (lanes 2–7) of recombinant ABI4 protein. Competition experiments were performed using a 20× (lane 3), 40× (lane 4), or 80× (lane 5) molar excess of unlabelled wild-type probe; or using 20× (lane 6) or 80× (lane 7) unlabelled mutated probe.

Overexpression of ABI4 enhances the promoter activity of ZmSSI in maize endosperms

The above results show that the CACCG motif in the *ZmSSI* promoter is responsible for the ABA inducibility of this gene and interacts with the ABI4 protein *in vitro*. To determine whether

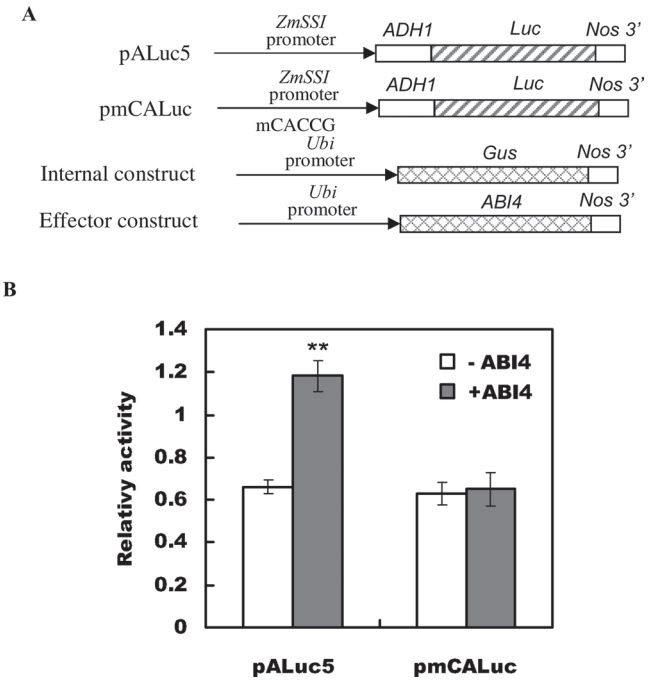

Fig. 6. Overexpression of *ABI4* induces *ZmSSI* promoter activity in maize endosperm. (A) Schematic representation of the reporter and effector constructs used in the experiment. (B) The reporter construct, pALuc5 and pmCALuc, and the internal control construct, pUbi:Gus, were co-bombarded into maize endosperm cells either with (+) or without (–) the effector construct (pUbi:ABI4). LUC activity was normalized to GUS activity in every independent transformation. The data are given as the means ±SE of three replicates. The significance of the difference between the –ABI4 and +ABI4 conditions was analysed using a one-sided paired *t*-test (**$P < 0.01$).

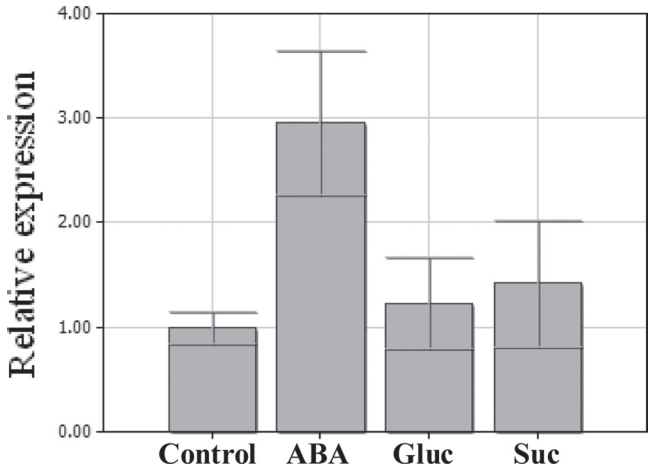

Fig. 7. qRT-PCR analyses of *ABI4* expression in maize endosperm in response to ABA and sugar treatments. Twelve DAP maize endosperms were separated and treated with 100 μM ABA, 200 mM glucose, or 200 mM sucrose, as described in the *ZmSSI* analysis (Fig. 1). The RNA levels were quantified and normalized to *18S* rRNA. The error bars represent the standard deviation of three independent experiments.

ABI4 is involved in the induction of *ZmSSI* through ABA *in vivo*, the maize *ABI4* coding sequence, spanning from the ATG to the stop codon, was cloned into an expression vector driven by the maize *Ubiquitin* promoter (pUbi:ABI4), which served as an effector construct (Fig. 6A). Because the activity of the pALuc5 reporter construct was induced after incubation of the developing endosperm with ABA and the CACCG motif mutated reporter construct, pmCALuc lost inducibility. The pUbi:ABI4 effector construct was co-bombarded into maize endosperm with the reporter constructs pALuc5 or pmCALuc. As shown in Fig. 6B, in the absence of ABA, the LUC activity driven by the pALuc5 construct in the ABI4-overexpressing endosperms was higher than that of endosperms not expressing ABI4 (~78% increased activity); however, the induction did not occur in the pmCALuc construct-bombarded endosperms. These results indicate that the ABI4 protein interacts with the CACCG motif in the *ZmSSI* promoter and activates its transcription in response to ABA.

Expression of ABI4 in maize endosperm is induced by ABA

The results of previous studies have indicated that ABI4 binds to the CACCK motif and activates the transcription of the *APL3* and *SBE2.2* genes in *Arabidopsis* vegetative tissue in response to sugars (Bossi *et al*., 2009). However, the present results indicated that ABI4 is involved in the ABA inducibility of the *ZmSSI* gene in maize endosperm. Therefore, the expression of *ABI4* in maize endosperm stimulated with sugar and ABA treatments was further tested. RNA was isolated from developing maize endosperm treated with ABA, glucose, or sucrose, as previously described for the *ZmSSI* expression analysis, and qRT-PCR was conducted. As shown in Fig. 7, the expression pattern of *ABI4* in developing maize endosperm was similar to that of the *ZmSSI* gene—it was enhanced by ABA treatment but not significantly affected by glucose or sucrose treatment.

Discussion

Some starch synthesis genes are known to be transcriptionally up-regulated in response to sugars (Li *et al*., 2002; Sun *et al*., 2003; Crevillén *et al*., 2005; Baguma *et al*., 2008; Ahn *et al*., 2010) and plant hormones (Miyazawa *et al*., 1999; Rook *et al*., 2001 Baguma *et al*., 2008). In the present study, the effect of different sugars and plant hormones on *ZmSSI* gene expression in maize endosperm was investigated. The results show that *ZmSSI* was expressed at a basal level in the developing maize endosperm under all conditions tested. *ZmSSI* transcript levels were increased in the presence of 100 μM ABA; however, *ZmSSI* transcription was hardly induced by sucrose, glucose, or GA treatments. Importantly, as signalling molecules and precursors of starch biosynthesis, sugars promote the induction of several starch biosynthetic genes, such as *APL3* and *APL4* in *Arabidopsis* (Rook *et al*., 2001; Crevillén *et al*., 2005), rice (Akihiro *et al*., 2005), and tomato (Li *et al*., 2002), *SS* in potato (Visser *et al*., 1991) and rice (Dian *et al*., 2005), *SBE* in potato (Kossmann *et al*., 1991), maize (Kim and Guiltinan, 1999), and

Arabidopsis (Rook *et al*., 2001), and *ISO* in barley (Sun *et al*., 2003). The sugar inducibility of the *SSI* gene in rice leaves was also reported (Dian *et al*., 2005). However, the present results showed that sucrose and glucose have little or no effect on *ZmSSI* gene expression in maize endosperm. This suggests that the expression of some starch synthesis genes, such as *SSI*, may be differentially regulated in endosperm tissue and in vegetative tissues such as leaf. This difference was also observed in *APL3* and *APL4* gene expression, for which sugar induction is leaf specific and does not take place in sink tissues (Crevillén *et al*., 2005). The expression of *ZmSSI* was induced through the exogenous application of ABA but was not affected by GA (Fig. 1), although GA is a well-known antagonist of ABA during plant development. ABA treatment enhanced the expression of starch synthetic genes, including the *APL3* gene, in *Arabidopsis* leaves (Rook *et al*., 2001) and rice suspension cells (Akihiro *et al*., 2005); ABA also promoted the expression of the *ZmSSIIIa* gene in developing maize endosperm (Hu *et al*., 2011) and the *SBE* gene in cassava sink cells (Baguma *et al*., 2008).

As an important plant hormone, ABA can enhance the expression of some starch synthetic genes and accelerate grain filling (Yang *et al*., 2001, 2004; Zhu *et al*., 2011; Zhang *et al*., 2012), but little is known about how this process occurs. The promoters of ABA-responsive genes contain conserved *cis*-elements, known as ABA-responsive elements (ABREs). Several ABREs have been identified (Busk and Pages, 1998; Gómez-Porras *et al*., 2007; Fujita *et al*., 2012). Typically, ABRE motifs contain an ACGT core and a (C/T)ACGTGGC consensus sequence that can be considered a member of the 'G box' (CACGTG) family (Menkens *et al*., 1995). Other *cis*-acting regulatory elements in ABA-responsive genes include the 'coupling element 3' (CE3) motif (ACGCGTGTCCTC) in the barley *HVA1* gene, 'motif III' (GCCGCGTGGC) of the rice *rab16B* gene, and a synthetic element, hex-3 (GGACGCGTGGC) (Busk and Pages, 1998). These elements share a CGCGTG consensus sequence similar to typical ABREs, except that the A of the ACGT element is substituted with G in the latter. ABREs that do not belong to G box or G box-like ABRE families have also been reported, including the Sph element-containing sequence (CGTGTCGTCCATGCAT) of the maize *C1* gene, the MYB and MYC binding sites of the *Arabidopsis rd22* gene, the element in the *CdeT27-45* gene of *Craterostigma plantagineum* (Choi *et al*., 2000), and the CE1 element of the barley *HVA22* gene (Shen and Ho, 1995). The isolation and functional analysis of the *ZmSSI* promoter confirmed the ABA-mediated activation of this protein, as expected based on its expression profile (Fig. 2B). The results of the deletion analysis of the *ZmSSI* promoter region demonstrated that the sequence from –369 to –301 is required for ABA inducibility. The *cis*-acting element analysis of this region identified a CACCG motif that is similar to the CE1 element and a GCCACGCTGC motif that is similar to motif III, albeit in an antisense orientation. Further mutational analysis indicated that the CACCG motif is crucial for ABA inducibility (Fig. 4B). However, it was not possible to identify the fragment conferring the ABA-repressed activity of the promoter downstream of the –185 position. The qRT-PCR analysis showed that *ZmSSI* expression in the developing endosperm was increased ~130% (2.3-fold) upon ABA treatment (Fig. 1), but the transient expression analysis of the promoter activity of the pALuc1 construct, which contains the full-length promoter of *ZmSSI* and with an *ADH1* intron as enhancer, only showed a 60% increase after ABA treatment (Fig. 2). Therefore, it is speculated that the ABA-repressed promoter activity of pALuc1 might be caused by the *ADH1* intron, although the *ADH1* intron was widely used as an enhancer in the expression of many monocot genes.

The CE1 element was initially identified as a *cis*-element in combination with the ABRE in promoters that exhibit synergistic responses to ABA treatment (Shen and Ho, 1995). Niu *et al*. (2002) demonstrated that the transcription factor ABI4 in maize binds to a 5 bp consensus sequence (CACCK) that resembles the CE1 element, which had previously been identified in a number of ABA-related genes. The functional interaction between ABI4 and the CACCK motif appears to be conserved in the promoters of *Arabidopsis* sugar-responsive genes such as *Em6*, *ADH1*, *PC*, *APL3*, and *SBE2.2* (Niu *et al*., 2002). The present *in vitro* binding studies demonstrate that ABI4 binds directly to the CACCG motif in the *ZmSSI* promoter (Fig. 5), suggesting that ABI4 might also regulate the expression of *ZmSSI* and mediate its activation in response to ABA.

It is generally accepted that ABI4 binds to the CACCK motif under certain conditions, and functions as a positive regulator of gene expression. Most ABI4-regulated genes are up-regulated by ABI4, including *APL3*, *SBE2.2*, *ABI4*, and *ABI5* (Bossi *et al*., 2009). However, some genes, such as *RBCS* and *Lhcb*, are repressed by ABI4 (Acevedo-Hernández *et al*., 2005; Koussevitzky *et al*., 2007). The results of the sequence analyses indicated that the ABI4 binding sequence is closely associated with a G box in the promoters of genes that are repressed by ABI4, but not in those that are activated by ABI4 (Acevedo-Hernández *et al*., 2005; Rook *et al*., 2006). Although ABI4 is a transcriptional activator, its activity could be much weaker than that of the G box-binding factor with which it competes, resulting in the relatively reduced activation of gene expression (Rook *et al*., 2006). Promoter analysis in this study showed that there was no G box closely associated with the CACCG motif in the *ZmSSI* promoter.

ABI4 was originally identified as a critical factor for transcriptional regulation by ABA during seed germination, and has been isolated in *Arabidopsis* (Finkelstein *et al*., 1998). The *ABI4* expression pattern has been reported to be under discrete developmental control. In *Arabidopsis*, it is expressed most strongly in seeds during seed maturation and at low levels in vegetative tissue (Finkelstein *et al*., 1998; Söderman *et al*., 2000; Arroyo *et al*., 2003; Nambara *et al*., 2010). The expression of *ABI4* in germinating seedlings is strongly induced by glucose but not by ABA or stress (Arroyo *et al*., 2003). However, in *Arabidopsis* roots, the expression of *ABI4* is induced by ABA and cytokinin and inhibited by auxin (Shkolnik-Inbar and Bar-Zvi, 2010). Niu *et al*. (2002) isolated the maize *ABI4* gene and reported that it is expressed specifically in seeds; *ABI4* transcripts were not detectable in maize vegetative tissues and were not induced by ABA or sugars in the leaves, roots, or early kernel tissues. Here, a more sensitive method, qRT-PCR, was used to detect the expression of the *ABI4* gene in endosperm. It was also shownn here that *ABI4* expression was induced by ABA in maize developing endosperm and that its expression was not significantly

affected by sugars such as sucrose and glucose (Fig. 7). These results imply that as a transcription factor with pleiotropic effects in plant development, *ABI4* might be regulated through several different signalling pathways and that the regulation of *ABI4* expression might differ across species, tissues, and even developmental stages.

In the present work, EMSA indicated that recombinant ABI4 protein could bind to the CACCG motif in the –369 to –301 region of the *ZmSSI* promoter. Further transient expression analysis showed that the overexpression of ABI4 in bombarded endosperm cells enhanced the activity of *ZmSSI* promoters containing the CACCG motif, even in the absence of exogenous ABA, but could not enhance the activity of a CACCG motif mutated promoter (Fig. 6B). These results indicate that ABI4 acts as a transcriptional activator of *ZmSSI* in response to ABA treatment. It was also noticed that the *ZmSSI* promoter activity in the *ABI4*-overexpressing endosperms and the ABA-treated endosperms was similar, suggesting the inducibility of *ZmSSI* expression by ABI4 might have an upper limit, as the expression of *ABI4* in the overexpressing endosperm cells was probably higher than that in the ABA-treated endosperm cells. The present results might provide an explanation of how ABA accelerates the filling rate during the grain-filling period.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The isolation of *ZmSSI* promoter sequences using SON-PCR.

Figure S2. Nucleotide sequence of the putative *ZmSSI* promoter region (–1586/+51) and analysis of the potential ABA response sites.

Figure S3. Effect of ABA on the activity of the *ZmSSI* promoter.

Figure S4. Purification of recombinant His-tagged ABI4 protein.

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