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ORIGINAL RESEARCH PAPER

PzsS3a, a novel endosperm specific promoter from maize (*Zea mays* L.) induced by ABA

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Abstract The maize *zsS3a* gene codes for starch synthase. Transcriptional analysis revealed that it is mainly expressed in endosperm and is induced by abscisic acid (ABA). The 5′-flanking region of *zsS3a* was isolated, and a 1772 bp *zsS3a* promoter (*PzsS3a*) was fused to a *Luc* reporter gene with a maize *Adh1* intron. Transient expression assay by bombardment transformation showed that, although the addition of the *Adh1* intron enhanced the promoter activity approx. 52-fold, it did not alter the promoter specificity. *PzsS3a* with the *Adh1* intron drove the *Luc* gene preferentially and it was highly expressed in the endosperm relative to the embryo but not in the leaf or root. Furthermore, the promoter activity in the endosperm was enhanced four fold by 100 mM ABA.

Keywords Abscisic acid · Endosperm · Maize · Promoter · *zsS3a* Gene

Introduction

Endosperm is the main storage organ of cereal crops and provides the primary source of carbohydrate and protein for humans and animals. Improvement of endosperm composition and quality by genetic engineering is attractive, and great achievements have been made in this area (Bajaj and Mohanty 2005). Cereal endosperm is also an ideal platform for the production of recombinant proteins, offering many advantages including cost-effective and easy agricultural scale-up, high rates of protein synthesis and easy long-term storage of recombinant protein to avoid degradation at ambient temperatures (Takaiwa et al. 2007; Xie et al. 2011).

Endosperm specific promoters play important roles in the genetic modification of endosperm composition and in using endosperm as bioreactors. At present, although several endosperm specific promoters have been cloned and used in these applications, there are still few available endosperm specific promoters that cannot satisfy all the practical demands (Qu et al. 2008). Moreover, most endosperm specific promoters are isolated from storage protein genes. Investigations

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have revealed that the activity of storage protein gene promoters is influenced by the availability of nitrogen (Muller and Knudsen 1993), thus the use of nitrogen-based fertilizers is bound to increase to improve recombinant protein accumulation when such a promoter is utilised. It is reported that heavy use of nitrogen fertilizers delays plant senescence, slows grain filling, decreases the harvest index and leaves carbohydrate unused in the straw. On the other hand, the accumulation of ABA in grains during grain development promotes grain filling and increases harvest index (Zhang et al. 1998; Yang et al. 2001). Thus, it is desirable to identify new endosperm specific promoters that respond to ABA, rather than nitrogen.

The maize *zsS3a* gene is one of the most important starch synthase genes and is mainly expressed in maize endosperm during starch biosynthesis (Gao et al. 1998). In the present study, we analysed the effect of plant hormones and sugars on the expression of *zsS3a* and found that expression is induced by ABA in endosperm. We then isolated the promoter region of the *zsS3a* gene (*PzsS3a*) and determined its expression patterns by bombardment transformation assay. The effect of the maize *Adh1* intron on the expression pattern of *PzsS3a* was also investigated.

Materials and methods

Plant materials and growth conditions

Maize inbred line 18R (bred by Sichuan Agricultural University) was grown in the field under recommended agronomic practices and self-pollinated. To obtain etiolated seedlings, maize seeds were imbibed for 48 h before being transferred into dark, moist chambers for a period of 7–14 days after germination (DAG).

Promoter isolation and sequence analysis

The 5′-flanking region of *zsS3a* was isolated from genomic DNA of 18R using the single oligonucleotide nested (SON) PCR described by Antal et al. (2004). The *zsS3a* specific primer (zSP1: 5′-CTCCGAAGG CAGAGAGGGCTCTG-3′) and the nested *zsS3a* (zSP2: 5′-GTAGGACCATCTCCATTTCTAG-3′) were designed from the mRNA sequence of *zsS3a* published by Gao et al. (1998) (GenBank access No. AF023159). The SON-PCR reactions (50 µl) were performed with 200 µmol of each dNTP, 2 µmol primer and 2 units LA Taq DNA polymerase (Takara, Dalian, China). The DNA template of the primary reaction consisted of 20 ng maize genomic DNA. The secondary reaction consisted of 2 µl of a 1:50 dilution of the first reaction; the reaction began 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) new 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. A resulting ~3 kb PCR fragment was cloned into a pMD19-T vector (Takara, Dalian, China), sequenced, and named as pzsS3a-3KT. The *zsS3a* promoter sequence was analysed by the PLACE Web Signal Scan program(http://bioinformatics.psb.ugent.be/webtools/plantCARE; Higo et al. 1999).

Semi-quantitative RT–PCR analysis

Total RNA from various tissue samples was extracted with Trizol and treated with DNase I to remove any genomic DNA. The RT–PCR was performed with 1 µg total RNA using PrimeScript RT reagent Kit (Takara, Dalian, China). Semi-quantitative RT–PCR was employed to investigate the tissue expression profiles of *zsS3a*. One 220 bp *zsS3a* cDNA fragment was amplified by the primers: zsS3a-F (5′-CACTC GCCTGACAGCCCAAAAG-3′) and zsS3a-R (5′-GCC AGCGTATATCAGATGCGAGAG-3′). Another 250 bp housekeeping 18S rRNA fragment was amplified by the primers: 18S-F (5′-CTGAGAAACGGCTACCA CA-3′) and 18S-R (5′-CCCAAGGTCCAACTAC GAG-3′) to determine if equal amounts of total RNA were used in the RT–PCR reactions among different samples.

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

Experiments studying the effect of exogenous plant hormones and sugars on *zsS3a* expression were performed by incubating five detached maize endosperms in 5 ml buffer (20 mM $CaCl_2$, 20 mM sodium succinate, pH 5.0 and 10 µM chloramphenicol) containing either 200 mM mannitol, glucose, sucrose or buffer containing 200 mM mannitol and either

10^{-6} M GA, or 5×10^{-5} M ABA in a petri plate (Chen et al. 2006). After 36 h of incubation in the dark at 28°C, total RNA was extracted and first strand cDNA was synthesised. Then qRT–PCR analysis was conducted using gene specific primers as described above in a total reaction volume of 25 µl SYBR Green PCR Master Mix (Takara, Dalian, China).

Plasmid construction

To analyse promoter activity, the region 1772 bp upstream of the ATG start codon was amplified from pzsS3a-3KT using primers PzsS3a-F (5′-GCGAAGCTTCACTCGTCGACGCCTTTGAGACTG-3′) and PzsS3a-R (5′-TCTCTCGAGCTAGAAGGGGAAGAAAAGAAG-3′) and digested with *Hin*dIII and *Xho*I. The modified firefly luciferase gene (*Luc*) derived from pGL3-Basic (Promega) was amplified by PCR with primers Luc-F (5′-CGAGCTCTTACGCGTGCTAG-3′) and Luc-R (5′-CTGAGAGCTCATTACACGGCGATCTTTCCG-3′) and digested with *Xho*I and *Sac*I. Promoter and *Luc* gene digestion products were simultaneously cloned into *Hin*dIII and *Sac*I digested pBI221 (Clontech) to produce pzsS3a-Luc. To enhance the expression of pzsS3a-Luc, the first intron of the maize alcohol dehydrogenase gene (*Adh1*) was amplified from 18R genomic DNA using the primers Adh1-F (5′-GTCTCTCGAGAAGTGCAAAGGTCCGCCTTG-3′) and Adh1-R (5′- CTCACCATGGTGGCCGCAGCTGCACGGGTC-3′), digested with *Xho*I and *Nco*I, and cloned into *Xho*I and *Nco*I digested pDu1-Luc to yield the plasmid pzsS3a-ALuc.

For the internal control construct, the maize *Ubiquitin* promoter was amplified by the primers Ubi-F (5′-GTGGCATGCTGCAGCGTGACCCGGTCGTG-3′) and Ubi-R (5′-GCGGGATCCCTGCA-GAAGTAACACCAAACAAC-3′), and the PCR product was digested and inserted into the *Pst*Iand *Bam*HI sites of pBI221 to yield pUbi-Gus. All constructs were confirmed by sequencing.

Particle bombardment and transient expression assay

Maize kernels were surface-sterilised by 75% (v/v) ethanol. Developing endosperms and embryos were isolated from immature kernels 8–10 and 13–16 days after pollination (DAP), respectively. The kernel top 1 mm was sliced off, and endosperm or embryo tissues were separated and placed on MSO medium (MS salts containing 1% agar and 10% v/v sucrose). The upper halves of the second leaves from 14 DAG maize seedlings were cut in 2 cm long sections and transferred to MSO medium with the lower epidermis facing upward. Roots from 5 DAG maize seedlings were cut longitudinally and also kept on MSO medium. All preparations were done under sterile conditions; all tissues were plasmolysed on media for 4 h prior to bombardment.

A Biolistic PDS-1000/He (Bio-Rad) was used to deliver gold particles (1.6 µm) coated with the DNA constructs by using 1100 psi He pressure with the sample holder placed 6 cm from the stopping screen. The molar ratio of the test DNA (pzsS3a-ALuc or pzsS3a-Luc) to the internal control plasmid DNA (pUbi-Gus) was 4:1. Each independent experiment consisted of three replicates and was repeated two to three times with similar results.

After bombardment, the Petri dishes containing the bombarded tissues were either sealed and incubated at 28°C in the dark for 24 h for tissue specific expression analysis, transferred to the buffers as described in qRT-PCR analysis, or were incubated at 28°C in the dark for 36 h for analysis of the ABA effect.

LUC and GUS Activity Assay

Total proteins were extracted as previously described (Chen et al. 2006). Bombarded maize tissues with 0.2 ml CCLR buffer (100 mM KH_2PO_4, pH 7.8; 1 mM EDTA; 10% v/v glycerol; 1% Triton X-100 and 7 mM β-mercaptoethanol). The fluorogenic assay for GUS activity was performed with methods described by Lu et al. (1998). LUC activity was determined by a luciferase assay system (Promega), the photons emitted were integrated over 10 s. Fluorescence and luminescence were determined using a Luminoskan Ascent (Thermo).

Results and discussion

Expression pattern of the *zsS3a* gene

zsS3a transcripts are present mainly in the endosperm of maize during starch biosynthesis (Gao et al. 1998). To confirm the preferential tissue expression of

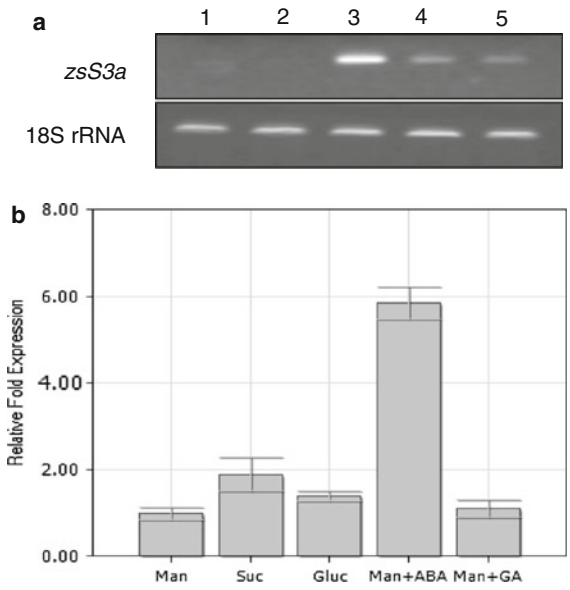

Fig. 1 Expression pattern of the *zsS3a* gene. **a** Semi-quantitative RT-PCR analyses of tissue-specific expression patterns of *zsS3a*. Leaves (*Lane 1*), roots (*Lane 2*), endosperm (*Lane 3*) and embryos (*Lane 4*) were harvested from 15 DAP maize and pollen (*Lane 5*) from shedding tassels. 18S rRNA was amplified as a control. The PCR products were determined by electrophoresis on a 2% (w/v) agarose gel. **b** qRT-PCR analyses of *zsS3a* expression in maize endosperm respond to various plant hormone and sugar treatments. Ten DAP maize endosperms were separated and treated with various plant hormones and sugars [200 mM mannitol, 200 mM sucrose, 200 mM glucose, 200 mM mannitol supplemented 50 mM ABA, 200 mM mannitol supplemented 1 mM GA] at 28°C in the dark for 36 h. Total RNA was isolated and subjected to qRT-PCR; RNA levels were quantified and normalised to the level of 18S rRNA

zsS3a, total RNA was extracted from endosperm, embryo, leaf, root and pollen from 15DAP maize, and the presence of *zsS3a* mRNA was examined by semi-quantitative RT-PCR. As reported previously, the transcripts of *zsS3a* were mainly present in the developing endosperm, not in the leaf or root (Gao et al. 1998). Transcripts were also detected low levels in the embryo and pollen (Fig. 1a). These results indicate that *zsS3a* is preferentially expressed in the endosperm.

Some starch synthase genes are inducible by ABA and sugars (Akihiro et al. 2005; Ahn et al. 2010). Our goal was to determine whether the expression of *zsS3a* was also affected by various plant hormones and sugars. Developing maize endosperms were detached and treated with ABA, GA, glucose and sucrose and then subjected to qRT-PCR analysis. The results showed that *zsS3a* was induced (sixfold) by a 36 h treatment with 100 mM ABA and was slightly increased by glucose, sucrose or GA (Fig. 1b).

Isolation and analysis of the promoter region of *zsS3a*

The isolation of the promoter region of the *zsS3a* gene was conducted by SON-PCR. After two rounds of high-stringency SON-PCR, a few distinct DNA fragments were generated. One band approximately 3 kb in size (positioned by arrows) was found to be very abundant (Fig. 2) and was extracted and sequenced. Sequencing analysis showed that the fragment was 3474 bp (GenBank accession no. EF988335). Comparison with the complete mRNA sequence of *zsS3a* confirmed the presence of a 137 bp mRNA sequence. To identify potential cis elements present in the *zsS3a* promoter, the 1772 bp region upstream of the ATG start codon was searched for homology to published cis elements using the PLACE and PlantCARE databases (Higo et al. 1999). The results showed that this fragment contained several potential specific cis elements including GCN4 motif, Skn-1 motif, ABRE element and G-box. However, no typical TATA or CAAT boxes were found in this region (Table 1).

Analysis of *PzsS3a* activity in various maize tissues

The forgoing results show that the *zsS3a* gene is mainly expressed in the developing endosperm and with little or no gene expression in other tissues.

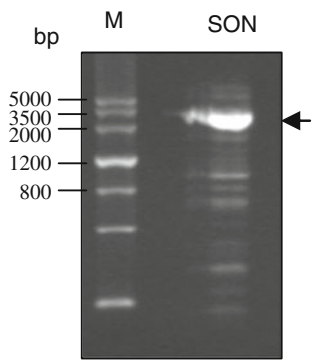

Fig. 2 The isolation of *zsS3a* promoter sequences using SON-PCR. *Lane M* DNA molecular size marker. *Lane SON* the secondary round of SON-PCR product. The *arrow* indicates the result of exponential amplification of the SON-PCR product

Table 1 Putative cis elements in the *zsS3a* promoter sequence using PlantCARE

Cis element	Organism	Consensus	Position in pdu1	Function
Skn-1 motif	*Oryza sativa*	GTCAT	−1623, −1562, −428	Endosperm expression
GCN4 motif	*Oryza sativa*	CAAGCCA	−142	Endosperm expression
ABRE	*Arabidopsis thaliana*	CACGTG	−853, −836, −274	Abscisic acid responsiveness
G-Box	*Arabidopsis thaliana*	CACGTG	−309, −129	Light responsiveness
GARE motif	*Brassica oleracea*	AAACAGA	−55	Gibberellin-responsive

To test whether the isolated promoter region is preferentially active in the endosperm, the 1772 bp region upstream of the ATG start codon was fused to the *Luc* gene and the nopaline synthase (*Nos*) polyadenylation site. The resulting construct was named pzsS3a-Luc. Because the presence of maize *Adh1* intron 1 has been found to increase the expression of genes in maize (Mascarenhas et al. 1990) and other cereals (Cornejo et al. 1993), we created another cassette, pzsS3a-ALuc, by inserting the maize *Adh1* intron between *PzsS3a* and *Luc* for improved expression. First, the two constructs pzsS3a-Luc and pzsS3a-ALuc were introduced into the developing maize endosperm by particle bombardment to test their promoter efficiencies. Because transfection efficiency varies from sample to sample using this method (Chiou et al. 2008), the plasmid pUbi-Gus, in which *uidA* was driven by the strong and constitutive maize *Ubiquitin* promoter, served as an internal control plasmid (Fig. 3a). As expected, modification of the promoter by adding the *Adh1* intron increased the promoter strength approximately 52-fold (Fig. 3b). Thus, the pzsS3a-ALuc construct was used to test the promoter active in different tissues. We assayed the promoter efficiency of pzsS3a-ALuc in maize leaf, root, endosperm and embryo, though we were not able to express the foreign gene in maize pollen due to technical difficulties. As shown in Fig. 3c, abundant LUC activity was detected in maize endosperm. Though a relatively low level of activity was detected in the embryo, no activity was detected in the leaf or root, which mimicked the expression pattern of *zsS3a*. This finding suggests that *PzsS3a* is an endosperm specific promoter. The addition of the *Adh1* intron enhanced promoter activity but did not alter promoter specificity.

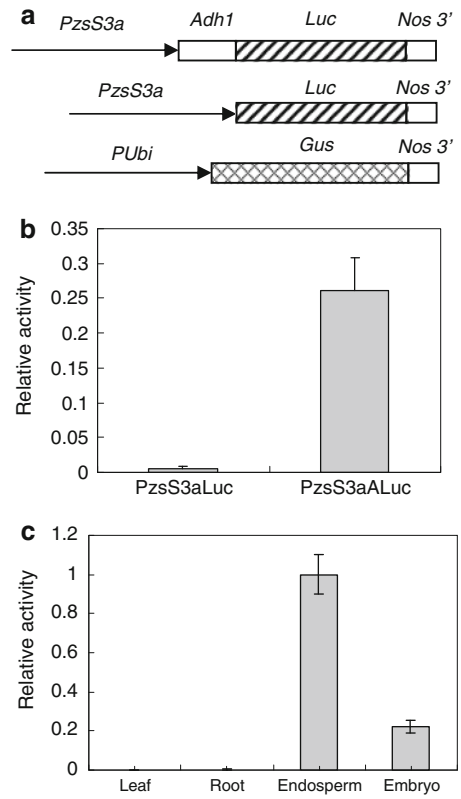

Fig. 3 Activity of *PzsS3a* in various maize tissues. **a** Diagram of the reporter and internal control constructs used in the experiment. **b** Activity of *PzsS3a* with or without an *Adh1* intron in 10 DAP maize endosperm. **c** Tissue specific expression of *PzsS3a* with the *Adh1* intron in various maize tissues. Maize endosperms, embryos, leaves and roots were co-bombarded with the reporter construct, pzsS3a-Luc/pzsS3a-ALuc and the internal control construct, pUbi-Gus, and were incubated in the dark for 24 h. LUC activity was standardised to GUS activity. Each result represents an average of six bombardments. Relative LUC/GUS activity is expressed as endosperm = 1. Data are mean ± SD ($n = 6$)

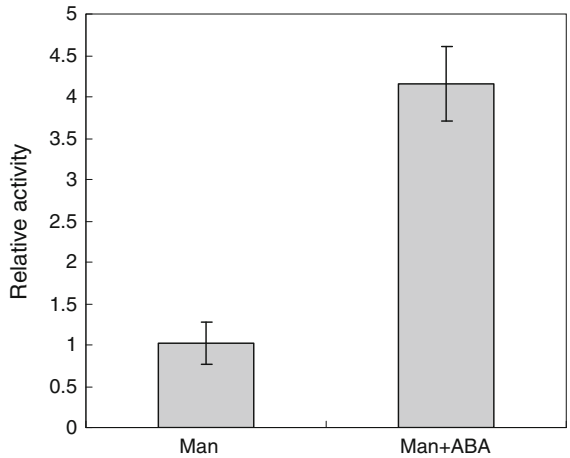

Fig. 4 The response of *PzsS3a* activity to ABA treatment. The reporter construct, pzsS3a-ALuc, and internal control construct, pUbi-Gus, were co-bombarded into maize endosperms. LUC activity was normalised relative to GUS activity after 40 h of incubation of the bombarded endosperms with or without 50 μM ABA at 28°C. Relative LUC/GUS activity is expressed as mannitol = 1. Data are mean ± SD ($n = 6$)

The *PzsS3a* is an ABA-inducible promoter

qRT-PCR showed that the expression of *zsS3a* is induced by ABA. To test the ABA-inducible activity of *PzsS3a* in maize endosperm, the transient expression analysis system was used as described above. After bombardment, maize endosperms were divided into halves; each half was incubated with or without ABA for 36 h, and the relative LUC activities were determined. As expected, LUC activity in the ABA-treated endosperms was approx. fourfold higher than in the untreated groups (Fig. 4). When we incubated the bombarded endosperm for 72 h to deplete endogenous sugars, relative LUC activity was similar to the results seen with 36 h incubation (data not shown). This finding suggests that the endosperm specific promoter, *PzsS3a*, is an ABA inducible promoter.

In transgenic plants, high-level accumulation of foreign protein in all tissues at all developmental stages is a waste of energy and is often harmful (or lethal) to the host plant. Such problem may be solved by using tissue-specific promoters and inducible systems for regulated gene expression. In cereal endosperm, it is well known that ABA content is low at early developmental stages when cell division is active and accumulates to a high level during middle to late development to induce grain-filling. When foreign genes are driven by *PzsS3a*, the exogenous proteins did not significantly accumulate in the early developmental endosperm and other plant tissues. Rather, the protein only accumulated during mid to late development of cereal endosperm with the accumulation of endogenous ABA. Hence, *PzsS3a* is an appropriate promoter that can decide the timing, tissue-specificity and foreign protein expression levels in cereal endosperm. *PzsS3a* might be an ideal endosperm specific promoter and may offer potential applications for the improvement of endosperm composition or for using endosperm as bioreactors.

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