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RESEARCH PAPER

OsbZIP58, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm

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Abstract

Starch composition and the amount in endosperm, both of which contribute dramatically to seed yield, cooking quality, and taste in cereals, are determined by a series of complex biochemical reactions. However, the mechanism regulating starch biosynthesis in cereal seeds is not well understood. This study showed that OsbZIP58, a bZIP transcription factor, is a key transcriptional regulator controlling starch synthesis in rice endosperm. *OsbZIP58* was expressed mainly in endosperm during active starch synthesis. *osbzip58* null mutants displayed abnormal seed morphology with altered starch accumulation in the white belly region and decreased amounts of total starch and amylose. Moreover, *osbzip58* had a higher proportion of short chains and a lower proportion of intermediate chains of amylopectin. Furthermore, OsbZIP58 was shown to bind directly to the promoters of six starch-synthesizing genes, *OsAGPL3*, *Wx*, *OsSSIIa*, *SBE1*, *OsBEIIb*, and *ISA2*, and to regulate their expression. These findings indicate that OsbZIP58 functions as a key regulator of starch synthesis in rice seeds and provide new insights into seed quality control.

Key words: Endosperm, coordination, OsbZIP58, rice, starch biosynthesis.

Introduction

Starch, the most abundant reserve polysaccharide in nature, mainly comprises amylose and amylopectin. Amylose is a linear molecule containing α-1,4-linked D-glucopyranosyl units, and amylopectin consists of short α-1,4-linked D-glucosyl chains with 5–6% α-1,6 bonds (Juliano, 1998; Smith, 1999). In crop plants, a large portion of starch is deposited in storage tissues, such as the endosperm in rice and maize, accounting for the main carbon sources for humans and livestock (Burrell, 2003).

Starch biosynthesis in plant seeds includes a series of complex and coordinated biochemical reactions. Multiple enzymes such as ADP-glucose pyrophosphorylase (AGPase), plastidial starch phosphorylase (PHO), granule-bound starch synthase [GBSS, also called Waxy (Wx)], soluble starch synthase (SS), starch branching enzyme (SBE), and starch debranching enzyme (DBE) are involved in this procedure (Hannah and James, 2008; James *et al.*, 2003; Jeon *et al.*, 2010). At the first step of starch biosynthesis, AGPase catalyses the conversion of glucose-1-phosphate into ADP-glucose, and PHO is hypothesized to play an important role in the glucan initiation process by synthesizing glucan primers with long degrees of polymerization (DP). GBSSI/Wx is responsible for amylose synthesis. Amylopectin biosynthesis is controlled by a series of starch

Abbreviations: AAC, apparent amylose content; bZIP, basic leucine zipper; ChIP, chromatin immunoprecipitation; CL, complemented line; DAF, days after flowering; DP, degrees of polymerization; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; ORF, open reading frame; qRT-PCR, quantitative reverse transcription; REB, rice endosperm bZIP; SD, standard deviation; SEM, scanning electron microscopy.

biosynthetic enzymes such as SS, SBE, and DBE in the cereal endosperm (Tian *et al.*, 2009). Rice seed development can be divided into four stages: the initiation stage [1–3 d after flowering (DAF)], during which starch is synthesized exclusively in the pericarp; the early developmental stage (3–5 DAF), indicated by endosperm starch accumulation with an obvious increase in seed weight; the middle stage (5–10 DAF), with a rapid increase in starch deposition and grain weight; and the late stage (10 DAF and beyond), in which seed maturation occurs (Counce *et al.*, 2000). Among the rice genes involved in starch biosynthesis, 14 genes, including *AGPase* (*OsAGPL3*, *OsAGPS2b*, *OsAGPL2*), *PHO* (*PHOL*/*OsPHO1*), *GBSS* (*OsGBSSI*/*Wx*), *SS* (*OsSSI*, *OsSSIIa*, *OsSSIIIa*, *OsSSIVb*), *SBE* (*OsBEI*/*SBE1*, *OsBEIIb*), and *DBE* (*OsISA1*,*OsISA2*, *OsPUL*), exhibit high levels of expression at approximately 5–7 DAF (Hirose and Terao, 2004; Dian *et al.*, 2005; Ohdan *et al.*, 2005), suggesting that these genes are closely associated with starch accumulation in rice seeds. Changes in the expression level of various starch biosynthetic enzymes are closely associated with the physicochemical properties of starch in rice endosperm. Moreover, amylose content is one of the key factors used for evaluating rice grain quality (Fitzgerald *et al.*, 2009; Jeon *et al.*, 2010). However, how these genes are regulated during rice seed development remains poorly understood.

The rice *Wx* gene encodes the OsGBSSI, a key enzyme for amylose synthesis in rice endosperm; *wx* mutants almost completely lack amylose (Sano *et al.*, 1985). The expression of *Wx* is regulated at the transcriptional and post-transcriptional levels. The MYC transcriptional factor OsBP-5 can form a heterodimer with an ethylene-responsive element binding protein (EREBP), OsEBP-89, to regulate *Wx* expression synergistically. Knockdown of *OsBP-5* using RNA interference results in reduced expression of *Wx*, leading to a reduction in the amylose content of mature seeds (Zhu *et al.*, 2003). RSp29 and RSZp23, two Ser/Arg-rich proteins, have been shown to enhance the splicing of the Wx^b mRNA precursor and alter the alternative 5′ splicing site of *Wx* at the intron 1 position (Isshiki *et al.*, 2006). Du1, a member of the pre-mRNA processing complex, is also able to affect the splicing efficiency of Wx^b pre-mRNA and regulate starch biosynthesis (Zeng *et al.*, 2007). Moreover, nuclear proteins extracted from immature rice seeds bind DNA motifs in the *Wx* promoter (Chen *et al.*, 1996a). In addition to the regulators of the *Wx* gene, other factors involved in starch synthesis have also been reported. For example, *FLOURY ENDOSPERM2* (*FLO2*), encoding a protein with a tetratricopeptide repeat motif, regulates rice grain size and starch quality in the endosperm (She *et al.*, 2010). FLO2 may interact with a basic helix–loop–helix protein to directly or indirectly modulate the expression of genes involved in the production of storage starch and storage proteins (She *et al.*, 2010). RSR1, an AP2/EREBP family transcription factor, negatively regulates the seed-specific expression of genes involved in starch metabolism, and mutation of *RSR1* results in enhanced expression of all type I starch synthesis genes in seeds (Fu and Xue, 2010). Nevertheless, many questions regarding the regulation of starch biosynthesis in rice endosperm remain unanswered.

REB [rice endosperm basic leucine zipper (bZIP)/OsbZIP33] interacts with the ACGT elements in the promoters of both *Wx* and *SBE1* and is involved in starch synthesis (Cai *et al.*, 2002). RITA (rice transcription activator-1)/OsbZIP20 (Izawa *et al.*, 1994) and RISBZ1/OsbZIP58 (Onodera *et al.*, 2001) bind to the ACGT element *in vitro*. In addition, RISBZ1/OsbZIP58 regulates seed storage protein synthesis (Yamamoto *et al.*, 2006; Kawakatsu *et al.*, 2009) and free lysine content (Kawakatsu and Takaiwa, 2010). All of these reports indicate that some seed-specific bZIP proteins may be involved in the regulation of starch synthesis in the endosperm.

In this study, we determined that four bZIP transcription factors including OsbZIP58 are capable of binding to the promoters of both *Wx* and *SBE1*. *osbzip58* seeds exhibited defects in starch composition and morphology, and altered expression of starch biosynthetic genes. This study reveals a new function of *OsbZIP58* in starch synthesis in rice seeds.

Materials and methods

Plant materials

Japonica rice (*Oryza sativa* L.) cultivar Dongjin and *osbzip58-1* (PFG_1B-15317.R) and *osbzip58-2* (PFG_3A-09093.R) were obtained from Pohang University of Science and Technology, Korea (Jeong *et al.*, 2002). The genetic backgrounds of these two mutants are the *japonica* rice cultivar Dongjin. The plants were grown during the summer under natural environmental conditions in the Song Jiang experimental field at the Shanghai Institute of Plant Physiology and Ecology, China, or in plastic pots filled with paddy field soil in a greenhouse under a 13 h light (28 °C)/11 h dark (26 °C) photoperiod.

Cloning of OsbZIPs and yeast one-hybrid assays

The open reading frames (ORFs) of ten *OsbZIP* transcription factor genes were cloned by reverse transcription (RT)-PCR using RNA from immature seeds of rice *japonica* variety Zhonghua 11 (ZH11) with primers designed according to the MSU coding sequences (Table 1 and Supplementary Table S1 at *JXB* online). The genes were confirmed by sequencing analysis and subsequently fused in frame to yeast GAL4-AD to construct the pPC86-bZIP plasmids (Invitrogen; Fig. 1A).

The reporter construct p178 was generated by modifying the plasmid pLGD-265UP1, which contains the *CYC1* core promoter and the *lacZ* gene (Chen *et al.*, 1996b). The Ha-2 fragment was from the *Wx* promoter (LOC Os06g04200, s1651–1399) and the C53 fragment was from the *SBE1* promoter (LOC_Os06g51084, L116OC_O. Yeast strain EGY48 (*MATα*, *trp1*, *his3*$^-$, *ura3*$^-$, *leu2*::6 LexAops-*LEU2*; Invitrogen) was used for transformation. The yeast assays were performed according to the manufacturer's protocol with the substrate chlorophenol red-β-D-galactopyranoside (Matchmaker One-hybrid System; Clontech).

To test the binding ability of OsbZIP58 to the 15 fragments in the promoter of ten rice starch biosynthetic genes (Supplementary Table S2 at *JXB* online), two copies of the fragments were amplified and inserted into the *Xho*I site of the p178 vector in front of pCYC1 (*iso*-1-cytochrome *c*) to generate reporter plasmids. Yeast strain EGY48 was transformed with the vector pPC86-OsbZIP58 and one of the 15 reporter plasmids per transformation, and colonies were selected on selection plates (SD/–Ura–Trp+X-gal).

Table 1. *Information about OsbZIP genes used for binding activity assay. The data are based on information from http://signal.salk.edu/cgi-bin/RiceGE*

OsbZIP no.	MSU locus ID	Alternate name	Gene expression pattern	ORF (bp)
OsbZIP15	LOC_Os02g07840	RISBZ4	Universal, increased in seed	837
OisbZIP20	LOC_Os02g16680	RITA-1,RISBZ3	Universal	897
OsbZIP33	LOC_Os03g58250	REB,RISBZ2	Universal	1278
OsbZIP34	LOC_Os03g59460		Specific in seed	990
OsbZIP35	LOC_Os04g10260		Specific in seed	1281
OsbZIP40	LOC_Os05g36160		Universal, increased in seed	804
OsbZIP50	LOC_Os06g41770		Universal, increased in seed	1119
OsbZIP52	LOC_Os06g45140	RISBZ5	Universal, increased in seed	888
OsbZIP58	LOC_Os07g08420	RISBZ1	Specific in seed	1311
OsbZIP76	LOC_Os09g34880		Specific in seed	1125

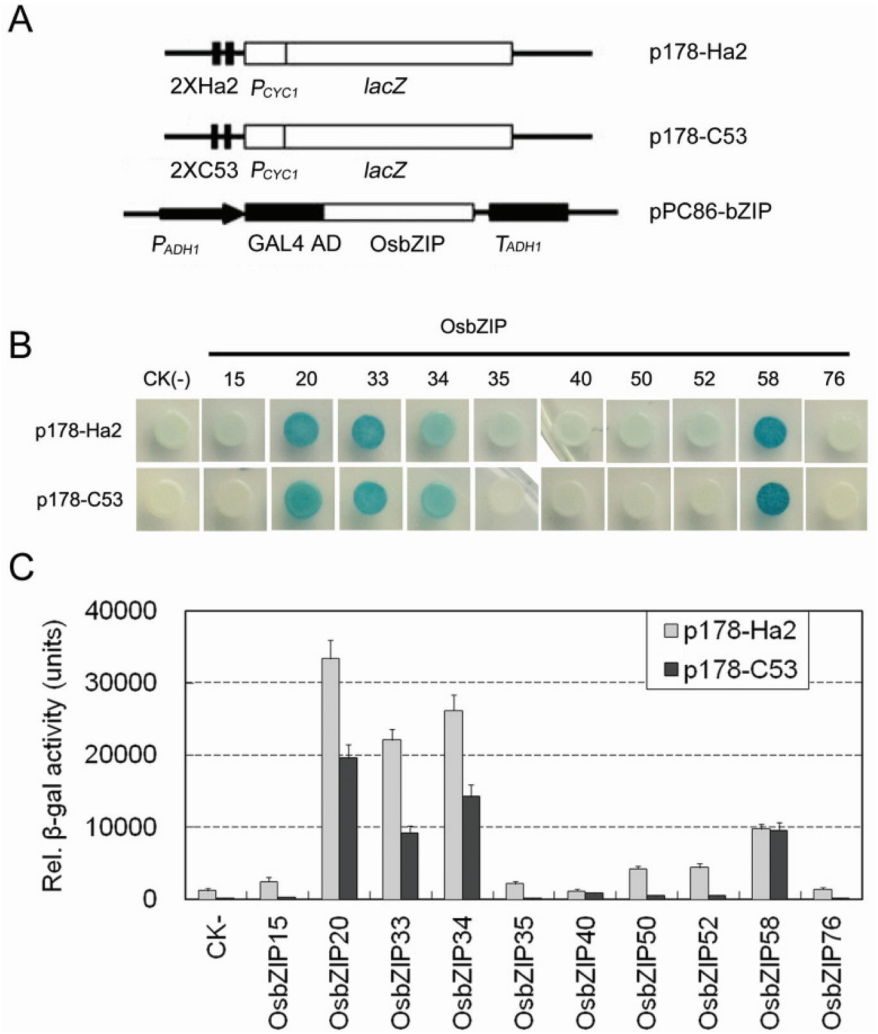

Fig. 1. Assay of binding of OsbZIP transcription factors to the Ha-2 fragment of the *Wx* promoter and the C53 fragment of the *SBE1* promoter in yeast. (A) Diagram of the p178-C53/p178-Ha2 reporter constructs and pPC86-bZIP bait construct. *PCYC1*, the minimal promoter of the yeast *cytochrome C1* gene; GAL4 AD, GAL4 activation domain; *PADH1*, a constitutively active *ADH1* promoter; *TADH1*, *ADH1* transcription termination signal. (B) Detection of interaction between OsbZIP transcription factors and the chimeric promoters by yeast one-hybrid analysis. The blue yeast colonies indicate positive interactions. (C) Quantitative assays of β-galactosidase (β-gal) activity in different yeast transformants. Data are presented as means ±standard deviation (SD) from six replicates in two assays. Light grey columns indicate pPC86-bZIP transformed into EGY48 (p178-Ha2); dark grey columns indicate pPC86-bZIP transformed into EGY48 (p178-C53). (This figure is available in colour at *JXB* online.)

Isolation of OsbZIP58 mutants

Two alleles of *OsbZIP58* mutants, PFG_1B-15317.R and PFG_3A-09093.R, were identified from the rice T-DNA Insertion Sequence Database (Jeong *et al.*, 2002; http://signal.salk.edu/cgi-bin/RiceGE).

Complementation of the osbzip58-1 mutant

A 6149 nt genomic fragment of the wild-type plant corresponding to LOC_Os07g08420 containing the region between –1843 and +4281 was cloned into the binary vector pCAMBIA2300 and this resultant construct was introduced into *Agrobacterium tumefaciens* strain EHA105 and subsequently transfected into immature embryos of *osbzip58-1* by *Agrobacterium*-mediated transformation as described previously (Liu *et al.*, 1998).

Observation of starch granules of endosperm

The starch granules were observed by scanning electron microscopy (SEM) (JSM-6360LV; JEOL) according to the methods of (Fu & Xue, 2010).

Anatomical analysis

Immature seeds were fixed in 50% FAA (50% ethanol, 10% formaldehyde, 5% acetic acid) at 4 °C overnight after vacuum infiltration. After serial dehydration in several concentrations of ethanol, the samples were embedded in epoxide resin and cut into 2–3 μm sections. Strips of these sections were spread on a 42 °C platform and incubated overnight, stained with 0.5% toluidine blue, and sealed for observation under a microscope (BX51 plus DP70; Olympus).

Measurement of grain quality

Embryos and pericarps were removed from the dehulled grains, and the endosperms were ground to a powder. The starch content was measured using a starch assay kit (K-TSTA; Megazyme) according to the manufacturer's instructions. Apparent amylose content (AAC) was measured according to the method described by Tan *et al.* (1999). For analysis of soluble sugars with anthrone reagent, 50 mg of powder was washed twice in 80% (v/v) ethanol at 80 °C for 40 min. The supernatant was collected and diluted to a volume of 15 ml with water. An aliquot (0.1–0.3 ml) of this solution was analysed for sugar content using the anthrone method.

To determine the chain length distributions of amylopectin, 5 mg of rice powder was digested with *Pseudomonas amyloderamosa* isoamylase (Sigma-Aldrich) and then analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS3000 model (Dionex) equipped with a pulsed amperometric detector and a CarboPac PA-20 column (Nagamine and Komae, 1996).

In situ hybridization

Non-radioactive *in situ* hybridization was performed as described previously (Dong *et al.*, 2005). For synthesis of *OsbZIP58* RNA probes, a gene-specific fragment (nt 1–699) was amplified with primers GE0336 and GE0311 (Supplementary Table S1) and cloned into the pSK vector (Stratagene).

RT-PCR and quantitative (q)RT-PCR analysis

Seed samples used for RT-PCR and qRT-PCR were obtained from greenhouse-grown plants; the spikelets were harvested at 3, 5, 7, 10, 15, and 20 DAF. Seed samples were immediately frozen in liquid nitrogen and stored at –80°C until use. Total RNA was extracted from immature rice seeds with RNAplant plus reagent (Tiangen) and treated with RNase-free DNaseI (TaKaRa). Two micrograms of total RNA were used for first-strand cDNA synthesis with an oligo-dT primer and an ImProm-II™ Reverse Transcription System (Promega).

For RT-PCR, *OsACT1* was amplified with primers GE0013 and GE0014 as an internal control. *OsbZIP58* was amplified with primers GE0332 and GE0333. The primer sequences are listed in Supplementary Table S1.

The qRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa) on a Bio-Rad My-IQ 2 system (Bio-Rad). The reactions were performed following the manufacturer's protocol. Each real-time PCR analysis was repeated five times. The expression level of each gene was normalized to *UBQ10* as the reference. Of the ten housekeeping genes, *UBQ10* exhibits the most stable expression in immature seeds of different stages (Jain *et al.*, 2006). The starch synthesis genes were amplified as described previously (Ohdan *et al.*, 2005). The primer sequences are listed in Supplementary Table S1.

Chromatin immunoprecipitation (ChIP) PCR

Antibodies were raised in rabbit against a purified fusion protein produced with vector pET32a, corresponding to aa 1–233 of OsbZIP58 (using the primer sequences listed in Supplementary Table S1). The antibodies were affinity purified, and 10 μl aliquots were used for the ChIP experiments. The DNA–protein complex was isolated at 7 DAF from immature rice seeds according to the method of Haring *et al.* (2007), and DNA was released using the method in the Chromatin Immunoprecipitation kit (Millipore) handbook. Relative enrichment was measured by comparing the input and ChIP values. Normal rabbit IgG was used for the negative control Ab. The *Actin1* ORF (GenBank accession no. AK100267) was used as a negative control sequence. All primers used in the ChIP assays are listed in Supplementary Table S2.

Results

OsbZIP transcription factors bind the promoters of Wx and SBE1

Our previous study revealed that nuclear proteins extracted from immature rice endosperm can specifically bind to the 53 bp (C53) DNA fragment located in the 5′ upstream region of *SBE1*, and the Ha-2 fragment of *Wx* can compete with this binding activity, suggested that the biosynthesis of amylose and amylopection may be co-regulated by certain factors such as REB (Cai *et al.*, 2002).

To identify the transcription factors that regulate both amylose and amylopectin synthesis, we generated two fused constructs: p178-Ha2, containing two copies of the Ha-2 fragment of the *Wx* promoter with three ACGT elements inserted at the 5′ end of pCYC1 mini-promoter, and p178-C53, containing two copies of the C53 fragment of the *SBE1* promoter with two ACGT elements inserted at the 5′ end of pCYC1 mini-promoter (Fig. 1A). Previous expression analysis has shown that there are ten bZIP transcriptional factors that are either homologous with REB/OsbZIP33 or have seed-specific expression patterns (http://signal.salk.edu/cgi-bin/RiceGE) (Onodera *et al.*, 2001; Nijhawan *et al.*, 2008) (Table 1). To test whether these ten OsbZIPs were capable of binding to the two *cis* elements Ha-2 and C53, we performed yeast one-hybrid analysis using pPC86-bZIP vectors, which individually contain the ORFs of these genes fused in frame with yeast GAL4-AD (Fig. 1A). Compared with the controls, four of OsbZIPs – OsbZIP20, REB/OsbZIP33, OsbZIP34, and OsbZIP58 – induced higher expression of β-galactosidase activity in both EGY48 (p178-Ha2) and EGY48 (p178-C53), while OsbZIP50 and OsbZIP52 slightly

induced β-galactosidase activity in EGY48 (p178-Ha2) but not in EGY48 (p178-C53) (Fig. 1B, C). These results suggested that OsbZIP20, REB/OsbZIP33, OsbZIP34, and OsbZIP58 can bind to both the Ha-2 and C53 fragments and may regulate the expression of *Wx* and *SBE1*.

Seeds of osbzip58s display altered starch accumulation

To determine the function of these four *OsbZIPs* in seed starch accumulation, we searched the T-DNA insertion mutant database (Jeong *et al.*, 2002) and the rice Tos17 retrotransposon insertion database (Miyao *et al.*, 2007) and obtained six mutant lines (Table 2). Among these, two T-DNA insertion lines of *OsbZIP58*, *osbzip58-1* (PFG_1B-15317.R) and *osbzip58-2* (PFG_3A-09093.R), both harboured a pGA2715 T-DNA insertion in the first intron of *OsbZIP58* (Fig. 2A). Homozygotes of these two mutants were isolated by PCR screening from the segregating progeny populations (Fig. 2A). Southern blot analysis revealed the presence of a single T-DNA insertion in homozygous plants (Supplementary Fig. S1A at *JXB* online), and all of these plants exhibited white, floury endosperm (Fig. 3E, I). No transcripts from *OsbZIP58* were detected by RT-PCR in 7 DAF seeds of the homozygous mutants, while they were detected in the heterozygous and in wild-type plants (Supplementary Fig. S1B), suggesting that the expression of *OsbZIP58* was completely abolished by the T-DNA insertion in the two mutant lines.

The two *osbzip58* mutants showed several defective seed phenotypes, including reduced mass per 1000 seeds, reduced grain width, abnormal seed shape, and a white belly, which is a floury-white core that occupies the centre to the ventral region of the seed; (Figs 2B–E and 3F, J). The *osbzip58-1* mutant also had an apparently shrunken belly in the grain (Fig. 3E). SEM images of transverse sections of *osbzip58-1* and *osbzip58-2* grains indicated that the dorsal endosperm consisted of densely packed, polyhedral starch granules (Fig. 3G, K), which were similar to those of the wild-type Dongjin (Fig. 3C, D), while the ventral endosperm was filled with loosely packed, spherical starch granules with large air spaces (Fig. 3H, L), corresponding to the chalky region of endosperm. The morphology of starch granules in the ventral regions of the immature *osbzip58-1* seeds was analysed in semi-thin sections. Endosperm cells of the wild type were full of amyloplasts, and each amyloplast consisted of densely packed starch granules (Fig. 4A, C), while in endosperm cells of *osbzip58-1*, the envelope of the amyloplast was not distinct, and starch granules were loosely packed and spread apart (Fig. 4B, D). This phenotype is consistent with the phenotype of mature seeds observed by SEM. In addition, the number of proteosomes was significantly reduced in the *osbzip58-1* endosperm (Fig. 4B, D). These analyses indicated that the mutant seeds exhibited altered starch accumulation. The changes in starch granule morphology in the *osbzip58* mutants may have resulted in grain morphology defects.

To further confirm the phenotype of *osbzip58*, we introduced a wild-type copy of *OsbZIP58* into the *osbizp58-1* mutant. Forty-four independent transgenic lines were obtained, 20 of which exhibited a nearly wild-type seed phenotype. Two complemented lines (CL1 and CL2) with single insertions (Supplementary Fig. S1C) were chosen for further analysis. The two CL set seeds had normal sizes and shapes (Figs 2B–E and 3M, Q). Transverse sections of CL grains revealed normal to slight chalkiness in the ventral region (Fig. 3N, R). SEM of transverse sections of CL grains in the ventral region showed that most of the starch granules were densely packed and regularly polyhedral (Fig. 3P, T), which was similar to those of the wild-type Dongjin (Fig. 3C, D). The expression of *OsbZIP58* in the CL lines was also restored to wild-type levels (Supplementary Fig. S1D). These results indicated that the defective seed phenotype was caused by the *OsbZIP58* mutation.

Disruption of OsbZIP58 alters the starch content and chain length distribution of amylopectin

To understand further the role of *OsbZIP58* in starch synthesis, we measured the seed starch content and the chain length distribution of amylopectin. Total starch content and AAC in the *osbzip58-1* and *osbzip58-2* mutants were slightly decreased compared with those in the wild type (Fig. 5A, B), while the soluble sugar content was significantly increased in the mutants (Fig. 5C). The total starch content, AAC, and soluble sugar content in the seeds of the two CLs were mostly similar to wild-type levels. To examine the changes in the fine structure of amylopectin caused by altered expression of *OsbZIP58*, we determined the chain length distribution of the endosperm starch in the wild type, two *osbzip58* mutants, and two CLs using HPAEC-PAD followed by isoamylase debranching (Nagamine and Komae, 1996; Yamakawa *et al.*,

Table 2. *Information about OsbZIP mutant lines. ND0017 and ND0015 are from the rice Tos17 retrotransposon insertion database (http://www.rgrc.dna.affrc.go.jp/)*

The other mutants were obtained from Pohang University of Science and Technology (Korea) (http://www.postech.ac.kr/life/pfg/risd/index.html).

Gene Name	Mutant line	Insertion site	Background	Phenotype
OsbZIP20	PFG_2A-00762.L	5th exon	Hwayoung	Viviparous
OsbZIP33	PFG_2A-40577.L	1st intron	Dongjin	Dwarf
OsbZIP34	ND0017	8th exon	Nipponbare	Normal
	ND0075	7th intron	Nipponbare	Normal
OsbZIP58	PFG_1B-15317.R	1st intron	Dongjin	White belly seeds
	PFG_3A-09093.R	1st intron	Dongjin	White belly seeds

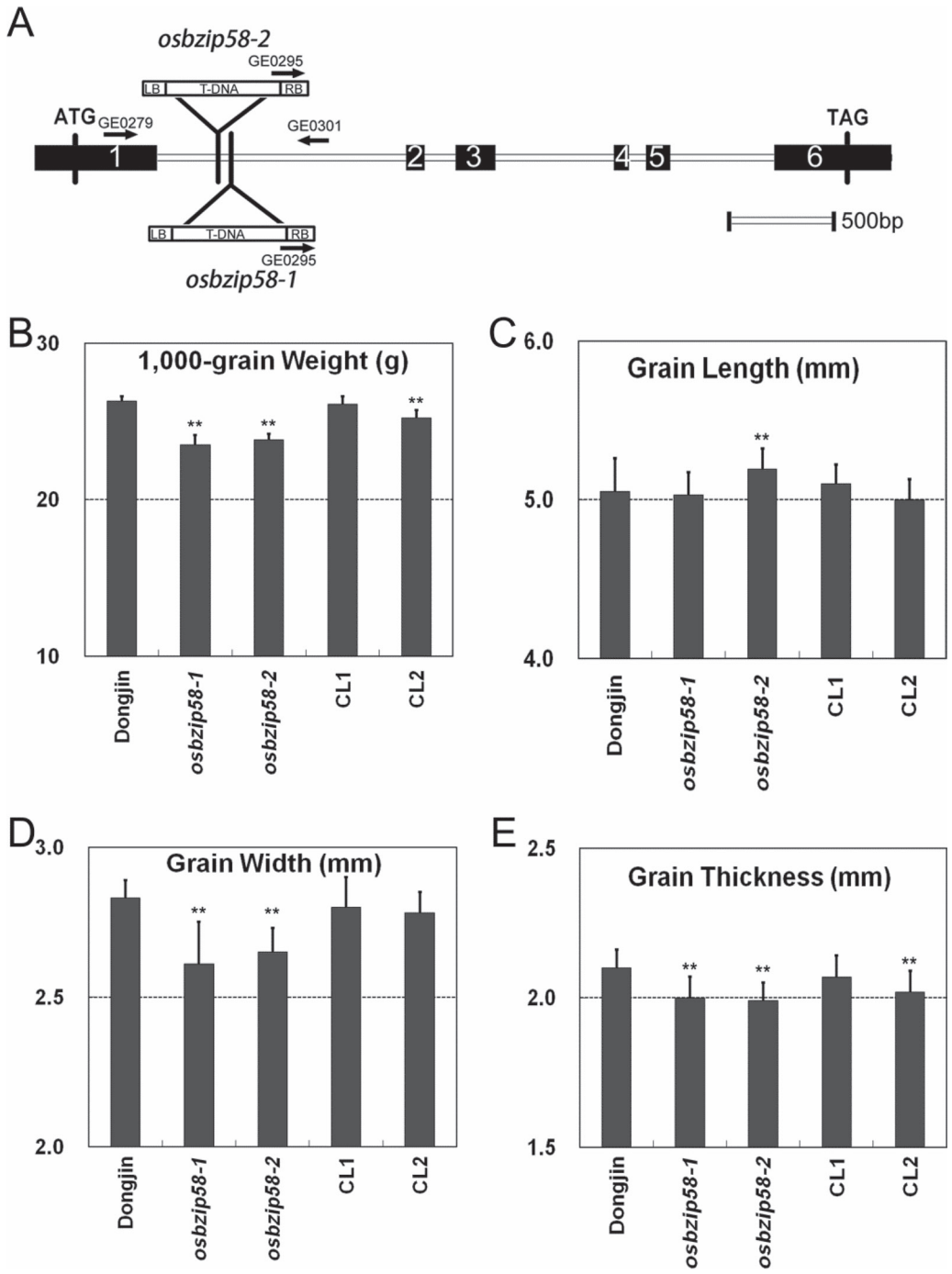

Fig. 2. Gene structure of *OsbZIP58*, and grain size and weight of the *osbzip58* mutants and CLs. The CLs are transformants containing a wild-type *OsbZIP58* gene in the *osbzip58-1* mutant background. (A) Diagram of the *OsbZIP58* gene structure and T-DNA insertion positions in *osbzip58-1* (PFG_1B-15317.R) and *osbzip58-2* (PFG_3A-09093.R). Exons are shown as black boxes. Both mutants had a T-DNA insert in the first intron. Primers used in the genotype analysis are indicated by black arrows. GE0279 and GE0301 are gene-specific primers, and GE0295 is the T-DNA specific-primer. (B–E) Grain weight (B), grain length (C), grain width (D), and grain thickness (E). Fifty seeds were analysed for seed size, and data are presented as means ±SD. The 1000-grain weight was determined by counting ten replicates of 100-grain samples independently on an electronic balance. Data are shown as mean ±sd. Two-tailed unpaired tests indicate significant differences in 1000-grain weight and seed size. $^{**}P < 0.01$.

2007). Compared with the wild-type Dongjin, the *osbzip58* mutants had a higher proportion of short chains with DP values between 6 and 11, and a lower proportion of intermediate chains with DP values between 13 and 21 (Fig. 5D). In the two CLs, the distribution profile of short and intermediate chains of amylopectin shifted towards that of the wild type (Fig. 5E). These data confirmed that the altered starch content and chain length distribution of amylopectin were caused by

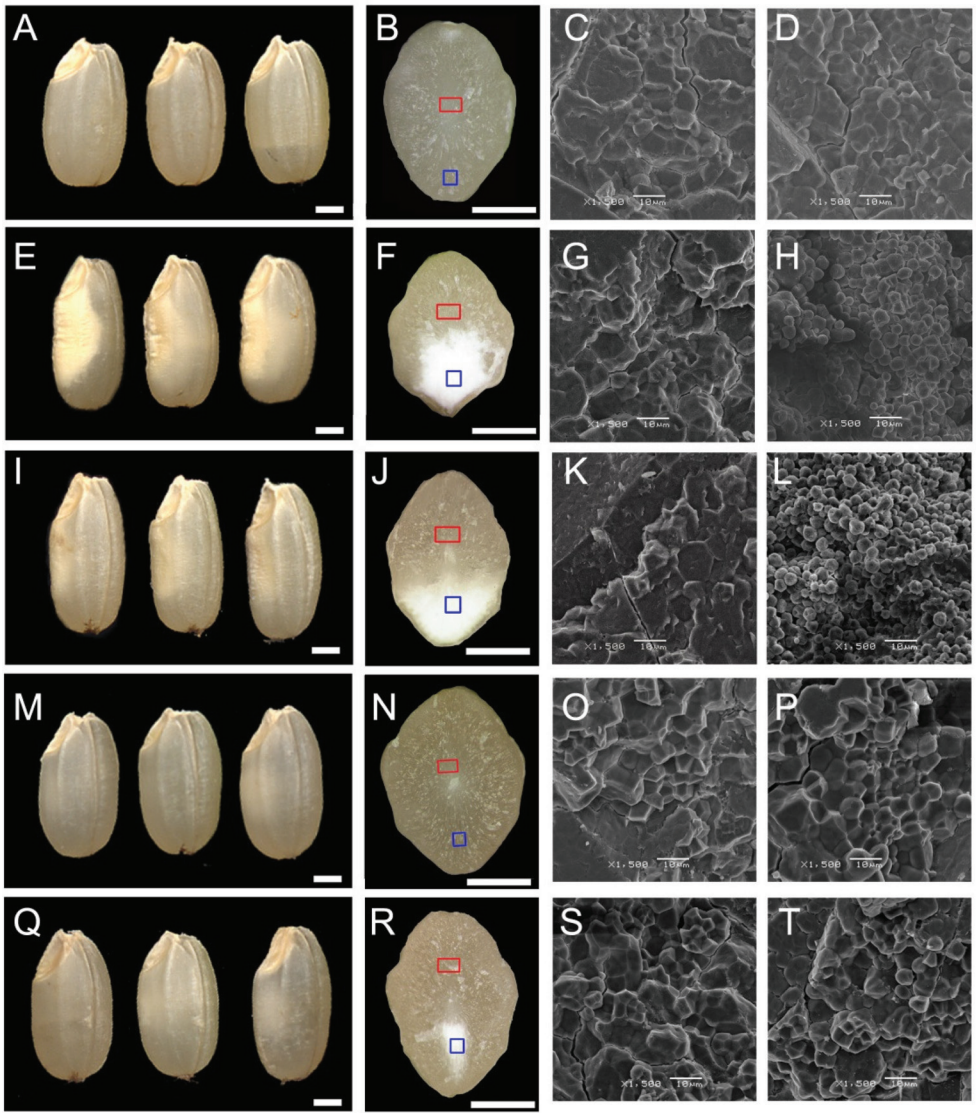

Fig. 3. Altered seeds phenotype of the *osbzip58* mutants and CLs. (A–D) Dongjin; (E–H) *osbzip58-1*; (I–L) *osbzip58-2*; (M–P) CL1; (Q–T) CL2. The appearance of mature seeds is shown in (A), (E), (I), (M) and (Q). Cross-sections of mature seeds are shown in (B), (F), (J), (N) and (R). SEM of the central area of mature endosperm is shown in (C), (G), (K), (O) and (S), from the cross-sections in (B), (F), (J), (N), and (R), respectively, indicated by a red square. SEM of the ventral area of mature endosperm is shown in (D), (H), (L), (P) and (T), from the cross-sections in (B), (F), (J), (N), and (R), respectively, indicated by a blue square. Bars: 1 mm (A, B, E, F, I, J, M, N, Q, R; 10 μm (C, D, G, H, K, L, O, P, S, T).

the *OsbZIP58* mutation. The altered composition and structure of starch suggested that *OsbZIP58* modulates not only amylose but also amylopectin synthesis, especially α-1,4 chain elongation of amylopectin, which comprises several concerted reactions catalysed by distinct SS, SBE, and DBE isoforms.

Expression pattern of OsbZIP58

To understand further the role of *OsbZIP58*, RT-PCR analysis was performed to determine the expression pattern of *OsbZIP58*. The expression of *OsbZIP58* was specifically in seeds, with a maximum expression level at 5–10 DAF (Fig. 6A). In addition, *in situ* hybridization showed that, at 5 DAF, the expression of *OsbZIP58* was detected at a relatively high level in the pericarp and weakly in the endosperm (Fig. 6B). At 7 DAF, *OsbZIP58* mRNA expression appeared to increase in the central region of the endosperm and decreased in the pericarp (Fig. 6C). In addition, *OsbZIP58* mRNA was detected in the dorsal vascular bundles of rice grains at 5 DAF. No signal was observed in these tissues using the sense probe (Fig. 6D). Some genes functioning in starch biosynthesis, such as *OsSSI*, *OsSSIIa*, and *OsSSIIIa*, are expressed in the pericarp at the early stage of seed development and are increasingly expressed in the endosperm at the middle stage of seed development (Hirose and Terao, 2004). The similar expression pattern of these genes and *OsbZIP58* suggests that *OsbZIP58* plays a role in regulating storage starch biosynthesis.

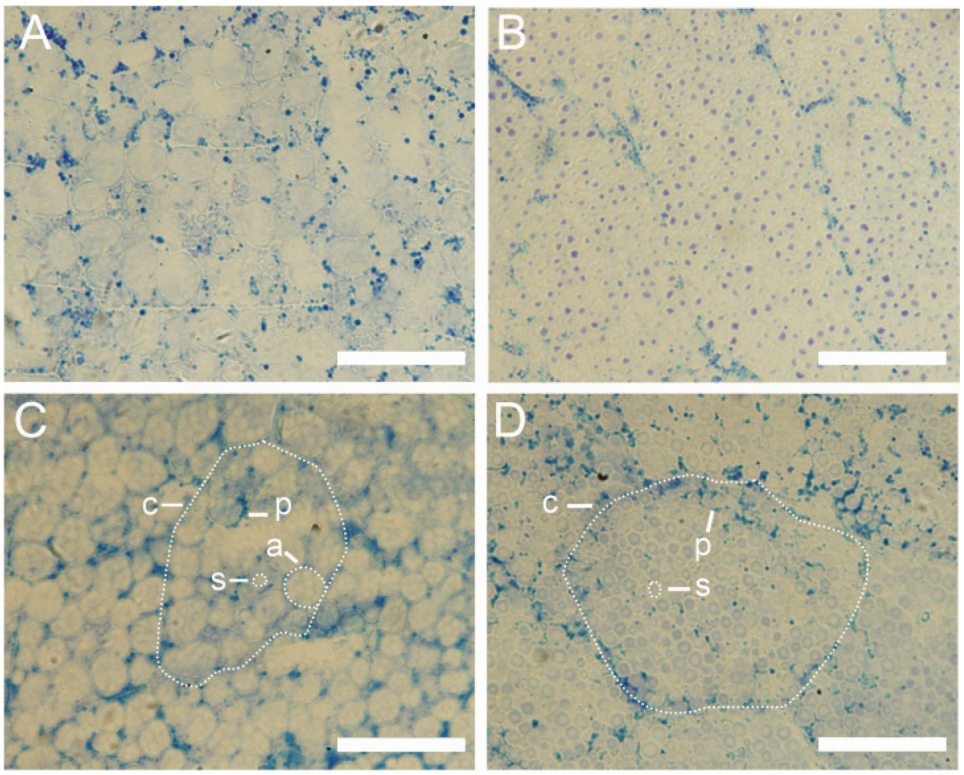

Fig. 4. Altered starch granules morphology in the wild-type Dongjin and the *osbzip58-1* mutant examined using semi-thin sections. Immature seeds were fixed in FAA and stained with ammonium methylbenzene blue. (A, C) Dongjin; (B, D) *osbzip58-1*. (A, B) 10 DAF; (C, D) 15 DAF. a, Amyloplast; c, endosperm cell; p, protein body; s, starch granule. Bars, 50 μm.

OsbZIP58 regulates the expression of starch biosynthetic genes in rice endosperm

To understand how OsbZIP58 regulates starch synthesis, we examined the expression of 14 starch synthesis genes in the *osbzip58-1* mutant using qRT-PCR. Compared with the wild type, these 14 genes displayed four groups of altered expression profiles in *osbzip58-1* from 5 to 15 DAF during the grain-filling stage. Thus, the expression of *OsAGPS2b*, *OsAGPL2*, *OsSSI*, *OsSSIIIa*, *OsSSIVb*, *OsBEIIb*, and *OsISA2* was obviously upregulated, while expression of *OsAGPL3*, *OsPHO1*, *Wx*, and *SBE1* was obviously downregulated. The expression of *OsISA1* and *OsPUL* was upregulated from 5 to 7 DAF but subsequently downregulated, and there was no significant change for *OsSSIIa* from 5 to 15 DAF (Fig. 7). These data revealed that *OsbZIP58* regulates the expression of most starch synthesis genes in rice seeds during the grain-filling stage.

OsbZIP58 directly regulates genes involved in starch biosynthesis

To reveal whether OsbZIP58 was capable of directly binding to the promoter sequences of starch biosynthetic genes, we examined the distribution of ACGT elements in the promoters of the 14 rice starch biosynthetic genes including genes encoding AGPase, PHO, GBSS, SS, SBE, and DBE, which exhibit a high level of expression at approximately 5–7 DAF during seed development (Hirose and Terao, 2004; Dian *et al.*, 2005; Ohdan *et al.*, 2005). The region from –2000 bp upstream of the putative transcription initiation site to the translation start site ATG was used to search for ACGT elements for each gene. Fifteen fragments were observed to contain three or more copies of the ACGT element within 300 bp 5′ of transcription initiation in ten genes. Strikingly, the *Wx* promoter contained 16 ACGT elements in the 300 bp fragments (Fig. 8A and Supplementary Table S2). The high frequency of the ACGT elements in rice starch biosynthetic genes suggested that these genes may be coordinately regulated by the same transcription factors via their common *cis* element.

We used ChIP assays to examine whether OsbZIP58 bound to the promoters of *in vivo*. A specific antibody against OsbZIP58 demonstrated by Western blot analysis (Supplementary Fig. S2 at *JXB* online) was used for pulling down the OsbZIP58-associated complex from immature rice seeds at 7 DAF. ChIP-PCR analysis revealed that 11 fragments in the promoters of eight genes (*OsAGPL3*, *Wx*, *OsSSIIa*, *OsSSIIIa*, *OsSSIVb*, *SBE1*, *OsBEIIb*, and *OsISA2*) could be enriched by the anti-OsbZIP58 antibody individually (Fig. 8B). Furthermore, the Ha-2 fragment of the *Wx* promoter was in the Wx-a fragment (–1651 to –1399), and the C53 fragment of the *SBE1* promoter in the SBE1-b fragment (–116 to –42), and both fragments were significantly enriched by the anti-OsbZIP58 antibody.

Additionally, yeast one-hybrid analysis was used to further test the binding ability of OsbZIP58 to the 15 loci used in ChIP-PCR assay. As shown in Fig. 8C and D, six of these fragments, *OsAGPL3*, *Wx-a*, *OsSSIIa-b*, *SBE1-b*, *SBEIIb-a*, and

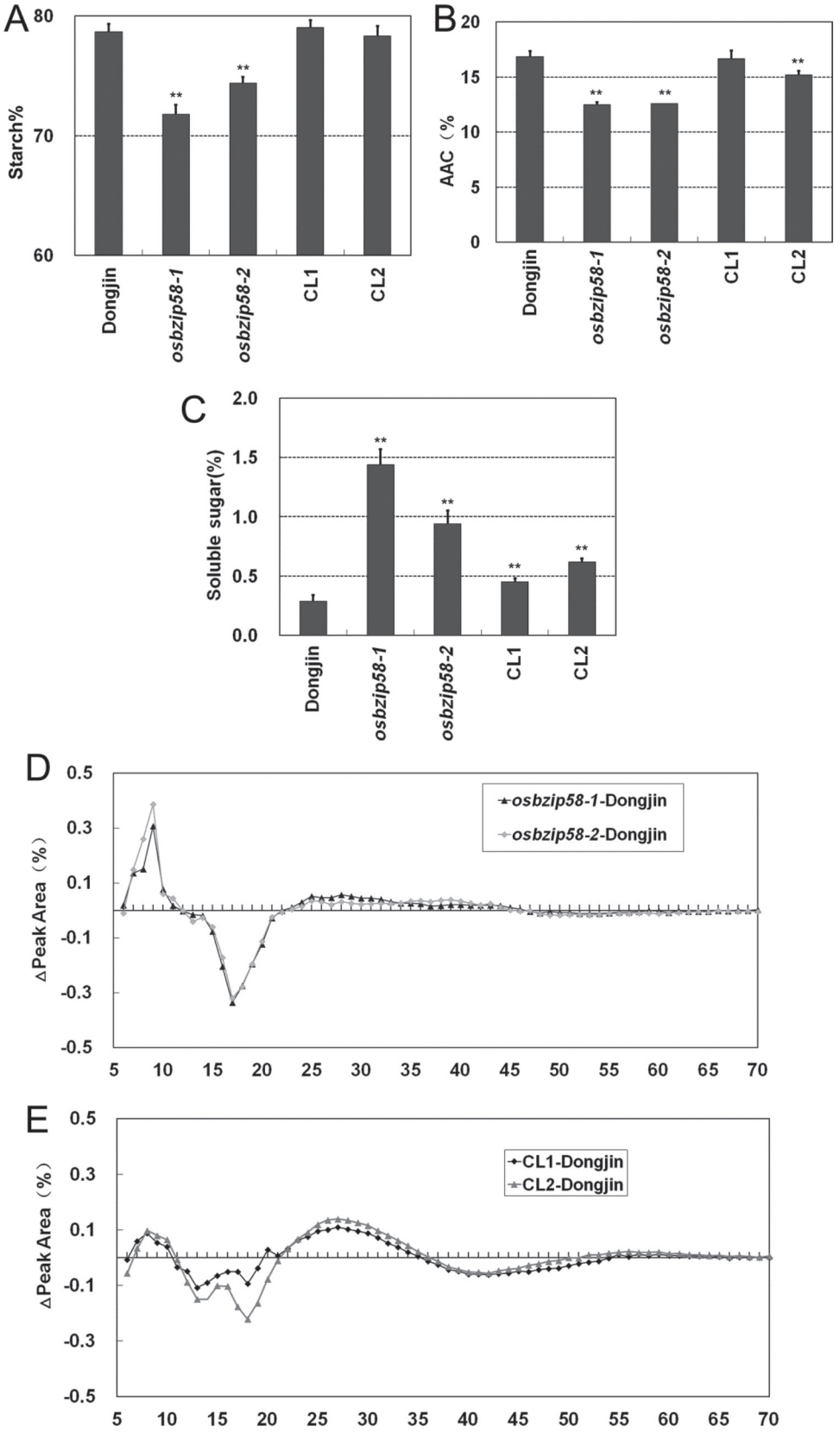

Fig. 5. Altered starch content and fine structure of amylopectin in mutants of *OsbZIP58*. (A) Total starch content in endosperm (*n*=5). (B) Apparent amylose content in endosperm (*n*=5). (C) Soluble sugar content in endosperm (*n*=5). (D) Differences in the chain length distributions between Dongjin and *osbzip58-1* / *osbzip58-2*. (E) Differences in the chain length distributions between Dongjin and CL1/CL2.

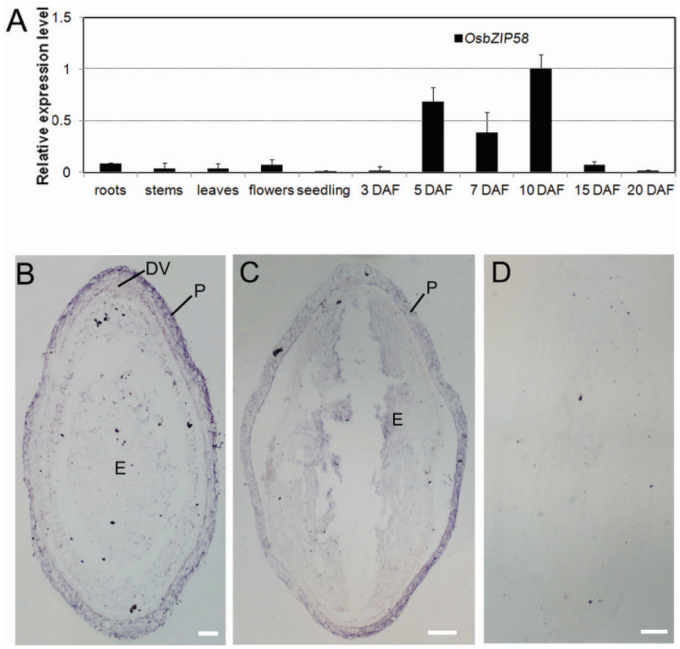

Fig. 6. Expression pattern of *OsbZIP58*. (A) Expression patterns of *OsbZIP58* in roots, stems, leaves, flowers, seedlings, and seeds analysed by qRT-PCR. The developmental stage of the seed is indicated by DAF. Rice *OsAct1* was used as a control. (B, C) Detection of *OsbZIP58* mRNA in cross-sections of a maturing rice seed by *in situ* hybridization at 5 DAF (B) and 7 DAF (C). The region expressing *OsbZIP58* is shown in purple. Antisense strand was used as a probe. (D) *In situ* hybridization with a sense-strand probe in maturing rice seed at 7 DAF. P, Pericarp; DV, dorsal vascular; E, endosperm. Bars, 100 μm (B); 200 μm (C, D).

OsISA2, were strongly recognized by the OsbZIP58 protein. Four other fragments, *Wx-b*, *Wx-c*, *SBE1-a*, and *SBEIIb-b*, showed weaker binding with OsbZIP58. These data indicated that ten fragments in six promoters, including *OsAGPL3*, *Wx*, *OsSSIIa*, *SBE1*, *SBEIIb*, and *OsISA2*, could be recognized by OsbZIP58 in yeast. These results suggested that OsbZIP58 directly regulates six starch synthetic genes, controlling the accumulation of starch during seed development.

Thus, OsbZIP58 binds to the promoters of multiple rice starch biosynthetic genes *in vivo*, and this association is probably mediated by the ACGT elements.

Discussion

OsbZIP58 directly regulates starch synthesis

In this study, we identified a rice bZIP transcription factor, OsbZIP58, as a key regulator modulating different steps of starch synthesis in rice endosperm by promoting the expression of multiple rice starch biosynthetic genes (Fig. 8). Mutations of *OsbZIP58* led to altered expression of rice starch biosynthetic genes (Fig. 7) and altered starch composition and structure (Figs 3 and 5).

The observation that a reduction in *OsbZIP58*/*RISBZ1* expression caused opacity in seeds has been reported in KD-RISBZ1 seeds, where the expression of *OsbZIP58* is lower than that of wild-type seeds (Kawakatsu *et al.*, 2009). The seed phenotypes in KD-RISBZ1 were weaker compared with the *osbzip58* mutants described in this study, possibly due to the remaining expression of *OsbZIP58* in KD-RISBZ1 plants.

OsbZIP58 has pleiotropic effects on starch synthesis

Our genetic and biochemical analyses indicate that OsbZIP58 regulates the expression of starch biosynthesis genes (Fig. 7) and hence modulates starch metabolism and starch-related phenotypes in rice endosperm. The amylopectin composition of *osbzip58* mutant seeds was similar to that of the *sbe1* mutant and was opposite to those of the *ssI* and *beIIb* mutants (Nishi *et al.*, 2001; Satoh *et al.*, 2003; Fujita *et al.*, 2006). *SBE1* is downregulated in *osbzip58*, whereas *SSI* and *OsBEIIb* are dramatically upregulated. Thus, the aberrant features of amylopectin in the *osbzip58* mutant were the manifestation of the effects of several genes, including *SBE1*, *SSI*, and *OsBEIIb*.

Surprisingly, several mutants of various pathways exhibit *sbe1* mutant-like amylopectin properties, including *flo2*, *pho1*, and *sugar-1*. FLO2 harbours a tetratricopeptide repeat motif and is considered to mediate protein–protein interactions (She *et al.*, 2010). *PHOL*/*OsPHO1* is hypothesized to play a crucial role in the glucan initiation process, which occurs at an early stage of starch biosynthesis, by synthesizing glucan primers with long DP values (Satoh *et al.*, 2008). The *sugar-1* mutant is defective in ISA1 (Kubo *et al.*, 2005), which is a starch debranching enzyme directly involved in the synthesis of amylopectin. The amylopectin properties of inactive *japonica*-type SSIIa grains largely resemble those of the *sbe1* mutant (Nakamura *et al.*, 2005). This raises the possibility that SBE1 is part of a protein complex of multiple enzymes that play important roles in the formation of A chains, B1 chains, and clusters connecting B chains of amylopectin (Jeon *et al.*, 2010). The current study suggests that *OsbZIP58* is probably one of the regulators of this enzyme complex.

The *osbzip58* mutants exhibited loosely packed, spherical starch granules on the ventral region of endosperm and contained reduced amounts of starch. In the *sbe1* mutant, the loss of SBE1 activity did not affect the accumulation of starch or the morphological properties of the seeds (Satoh *et al.*, 2003). This indicates that a low level of *SBE1* is not the sole cause of the *osbzip58* starch phenotype in endosperm. The *osbzip58* starch phenotype may be ascribed to the combined effects of altered expression of multiple rice starch synthesis genes.

The broad binding specificity of OsbZIP58

Here, we showed that OsbZIP58 could bind to the promoter regions of multiple rice starch synthesis genes *in vivo*, possibly via the ACGT motifs. An electrophoretic mobility shift assay was used to demonstrate that OsbZIP58/RISBZ1 is able to bind to the GCN4 motif located in seed storage protein

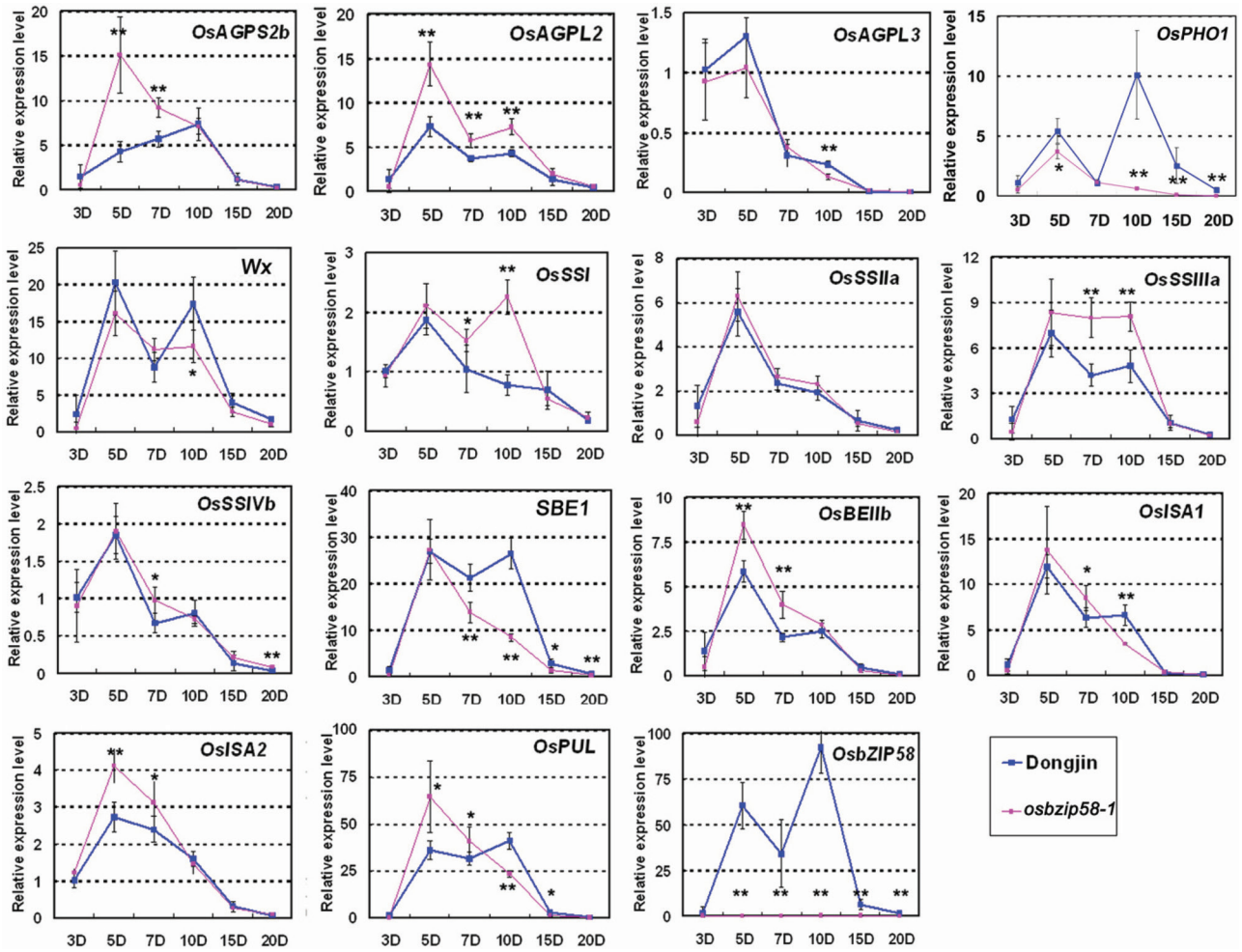

Fig. 7. Expression profiles of rice starch synthesis genes during seed development in wild-type Dongjin and *osbzip58-1* mutant. Total RNA was extracted from seeds at 3, 5, 7, 10, 15, and 20 DAF. The expression of each gene in the 3 DAF seeds of Dongjin was used as a control. All data are shown as means ±SD from five biological replicates. Two-tailed unpaired *t*-tests were used to determine significant differences. **P* <0.05; ***P* <0.01.

gene promoters, and transient assays demonstrated that this protein can activate the transcription of several seed storage protein synthesis genes via the GCN4 motif (Onodera *et al.*, 2001; Yamamoto *et al.*, 2006). In addition, the electrophoretic mobility shift assay was used to demonstrate that OsbZIP58/RISBZ1 binds to the O2 target sequences [TCCACGT(a/c)R(a/t) and GATGYRTGG] located in the promoters of seed storage protein genes (Onodera *et al.*, 2001). Taken together, these data suggest that OsbZIP58 possesses broad binding specificity for genes related to seed maturation.

Several other bZIP proteins exhibit broad binding ability. For example, RITA/OsbZIP20 displays broad binding specificity for palindromic ACGT elements (Izawa *et al.*, 1994). The maize Opaque2 protein interacts with the promoter regions of *b-32* and *cyPPDK1* at their binding sites (GA/TGAPyPuTGPu), and also interacts with 22 kDa zein by binding to the sequence TCCACGTAGA and activates transcription of these genes *in vivo* (Lohmer *et al.*, 1991; Schmidt *et al.*, 1992; Maddaloni *et al.*, 1996). Another rice bZIP protein, OsbZIP33/REB, can recognize and bind to the GCN4 element in the *Wx* gene in addition to the ACGT element in the promoter of α-globulin (Nakase *et al.*, 1997; Cheng *et al.*, 2002). The above-mentioned bZIP transcription factors have a close phylogenetic relationship. OsbZIP58/RISBZ1 is the closest homologous protein of maize Opaque2 in rice, while OsbZIP58 and OsbZIP33/REB are classified into one minimum cluster, and OsbZIP20 is outside of this cluster in an unrooted phylogenetic tree (Nijhawan *et al.*, 2008). These data suggest that these bZIP transcription factors play broad roles during seed maturation.

Fourteen genes encoding starch biosynthesizing enzymes have been shown to have similar expression patterns during seed development, with high expression levels at approximately 7 DAF; there may be a coordination mechanism that regulates these seed-specific genes (Ohdan *et al.*, 2005). The current study revealed, for the first time, that OsbZIP58 is one of these regulators. This study elucidated the unknown regulatory mechanism underlying rice starch synthesis and will potentially assist rice breeding and engineering efforts.

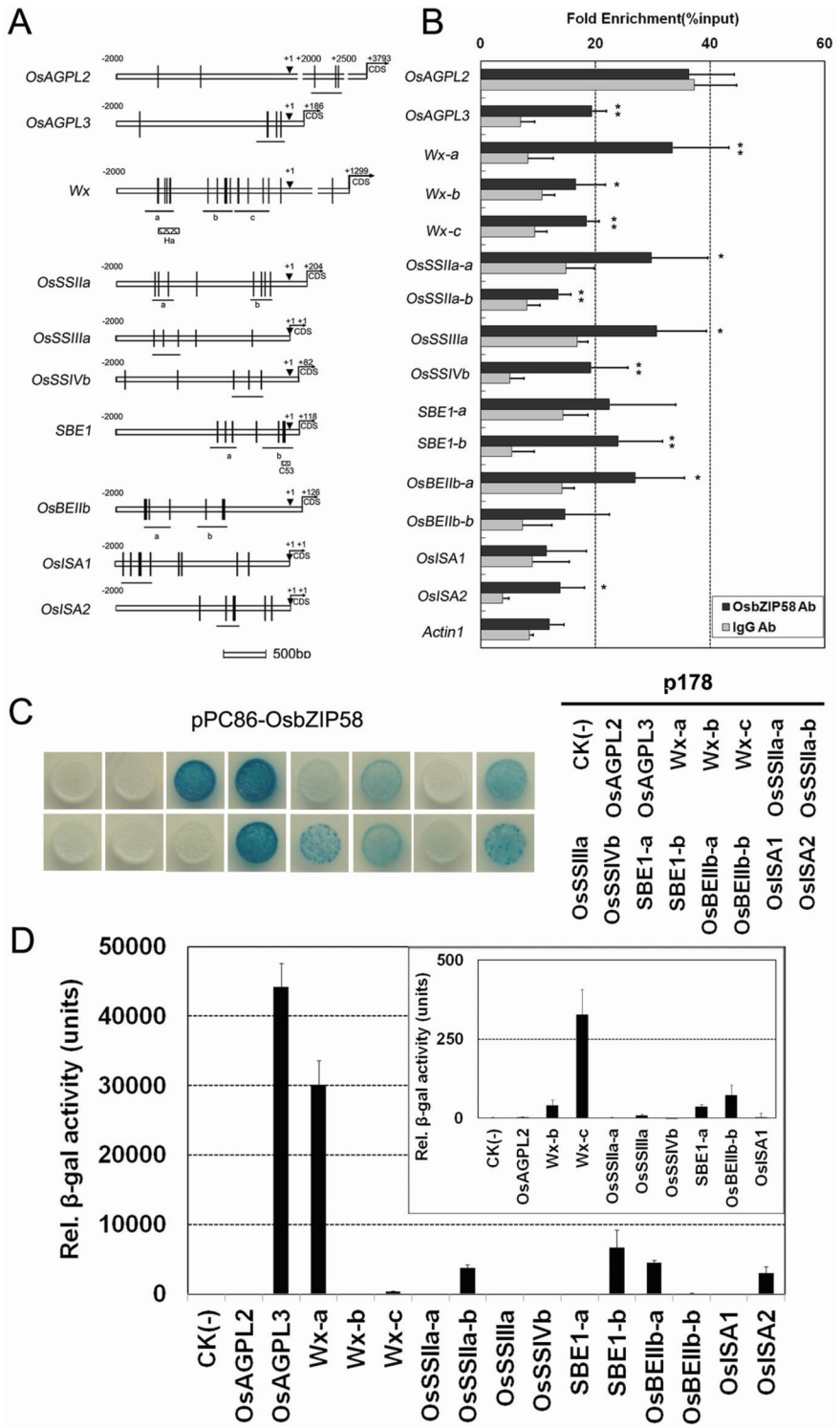

Fig. 8. OsbZIP58 broadly bind to the promoters of rice starch metabolism genes *in vivo*. (A) Diagram of the promoter region from –2000 bp upstream of the putative transcription initiation site to the translation start site ATG in the ten rice starch metabolism genes. Vertical black lines indicate the ACGT elements. Arrowheads indicate the putative transcription initiation site. Vertical arrows indicate the translation start site ATG. PCR fragments are indicated by thick lines. (B) Quantitative real-time PCR assay of chromatin immunoprecipitated DNA. Normal rabbit IgG was used for the negative control. '% input' represents the qPCR signals that were derived from the ChIP samples versus qPCR signals that were derived from the input sample taken early during the ChIP procedure. All data are shown as means ±SD from six biological replicates. Two-tailed unpaired *t*-tests were used to determine significant differences. **P* <0.05; ***P* <0.01. (C) Detection of interactions between OsbZIP58 and the chimaeric promoters by yeast one-hybrid analysis. The plasmids pPC86-OsbZIP58 and p178 were transformed into EGY48, and colonies were selected on selection medium (SD/–Ura–Trp+X-gal). The blue yeast colonies indicate positive interactions. (D) Quantitative assays of β-galactosidase (β-gal) activity in different yeast transformants. Data are presented as means ±SD from six replicates in two assays. (This figure is available in colour at *JXB* online.)

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Identification and characterization of the *osbzip58* mutants and CLs.

Supplementary Fig. S2. Western blot detecting the specificity of the anti-OsbZIP58 antibody.

Supplementary Table S1. Information about primers used in this study.

Supplementary Table S2. Locations of promoter regions and sequences of primers used in the ChIP-PCR assays.

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