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Cloning and functional analysis of the promoter of a maize starch synthase III gene (*ZmDULL1*)

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ABSTRACT. The *ZmDULL1* gene encodes a starch synthase and is a determinant of the structure of endosperm starch in maize (*Zea mays* L.). However, little is known regarding the regulatory mechanism of the *ZmDULL1* gene. In this study, we isolated and characterized the *ZmDULL1* promoter (PDULL1), which is the 5' flanking region of *ZmDULL1* in maize. Sequence analysis showed that several *cis*-acting elements important for endosperm expression (GCN4_motif and AACA-element) were located within the promoter. A series of *PDULL1* deletion derivatives, PDULL1-1-PDULL1-4, from the translation start code (-1676, -1216, -740, and -343) were fused to the β-glucuronidase (GUS) reporter gene. Each deletion construct was transformed into rice using the *Agrobacterium*-mediated method, and then GUS activity was measured in transgenic plants. The results showed that *PDULL1* was an endosperm-specific promoter. Further analysis showed that the promoter sequence (-343 to -1 base pairs) was sufficient for mediating GUS gene expression in endosperm. These results indicate that the region from -343 to -1 base pairs

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of *PDULL1* is valuable for transgenic rice breeding and genetic engineering studies.

Key words: Endosperm-specific promoter; Functional analysis; Maize; Promoter cloning

INTRODUCTION

Endosperm is of enormous economic and nutritional importance, providing the primary source of carbohydrate and protein for the human population and animals. Improving the quality and composition of endosperm through genetic engineering has therefore been the subject of numerous studies, and great achievements have been made in this area (Bajaj and Mohanty, 2005). To increase crop yield, cloning and characterizing endosperm-specific genes or the promoter is important (Smidansky et al., 2003; Li et al., 2011). However, whether foreign genes introduced into target plants or receptor organs can show controlled and stabilized expression is unknown.

In most cases, desirable foreign genes were introduced into target plants, specifically into target tissues or cells under the control of a powerful constitutive promoter, such as the cauliflower mosaic virus 35S (CaMV35S) (Covey et al., 1981) and its derivatives (Rushton et al., 2002). However, the high expression of foreign genes in transgenic plants during all development increases the metabolic burden and wastes, which may lead to a loss of material and energy in transgenic plants (Hsieh et al., 2002; Malnoy et al., 2006). Thus, identifying a tissue-specific promoter to drive gene expression would overcome this limitation.

Many tissue-specially promoters have been isolated from various plants, including vascular bundles, roots, leaves, stems, seeds, and fruits. For instance, a fragment sequence from -1677 to -1380 base pairs (bp) was crucial for driving gene-specific expression in vascular bundles (Liu et al., 2001). In the upstream region of the *PEPC* promoter, a light-regulated and cell-specific methylation promoter in maize was identified (Tolley et al., 2012). Two novel positive *cis*-regulatory elements (*GSE1* and *GSE2*) were reported to be specifically expressed in green tissues in rice (Ye et al., 2012).

Endosperm has many advantages as a heterogeneous expression platform compared to other expression systems, including its low cost, stability in storage, and high safety (Takaiwa et al., 2007; Wu et al., 2007). Several endosperm-specific expression promoters have been isolated and analyzed from rice (Rasmussen and Donaldson, 2006), wheat (Wiley et al., 2007), maize (Russell and Fromm, 1997), and barley (Choi et al., 2003). However, few promoters with endosperm-specific expression have been examined in plant genetic transformation studies, as the transgenic plants showed unsatisfactory expression levels and patterns (Li et al., 2008; Qu et al., 2008).

In this study, we cloned the *PDULL1* gene from maize and characterized its function by detecting the expression level of the β-glucuronidase (GUS) gene in transgenic rice. Moreover, GUS activity driven by different promoter deletions were analyzed. The results of this study showed that *PDULL1-4* was an ideal endosperm-specific promoter because of its stable and effective expression in the transgenic plant.

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MATERIAL AND METHODS

Plant materials and growth conditions

Seeds of *Zea mays* (inbred line B73) were raised in pots containing mixed soil (soil/vermiculite/perlite, 4:1:1, v/v/v) in a greenhouse and grown under a 16-h light/8-h dark cycle at 28°C. Zhonghua 11 (*Oryza sativa* L. subsp *japonica*) was used for tissue culture in this study. The rice plants were sowed in controlled chambers at 30°C under a 16-h light/8-h dark cycle and then planted in a rice paddy field.

Promoter cloning and sequence analysis

Polymerase chain reaction (PCR) was carried out using primers (Table 1) with maize genomic DNA as a template. The PCR conditions were as follows: 94°C for 5 min and 30 cycles of 94°C for 50 s, 58°C for 40 s, 72°C for 2 min, and 72°C for 10 min. The products were inserted into the expression vector and positive clones were sequenced. The *cis*-elements of the *ZmDULL1* promoter (PDULL1) were analyzed using Plant *Cis*-Acting Regulatory Elements (PlantCARE) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/).

Table 1. Primers for PCR used in this study.

Name	Forward	Reverse	Size (bp)
PDULL1	TCACAAGTAGGAGGAGCG	AAGGGGAAGAAAAGAAGG	1927
PDULL1-1	TTGTAGTGCTACGAAAAGGAAAG	AAGGGGAAGAAAAGAAGG	1676
PDULL1-2	TAGGAGCCGCCAAGCAC	AAGGGGAAGAAAAGAAGG	1216
PDULL1-3	GACACGGATTCAAATACTTCAA	AAGGGGAAGAAAAGAAGG	740
PDULL1-4	ATGAGCTAGGGAACCCACA	AAGGGGAAGAAAAGAAGG	343

Production of recombinant vector and transgenic plants

Various truncated fragments of the *ZmPDULL1* promoter was obtained by PCR amplifications. The 4 deletion fragments were named PDULL1-1 (-1676 bp), PDULL1-2 (-1216 bp), PDULL1-3 (-740 bp), and PDULL1-4 (-343 bp) using the primers shown in Table 1. The different PCR products were cloned into the pCMBIA1301 plasmid to replace the CaMV35S promoter. Recombinant plasmids were transformed into *Agrobacterium tumefaciens* EHA105. Briefly, the genetic transformation steps were as follows: mature seeds were first dehulled and sterilized, then placed onto the induction medium to produce the callus. The callus was immersed in the *Agrobacterium* suspension carrying the recombinant construct and then cultured in selective medium.

Southern blot analysis

Genomic DNA was extracted from the leaves of T_0 transgenic plants using a DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). Genomic DNA was digested with *Hin*dIII, and then the digested DNA was separated in a 0.7% agarose gel and transferred to a nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech, Amersham, UK). Hybridization was

performed using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany).

Histochemical staining and fluorometric GUS assay

Different tissues from transformed plants and control plants were collected and analyzed to detect GUS activity. These tissues were vacuum-infiltrated using reaction buffer (3 mg/mL X-gluc, 40 mM sodium phosphate pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM ferricyanatum kalium, 0.5 mM ferrocyanatum kalium, and 20% methanol). After overnight incubation at 37°C, the stained samples were bleached with 70% (v/v) ethanol to remove the chlorophyll. Stained samples were photographed using a Nikon SMZ1000 microscope under white light (Nikon, Tokyo, Japan). For the fluorometric GUS assay, tissues were used to determine GUS enzyme activity by measuring the fluorescence of 4-methylumbelliferone produced by GUS cleavage of 4-methylumbelliferyl-β-D-glucuronide. GUS activity was expressed as nanomoles methylumbelliferone per minute per milligram protein. The amount of protein was determined using a Protein Assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. Fluorescence values were determined at excitation and emission wavelengths of 365 and 455 nm, respectively, using a Hi-Tech U2001 fluorescence spectrophotometer (TgK Scientific, Ltd., Bradford-on-Avon, UK).

RESULTS

Analysis of promoter sequence

To identify *cis*-acting regulatory elements, the 1927-bp upstream sequence before the translation initiation codon (indicated with +1, Figure 1) was analyzed using the PLACE and the PlantCARE database (Higo et al., 1999). Some putative *cis*-acting regulatory elements (Skn-1_motif, GCN4_motif, and AACA motif), which are important elements for endosperm expression, were detected in *PDULL1* (Figure 1). In addition, several corn elements and *cis*-acting regulatory motifs known to modulate gene expression were identified, including 15 TATA-box elements, 3 CAAT-box elements, 5 light responsive elements [including ACE (AAAACGTTTA), Box4 (ATTAAT), G-Box (CACGTG), GATA-motif (GATAGG), and CGTCA-motif (CGTCA)], 1 ABRE site (ACGTG), 1 MBS (CAACTG), and other *cis*-acting regulatory elements, such as LTRE and GT1GMSCAM4.

Production and molecular characterization of transgenic rice plants

To determine the regulatory mechanism controlling the expression of the *ZmDULL1* gene, the 1927-bp fragment before the DNA sequence was isolated from maize genomic DNA and inserted into the expression vector pCAMBIA1301 (Figure 2A). This construct was transferred into *A. tumefaciens* EHA105 cells, and then transformed into Zhonghua 11 (*Oryza sativa* L. subsp. *japonica*) (Figure 2B) using the *Agrobaterium*-mediated method.

To obtain transgenic plants, we extracted genomic DNA from transgenic rice plants and analyzed the *PDULL1* and hygromycin gene using PCR. Bands of the expected sizes were detected in the positive plants (Figure 2C). In addition, stable gene integration and copy num-

bers in T_0 transgenic rice were estimated using Southern blot analysis. Of the 4 independent transgenic rice lines examined, lines 1, 3, and 4 contained a single copy and lines 2 contained 2 copies (Figure 2D). Thus, lines 1, 3, and 4 were used in subsequent experiments.

Figure 1. Analysis of *ZmDULL1* promoter region. Putative *cis*-acting regulatory elements detected in the promoter fragment using PlantCARE and PLACE databases are indicated. The transcription initiation site is indicated with +1. Light gray box represents the TATA-box, while the black box represents the CAAT-box. The light responsive elements consist of ACE (AAAACGTTTA), Box4 (ATTAAT), G-Box (CACGTG), GATA-motif (GATAGG), and CGTCA-motif (CGTCA). Both Skn-1_motif and GCN4_motif are involved in endosperm expression, and the yellow nucleotide sequence AACAAAA (AACA motif) is an important element related to endosperm expression. The ABRE site is involved in ABA induction, MBS represents drought inducible controlling element, and LTRE is a *cis*-regulatory element responsive to low temperature. The GT1GMSCAM4 represents the element involved in regulating pectate lyase activity.

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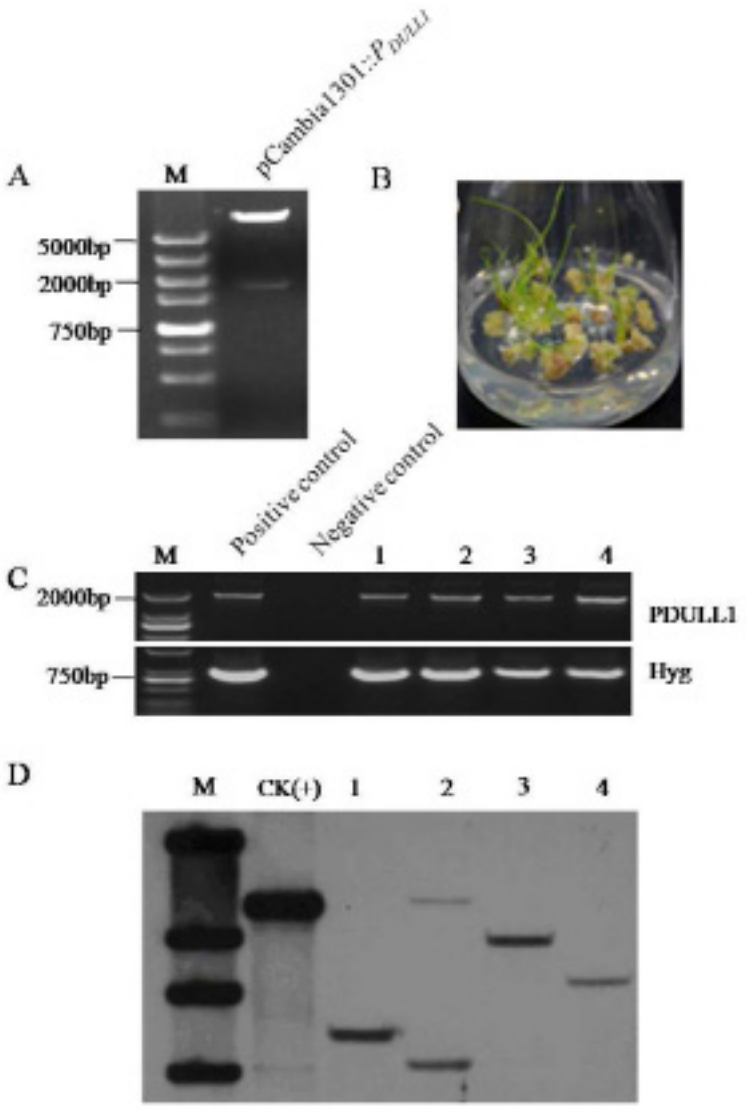

Figure 2. Production and molecular characterization of the *ZmDULL1* promoter transgenic rice plants. **A.** Construction of vector *pCambia1301::PDULL1*. **B.** Production of transgenic rice plants. **C.** PCR analysis the promoter *ZmDULL1* (*PDULL1*) and hygromycin gene (Hyg) using genomic DNA from the transgenic rice plant (1, 2, 3, and 4). Positive control: plasmid of *PDULL1*. Negative control: transgenic control plant. **D.** Southern blot analysis of *PDULL1* transgenic lines (*lanes 1*, *2*, *3*, and *4*); CK(+) represents the positive plasmid. *Lane M* = Lambda DNA markers.

GUS expression pattern under the control of *PDULL1*

To assay the GUS expression activity driven by *PDULL1* in transgenic rice, different tissues (root, leaf, stem, sheaths, glume, flowers, and endosperm) of T_2 transgenic rice plants were collected and analyzed by histochemical staining. As shown in Figure 3, in positive

control experiments, all tissues of transgenic plants containing the 35S promoter fused to the GUS exhibited high GUS activity (Figure 3O-U). In *PDULL1* transgenic plants, high levels of GUS activity were detected in endosperm (Figure 3G), whereas very weak GUS activity was measured in the root, leaf, stem, sheaths, glume, and flowers (Figure 3A-F).

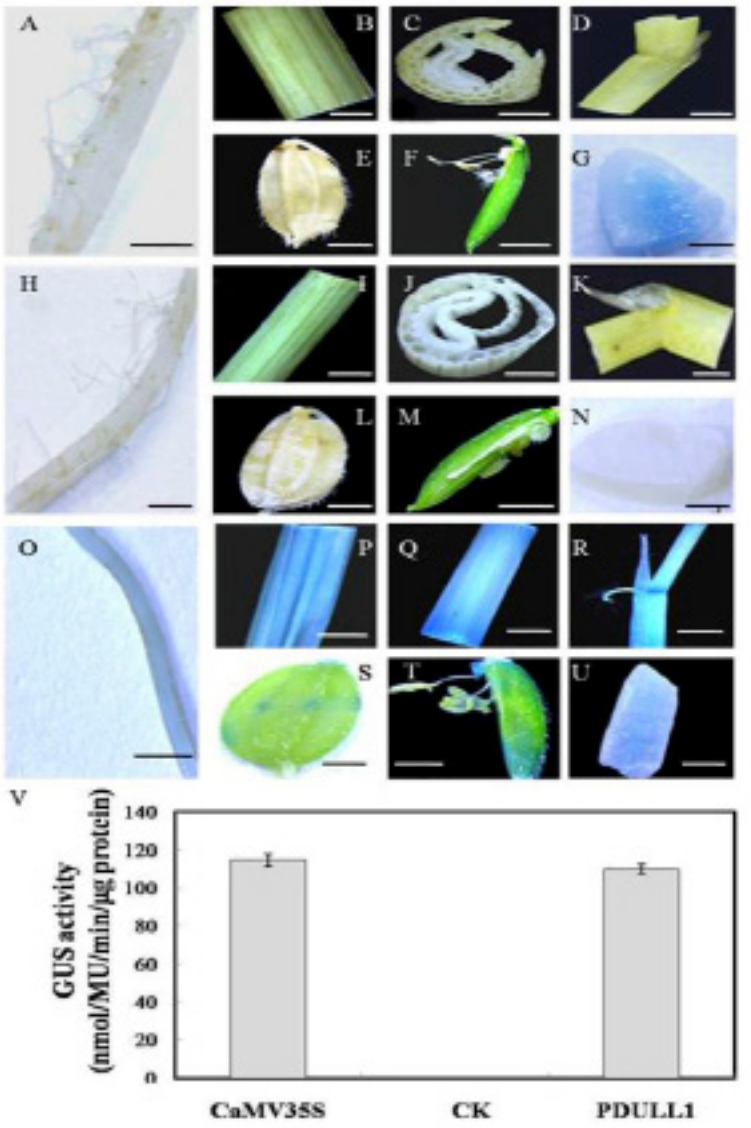

Figure 3. Expression patterns of *PDULL1* transgenic rice lines via GUS histochemical staining and GUS fluorescent quantitative analysis. **A.-U.** Histochemical analysis of GUS expression in different tissues of transgenic rice lines: **A.-G.** (*PDULL1* transgenic rice plant), **H.-N.** (transgenic control plant: Zhonghua11), **O.-U.** (35S transgenic rice plans). **A. H. O.**: root; **B. I. P.**: leaf; **C. J. Q.**: stem; **D. K. R.**: sheaths; **E. L. S.**: glume; **F. M. T.**: flowers; **G. N. U.**: endosperm. **V.** GUS fluorescent quantitative analysis of endosperm. CK = negative control (Zhonghua 11); CaMV35S: 35S transgenic rice lines. Values are reported as means ± SD of triplicate analyses. Bars = 1 mm.

To further verify these findings, the GUS expression level in endosperm was analyzed using a GUS fluorometric assay between control plants and *PDULL1* transgenic plants. The results showed that the GUS expression level in endosperm driven by *PDULL1* was similar to that in 35S transgenic plants (Figure 3V). These results demonstrated that *PDULL1* was a strong endosperm-specific promoter.

Activity analysis of *ZmDULL1* deletion promoters

To further identify sequences essential for high-level expression of the promoter in endosperm, deletion analysis of *PDULL1* was performed. Four truncated fragments covering the various regions of *PDILL1* based on the sequencing results were examined for their expression level in endosperm. Each was fused with the GUS gene in the pCAMBIA1301 vector (Figure 4A). The correct bands of *PDULL1-1* (-1676 bp), *PDULL1-2* (-1216 bp), *PDULL1-3* (-740 bp), and *PDULL1-4* (-343 bp) were produced using PCR (Figure 4B). Different PCR products were cloned into the pCMBIA1301 plasmid to replace the CaMV35S promoter and the digestion results are shown in Figure 4C.

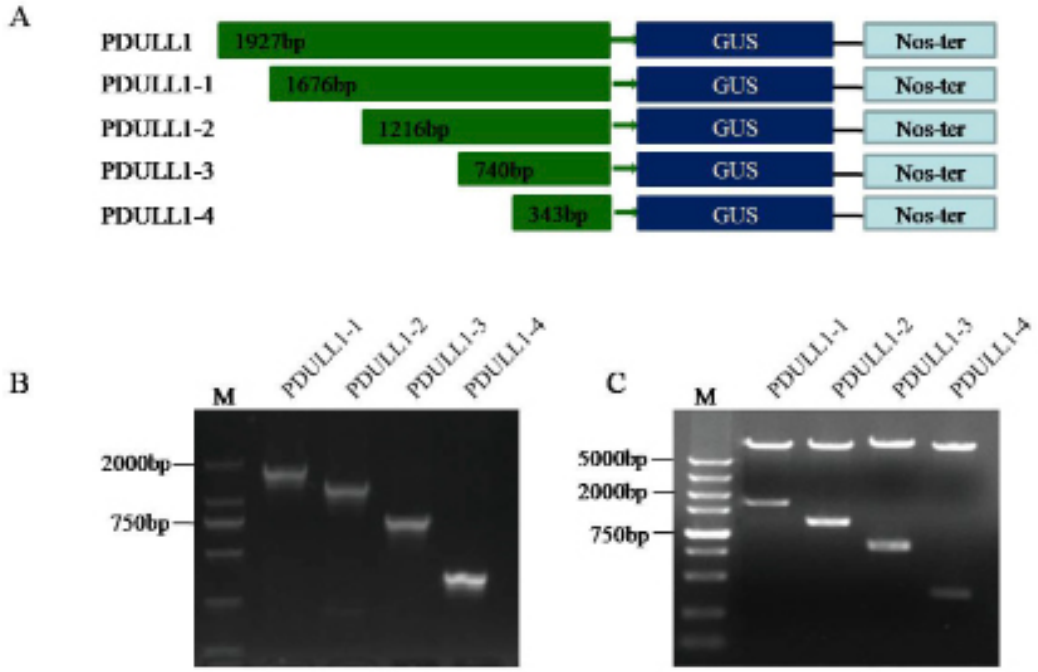

Figure 4. Cloning of different promoter deletions. **A.** Schematic representation of the size and position of each fragment of *PDULL1* for transgenic rice. GUS: β-glucuronidase gene. NOS-ter: nopaline synthase terminator. **B.** PCR results of different promoter deletions. **C.** Restriction results of the deletion promoter. *Lane M* = Lambda DNA markers.

Similarly, transgenic rice plants were obtained and assayed for expression driven by the truncated promoter of different lengths. The GUS expression level was analyzed in endosperm of different transgenic plants by histochemical staining and quantitative GUS fluores-

cence analysis (Figure 5). GUS activity increased as *ZmDULL1* promoter length increased from *PDULL1-1* through *PDULL1-4*. The highest GUS activity was detected in *PDULL1-4* transgenic plants among deletion promoters, showing a GUS expression level similar to that in transgenic plants driven by full length promoter sequence. However, very weak GUS expression was observed in *PDULL1-1* and *PDULL1-2* transgenic plants. These results indicate that the minimal regions (from -343 to -1 bp) of *PDULL1* direct strong gene expression in endosperm.

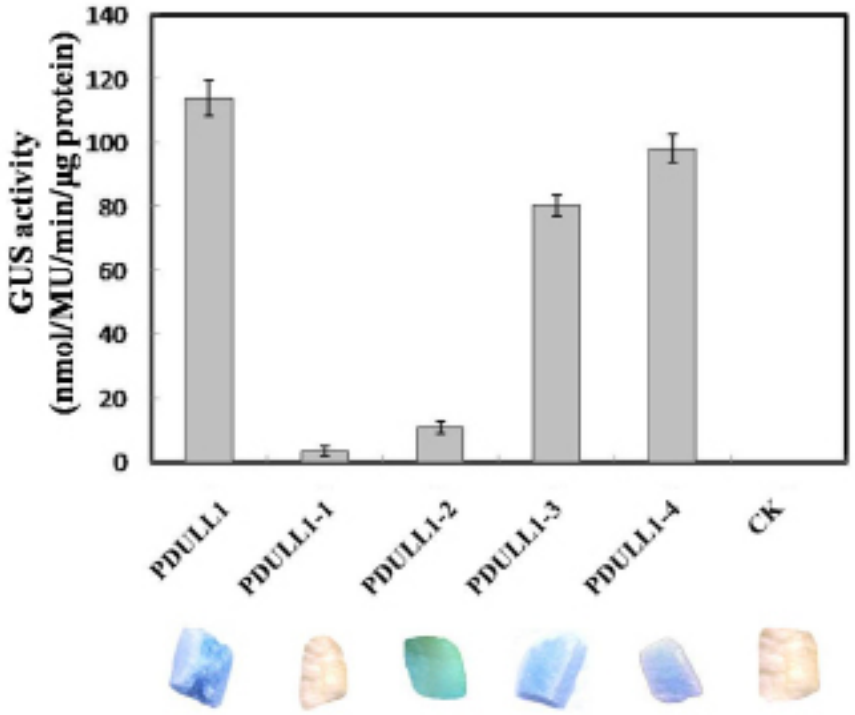

Figure 5. Expression patterns of endosperm in different promoter deletions transgenic rice plants via GUS staining and GUS fluorescent quantitative analysis. CK = negative control (Zhonghua 11).

DISCUSSION

In cereal, starch is not only mainly related to ingredients and quality characteristics of seeds, but also is closely related to productivity. Therefore, it is important to understand the process of starch biosynthesis and expression level of the starch synthase gene. Starch synthase genes were divided into 5 branches: *GBSS*, *SSI*, *SSII*, *SSIII*, and *SSIV*. *ZmDULL1* belongs to the subgroup ZmSSIII (Kang et al., 2012) and codes for a putative starch synthase that determines the structure of endosperm starch by regulating the expression level of 2 enzymes (starch synthase II and starch branching enzyme IIa) involved in starch biosynthesis. In addition, northern blot results showed that the *ZmDULL1* gene was specifically expressed in endosperm (Gao et al., 1998). However, this analysis was restricted to only a few tissues, and the expression pattern or important core elements of the ZmSSIII gene were not determined in detail.

In this study, to further analyze the expression activity of *PDULL1*, the fusion construct (*PZmDULL1*::*GUS*) was transformed into the rice genome and transgenic rice plants containing a single copy were produced to study promoter activity (Figure 2B and D). GUS activity driven by *PDULL1* was assayed in the root, leaf, stem, sheaths, glume, flowers, and endosperm. A previous study showed that maximum ZmDULL1 expression in endosperm occurred 12 days after pollination (Gao et al., 1998), and thus the endosperm in this stage was used as study material in our experiment. High GUS activity in endosperm (Figure 3G and V) and weak or no expression was tested in other tissues (Figure 3A-F). Similar results were reported for the homologous genes of the *ZmSSIII* gene in other species (Dian et al., 2005; Ohdan et al., 2005; Kang et al., 2012). In addition, several types of regulatory elements related to endosperm expression, including the GCN4_motif and AACA motif (Takaiwa et al., 1996), were found in *PDULL1* (Figure 1). These results suggest that *PDULL1* is an endosperm-specific promoter in cereal and may be used as a selectable marker protein in endosperm because of its stable and high level expression in transgenic plants.

Next, to determine the function of the *cis*-acting elements, deletion analysis of the *ZmDULL1* promoter was performed. The deletion promoter constructs were prepared (Figure 4) and transformed into rice using *Agrobacterium*-mediated transformation. As shown in Figure 5, the GUS activity of *PDULL1-4* plants was higher than that in *PDULL1-1*, *PDULL1-2*, and *PDULL1-3* plants. This suggests that the sequence between -343 to -1 bp of the *ZmDULL1* promoter may contain *cis*-elements involved in GUS expression. Sequence analysis showed that some important *cis*-acting elements related to the endosperm expression were found in *PDULL1-4*, including the GCN4_motif and AACA motif (Figure 1). The GCN4 motif, a *cis*-element is highly conserved in the promoters of cereal seed storage protein genes and plays a central role in controlling endosperm-specific expression (Onodera et al., 2001). Multimers of the rice glutelin GCN4 motif can direct endosperm-specific expression in stable transgenic rice. Wu et al. (1998) reported that a 21-bp *GluB-1* promoter fragment spanning the GCN4 motif, as a multimer, directed GUS gene expression in endosperm of transgenic rice plants. The AACA motif (AACAAAA), conserved in cereal glutelin genes, is also involved in controlling the endosperm-specific expression (Zheng et al., 1993; Washida et al., 1999). Interestingly, it was recently demonstrated in the cereal glutelin gene that AACA was closely associated with the GCN4 motif and together were shown to enhance expression in the endosperm (Yoshihara et al., 1996; Wu et al., 1998, 2000). In this study, we found that when the promoter was deleted to -343 bp, GUS activity increased to the maximum level, as this sequence contained the AACA motif sequence and GCN4 motif element (Figures 1 and 5). Unexpectedly, very weak GUS activity was observed in endosperm of the *PDULL1-1* and *PDULL1-2* transgenic rice. There may be repressor elements of *ZmDULL1* gene expression in the region from -1676 to 740 bp.

In conclusion, we showed that the upstream 5' flanking region of the *ZmDULL1* gene possesses important regulatory sequences (-343 to 1 bp) that determine endosperm-specific expression in transgenic rice based on the results of histochemical staining and fluorometric GUS assay. Therefore, the region from -343 to 1 bp of the *ZmDULL1* promoter may serve as a valuable tool for transgenic rice breeding and enhancing crop yields.

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