



# Analyzing the action of evolutionarily conserved modules on HMW-GS *1Ax1* promoter activity

Luning Duan<sup>1</sup> · Shichen Han<sup>1</sup> · Ke Wang<sup>2</sup> · Peihong Jiang<sup>1</sup> · Yunsong Gu<sup>1</sup> · Lin Chen<sup>1</sup> · Junyi Mu<sup>1</sup> · Xingguo Ye<sup>2</sup> · Yaxuan Li<sup>1</sup> · Yueming Yan<sup>1</sup> · Xiaohui Li<sup>1</sup>

Received: 14 October 2019 / Accepted: 4 December 2019 / Published online: 9 December 2019  
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## Abstract

**Key message** The specific and high-level expression of *1Ax1* is determined by different promoter regions. HMW-GS synthesis occurs in aleurone layer cells. Heterologous proteins can be stored in protein bodies.

**Abstract** High-molecular-weight glutenin subunit (HMW-GS) is highly expressed in the endosperm of wheat and relative species, where their expression level and allelic variation affect the bread-making quality and nutrient quality of flour. However, the mechanism regulating HMW-GS expression remains elusive. In this study, we analyzed the distribution of *cis*-acting elements in the 2659-bp promoter region of the HMW-GS gene *1Ax1*, which can be divided into five element-enriched regions. Fragments derived from progressive 5' deletions were used to drive *GUS* gene expression in transgenic wheat, which was confirmed in aleurone layer cells, inner starchy endosperm cells, starchy endosperm transfer cells, and aleurone transfer cells by histochemical staining. The promoter region ranging from –297 to –1 was responsible for tissue-specific expression, while fragments from –1724 to –618 and from –618 to –297 were responsible for high-level expression. Under the control of the *1Ax1* promoter, heterologous protein could be stored in the form of protein bodies in inner starchy endosperm cells, even without a special location signal. Our findings not only deepen our understanding of glutenin expression regulation, trafficking, and accumulation but also provide a strategy for the utilization of wheat endosperm as a bioreactor for the production of nutrients and metabolic products.

**Keywords** *1Ax1* · Expression regulation · HMW-GS · GUS · Protein body · Wheat

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Luning Duan and Shichen Han have contributed equally to this work.

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s11103-019-00943-6>) contains supplementary material, which is available to authorized users.

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✉ Xiaohui Li  
lixiaohui@cnu.edu.cn

<sup>1</sup> Key Laboratory of Genetics and Biotechnology, College of Life Science, Capital Normal University, Beijing 100048, China

<sup>2</sup> National Wheat Improvement Center, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

## Introduction

Wheat is the one of the three most widely planted and highest yielding cereal crops in the world. Its grains are rich in storage proteins (8–14%) providing approximately 62 million tons of protein for humans and livestock in each year. High-molecular-weight glutenin subunit (HMW-GS), an important component of storage proteins, can accumulate specially and highly in the endosperm of common wheat and its relatives, and plays an important role in determining bread-making quality (Payne 1987, 1988; Beasley et al. 2002; Uthayakumaran et al. 2002; Don et al. 2006; Yang et al. 2014; Zhang et al. 2014; Li et al. 2015; Liu et al. 2016; Wang et al. 2017; Jiang et al. 2019). Each HMW-GS accounts, on average, for about 2% of total grain proteins (Seilmeier et al. 1991; Halford et al. 1992), and single gene introgression can improve bread-making quality (Barro et al. 1997; Blechl et al. 2007; Li et al. 2012). However, the regulatory mechanisms behind the specific and high-level

expression of HMW-GS genes in wheat endosperm are still unclear.

Of the various alleles, only the activity of *Dx* and *Dy* promoters has been investigated by driving a reporter gene expression in wheat, oat, maize, rice, and other plants. A 1251-bp fragment upstream of the *IDx5* start codon was able to activate the expression of the *GUS* gene in endosperm at 10 days after flowering (DAF), but not in aleurone layer cells or other tissues of wheat (Lamacchia et al. 2001). However, under the control of the same sequence, *GUS* expression products were successfully detected at 12 DAF in the endosperm and aleurone layer of transgenic oat (Perret et al. 2003). Tandem repeats of an 89-bp fragment spanning from –225 to –136 upstream of the transcriptional initiation site of the *IDx5* gene conferred a strong, specific, and additive effect in maize endosperm (Norre et al. 2002). The 425-bp promoter region upstream of the *IDy12* start codon could direct the specific *GFP* gene expression in the developing endosperm and aleurone layer of transgenic wheat, but more generally in endosperm, aleurone layer, pericarp, vascular parenchyma, root, and leaf of transgenic rice (Furtado et al. 2008, 2009). A large fragment (2936 bp) upstream of the *IDy10* start codon conferred exclusive *GUS* expression in endosperms of wheat and *Brachypodium distachyon* transgenic plants (Thilmoney et al. 2014). Recently, the 1 kb upstream of the *IDx2* start codon has been dissected by progressive 5' deletion, and all but the shortest 101-bp fragments were found to confer endosperm-specific *GUS* expression in transgenic wheat (Li et al. 2018). However, similar research on the other alleles, e.g., *IAx*, *IBx*, and *IBy*, is still lacking.

In rice, seed-specific promoters having activity in different tissues of the seed have been used to drive the expression of various heterologous genes in rice seeds (Paine et al. 2005; Takaiwa 2007; Wu et al. 2007; Zhu et al. 2018a). Under the control of an endosperm-specific promoter given from wheat, heterologous proteins might also be produced in wheat seeds, as plant seeds have been recognized as an ideal bioreactor for the cost-effective large-scale production of recombinant proteins (De Wilde et al. 2000). Therefore, research on the regulation mechanism of HMW-GS gene can facilitate the improvement of bread-making quality and development of plant bioreactor.

Some conserved *cis*-acting elements involved in the expression of seed storage proteins have been identified in the promoter of HMW-GS genes by bioinformatics analysis (Wang et al. 2013; Ravel et al. 2014; Makai et al. 2015); those include the AACA motif, ACGT motif, GCN4 motif (N motif), P box (E motif) and RY repeat motif (reviewed by Kawakatsu and Takaiwa 2010). Additionally, A box, W box, and as-2 box have been found in the promoter of HMW-GS genes (Wang et al. 2013; Li et al. 2018), which might participate in the regulation of expression. In this study, we

investigated the distribution of *cis*-acting elements located in the 2659-bp promoter fragment upstream of the HMW-GS gene *IAx1* start codon, and divided the fragment into five enrichment regions of *cis*-acting element. Progressive 5' deletion fragments of this promoter region were generated and used to drive *GUS* gene expression in the common wheat cultivar Fielder. The spatial and temporal expression patterns of *IAx1* were studied by observing the accumulation of GUS protein in various tissues and cells. Functional promoter regions and the key *cis*-acting elements affecting the expression of *IAx1* gene are also discussed.

## Materials and methods

### Plant materials

The common wheats Lo3-227 and Fielder were grown in a greenhouse at 20 °C and 16/8 h photoperiod. Lo3-227 was obtained from Professor Colin Wrigley, University of Adelaide, Australia. The Lo3-227 carrying the HMW-GS *IAx1* gene provided the DNA template for the cloning of *IAx1* promoter and its partial coding sequence. Fielder was used as the recipient material for *Agrobacterium*-mediated transformation. Seedlings were collected at 2 weeks after seeding, and tissues including roots, stems, flag leaves, paleas, lemmas, and florets, were collected 1–2 days before pollination. Seeds were harvested at 8, 11, 14, 17, 20, 23, 26, 29, and 32 days after flowering (DAF) between 11:00 a.m and 2:00 p.m.

### Plasmid construction

HMW-GS *IAx1* fragments consisting of different promoter sequence length and 225 bp of coding sequence were fused to a GUS reporter gene to create the p1Ax1(2659)::GUS, p1Ax1(2089)::GUS, p1Ax1(1724)::GUS, p1Ax1(618)::GUS, and p1Ax1(297)::GUS plasmids. The primers used are listed in Online Resource Table S1.

### Identification of transgenic plants

Total genomic DNA was isolated from leaves of transformants using the CTAB method (Stacey and Isaac 1994). PCR was carried out using 50–400 ng of genomic DNA in a 25 µL total reaction volume containing 12.5 µL 2×Phanta Max Buffer, 0.5 µL dNTP Mix, 1 µL of primer mix 110F+GUSR, 8.5 µL ddH<sub>2</sub>O, and 0.5 µL Phanta Max Super-Fidelity DNA polymerase. The primer pair used was 110F, 5'-GTAAACGACGGCCAGTGCC-3', and GUSR, 5'-AAACTGCCTGGCACAGCAT-3'. Amplification products were separated by 1% agarose gel electrophoresis, and

target bands were excised for sequence confirmation (Shanghai Meiji Biotechnology Co., Ltd., Beijing).

### Histochemical staining and microscopy

Seedlings and tissues including roots, stems, leaves, paleas, lemmas, and florets, as well as hand-cut seed sections from different development stages, were prepared using a  $\beta$ -Galactosidase Reporter Gene Staining Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing) according to the manufacturer's protocol. Samples for general morphology were observed on a SV11 light microscope (Carl Zeiss AG, Jena, Germany). Transverse sections (1–2 mm thick) were cut in fixative from the middle of seeds, and fixed for 45 min at room temperature in the fixation buffer of the staining kit. After three buffer washes, samples were incubated in GUS staining solution overnight at room temperature. After dehydration in an ethanol series, sections were infiltrated with different proportions of LR white resin (Medium Grade, 14381-UC) and ethanol for 7 days, and polymerized at 4 °C in an UV embedding box. Semi-thin sections (10  $\mu$ m) were cut using a Leica EM UC7 ultramicrotome (Wetzlar, Germany), collected in drops of distilled water on glass slides, and dried on a hot plate at 60 °C. Sections of grains at 11 and 17 DAF were stained with 0.25% coomassie brilliant blue, dried on a hot plate at 60 °C for 2 to 3 h, and then rinsed repeatedly with distilled water to remove the excess dye. Sections were examined on an inverted fluorescence microscopy (Zeiss Axiovert 200, Oberkochen, Germany).

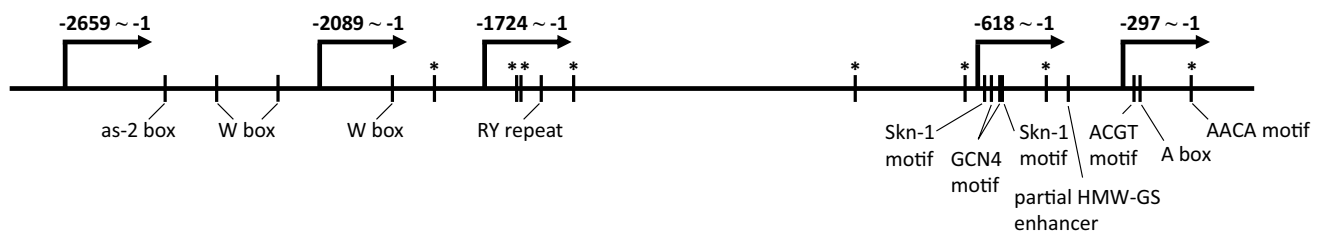
### Immunoelectron microscopy

Transverse sections were fixed for 2 days at room temperature within 4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline (PBS, prepared from  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  and  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ), pH 7.2. After three 10-min rinses in PBS, samples were dehydrated in an ethanol series. After infiltration with different proportions of LR white

resin and ethanol for over 7 days, sections were polymerized in a temperature gradient, i.e., 37 °C, 45 °C, 50 °C, and 65 °C. Ultrathin sections (~80 nm) were cut using the Leica EM UC7 ultramicrotome, collected on 300-mesh carbon-coated copper grids, and dried at room temperature. Sections were repeatedly rinsed with PBSG buffer (PBS, glycine, pH7.4) and then twice with PBGT (PBS, glycine, 0.1%v/v Tween 20, pH7.4). Sections were incubated in primary antibody (mouse monoclonal anti-GUS) diluted 1:25 in PBGT for 1 h at room temperature, followed by several rinses with PBGT for a total of 30 min, and then incubated for 1 h with secondary antibody diluted 1:20 in PBGT. Slides were rinsed twice in each of the following buffer, in order: PBGT, PBSG, and distilled water. The samples were treated with uranium oxyacetate for 15 min, followed by six rinses with distilled water. Slides were examined on a Zeiss Axiophot epifluorescence microscope and by transmission electron microscopy (Hitachi H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan).

### Real-time quantitative PCR (RT-qPCR) and semi-quantitative PCR

Extraction of total RNA from grains harvested at different stages and from various tissues of transgenic lines was performed according to the method described by Li et al. (2010). Reverse transcription of total RNA was performed following the protocols of an RT reagent kit (TaKaRa, Dalian, China). Using cDNA as templates and *ATG8d* as the internal reference gene (Mu et al. 2019), RT-qPCR and semi-quantitative PCR were performed to evaluate the transcription level of *GUS* in the developing endosperm of transgenic lines. The primer pair used for *GUS* was as in Li et al. (2018). The tissue expression of *GUS* in P1Ax1(297) was detected by semi-quantitative PCR.



**Fig. 1** Distribution of regulatory motifs in the HMW-GS gene 1Ax1 promoter. Those elements conserved in promoters of cereal seed storage protein genes were indicated (reviewed by Kawakatsu and Takaiwa 2010). as-2 box (GATAATGATG; Diaz-De-Leon et al. 1993), W boxes (TTGACC/T, Rushton et al. 1996; Ciolkowski et al. 2008), RY repeat (CATGCAT; Hoffman and Donaldson 1985; Dickinson et al. 1988), GCN4 motifs (TGAGTCA; Onodera et al. 2001),

Skn-1 motifs (GTCAT; Washida et al. 1999), partial HMW-GS enhancer (TTTGCAA; Thomas and Flavell 1990), ACGT motif (ACGTG; Takaiwa et al. 1996), A box (CCGTCC; Logemann et al. 1995), and AACA motifs (AACAA/GA; Takaiwa et al. 1996; Guo et al. 2015) indicated by stars. Element positions were indicated relative to the start codon

## Results

### Distribution of *cis*-acting elements in the HMW-GS *1Ax1* promoter region

The 2659 bp upstream of the *1Ax1* start codon was cloned from the common bread Lo3-227 (Online Resource Fig. S1). Using the general regulatory pattern for genes coding cereal storage proteins (Kawakastu and Takaiwa 2010), the major *cis*-acting elements considered to regulate tissue-specific expression were identified in the promoter region of *1Ax1*. These included an AACCA motif at –136, an A box at –256, an ACGT motif at –273, a partial HMW-GS enhancer at –413, Skn-1 motifs at –565 and –611, GCN4 motifs at –566 and –587, and an RY repeat at –1589. An as-2 box and three W boxes were located at the distal 5' end of the *1Ax1* promoter, i.e., –2433 and –2316, –2183, and –1925, respectively (Fig. 1). Taken together, the *cis*-acting elements were distributed among the following sections: –300 to –1, –700 to –301, –1700 to –701, –2100 to –1701, and –2659 to –2101.

### Construction of transgenic plasmids

Progressive 5' deletion fragments carrying varying lengths of the promoter and 225 bp of coding sequences for HMW-GS gene *1Ax1* were used to drive the expression of the *GUS* gene. Five fragments corresponding to 2659 bp, 2089 bp, 1724 bp, 618 bp, and 297 bp upstream of the start codon were cloned from Lo3-227 and inserted into the plasmid pCUB-*Ubi*-*GUS* to create *p1Ax1*(2659)::*1Ax1p*(225)::*GUS*, *p1Ax1*(2089)::*1Ax1p*(225)::*GUS*, *p1Ax1*(1724)::*GUS*, *p1Ax1*(618)::*1Ax1p*(225)::*GUS*, and

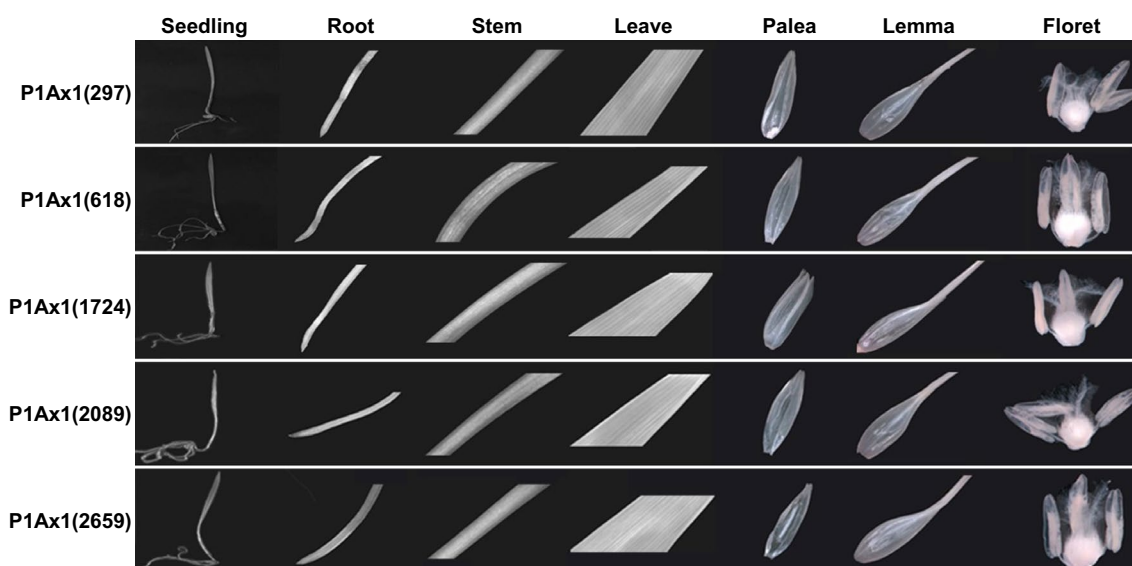
*p1Ax1*(297)::*1Ax1p*(225)::*GUS*. Except for the *p1Ax1*(1724)::*GUS* (which served as a control), all were fused to a 225-bp coding fragment from the 5' end of the *1Ax1* gene to track the effect of this portion of the coding region during trafficking of target proteins (Online Resource Fig. S2).

### Identification of transgenic plants

Five plasmids were introduced into Fielder by *Agrobacterium*-mediated transformation using a licensed procedure (<http://www.jti.co.jp/biotech/en/plantbiotech/index.html>), yielding five groups of transgenic materials termed P1Ax1(2659), P1Ax1(2089), P1Ax1(1724), P1Ax1(618), and P1Ax1(297). Genomic DNA from leaves of each transgenic plant was used as a template for PCR. The numbers of T<sub>0</sub> positive transgenic plants identified for P1Ax1(2659), P1Ax1(2089), P1Ax1(1724), P1Ax1(618), and P1Ax1(297) were 10, 11, 11, 5, and 3, respectively. Three independent homozygous lines isolated from each group were used for the expression analysis of the *GUS* reporter.

### Tissue-specific expression of P *1Ax1*::*GUS*

*GUS* expression was detected by staining seedlings, roots, stems, flag leaves, paleas, lemmas, pistils, and stamens, as well as grains harvested at 8, 11, 14, 17, 20, 23, 26, 29, and 32 DAF. Blue staining was not seen in seedlings, roots, stems, flag leaves, paleas, lemmas, pistils, or stamens from any of the transgenic lines (Fig. 2), but was visible in developing grains from P1Ax1(2659),



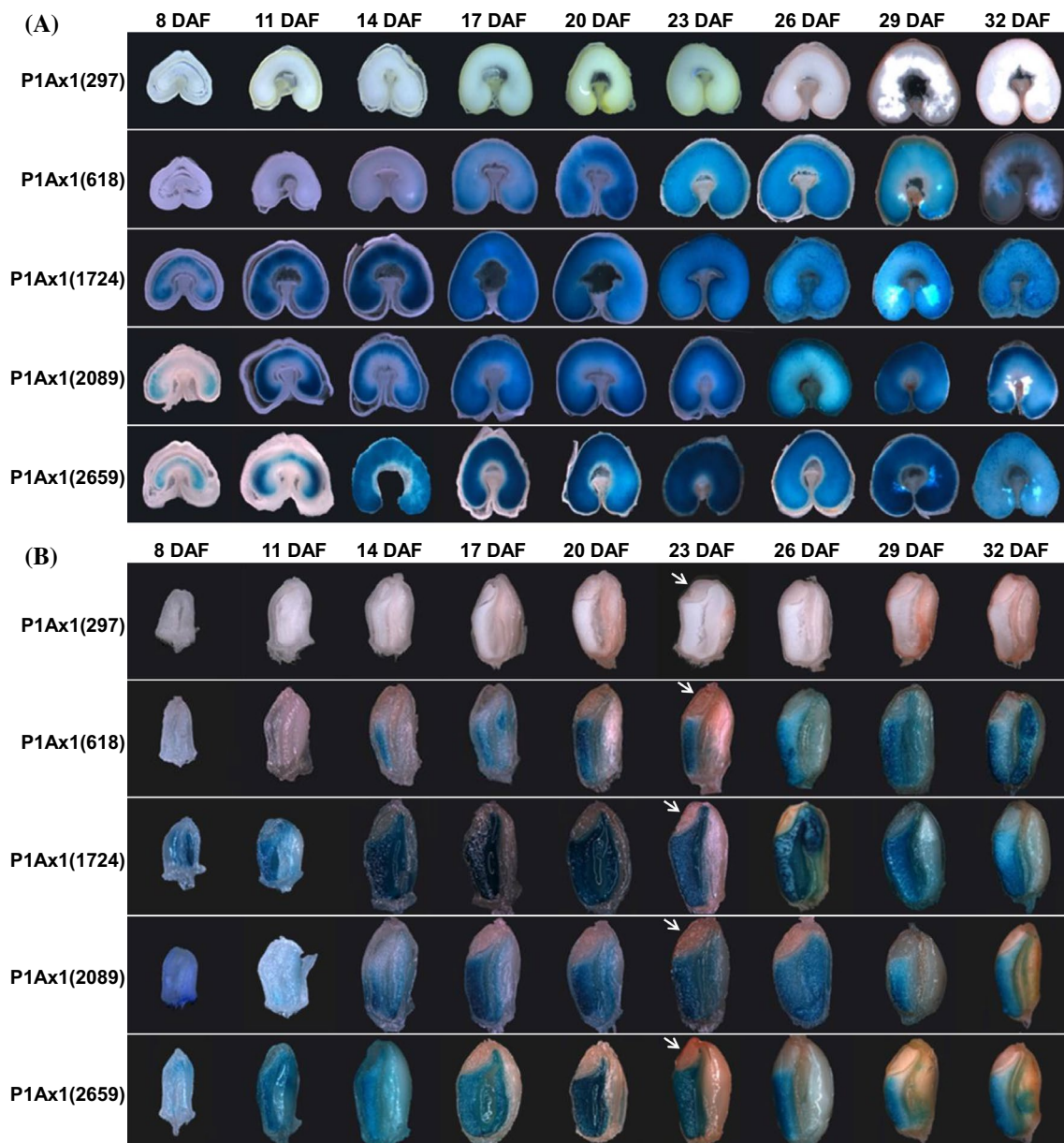
**Fig. 2** Histochemical staining of seedlings, roots, stems, leaves, palea, lemma, and floret from five transgenic lines

P1Ax1(2089), P1Ax1(1724), and P1Ax1(618) (Fig. 3). Dissected sections of grains at different developmental stage exhibited GUS staining in the endosperm (Fig. 3a, b), but not in the embryo (Fig. 3b). The above results indicate that GUS expression driven by the promoter of the *Iax1* gene was endosperm specific and that the 618-bp fragment was sufficient for specificity. Additionally, there were obvious differences in expressing among the four groups during endosperm development. Transverse sections of seeds at different stages showed that endosperms from P1Ax1(2659), P1Ax1(2089), and P1Ax1(1724) were stained from 8 DAF to 32 DAF, while those from P1Ax1(618) were not stained until 14 DAF. This suggests

that the *cis*-acting elements located on the fragment ranging from –1724 to –619 might regulate the expression level of the *GUS* gene. P1Ax1(297) failed to exhibit visible blue staining in developing endosperm (Fig. 3).

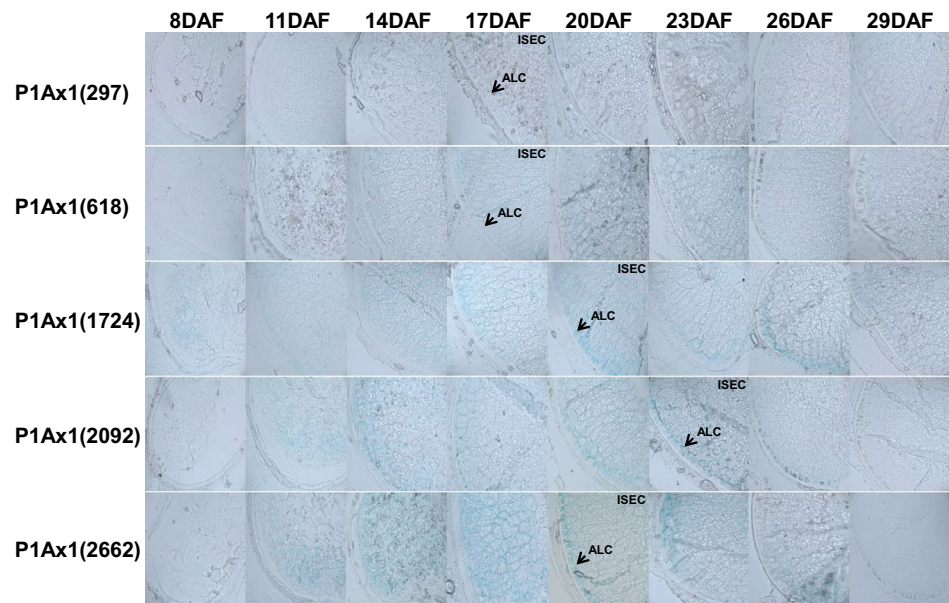
### Cell-specific expression of P1Ax1::GUS

Distribution of GUS proteins among the different cell layers of the endosperm were determined by observing the staining of resin-embedded sections under the microscope. GUS staining was not visible in endosperm cells developed from 8 to 32 DAF of P1Ax1(297), or from 8 to 11 DAF of

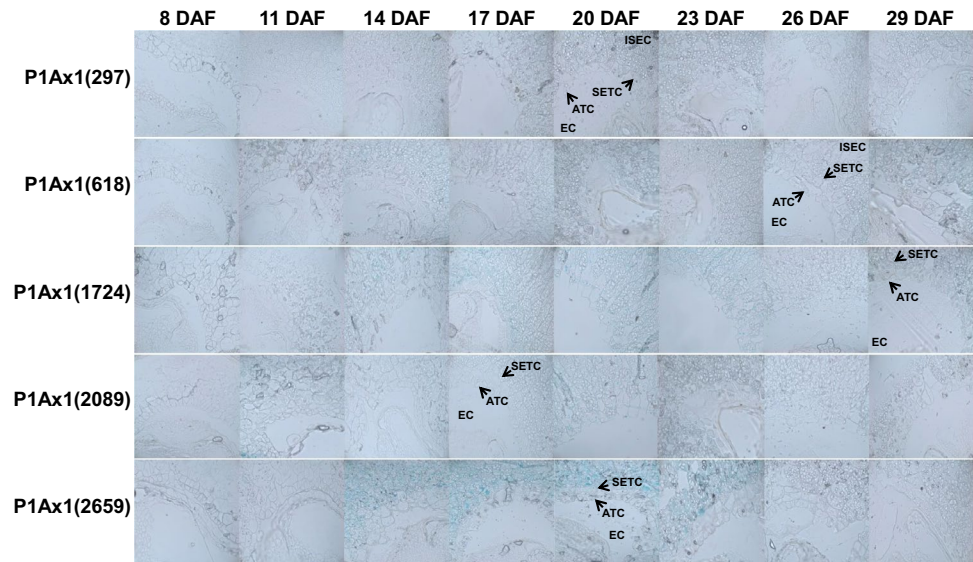


**Fig. 3** Histochemical staining of grains from five transgenic lines at different developmental stages. **a** Transverse sections; **b** longitudinal sections. Embryos were indicated by white arrows

**Fig. 4** Expression patterns of GUS in aleurone layer cells and inner starchy endosperm cells at different development stages of five transgenic lines. *ALC* aleurone layer cell, *ISEC* inner starchy endosperm cell



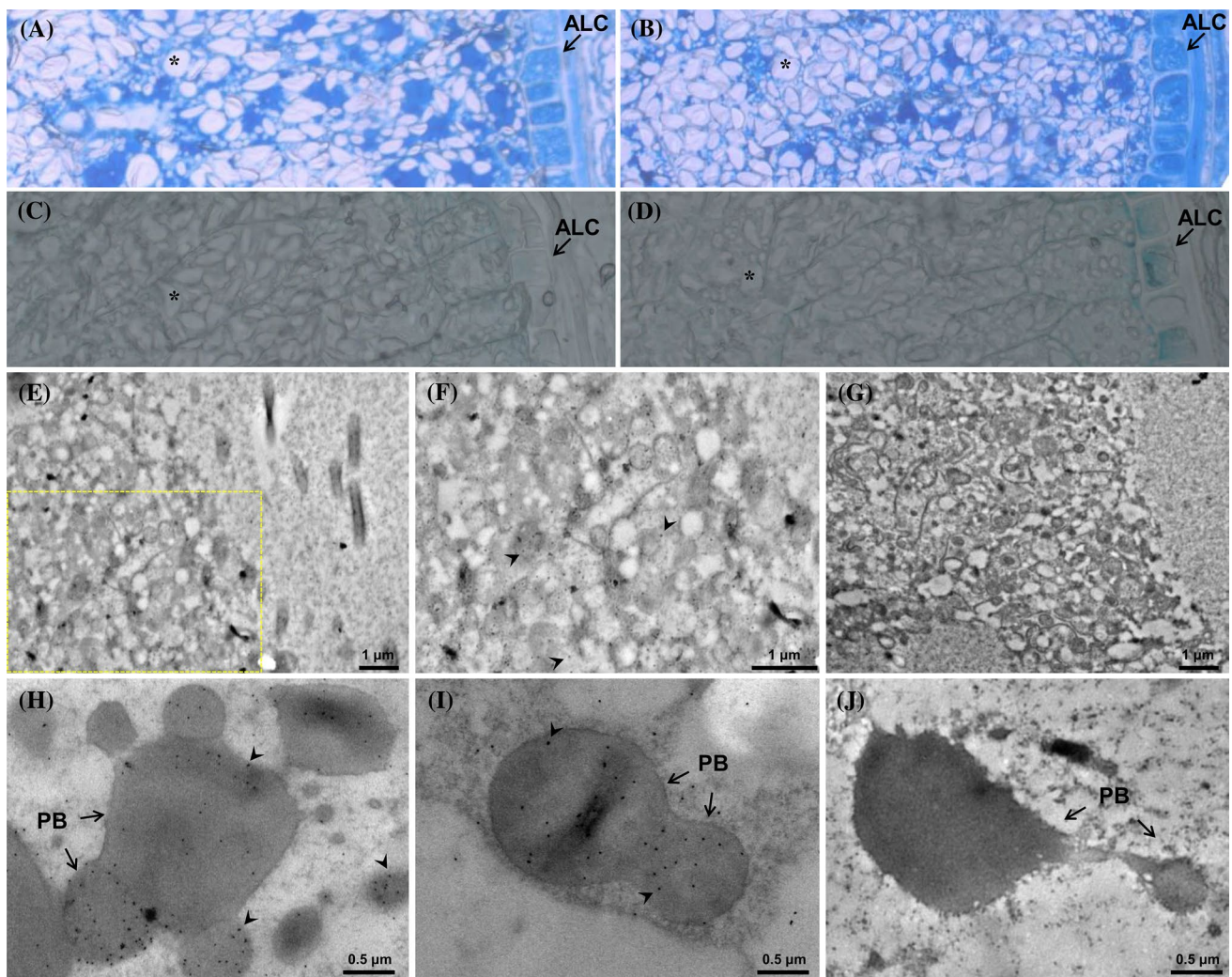
**Fig. 5** Expression patterns of GUS in transfer cells at different development stages of five transgenic lines. *ATC* aleurone transfer cell, *SETC* starchy endosperm transfer cell, *ISEC* inner starchy endosperm cell, *EC* endosperm cavity



P1Ax1(618) (Fig. 4), which was consistent with the results of hand-cut sections. As in the starchy endosperm cells, aleurone layer cells were also stained from 8 DAF to 32 DAF in P1Ax1(2659), P1Ax1(2089), and P1Ax1(1724), and from 14 DAF to 32 DAF in P1Ax1(618), indicating that the promoter fragments of *IAX1* were able to drive the *GUS* expression in those cells. Similarly, *GUS* was also expressed in aleurone transfer cells (ATCs) and starchy endosperm transfer cells (SETCs) (Fig. 5). In comparing the staining of transfer cells near the endosperm lumen, we found that *GUS* staining was clearly stronger in SETCs than in ATCs (Online Resource Fig. S3).

### Subcellular distribution of GUS

Grains harvested at 17 DAF from P1Ax1(618) and P1Ax1(1724) transgenic lines were selected to generate fixed sections. The location of protein bodies (PBs) was indicated by staining with coomassie brilliant blue (Fig. 6a, b). Spots (representative of PBs) distributed in the spaces between starch granules were also observed by histochemical staining in each cell of the starchy endosperm (Fig. 6c, d), but not in aleurone layer cells, which lack PBs (Xiong et al. 2013). Ultrathin sections were used to locate *GUS* proteins by immunocolloidal gold staining. Colloidal gold particles were evenly distributed in the cytoplasm of aleurone



**Fig. 6** Detection of GUS proteins at the 17 DFA endosperm from the P1Ax1(618) and P1Ax1(1724) transgenic lines. **a** and **b** sections were stained by coomassie brilliant blue from P1Ax1(618) and P1Ax1(1724), respectively. **c** and **d** samples were treated by histochemical staining from P1Ax1(618) and P1Ax1(1724), respectively. **e** immunocolloidal gold labelling sample from the aleurone layer cell of P1Ax1(618). **f** the enlarged block marked by the yellow frame

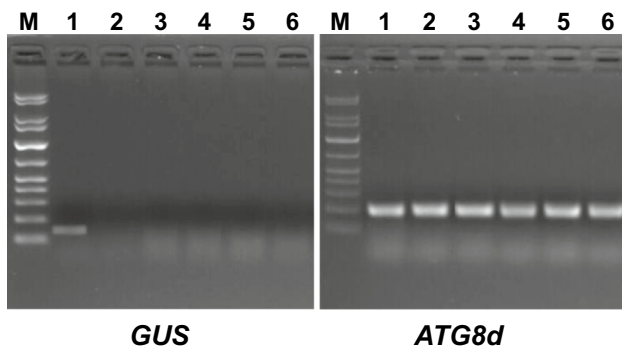
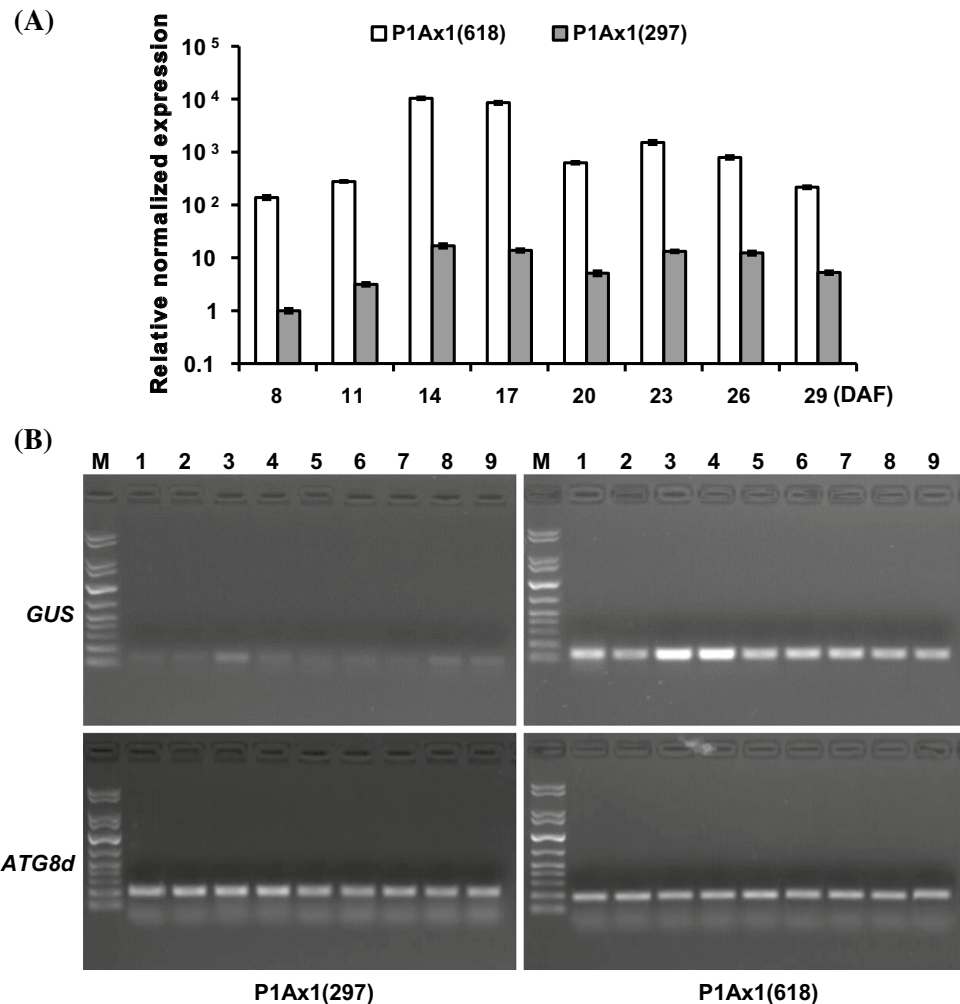
in **(e)**. **h** and **i** immunocolloidal gold labelling samples from inner starchy endosperm cells of P1Ax1(618) and P1Ax1(1724), respectively. **g** same treat as **(e)** but without GUS antibody. **j** same treat as **(h)** but without GUS antibody. *ALC* aleurone layer cells, *PB* protein bodies. Starch granules were indicated by stars. Arrow heads indicated immunocolloidal gold particles

layer cells from P1Ax1(618) and P1Ax1(1724) (Fig. 6e, f), but were enriched on PBs in the cytoplasm of inner starchy endosperm cells (Fig. 6h, i) as was seen with both coomassie brilliant blue and histochemical staining. This phenomenon was not observed in negative controls treated only with second antibody containing colloidal gold (Fig. 6g, j). PBs labeled by colloidal gold particles appeared to fuse with each other into large and irregular polymers at 17 DAF stage in the inner starchy endosperm cells of both P1Ax1(618) and P1Ax1(1724).

### Expression analysis of *GUS* in the P1Ax1(297) transgenic line

P1Ax1(297) transgenic plants were identified by PCR, but no GUS activity was detected by histochemical staining. To determine whether low-level expression of *GUS* was present, RT-qPCR was performed to compare the transcription level of the *GUS* gene between P1Ax1(297) and P1Ax1(618), using *ATG8d* as an internal reference gene (Fig. 7a). The specificity of primers used was verified by agarose gel electrophoresis (Fig. 7b). Transcripts of *GUS* could be detected in all stages of the endosperm from the P1Ax1(297) transgenic line, but these were present at a level approximately

**Fig. 7** Expression detection of GUS at different development stages of endosperm in P1Ax1(297). **a** RT-qPCR; **b** semi-quantitative PCR. PCR was performed 30 cycles in (b). M, 1 kb plus marker; lane 1–9 were 8, 11, 14, 17, 20, 23, 26, 29, and 32 DAF, respectively



**Fig. 8** Tissue specificity analysis of GUS in P1Ax1(297) by semi-quantitative PCR. Lane 1–9 were the endosperm, root, stem, leaf, lemma, and palea, respectively. M, 1 kb plus marker

100-fold lower than in P1Ax1(618) at each stage. Similarly, the expression difference between the two transgenic lines

at each stage was visible by agarose gel electrophoresis after 30 cycles of semi-quantitative PCR (Fig. 7b). These results indicate that the 297-bp promoter fragment of *IAx1* was able to drive the *GUS* gene to express at a low level in wheat endosperm. Additionally, *GUS* transcripts could be detected in endosperm, but not in roots, stems, leaves, paleas, or lemmas, suggesting that the 297-bp promoter fragment was able to confer endosperm-specific expression (Fig. 8).

## Discussion

### Distal cis-acting elements affect expression level; proximal elements determine tissue specificity

HMW-GSs are a kind of storage proteins, and their encoding genes have a characteristic of specific and high-level expression during endosperm development in wheat. The



phenomenon mainly controlled at the transcriptional level is attractive as desirable bread-making quality and nutrition quality of HMW-GSs. The HMW-GS gene promoters have been investigated first basing on bioinformatics analysis in the past ten years. As a result, lots of typical *cis*-acting elements and their variants related to the storage protein expression are found to tightly distribute on the 1 kb region upstream of initiation codon, of which the type and amount might affect the expression level of HMW-GS genes (Wang et al. 2013; Ravel et al. 2014; Guo et al. 2015; Makai et al. 2015). Some elements located in the 5' distal region, e.g. a 5'-UTR Py-rich stretch motif at position –1954 of *IBx7* promoter (Wang et al. 2013), also might contribute the high-level expression regulation of HMW-GS genes. Most of HMW-GS gene promoters contain 3–4 AACA motifs and the amount are significantly correlated with the abundance of HMW-GS proteins (Guo et al. 2015). However, there are still several unresolved issues; how long the length of promoter fragment, how many types of regulatory elements, or whether all copies are essential for high-level transcription of HMW-GS genes. In this study, the expression of *GUS* driven by any of the HMW-GS gene *IAX1* promoter fragments (including the 297-bp fragment) showed strict endosperm-specificity and was not observed in embryonic or other tissues (Figs. 2, 3, 8). The 297-bp fragment contains an AACA motif, an A box, and an ACGT element. Members of the *PAL* gene family, encoding phenylalanine ammonia-lyases, carrying an A box near the TATA box are widely expressed in various tissues or cells of parsley, e.g., leaves, roots, and cultured cells (Logemann et al. 1995), suggesting that the A box is not a key element for determining endosperm specificity. Although both AACA and ACGT motifs have been considered quantitative regulation elements for rice storage protein genes, only mutation of the AACA motif resulted in the failure to detect *GUS* activity in transgenic rice endosperm (Wu et al. 2000). Furthermore, TaGAMyB ectopically expressed in *Arabidopsis* activates the transcription of HMW-GS gene *IDy* via binding its promoter (Guo et al. 2015). This suggests that the AACA element located at –136 was a candidate for endosperm-specific expression. Confusingly, the endosperm specificity-determining motif AACA is absent in the promoter of the rice seed storage protein gene *GluC* (Qu et al. 2008), implying that other, non-annotated elements or a combination of several elements might be involved in determining endosperm-specific expression of *IAX1*. P1Ax1(618) transgenic lines exhibited a detectable *GUS* activity in endosperms and 100-fold high than P1Ax1(297) which mean that GCN4, Skn-1, AACA, and partial HMW-GS enhancer were involved in controlling expression level. GCN4 is considered as a critical *cis*-element for determining qualitative expression of the *IAX1*, as mutating GCN4

resulted in a nearly 90-fold reduction of *GluB-1* promoter activity in transgenic rice seeds (Wu et al. 1998), and overexpressing TaSHP that can bind the GCN4 motif significantly inhibits the expression of HMW-GSs under the high nitrogen treatment (Boudet et al. 2019). A complete HMW-GS enhancer contains a P box (TGT/C/AAAAG), of which the core sequence AAAG can be efficiently bound by the TaPBF for transcriptional activation of *IDx2* and *IBy8* (Zhu et al. 2018a). However, the lack of typical P box in the partial HMW-GS enhancer (TTTGCAAA) and even in 2689-bp length of *IAX1* promoter (Fig. 1) meaning that the expression regulatory of *IAX1* differed in that of the other HMW-GS genes. Additionally, *GUS* activity in endosperm from P1Ax1(1724) was clearly higher than in that from P1Ax1(618), but was not different from that of P1Ax1(2659) or P1Ax1(2089). It has been reported that TaFUSCA3 can specifically recognize and combine with the RY repeat located on the promoter of HMW-GS gene *IBx7* *in vitro*, and thus activates the transcription of reporter gene *gus* in onion epidermal cells (Sun et al. 2017). This implies that the 1724-bp fragment of the *IAX1* promoter was sufficient for high-level expression of *GUS* in endosperm and the RY repeat located at its 5' distal end was a *cis*-regulatory element responsible for quantitative regulation of the *IAX1* gene. However, besides the RY repeat, there were 5 AACA motifs distributed in the region ranging from –1724 to –618. These AACA motifs also possibly contributed to quantitative regulation of the *IAX1* gene. Therefore, it needs further experimental studies to explore which motif has beneficial effect on enhancing the expression level of *IAX1* gene.

### HMW-GS is synthesized in aleurone layer cells and transfer cells, as well as in inner starchy endosperm cells

There is no consensus on whether the HMW-GS is expressed in aleurone layer cells. A 1251-bp fragment of the *IDx5* gene promoter activated *uidA* reporter gene expression in inner starchy endosperm cells, but not in aleurone layer cells, in transgenic wheat (Lamacchia et al. 2001). However, the same construct resulted in expression of *uidA* in both inner starchy endosperm cells and aleurone layer cells of transgenic oat plants (Perret et al. 2003). A 425-bp promoter fragment from the *IDy12* gene conferred endosperm-specific expression of the green fluorescent protein gene in inner starchy endosperm cells and aleurone layer cells in transgenic wheat (Furtado et al. 2009), but non-specific endosperm expression in transgenic rice (Furtado et al. 2008). Under the control of a 2396-bp fragment of the *Dy10* gene promoter, *GUS* activity was not detected in the aleurone layer cells of transgenic wheat (Thilmony et al. 2014). In our study, except for the 297-bp fragment, all four

fragments larger than 297 bp could produce blue GUS staining in aleurone layer cells, visible by microscope, and some sections showed higher staining in the aleurone layer cells than in inner endosperm cells (Fig. 4, Online Resource Fig. S3A). Furthermore, colloidal gold particles were located to the cytoplasm of aleurone layer cells from P1Ax1(618) and P1Ax1(1724) transgenic lines. Besides, RNA-Seq data show all HMW-GS genes are highly expressed in aleurone layer cells (Gillies et al. 2012; Pfeifer et al. 2014; Pearce et al. 2015) (Online Resource Fig. S3B). The above results support the hypothesis that HMW-GS is synthesized in aleurone layer cells (Perret et al. 2003; Furtado et al. 2009). A reasonable explanation for observation failure in aleurone layer cells is that elements affecting cell-specific expression might be distributed in different locations among the various HMW-GS alleles, and inappropriate promoter length might generate a disorder of cell location. The synthesized HMW-GSs in aleurone layer cells possibly act as temporary nitrogen reserves for protein synthesis at the early stage of seed germination.

HMW-GS genes can be highly expressed in transfer cells (Pfeifer et al. 2014) (Online Resource Fig. S3B). Comparing two different types of transfer cells near the endosperm cavity, it was clear that the staining of SETCs was somewhat stronger than that of ATCs (Fig. 5, Online Resource Fig. S4). Possible reasons for this differential *GUS* expression are: (1) SETCs may take up sucrose by apoplastic and symplastic transport pathways. In addition to transport by ATCs (symplastic transport pathway), sucrose may be taken up directly by SETCs from the endosperm cavity, via spaces between ATCs performing apoplastic transport pathway (Online Resource Fig. S4). (2) There may be differences in the structure of transfer cells. The amount of wall ingrowth developed in SETCs increases the membrane surface area and subsequently enhances the uptake rates for sucrose from both endosperm cavity and ATCs (Wang et al. 1995; Wang et al. 2011).

### N-terminal region protein sequence does not affect the accumulation pattern of HMW-GS

It is generally believed that cereal storage proteins are deposited into PBs via transport to vacuoles (Levanony et al. 1992; Loussert et al. 2008; Tosi et al. 2009), or by accumulating immediately in the lumen of the endoplasmic reticulum (Parker 1982; Tosi et al. 2009). So far, no evidence has been found to suggest that glutenin contains a specific localization signal. We thought whether the N-terminal region of HMW-GS affected its accumulation pattern. The 225-bp fragment of the *IAx1* coding sequence, comprising 63 bp encoding a signal peptide and 162 bp encoding partial N-terminal region, was fused to the 5' end of the *GUS* reporter gene. Unlike previous studies, we used an antibody

specific to the target protein in immunoelectron microscopic assays, and found colloidal gold particles specifically labeling protein bodies, which formed fused structures in the cytoplasm of endosperm cells at 17 DAF from P1Ax1(618) and P1Ax1(1724) transgenic lines (Fig. 6). No obvious difference in membrane structure, subcellular distribution, or fusion pattern of PBs was produced by proteins with and without the N-terminal region of *IAx1*, indicating that the N-terminus did not direct the accumulation pattern of HMW-GS and that no specific localization signal was necessary for deposition of heterologous proteins as PBs in wheat endosperm.

### Wheat endosperm as a bioreactor

Plants are particularly attractive as bioreactors for heterologous protein production due to their ease of culture and the low cost of production at large scales (De Wilde et al. 2000). Among the three major world grain crops, endosperm of rice and maize seeds have been proved as ideal bioreactors for metabolic engineering and food biofortification (Paine et al. 2005; Takaiwa 2007; Wu et al. 2007; Farré et al. 2016; Zhu et al. 2017, 2018b; Cho et al. 2019; Mangel et al. 2019), whereas a role for wheat endosperm remains unclear. In this study, GUS and storage proteins were co-deposited into PBs, suggesting that trafficking of proteins with high-level expression might share the same pathway as storage proteins in inner starchy endosperm cells. In these cells, deposit of high-yield proteins in the form of PBs may be preferred to protect nutritional substrates from degradation by proteases, or to reduce the risk of cell damage attributed to proteins accumulation. It is easy to specifically express heterologous genes at a high level in wheat endosperm, where the synthesized proteins may be readily deposited into protein bodies in inner starchy endosperm cells. Thus, wheat seeds may be an appreciated bioreactor, with high protein synthesis ability, long-term and stable storage for recombinant proteins, and economic benefits for one-third of the world's population.

### Conclusion

The expression of a *GUS* reporter gene under the control of a series of HMW-GS gene *IAx1* promoter fragments in wheat demonstrated that glutenin is expressed in aleurone layer cells, inner starchy endosperm cells, starchy endosperm transfer cells, and aleurone transfer cells. The promoter region ranging from –297 to –1 determines the tissue-specific expression of *IAx1*, and the AACA element at position –136 may be a key regulator. Regions ranging from –1724 to –618 and from –618 to –297 affect transcriptional levels, and the GCN4, Skn-1, AACA, and RY repeat motif are candidate regulatory elements. Immunoelectron

microscopy indicated that GUS proteins co-deposit with storage proteins in protein bodies in the cytoplasm of inner starchy endosperm cells during the development of wheat grains. These results would be beneficial for our understanding of glutenin expression regulation, trafficking, and accumulation, and suggest that developing the wheat seed as a bioreactor could provide large amounts of nutrients and metabolic products.

**Acknowledgements** This research was supported by grants from National Key R&D Program of China (2016ZX08009003-004), National Natural Science Foundation of China (31571652), the Natural Science Foundation of Beijing (6162002), and the Youth Innovative Research Team of Capital Normal University.

**Author contributions** XHL conceived and designed the experiment. LND, SCH, KW, PHJ, YSG, LC, and JYM performed the experiment. XGY, YXL, and YMY provided technical assistance and scientific discussion. LND and XHL wrote the paper. All authors read and approved the final manuscript.

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