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Dissecting conserved *cis*-regulatory modules of *Glu-1* promoters which confer the highly active endosperm-specific expression via stable wheat transformation

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ABSTRACT

Wheat high-molecular-weight glutenin subunits (HMW-GS) determine dough elasticity and play an essential role in processing quality. HMW-GS are encoded by *Glu-1* genes and controlled primarily at transcriptional level, implemented through the interactions between *cis*-acting elements and *trans*-acting factors. However, transcriptional mechanism of *Glu-1* genes remains elusive. Here we made a comprehensive analysis of *cis*-regulatory elements within 1-kb upstream of the *Glu-1* start codon (–1000 to –1) and identified 30 conserved motifs. Based on motif distribution pattern, three conserved *cis*-regulatory modules (CCRMs), CCRM1 (–300 to –101), CCRM2 (–650 to –400), and CCRM3 (–950 to –750), were defined, and their functions were characterized in wheat stable transgenic lines transformed with progressive 5′ deletion promoter::GUS fusion constructs. GUS staining, qPCR and enzyme activity assays indicated that CCRM2 and CCRM3 could enhance the expression level of *Glu-1*, whereas the 300-bp promoter (–300 to –1), spanning CCRM1 and core region (–100 to –1), was enough to ensure accurate *Glu-1* initiation at 7 days after flowering (DAF) and shape its spatiotemporal expression pattern during seed development. Further transgenic assays demonstrated that CCRM1-2 (–300 to –209) containing Complete HMW Enhancer (–246 to –209) was important for expression level but had no effect on expression specificity in the endosperm. In contrast, CCRM1-1 (–208 to –101) was critical for both expression specificity and level of *Glu-1*. Our findings not only provide new insights to uncover *Glu-1* transcription regulatory machinery but also lay foundations for modifying *Glu-1* expression.

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1. Introduction

Wheat (*Triticum aestivum* L.), an important staple crop worldwide, can be processed into a wide range of food products. This unique property results from its seed storage proteins (SSP) which form a polymer complex and provide both elasticity and extensibility for dough [1,2]. High-molecular-weight glutenin subunits (HMW-GS), the major component of wheat SSP, determine dough elasticity, and their differences in content and composition can explain up to 70% of the variation in the processing quality [3–6]. Therefore, it is very important to mine the desirable alleles encoding HMW-GS and uncover the regulatory machinery underlying their expression. HMW-GS are encoded by *Glu-1* loci on chromosomes 1AL, 1BL, and 1DL, and each locus consists of two tightly linked genes (*Glu-1-1* and *Glu-1-2*) encoding x-type and y-type HMW-GS, respectively [7]. Numerous variant alleles of *Glu-1* genes have been identified and the relationships between these alleles and processing properties have been well studied [1,7,8].

Glu-1 genes are specially expressed in the endosperm at the mid and late stages of wheat seed development [9,10]. Just like other SSP genes, the spatiotemporal expression pattern of *Glu-1* is primarily controlled at the transcriptional level involving a series of *cis*-acting motifs and *trans*-acting factors [11,12]. In term of *trans*-acting factors, four families of transcription factors (TFs), bZIP, DOF, MYB, and B3, were reported to be involved in SSP regulation [13–18]. Numerous *cis* motifs have also been identified in the promoters of SSP genes and several of them have been characterized functionally in model plants. The prolamin box (P-box) and N-motif (GCN4-like motif) complex are usually identified as the –300 element or endosperm box at around 300 bp upstream of the transcription start site for many SSP genes [19–22]. Mutation analyses in rice revealed that the N-motif regulates both gene expression specificity and quantity whereas the P-box controls only the expression level [23,24]. The AACA and ACGT motifs govern SSP expression quantitatively and their mutations can lead to dramatic reductions in promoter ability but have no effect on endosperm specificity [24,25]. The RY motif is another important element controlling seed-specific expression of SSP genes in dicots [26–28].

Previous functional characterizations about *Glu-1* promoter were mainly carried out in heterologous systems due to the lack of efficient stable wheat transformation technique. By promoter deletion assays, a 38-bp element designated as the Complete HMW Enhancer was identified within wheat *Glu-1* promoter and it could regulate both gene expression level and specificity in tobacco [29]. The quantitative roles of P-box and ACGT motif for *Glu-1* expressions were characterized by transient assays in maize endosperm [30]. *Glu-1* promoters have also been functionally analyzed in rice [31] and *Brachypodium distachyon* [32]. Although heterologous systems play an important role in understanding *Glu-1* promoters, they do not necessarily reflect their native functions. Wheat *Glu-1* promoter was unable to maintain tissue specificity when transformed in rice [33]. Therefore, it is necessary to decipher the functions of *Glu-1* promoters in homologous systems. Recent advances in transgene technology allow us to systematically dissect the functions of *Glu-1* promoters using wheat stable transformation.

The current study provides a comprehensive analysis of conserved *cis* motifs in ten representative *Glu-1* promoters and defines the conserved *cis*-regulatory modules (CCRMs). The regulatory functions of CCRMs are characterized by deletion assays in wheat stable transgenic lines. This work not only deepens our understanding on the transcription regulatory machinery underlying *Glu-1* expression, but also sets the groundwork to alter HMW-GS contents at the transcriptional level as a model system for modifying flour quality attributes.

2. Materials and methods

2.1. Collection of Glu-1 promoters

One-kb regions upstream of the start codon in 10 representative *Glu-1* genes were selected to characterize conserved *cis*-regulatory elements. Promoter sequences for the *Glu-1Ax1*, *Glu-1Bx7*, *Glu-1By8*, *Glu-1Dx5*, and *Glu-1Dy10* alleles were retrieved from public databases (KC820627, X13927, DQ537336, X12928, and X12929 in GenBank: https://www.ncbi.nlm.nih.gov/genbank/). The promoters for the *Glu-1Ay null*, *Glu-1Bx14*, *Glu-1By15*, *Glu-1Dx2*, and *Glu-1Dy12* alleles were amplified from the wheat cultivar Fielder (*Triticum aestivum* L.) using *rTaq* DNA polymerase (Takara Bio, Ohtsu, Japan). PCR were carried out in 20 μL reaction volumes containing 10 μL 2× GC buffer I, 100 μmol L^{-1} of each dNTP, 2 pmol of each primer, 100 ng of genomic DNA and 1 U of *rTaq* polymerase. The specific primer pairs for each promoter are listed in Table S1. PCR products were separated in agarose gels and recovered using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA). After purification, target fragments were inserted into the pEASY-T5 vector (TransGen Biotech, Beijing, China) and eight positive clones were identified for sequencing.

2.2. Sequence analysis

Genes with similar transcriptional characteristics are often regulated by consensus *cis* elements and this can be used to identify important motifs in gene promoters [21,34–36]. *Glu-1* promoter sequences were aligned using DNAMAN5.2 (http://www.lynnon.com/). Motif annotation was carried out with the PLACE database (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace) [37] to define the CCRMs. For motifs with overlapping signal sequences and similar functions, only the longest one was taken into account for further analysis. For the motif variants, we also named them as the typical motifs deposited in PLACE database (Table 1).

2.3. Vector construction and transgenic arrays

The *Glu-1Dx2* promoter was selected for vector construction and functional validation. To assess the function of CCRMs, progressive 5′ deletion fragments were generated from *Glu-1Dx2* promoter and fused to the GUS reporter gene. Five fragments corresponding to 100-bp (core region), 208-bp (core region plus CCRM1-1), 300-bp (core region plus CCRM1), 650-bp (core region plus CCRM1 and CCRM2), and 950-bp upstream

Table 1 – Conserved motifs identified in *Glu-1* promoters.

CCRM	Name	PLACE name	Sequence	TF	Location[c]
CCRM1					
	AACA[a]		AACA/TAAC	MYB	–142 (+)
	POA[b]	PYRIMIDINEBOXOSRAMY1A	CCTTTT	DOF	–186 (+)
	(CA)n	CANBNNAPA	CNAACAC	–	–203 (–)
	TOA	TATCCAOSAMY	TATCCA	MYB	–218 (+)
	I-box	IBOX	GATAAG	MYB	–220 (–)
	E-box	EBOXBNNAPA	CANNTG	bHLH	–233 (+)
	CCAAT	CCAATBOX1	CCAAT	NF-Y	–234 (+)
	P-box[b]	PROLAMINBOXOSGLUB1	TGCAAAG	DOF	–243 (+)
	SEF4	SEF4MOTIFGM7S	RTTTTTR	SEF	–256 (–)
	A-box[b]	PALBOXAPC	CCGTCC	–	–261 (+)
	ABRE	ABRELATERD1	ACGTG	bZIP	–277 (+)
CCRM2					
	AMY[b]	AMYBOX1	TAACARA	MYB	–403 (+)
	P-box[b]	–300ELEMENT	TGHAAARK	DOF	–418 (+)
	RY	RYREPEATBNNAPA	CATGCA	B3	–475 (+)
	Skn-1[b]	WRKY71OS	GTCAT	bZIP	–529 (–)
	RAV1	RAV1AAT	CAACA	AP2	–550 (–)
	N-motif	GCN4OSGLUB1	TGAGTCA	bZIP	–570 (+)
	TOA[b]	TATCCAOSAMY	TATCCA	MYB	–583 (–)
	W-box	WBOXNTERF3	TGACY	WRKY	–589 (–)
	N-motif[b]	GCN4OSGLUB1	TGAGTCA	bZIP	–591 (+)
CCRM3					
	PAP	PREATPRODH	ACTCAT	bZIP	–780 (+)
	CLL	CIACADIANLELHC	CAANNNNATC	–	–802 (+)
	CCAAT	CCAATBOX1	CCAAT	NF-Y	–831 (–)
	E-box	EBOXBNNAPA	CANNTG	bHLH	–859 (+)
	RY	RYREPEATBNNAPA	CATGCA	B3	–888 (+)
	AMY	AMYBOX1	TAACARA	MYB	–898 (–)
	E-box[b]	EBOXBNNAPA	CANNTG	bHLH	–917 (–)
	ABRE[b]	ABRELATERD1	ACGTG	bZIP	–924 (–)
Others					
	TATA[a]		TATAAA	TBP	–91 (+)
	BO	BIHD1OS	TGTCA	Homeo-domain	–670 (+)

The dash lines indicate TFs were not identified. The underlined motifs were reported to regulate SSP expression levels.
[a] Motifs were not included in PLACE database, but these are involved in SSP regulation [43–45].
[b] There were polymorphic nucleotides in motif sequences and the variants were named as the typical motifs deposited in PLACE database.
[c] Symbols in brackets indicate the motif location in the plus strand (sense strand) or minus strand (antisense strand). Motif locations refer to the *Glu-1Dx2* promoter.

(core region plus CCRM1, CCRM2, and CCRM3) of the start codon were amplified from Fielder genomic DNA and inserted into plant expression vector pUbi-GUS to create pGUS100, pGUS208, pGUS300, pGUS650, and pGUS950, respectively. These constructs were introduced into Fielder by *Agrobacterium tumefaciens* mediated transformation using a licensed procedure (http://www.jti.co.jp/biotech/en/plantbiotech/index.html). Transgenic lines were grown in the greenhouse at 20 °C and 16/8-h photoperiod with supplementary lighting provided by high-pressure sodium vapor lamps (Powertone SON-T AGRO 400W; Philips Electronic UK). Positive transgenic lines were identified by PCR with promoter-specific forward and common *GUS*-specific reverse primers. Primer sequences for vector construction and transgenic lines screening were shown in Table S1.

2.4. Analysis of GUS expression

Nine independent T_2 transgenic lines for pGUS300 construct and 10 for each of pGUS100, pGUS208, pGUS650, and pGUS950 constructs were used to investigate *GUS* expression. Plant tissues such as roots, leaves, leaf sheaths, spikelets and stems were collected two weeks after flowering, and seeds were sampled at the middle of spikes every 5 DAF until maturity (30 DAF). Seeds were also collected every day from 6 to 9 DAF to define point of the initiation of *Glu-1* expression. All samples were collected from 3:00 to 5:00 pm to avoid diurnal fluctuations in gene expression. One part of the samples was used for GUS staining and the remainder was stored at –80 °C for *GUS* gene expression and enzyme activity assays. For histochemical analysis of GUS activity, hand-cut seed pieces (longitudinally and laterally) and other tissues were incubated in 0.1 mol L^{-1} sodium phosphate buffer (pH 7.0) containing 1 mmol L^{-1} X-Gluc at 37 °C for 0.5 h to overnight [24,29,38]. GUS-stained tissues were imaged with a Leica M165 FC stereo microscope (Leica, Wetzlar, Germany).

GUS activity was measured by fluorometric quantification of 4-methylumbelliferone (4-MU) following Jefferson [39]. Mature seeds (30 DAF) were ground in GUS extraction buffer

and the supernatants were used for quantification of GUS activity. Protein concentrations were determined using bovine serum albumin (BSA) as control [40]. Fluorescence of 4-MU was measured at 365 nm excitation and 455 nm emission using the Synergy H1 microplate reader (BioTek, Winooski, VT, USA). GUS activity was calculated as pmols 4-MU/min/mg protein. *GUS* gene expression was examined by qPCR. Total RNA was isolated from frozen tissues using RNAprep Pure Plant Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. First strand cDNA was synthesized by a PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Ohtsu, Japan) following the manufacturer's protocol. qPCR was performed on a BioRad CFX system using i*Taq* Universal SYBR Green Supermix (BioRad, Hercules, CA, USA). Relative gene expression was normalized to *elongation factor 1 alpha* (*eF1α*) using the $2^{-\Delta\Delta Ct}$ equation [41]. Primer sequences for qPCR were shown in Table S1.

2.5. Statistical analyses

The one-way ANOVA analysis was performed with the SPSS Statistics 20 software (SPSS, Inc., Chicago, IL, USA). Multiple range test was used to compare mean values at the $P < 0.05$ probability level.

3. Results

3.1. Three conserved cis-regulatory modules (CCRMs) are defined in Glu-1 promoters

Previous studies showed that a 1-kb region upstream of the start codon enabled *Glu-1* highly expressed in endosperm [9,42]. The regions of ten representative *Glu-1* promoters were used to identify conserved *cis* motifs and define CCRMs. Multiple alignments showed that *Glu-1* promoters had a high sequence identity although some insertion or deletion differences were present (Fig. S1). Based on previous reports and PLACE annotation, we identified 30 conserved motifs, most of which were reported to be involved in SSP regulation (Table 1). According to motif distribution pattern, three CCRMs, CCRM1 (–300 to –101), CCRM2 (–650 to –400), and CCRM3 (–950 to –750) were defined (Fig. 1). CCRM1 contained 11 conserved motifs, five of which were included in the Complete HMW Enhancer, a critical element for *Glu-1* expression in transgenic tobacco [29]. CCRM2 comprised 9 conserved motifs. Among them, the N-motif regulated the endosperm specificity of SSP expression in rice [23] and RY controlled the seed-specific expression of SSP in dicots [26,28]. CCRM3 included 8 conserved motifs. In addition to RY, the ABRE, E-box and AMY were involved in seed-gene regulation [24,46,47]. Notably, the core region (–100 to –1) was also quite conserved, harboring basal transcription motifs such as a TATA box and the transcription start site (TSS) (Figs. 1; S1). The detail locations about these motifs were listed in Table S2.

3.2. CCRM1 plus the core region is able to confer Glu-1 endosperm-specific expression

To investigate the function of CCRMs, progressive 5′ deletion fragments were generated from the *Glu-1Dx2* promoter and fused with the GUS reporter gene to create constructs pGUS300, pGUS650, and pGUS950, which were transformed into wheat cultivar Fielder mediated by *Agrobacterium tumefaciens*. No GUS staining was detected in roots, leaves, leaf sheaths, spikelets or stems from all transgenic plants (Fig. S2). GUS expression was restricted to endosperm and prolonged incubation did not lead to staining in the embryo (Fig. 2-a), indicating that deletions of CCRM2 and CCRM3 had no effect on endosperm-specific expression of *Glu-1*. In short, the 300-bp promoter spanning CCRM1 and core region (–100 to –1) guaranteed *Glu-1* expression specificity.

3.3. CCRM2 and CCRM3 can elevate Glu-1 expression level

To determine the effects of CCRMs on expression activity, GUS expression patterns directed by pGUS300, pGUS650 and pGUS950 were monitored during seed development. GUS staining was detected in the endosperms from 10 DAF until 30 DAF and its intensity visibly increased with seed development (Fig. 2-a). Additionally, longer promoters drove stronger staining intensity, which was confirmed by GUS activity assays in the mature seeds (30 DAF) (Fig. 2-b). On average, GUS activities driven by pGUS650 and pGUS950 were about 4 and 6 folds higher than that by pGUS300, respectively, indicating that CCRM2 and CCRM3 greatly promoted *Glu-1* expression. However, there was still considerable GUS accumulated in seeds of pGUS300 transformants.

To define the accurate GUS expression pattern, we performed real-time quantitative PCR (qPCR) during seed

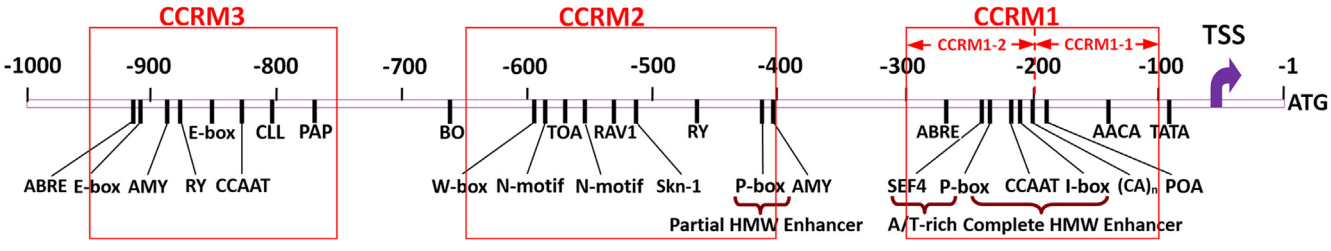

Fig. 1 – Conserved *cis*-regulatory modules (CCRMs) in *Glu-1* promoters. Motif positions are indicated relative to the start codon. In CCRM1, TOA, E-box and A-box were overlapped with I-box, CCAAT and SEF4, respectively, and they were not labeled due to limited space. CCRM1 was classified into two sub-CCRMs, CCRM1-1 (–208 to –101) and CCRM1-2 (–300 to –209).

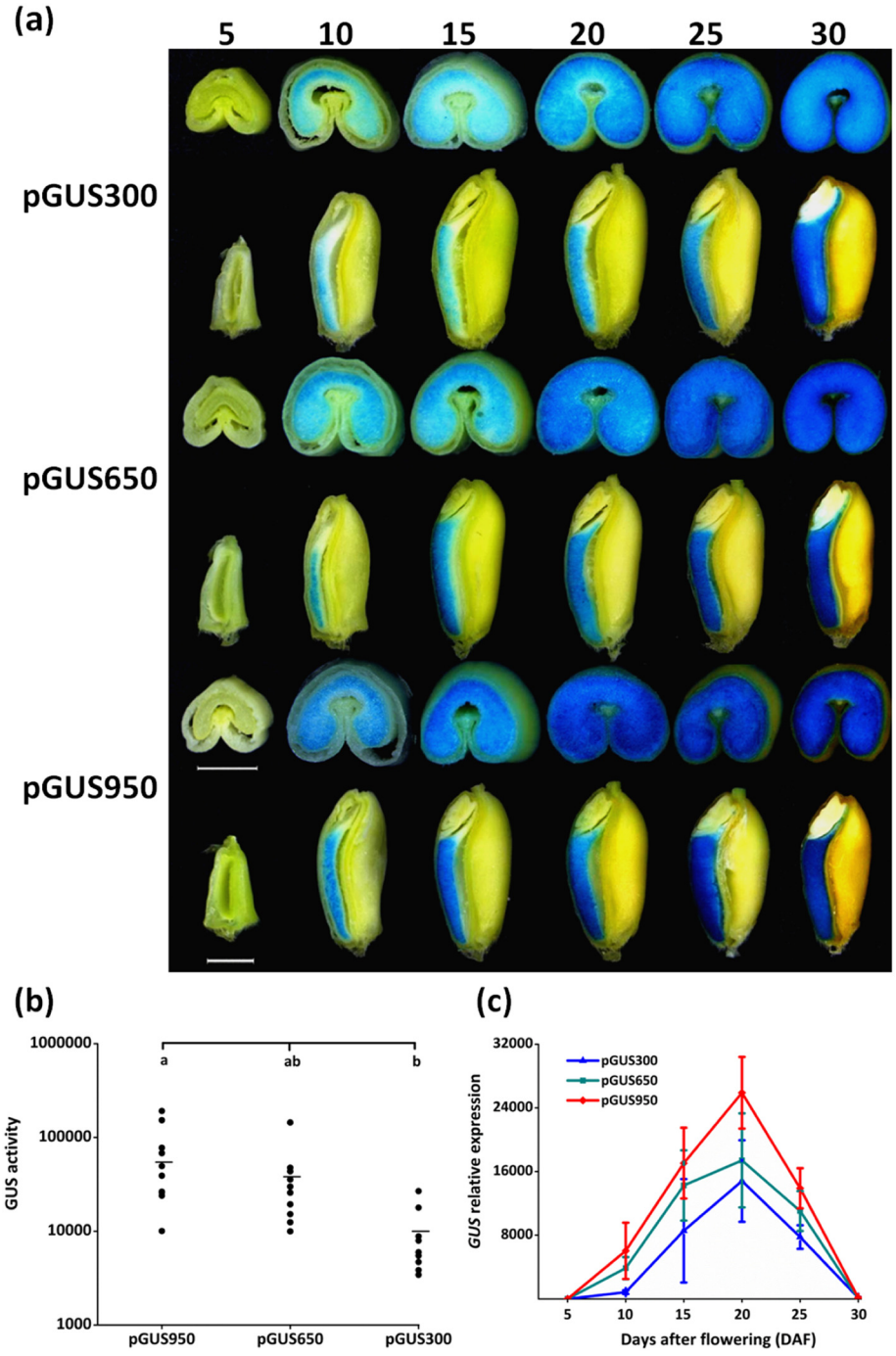

Fig. 2 – GUS expression in the transgenic seeds carrying pGUS300, pGUS650, or pGUS950 constructs. (a) Histochemical staining for the transgenic seeds during development. Seeds were collected every 5 DAF and incubated in 1 mmol L^{-1} X-Gluc solution. Bars indicate 2 mm and are used to scale the image captured longitudinally and laterally. (b) GUS activity in the seeds at 30 DAF. Each point shows GUS activity from an independent line among the 9 or 10 lines. GUS activity was calculated as pmols 4-MU/min/mg protein. Horizontal bars indicate average GUS activity. Different letters show the significant differences ($P < 0.05$). 4-MU, 4-methylumbelliferone. Data are present on a logarithmic scale. (c) Transcriptional patterns of the *GUS* reporter gene in developing seeds. Transcriptional levels were normalized to *elongation factor 1 alpha* (*eF1α*). Data are means ± SE (n = 9–10).

development in different transformants. All three constructs directed similar *GUS* expression patterns with transcripts gradually increasing up to 20 DAF and then declining sharply until 30 DAF (Fig. 2-c). This inconsistency between *GUS* gene expression (Fig. 2-c) and GUS staining (Fig. 2-a) may be explained by the long half-life of GUS protein [48]. Consistent with GUS staining, the longer promoters could drive a higher transcriptional activity. Compared to pGUS950, pGUS650, and

pGUS300 *GUS* transcripts at 20 DAF were decreased by about 20% and 50%, respectively (Fig. 2-c). Thus, CCRM2 and CCRM3 could increase *Glu-1* expression level, whereas the 300-bp promoter was sufficient to shape *Glu-1* expression pattern during seed development.

3.4. The 300-bp promoter can ensure accurate initiation of *Glu-1* expression

To further specify the effects of CCRMs on the transcription initiation of *Glu-1*, we performed a precise comparison of *GUS* expression in transgenic seeds during the early seed developmental phase. In the initial experiments, no GUS was detected in all transgenic lines at 5 DAF (Fig. 2-a, c). Therefore, histochemical staining and qPCR assays were carried out to test GUS expression in young seeds every day from 6 to 9 DAF. The detection results showed that GUS staining first appeared at 8 DAF (Fig. 3-a), whereas *GUS* transcripts had accumulated to a certain level at 7 DAF in all transformants (Fig. 3-b). Thus *Glu-1* expression was initiated no later than 7 DAF. We also observed that GUS was initially expressed at the distal part of the endosperm and then spread towards the embryo in all transgenic lines (Fig. 3-a). In summary, CCRM3 and CCRM2 did not affect onset of *Glu-1* expression and the 300-bp promoter was enough to direct accurate initiation of *Glu-1* expression.

3.5. CCRM1-2 containing the Complete HMW Enhancer only regulates *Glu-1* expression level

Since the 300-bp promoter was sufficient to drive *Glu-1* spatiotemporal expression, we conducted further functional dissection of the region. Based on the distribution pattern of motifs, CCRM1 were further divided into two sub-CCRMs, CCRM1-1 (–208 to –101), and CCRM1-2 (–300 to –209) (Fig. 1). CCRM1-2 was represented by the Complete HMW Enhancer, which extended from –246 to –209 and had previously been reported to control both gene expression level and tissue-specificity in transgenic tobacco [29]. To test the functions of CCRM1-2, a pGUS208 construct was generated and transformed into wheat cultivar Fielder. Histochemical staining indicated that GUS protein was restricted to the endosperm and could not be detected until 15 DAF (Figs. 4-a; 5). Additionally, GUS activity in seeds of pGUS208 transformants was only one-third of pGUS300 at 30 DAF (Fig. 4-b). qPCR assays also confirmed that deletion of CCRM1-2 greatly reduced the *GUS* transcriptional level (Fig. 4-c). Overall, CCRM1-2 significantly affects the expression level but not endosperm-specificity of *Glu-1*.

3.6. CCRM1-1 is indispensable for *Glu-1* expression specificity and quantity

A pGUS100 was created and introduced into Fielder to investigate the function of CCRM1-1 (–208 to –101). Histochemical staining showed that the 100-bp core promoter (–100 to –1) failed to drive GUS expression in seeds (Fig. 4-a), but there was some *GUS* transcript accumulation in seeds detected by qPCR assays (Fig. 4-c). Apparently the 100-bp core promoter could still drive transcription of *Glu-1*, albeit at quite a low level. However, the transcriptional activity of the 100-bp core promoter (–100 to –1) was negligible compared to

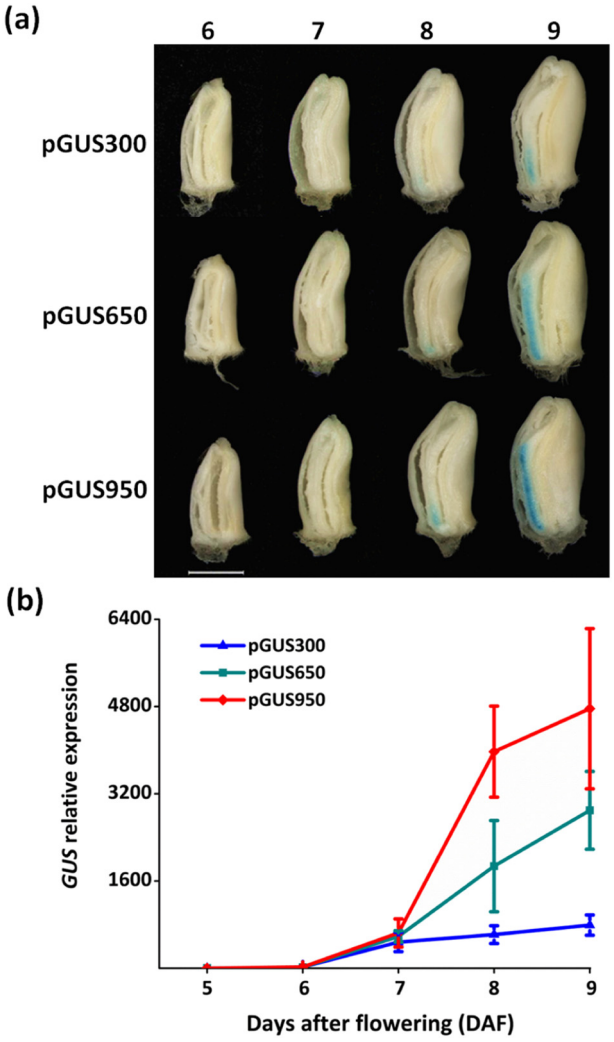

Fig. 3 – GUS expression in the transgenic seeds carrying pGUS300, pGUS650, or pGUS950 constructs at 6 to 9 DAF. (a) Histochemical analyses of the transgenic seeds. Seeds were collected every day and incubated in 1 mmol L^{-1} X-Gluc solution. Bars, 2 mm. (b) Transcriptional patterns of *GUS* reporter gene in developing seeds. The relative gene expression was normalized to *elongation factor 1 alpha* (*eF1α*). Data are means ± SE (*n* = 9–10).

pGUS208 (Fig. 4-c). No GUS activity was detected in all tested tissues except the rachilla of the spikelet (Fig. 5). As such, the 100-bp core promoter was unable to maintain endosperm-specific expression. Taken together, CCRM1-1 was critical for both expression level and endosperm-specificity of *Glu-1*.

4. Discussion

4.1. The N-motif, RY and Complete HMW Enhancer in CCRMs are not critical for endosperm-specific expression of *Glu-1*

In rice, the N-motif acts as an essential element regulating endosperm-specific expression and its internal removal or site-specific mutation abolishes endosperm-specific expression

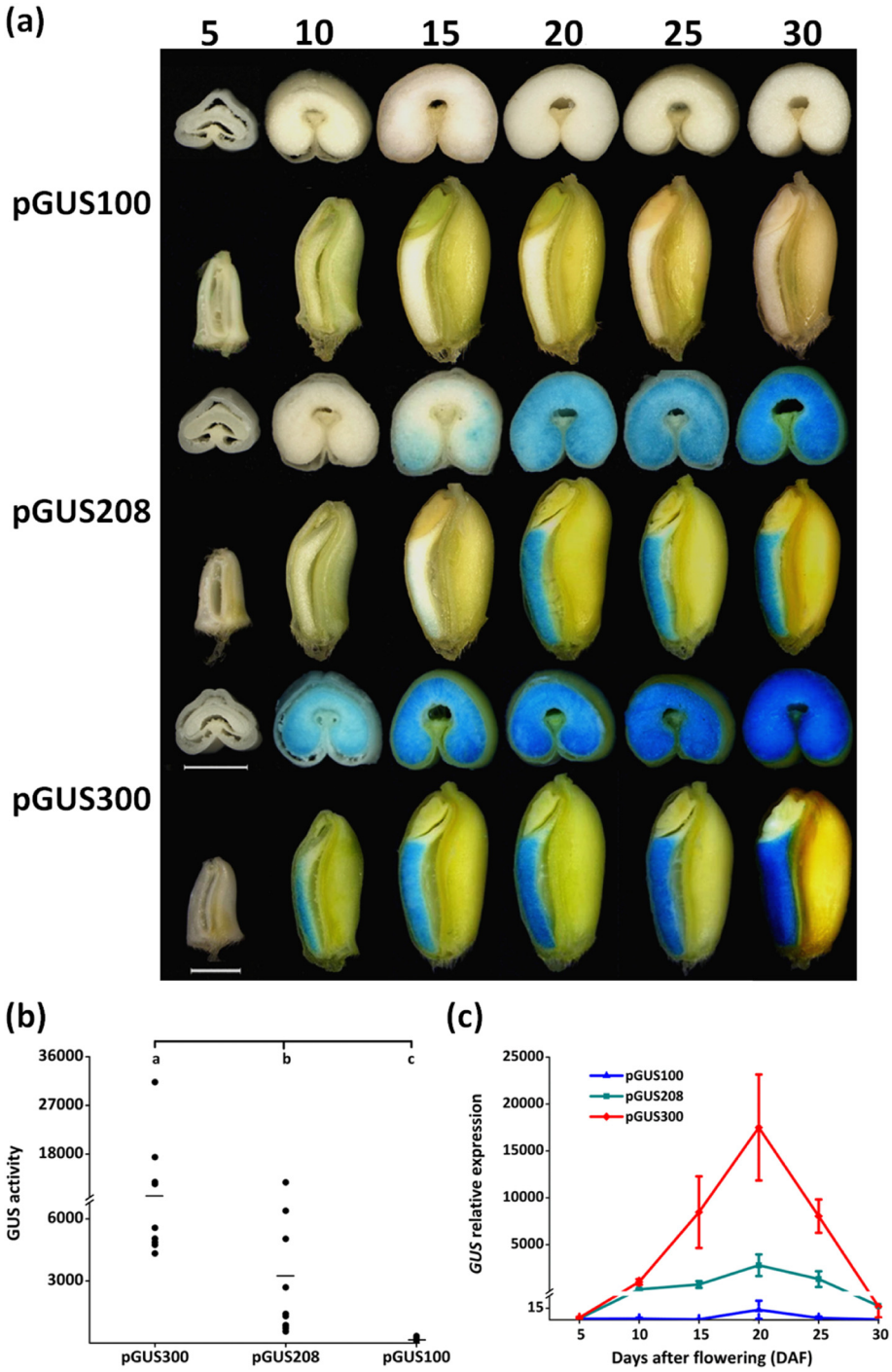

Fig. 4 – GUS expression in the transgenic seeds carrying pGUS100, pGUS208 or pGUS300 constructs. (a) Histochemical analyses of the transgenic seeds. Seeds were collected every 5 DAF and incubated in 1 mmol L^{-1} X-Gluc solution. Bars, 2 mm. (b) GUS activity in mature seeds at 30 DAF. Each point shows GUS activity from an independent line among the 9 or 10 lines. GUS activity was calculated as pmols 4-MU min^{-1} mg^{-1} protein. Horizontal bars indicate the average GUS activity. Different letters show significant differences ($P < 0.05$). 4-MU, 4-methylumbelliferone. (c) *GUS* gene expression in developing seeds. Relative gene expression was normalized to *elongation factor 1 alpha* (*eF1α*). Data are means ± SE (n = 9–10).

[24,25]. The RY element controls seed-specific expression in dicots, and its mutation eliminates promoter activity in seeds and causes gene expression in leaves [26,49]. In the present work, PLACE annotations of *Glu-1* promoters showed that all conserved RY and N-motif are located in CCRM2 and CCRM3 (Figs. 1; S1) and their deletion does not alter the endosperm-specific nature of the *Glu-1* promoter (Figs. 2-a, S2). The 300-bp promoter is able to confer endosperm-specific expression of

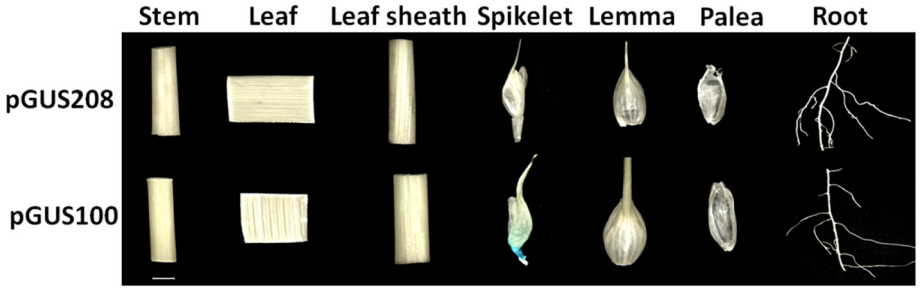

Fig. 5 – GUS staining of different tissues from the transgenic wheats carrying pGUS100 and pGUS208 constructs. Plant tissues were collected two weeks after flowering and incubated in 1 mmol L^{-1} X-Gluc solution. Bar, 2 mm.

Glu-1. Thus the N-motif and RY are not necessary for the endosperm-specific expression of *Glu-1*. This result is supported by previous work showing that a 338-bp *Glu-1* promoter was able to confer endosperm-specific expression in transgenic tobacco [50]. Moreover, seed-specific promoters containing the N-motif from wheat and barley were unable to direct seed-specific gene expression in transgenic rice [33]. Therefore, the N-motif and RY do not maintain a conserved function in directing endosperm-specific expression between wheat and other plants.

In transgenic tobacco, the Complete HMW Enhancer controls *Glu-1* expression specificity and levels [29]. To validate the function of the element, CCRM1-2 carrying the Complete HMW Enhancer was deleted from the 300-bp promoter. GUS staining showed that deletion of CCRM1-2 did not affect GUS expression specificity (Figs. 4-a; 5), indicating that the Complete HMW-Enhancer cannot regulate *Glu-1* expression specificity in wheat, which is different from Thomas and Flavell [29]. This result also demonstrates that the heterologous system cannot faithfully or fully reflect the real functions of *Glu-1* promoters.

4.2. $(CA)_n$, POA, and AACA motifs in CCRM1-1 are putative elements regulating Glu-1 expression specificity

Since pGUS208 still directed an endosperm-specific property, CCRM1-1 was further deleted from pGUS208 and the resultant pGUS100 construct was transformed into Fielder. No GUS staining was detected in all tested tissues in pGUS100 transformants except the rachilla of spikelets (Fig. 5). Therefore, the elements in CCRM1-1 should confer endosperm-specific expression of *Glu-1* but only three conserved motifs were identified in this region (Fig. 1). Among them, the POA (similar to the P-box) and AACA motifs only control gene expression level in seed [24], whereas the $(CA)_n$ motif regulates gene seed-specific expression in dicots [51]. Thus, (CA)n motif might have a role in *Glu-1* specific expression in endosperm. However, we did not rule out the possibility that the POA and AACA motifs or other non-annotated elements could function in *Glu-1* endosperm-specific expression. We generated a series of site-specific mutant versions of the 208-bp promoter for each of the $(CA)_n$, POA, and AACA motifs, as well as a 125-bp promoter (–125 to –1). The constructs containing these fragments fused with the GUS gene were firstly introduced into *Arabidopsis*. However, 208-bp promoter and its mutant or truncated derivatives were unable to drive GUS expression in seeds, indicating that the promoter was not activated in *Arabidopsis*. We further conducted transient expression assays in developing wheat seeds by biolistic-mediated transformation. All of the resulting promoters above were able to confer endosperm-specific property (Fig. S3). This result is consistent with transient assays in maize which showed that the 117-bp promoter of *Glu-1* was sufficient to drive gene expression in endosperm [30]. Transient gene expression by particle bombardment is affected by both the particle and DNA amount and is positively correlated with gene copy number, so it may not be a suitable approach to assess the effects of POA, $(CA)_n$, and AACA motifs, as well as the 125-bp promoter, on *Glu-1* expression. Thus, stable wheat transformation is prerequisite to identify the motifs responsible for endosperm-specific expression.

4.3. Many conserved motifs contribute to quantitative roles of CCRMs

The CCRMs were defined by 30 conserved motifs, most of which were reported to regulate SSP expression levels (Table 1). GUS activity and qPCR assays revealed that all CCRMs could promote *Glu-1* expression levels in the endosperm. The ABRE, P-box (POA), Skn-1, and AACA motifs have been reported to be quantitative motifs, and their roles in regulating *GluB-1* have been well characterized in rice [24,52]. Site-specific mutations of these motifs can result in at least a twofold decline in promoter activity. The RY and N-motif control both gene expression specificity and level, and their mutation also lead to loss of promoter activity in seed [23,28]. Although no TFs binding to the E-box and CCAAT box in *Glu-1* promoters have been isolated, evidence from other SSP genes indicates that they may participate in *Glu-1* expression [46,53]. Moreover, I-box, W-box, TOA and AMY might mediate crosstalk between *Glu-1* expression and other signaling pathways. HvMCB1, a light induced MYB TF in barley, can activate SSP synthesis and repress germination-related gene expression through interaction with the I-box [54,55]. The W-box, TOA, and AMY are motifs related to sugar signaling and activate or repress gene expression in sugar metabolism through interactions with TFs [47,56–58]. A recent study also demonstrated that O2 and PBF directly bind to promoters of starch biosynthetic genes in maize and regulate starch synthesis thus connecting sugar and protein metabolism [59]. Therefore, sugar metabolism and *Glu-1* expression may

be linked by *cis* motifs and their binding TFs. Collectively, the I-box, AMY, TOA, and W-box maybe mediate the cross-talk between *Glu-1* expressions and sugar metabolism, whereas the AACA, ABRE, P-box, E-box, RY, Skn-1, and N-motif probably regulate *Glu-1* expression in a quantitative manner.

4.4. CCRM1-2 is critical for accurate initiation of Glu-1

Previous studies showed that *Glu-1* genes initiate at the mid and late stages of seed development, ranging from 8 to 12 DAF in tobacco and durum wheat [9,29]. We first detected GUS staining at 8 DAF, but transcripts had already accumulated to a detectable level at 7 DAF in pGUS950, pGUS650, and pGUS300 transformants (Fig. 3-a, b). Thus the precise onset of *Glu-1* expression is defined as 7 DAF, earlier than previous reports. Deletions of CCRM2 and CCRM3 had no effect on initiation of *Glu-1* expression. The 300-bp promoter with CCRM1 was sufficient to ensure accurate initiation of *Glu-1* expression at 7 DAF (Fig. 3-a). However, appearance of GUS activity was delayed to 15 DAF when CCRM1-2 was removed from the 300-bp promoter (Fig. 4-a). Thus CCRM1-2 is probably important for *Glu-1* initiation. This delay in GUS appearance might be attributed to low transcriptional activity of the 208-bp promoter. pGUS208 directed only about one-third of *GUS* transcripts compared to pGUS300, and no GUS activity was observed until 10 DAF (Fig. 4-a, c).

4.5. Regulation of Glu-1 differs from other SSP genes

In this study, the 300-bp promoter was sufficient to direct spatial and temporal expression of *Glu-1*. Additionally, the endosperm box (–300 element) at around 300 bp upstream of the transcription start sites was identified as a critical *cis*-regulatory element for endosperm-specific expression of many other SSP genes [19–22]. To further uncover the common regulatory machinery, or key *cis*-regulatory elements for wheat SSP expression, we carried out promoter alignments between *Glu-1* and other major SSP genes such as *Glu-3*, *Gli-1*, and *Gli-2*, which code for low-molecular-weight glutenin subunits (LMW-GS) or gliadins, respectively. Unexpectedly, low sequence homology was detected among these promoters (Fig. S4). Although a few common motifs were identified among the promoters of the four gene families, they were arranged with different organizing modes (Tables S3, S4). Therefore, in terms of sequence alignment and motif distribution patterns, endosperm-specific expression of *Glu-1* may be regulated in a different transcriptional manner compared to other SSP genes in wheat.

5. Conclusions

In this study, we defined three CCRMs, CCRM1 (–300 to –101), CCRM2 (–650 to –400), and CCRM3 (–950 to –750) based on characterization of *cis* motifs within ten *Glu-1* promoters. A series of constructs, pGUS300, pGUS650, and pGUS950 were generated to evaluate the regulatory function of each CCRM in stable wheat transformants. GUS expression analysis showed that CCRM2 and CCRM3 enhanced transcriptional activity but had no effect on endosperm-specific expression of *Glu-1*. The 300-bp promoter spanning CCRM1 and core region (–100 to –1) was sufficient to direct the *Glu-1* expression pattern during seed development. Further dissections of the 300-bp promoter indicated that CCRM1-2 (–300 to –209) regulated only *Glu-1* expression level, whereas CCRM1-1 (–208 to –101) was not only indispensable for endosperm specificity but also critical for the level of *Glu-1* expression. This is the first time that this level of precision has been achieved in the functional dissection of *Glu-1* promoter in wheat. These results could enhance our understandings about *Glu-1* transcriptional regulation and set the groundwork to alter HMW-GS contents at the transcriptional level.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2018.08.003.

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