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ORIGINAL ARTICLE

bZIP transcription factors repress the expression of wheat (*Triticum aestivum* L.) high molecular weight glutenin subunit genes in vegetative tissues

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Abstract
High molecular weight glutenin subunits (HMW GS) represent an important fraction of endosperm-specific seed-storage proteins that provide elasticity to bread dough. Previously, the second *cis*-regulatory module (CRM2) was found to be one of the most conserved part of HMW GS promoters, which indicated its pre-eminent role in their gene regulation. Here, we observed that deletion of CRM2 from the promoters of the *Bx7* and *By8* HMW GS genes increased the leakage of their transient expression in wheat leaf tissue. The effect of a VP1, an Myb and an antisense bZIP transcription factor (TF)-binding site, potentially involved in endosperm-specific regulation within CRM2, was then studied. The activity of a *Bx7* gene promoter containing a mutant CRM2 with altered VP1 and Myb TF-binding sites, but an intact bZIP TF-binding site, was similarly low to that of the wild type in leaves. Transactivation analysis and EMSA indicated the binding of TFs TabZIP34 and TabZIP115 to the Skn-1 motif GTCAT in CRM2 and the repression of *Bx7* and *By8* HMW GS gene promoter activity in leaves. TabZIP34 and TabZIP115 may be involved in the downregulation of HMW GS gene expression in vegetative tissues and early-stage endosperm as well its modulation during seed maturation.

Keywords Gluten · Tissue-specific expression · EMSA · Prolamin

Introduction

Prolamins are important cereal seed-storage proteins supplying the germinating seedling with nitrogen and sulphur (Shewry and Halford 2002). Among the wheat prolamins, high molecular weight glutenin subunits (HMW GS) provide the elasticity in bread dough and are also a pivotal source of human protein uptake (Shewry and Halford 2002; Delcour et al. 2012). An ancient gene duplication event resulted in two paralogues (x and y) of HMW GS genes present in each of the A, B and D bread wheat genomes, of which Ax and Ay are frequently inactive (Payne et al. 1981; Jiang et al. 2009). The x-type HMWGS proteins are larger in size and accumulate at a higher level (Shewry and Halford 2002; Delcour et al. 2012). HMW GS genes coded at each of the A, B and D genomes may substantially contribute to bread-baking quality (Utebayev et al. 2019), while earlier studies underlined the role of Dx5 and Dy10 (Liang et al. 2010).

Given the importance of HMW GS proteins in end-use quality, it is essential to understand the regulation of their expression and possibly to increase their abundance within the wheat endosperm. Epigenetic and transcriptional regulation both have a role; however the latter is considered more important for HMW GS expression. A major feature of HMW GS gene promoters is their strength: only four or five active genes account for about 12% of the total seed protein content (Seilmeier et al. 1991; Halford et al. 1992). The functional regions of HMW GS gene promoters are also relatively long (around 2 Kb) and contain a number of different promoter motifs and predicted TF-binding sites such as RY, ABRE, bZIP, DOF, Myb, VP1, NAC and CBF (Wang et al. 2013; Makai et al. 2014; Ravel et al. 2014). Six *cis*-regulatory modules (CRMs) were identified in the

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1600-bp promoter region of HMW GS genes (Makai et al. 2014, 2015). Several components characteristic for other prolamin promoters, including the Endosperm box generally located around at −300 nucleotides from the start codon, are absent here (Makai et al. 2014). However, promoter motifs are enriched in DOF-binding sites (CRM1), and bZIP-binding sites (CRM3) are present in HMW GS gene promoters at a relatively short (ca. 200 bp) distance. Their cognate DOF and bZIP factors may interact via DNA bending, as DOF–bZIP interactions are characteristic in plant seeds (Agarwal et al. 2011). The connection between CRM1 and CRM3 is likely to be mediated by the intervening CRM2 module that contains a Viviparous1 [VP1, (Hoecker et al. 1995)], Myb, and bZIP TF-binding site (Makai et al. 2014, 2015). A DNA loop may be formed by a TF complex at CRM2 which brings CRM1 and CRM3 in proximity (Makai et al. 2014, 2015).

Other known elements of HMW GS gene promoters include the HMW enhancer, which is similar to the P-box. This motif provided transgenic plants with endosperm-specific expression, even when placed downstream of the transgene (Thomas and Flavell 1990). Distal promoter regions, too, were suggested to enhance HMW GS gene expression (Wang et al. 2013; Makai et al. 2014). The previously mentioned hairpin-like model (Makai et al. 2014) might explain the strength of these promoters, because transcription factors binding far from the transcription start site might also increase the gene expression.

HMW GS are endosperm-specific proteins under temporal and spatial control: their accumulation starts after 5 days and peaks between 15 and 20 days after anthesis (Makai et al. 2014). The role of storage protein activator (SPA) bZIP and GAMyb TFs is essential in the activation of their expression (Ravel et al. 2009; Guo et al. 2015). GAMyb has been shown to recruit histone acetyltransferases for epigenetic induction of HMW GS genes (Guo et al. 2015). The synthesis of seed-storage proteins like HMW GS is also influenced by environmental factors such as the temperature, as well as water, sulphur and nitrogen availability (Luo et al. 2000; Wardlaw et al. 2002; Xu et al. 2020). A focused transcriptional co-expression network constructed for HMW GS genes and regulating factors (Makai et al. 2015; Éva et al. 2018) revealed that the *Glu-1* paralogues (*x*- and *y*-type genes) belong to different regulatory circuits. The *x*-type genes were suggested to rely on Myb TFs, while the *y*-type genes are driven by NAC TFs during the late stage of grain filling (Makai et al. 2015), which may account for their differential regulation. The B3 TF TaFUSCA3 was also shown to activate *Glu-1Bx7* gene in the endosperm and interact with Spa bZIP TF (Sun et al. 2017).

As it was mentioned, HMW GS gene expression is blocked in vegetative tissues and early (0–5 days post-anthesis, DPA) endosperm. The lack of histone acetylation has been proven as being partially responsible (Guo et al. 2015; Éva et al. 2018). The inhibiting effect of Myb TFs in HMW GS expression in vegetative tissues has not been demonstrated yet, though it is common for other prolamin genes (Suzuki et al. 1998; Chen et al. 2005). Our transcriptional co-expression network analysis (Makai et al. 2015) revealed—among others—an early gene circuit of TFs expressing at the very beginning of endosperm development when HMW GS genes are not active yet; this circuit therefore may contain inhibitory TFs (Makai et al. 2015; Éva et al. 2018).

To clarify the mode of activation of HMW GS genes in the endosperm and their inhibition in other tissues, the functions of many HMW GS gene promoter elements, *cis*-regulatory modules and binding TFs have to be elucidated. These studies may be backed by the increasingly available wheat gene expression data (Borrill et al. 2016; Ramírez-González et al. 2018). Previously the mutation of the ABRE|CBF motif cluster (part of CRM1, from −280 to −177) enhanced leakage of HMW GS gene expression in leaves, a non-target organ (Makai et al. 2015), which indicates the role of the ABRE|CBF cluster in determining endosperm-specific expression. Here we studied the second *cis*-regulatory motif (CRM2), the most conserved part of HMW GS gene promoters (Makai et al. 2014, 2015) and possible interacting transcription factors using transient expression, and transactivation assays which are widely used methods to test promoter function, using either sense and antisense constructs (Oñate et al. 1999; Ravel et al. 2014; Makai et al. 2015).

Materials and methods

Generation of HMW GS promoter mutants

The activity of different HMW GS promoter mutants (see Fig. 1) was tested in transient expression assays and the binding TFs were studied (see below). All HMW GS promoter variants were custom synthesized in pIDTSmart (Amp) plasmid (Integrated DNA Technologies, Inc.). The first 1000 bp upstream of the start codon were taken as a promoter sequence, with the exception of the basal *Bx7* promoter, which was 177 bp in size. Promoter variants besides the wild-type *Bx7* promoter (GenBank ID: KC820629.1) as well as the wild-type *By8* promoter (GenBank ID: KC820630.1) controls included the basal (177 bp) *Bx7*, the CRM2del *Bx7*, the mutant CRM2m *Bx7*, and the double deletion *Bx7* promoter (CRM2 and the 103 bp-long ABRE|CBF|PBF motif cluster from − 288 to − 177 were both deleted). According to our previous analyses (Makai et al. 2014, 2015), the CRM2 region of *Bx7* promoter (− 588 to − 519) is the following (5´–3´ direction): A*ATGAC*AAGCAACAAAACCTGA AATGGGCTTTAGGAGAGATGGTTTATCAATTTACAT

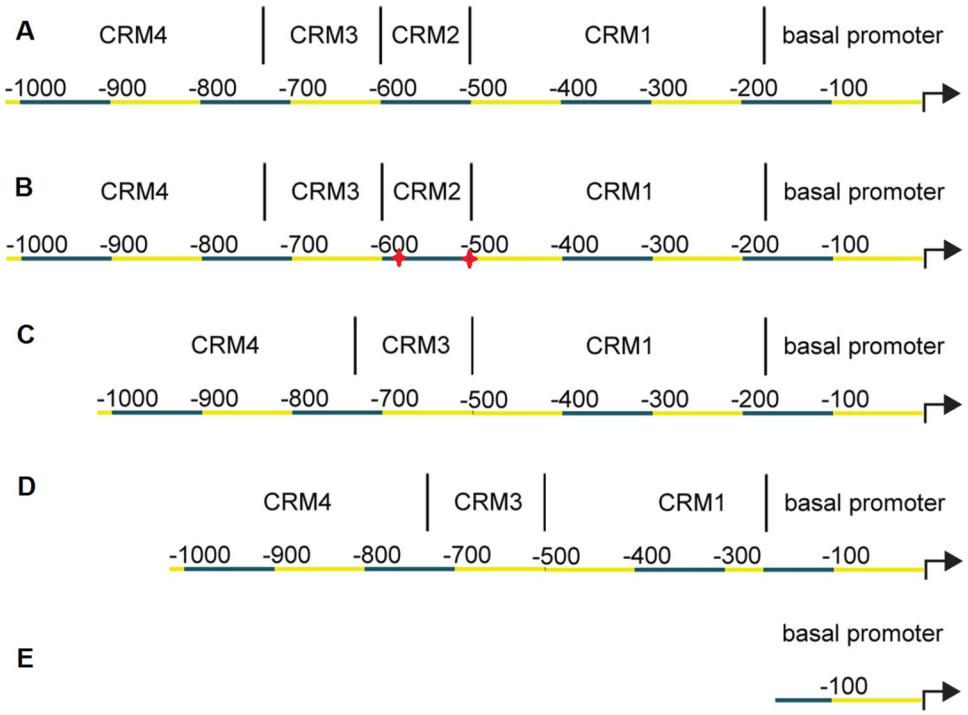

Fig. 1 *Bx7* HMW GS gene promoter variants tested in the study. Wild type (**A**), with mutant CRM2 region (**B**, CRM2m) containing mutated Myb (TGTGCC instead of AAC AAA) and VP1 sites (CTGT instead of CATGCA) positioned with red labels, CRM2 deletion (**C**, CRM2del), both CRM2 and ABRE|CBF deleted (**D**) and basal (177 bp) promoter (**E**)

GTTC**CATGCA**, whereas CRM2 of *By8* promoter (-521 to -465): T*ATGAC*AAGAAACAAAACATAAATGGGCTTT TGAAAGATGATTTATCAACTTACCTTATC**CATGCA** . Both CRM2 regions contain the predicted Myb-binding site AACAAA (underlined) and the predicted VP1 site CAT GCA (bold). There is also the predicted bZIP-binding site, the Skn-1 motif GTCAT in the other DNA strand (italic), in the reverse complement of both *Bx* and *By* CRM2. The full CRM2 region was deleted in CRM2del promoters, whereas in the mutant CRM2-containing promoter the Myb-binding site was changed to TGTGCC and the VP1 site to CTGT.

Transient expression assay

The high bread-making quality old Hungarian bread wheat variety 'Bánkúti 1201' served as plant material throughout the study. Activity of various HMW GS gene promoter variants was studied in leaves and bisected endosperms via transient expression of the GUS reporter gene using biolistic bombardment-mediated transformation. To this end, HMW GS gene promoter variants were subcloned to ShortCambia vector preceding the GUS reporter gene. The ShortCambia is our own derivative of the pCambia1391Z (GenBank ID: AF234312.1) and was constructed with the purpose of getting rid of background GUS activity of pCambia1391Z in plant tissues. For this purpose, the *Aat*II-*Hin*dIII fragment with the CAMV 35S promoter and the bacterial *lac* promoter and operon were removed and the small *Aat*II-*Hin*dIII fragment of the pWBVec8 (Wang et al. 1997) multicloning site was inserted in its place. This latter fragment does not contain any promoter. The studied promoters and their mutants were cloned between the *Hin*dIII (*Bx* promoters) or *Eco*RI (*By* promoters) and *Nco*I sites of ShortCambia, in front of the GUS gene. The promoter-containing ShortCambia vectors were bombarded into 'Bánkúti 1201' wheat leaves and bisected endosperms.

For leaf transformation, plants were grown in Jiffy pots in phytotron chambers (Conviron, Winnipeg, Canada) with 16 h day/8 h night photoperiod (80 µmol m^{-2} s^{-1} PAR) at 22 °C day/18 °C night and 75% relative humidity. Five-centimetre-long pieces of the oldest leaves from 2-week-old wheat plants were bombarded, five pieces being placed in the centre of a Petri dish. The leaves were placed on solid medium (0.5 wt% Phytoagar, 0.4 M mannitol, 10 µM thidiazuron, 1.65 g L^{-1} NH_4NO_3) 1 h before the transformation and remained there for 24 h afterwards (under the same light and temperature regime as before). Particle bombardment was carried out using the PDS 1000/He system (Bio-Rad). Gold particles of 1 µm diameter were coated according to the manufacturer's instructions. One and half µg of plasmid was coated onto 3 mg gold particles and suspended in 18 µL ethanol, and five µL suspension was used for a single shot. Each Petri dish containing five leaflets was shot once. The particle delivery system was adjusted to 1100 psi of helium pressure and 25 inches Hg of vacuum pressure inside the chamber. The distance between the stopping screen and the target was 6 cm. Transient expression was stopped 24 h after the bombardment by putting the leaves into GUS histochemical staining solution (1 mg mL^{-1} X-Gluc, 100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 1.2 mM

$K_4[Fe_2(CN)_6]$, 1.2 mM $K_3[Fe_2(CN)_6]$, 1 v% Triton X-100). The staining was performed overnight at 37 °C. Photosynthetic pigments were then removed by sequential washes with 20, 50, 70, and 100 v% ethanol. GUS spots were counted under a stereomicroscope.

For endosperm transformation, 'Bánkúti 1201' wheat plants were grown in pots in phytotron chambers (Conviron) using the Spring1 climatic programme (Tischner et al. 1997). Endosperms were aseptically isolated 2 h before bombardment from developing (21 DPA) wheat caryopses and placed at the middle of Petri dishes (eight endosperms in one Petri dish) containing Murashige–Skoog medium supplemented with maltose (100 g L^{-1}), casein hydrolysate (1 g L^{-1}) and 1 mM aurintricarboxylic acid, a nuclease inhibitor. Biolistic bombardment of endosperms was carried out identically to the leaves. The transient expression lasted for 4 days in the dark after the bombardment at room temperature. GUS staining was performed similarly to leaves, but the tissues were simply put in 70 v% ethanol after staining. Data are presented as mean values obtained from five independent experiments. Unpaired Student's *t* tests and ANOVA were performed on the data as statistical analysis.

Transactivation assay

To study the effect of various bZIP TF genes on HMW GS gene promoter activity in wheat leaves, antisense transactivation assays were carried out (Fig. 2). To this end, wheat bZIP TF coding sequences were cloned from 'Bánkúti 1201' wheat leaf cDNA. The leaf cDNA was obtained by reverse transcription (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) of total RNA extracted (RNeasy Plant Mini Kit, Qiagen) from the oldest leaves of 2-week-old wheat plants identically grown as for transient expression. Two primer pairs were designed to amplify and to clone the partial (half length) coding sequence of bZIP TF genes from wheat leaf cDNA (see Online Resource 1, available as Supplementary Information to this paper). As we assumed bZIP-based inhibition of HMW GS expression, preferably bZIPs with opposite expression characteristics (leaf, early-stage endosperm) than HMW GS were studied. One member was chosen for 8 of the 12 wheat bZIP phylogenetic groups (see Table 1), where we were able to amplify the TF gene (Li et al. 2015). Since antisense suppression is not entirely specific [see e.g., Moiseeva (2014)], suppressing with sequence of one member might suppress other members as well and provide information on which phylogenetic group contains the inhibitory TF. The second primer pair was basically the same as the first, but it also contained *Spe*I and *Nco*I restriction endonuclease recognition sites (see Online Resource 1, available as Supplementary Information to this paper). The partial bZIP coding regions were amplified from cDNA first using the restriction endonuclease siteless primers, then a second PCR was run on the PCR product, adding the restriction endonuclease sites. The second PCR products were cloned and sequenced using Clonejet PCR cloning kit (Thermo Scientific). The partial bZIP coding genes were then subcloned to a modified pGEM-T-Easy vector (Éva et al. 2014) containing the following gene construct: constitutive rice *actin* promoter (between *Apa*I and *Nco*I sites) fused to the *A. thaliana At2g37770.2* aldo–keto reductase gene (between *Nco*I and *Spe*I sites)::*Agrobacterium tumefaciens nos* poly(A) signal (between *Spe*I and *Not*I sites). Partial bZIP TF coding regions were cloned in antisense orientation between *Nco*I and *Spe*I sites, replacing the aldo–keto reductase gene. For transactivation assays, the pGEM-T-Easy

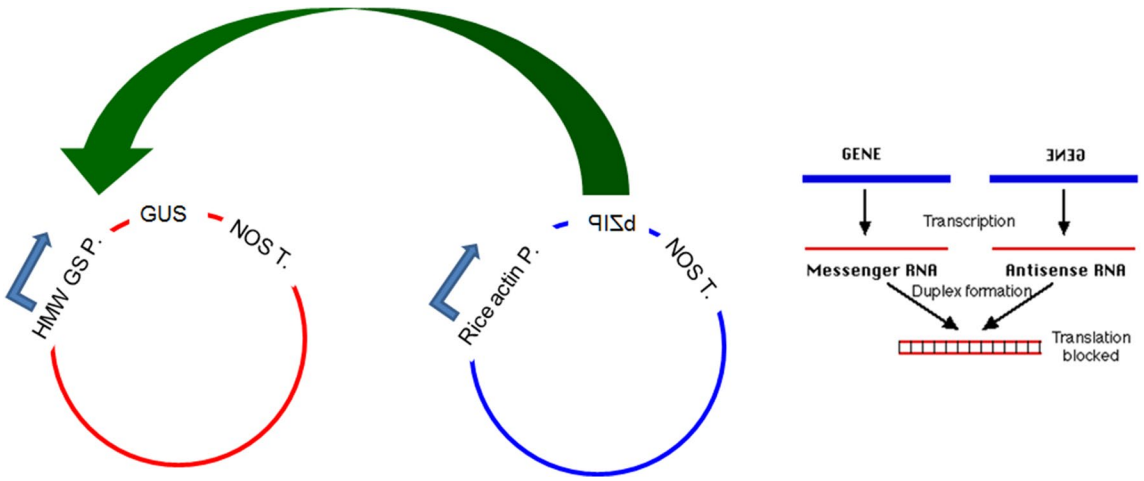

Fig. 2 Scheme of antisense transactivation assays for studying CRM2-binding TFs. For transactivation assays, the pGEM-T-Easy plasmids containing rice *actin* promoter::antisense bZIP CDS part::*nos* poly(A) constructs were co-bombarded into wheat leaves together with ShortCambia vectors containing HMW GS promoter-driven GUS reporter gene constructs. Antisense suppression of true repressors would release HMW GS promoters from inhibition and increase their activity

Table 1 Studied wheat bZIP transcription factors

Phylogenetic group	Studied member	2014 wheat genome survey seq ID	IWGSC Refseq v1.1 ID
A	TabZIP74	Traes_2BL_94E5996F7.1	TraesCS2B02G489900.1
B	Nd	Nd	Nd
C	TabZIP34	Traes_5AL_04D3E97F0.1	TraesCS5A02G440400.1
D	TabZIP94	Traes_4BL_DAFEC95DD.1	TraesCS4B02G178600.1
E	Nd	Nd	
F	TabZIP4	Traes_1AL_00A8A2030.2	TraesCS1A02G311900.1
G	TabZIP115	Traes_6BS_05264DAEA.2	TraesCS6B02G110100.1
H	Nd	Nd	
I1	TabZIP173	Traes_7DS_47B5A7FFF.1	TraesCS7D02G268400.1
S1	TabZIP43	Traes_5AL_D9A4DFF71.1	TraesCS5A02G179000.1
U1	TabZIP53	Traes_7AL_25850F96F.1	TraesCS7A02G398400.1
U3	Nd	Nd	Nd

Naming and classification were taken from by Li et al. (2015). 2018 Refseq IDs were also shown. We did not find leaf-specific members from phylogenetic group E and U3 in ExpVIP database and we could not amplify members from PG. B and H from'Bánkúti 1201' wheat leaf cDNA

plasmids containing rice *actin* promoter::antisense bZIP CDS::*nos* poly(A) signal constructs were co-bombarded to wheat leaves together with ShortCambia vectors containing HMW GS promoter-driven GUS reporter gene constructs (see Fig. 2). One µg of each vector (2 µg DNA in total) was coated onto 3 mg gold particles, which were then suspended in 18 µL ethanol. The biolistic bombardment, transient expression and GUS staining were otherwise performed as described above for transient expression. Data are presented as mean values obtained from five independent experiments. Unpaired Student's *t* tests and ANOVA were performed on the data as statistical analysis.

Electrophoretic mobility shift assay (EMSA)

To validate the binding of the TabZIP34 (TraesCS5A02G440400), TabZIP94 (TraesCS4B02G178600) and TabZIP115 (TraesCS6B02G110100) TFs to the CRM2 region of HMW GS gene promoters, full-length coding sequences of TabZIP34, TabZIP94 and TabZIP115 were amplified from 'Bánkúti 1201' wheat leaf cDNA using specific primers (Table 2). The TabZIP34 and TabZIP115 coding regions including stop codon were first amplified using restriction endonuclease siteless primers, then *Nde*I (to the 5' end) and *Not*I (to the 3' end) were added to the product during a second PCR with restriction endonuclease site-containing primers (Table 2). The second PCR products were cloned and sequenced using CloneJet PCR-cloning kit (Thermo Scientific). Full-length coding regions with correct sequence were then subcloned to the pET28C + expression vector, fusing them with N-terminal His-tag sequence. The sequence of TabZIP94 contains an *Nde*I restriction enzyme site; therefore, its full coding region was amplified without the stop codon, then re-amplified by adding an *Nco*I site and an *Not*I site to the 5´ and 3´ ends, respectively. It was eventually cloned between the respective sites of pET28C + expression vector, fusing with C-terminal His-tag, followed by the stop codon. The resulting plasmids were mobilized to Rosetta (DE3) *E. coli* cells for protein production. For protein expression, the Rosetta cells were grown in LB medium containing 50 mg L^{-1} kanamycin at 37 °C/200 rpm until reaching an optical density of 0.6–0.8 at 600 nm. Protein expression was then induced with 1 mM IPTG for 3 h while growing the bacteria further at 37 °C/200 rpm. The bacteria were then harvested and His-tagged proteins were extracted following the protocol of the Protino Ni–NTA agarose beads (Macherey–Nagel). Protein solubility was increased by adding 5 v% *n*-propanol at the cell lysis step. At the end, imidazole was removed from the samples and proteins were concentrated using Amicon Ultra-0.5 mL centrifugal filters (MWCO 10 kDa; Merck) while changing the solvent to 0.02 M potassium phosphate and 0.15 M sodium chloride (pH 7.2). Double stranded, 25 bp-long DNA probes for EMSA were prepared by mixing 2–2 µL 500 µM upper and lower DNA-strand oligos (Table 2) with 16 µL MQ water, heating the mixture at 65 °C for 5 min, then slowly cooling it down to room temperature and leaving for 24 h before use. The EMSA reactions were carried out according to Holden and Tacon (2011), with the following modifications: the reactions were prepared in a total volume of 15 µL containing 8 µg bZIP protein, 0.5 µL 50 µM dsDNA probe (see above), 0.1 µL (100 ng) LightShift Poly(dI-dC) competitor (Thermo Scientific), 1.4 µL 1 M DTT and 3 µL 5 × EMSA buffer (20 v% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl, 50 mM Tris–HCl, pH 7.4). For binding, the EMSA reactions were incubated for 30 min at room temperature, then separated

Table 2 Oligos used for cloning the full CDS of TabZIP34 and TabZIP115 and EMSA analysis

Oligo name	Sequence (5´–3´)	Product length
Tabzip34fullF	ATGCTCAAGCAGAAGCTCGA	900
Tabzip34fullR	CTATGAGGTCGATCCGGAC	
Tabzip34fullNdeIF	CATATGCTCAAGCAGAAGCTCGA	908
Tabzip34fullNotIR	GCGGCCGCTATGAGGTCGATCCGGAC	
Tabzip94fullF	ATGGCTGATGCCAGTTCGAG	1005
Tabzip94fullR	ACTCCCGTGGCCTCGCA	
Tabzip94fullNcoIF	CCATGGCTGATGCCAGTTCGAG	1015
Tabzip94fullNotIR	GCGGCCGCACTCCCGTGGCCTCGCA	
Tabzip115fullF	ATGGGGAGCAACGATCCTAGC	1050
Tabzip115fullR	TCAGTCAGGGCTCCTTCTGGT	
Tabzip115fullNdeIF	CATATGGGGAGCAACGATCCTAGC	1063
Tabzip115fullNotIR	GCGGCCGCTCAGTCAGGGCTCCTTCTGGT	
BxEMSA_Skn1us[a]	AATGACAAGCAACAAAACCTGAAAT	–
BxEMSA_Skn1ls[a]	ATTTCAGGTTTTGTTGCTTGTCATT	
BxEMSA_noSkn1us	ACAAAACAGCAACAAAACCTGAAAT	
BxEMSA_noSkn1ls	ATTTCAGGTTTTGTTGCTGTTTTGT	
ByEMSA_Skn1us	TATGACAAGAAACAAAACATAAATG	
ByEMSA_Skn1ls	CATTTATGTTTTGTTTCTTGTCATA	
ByEMSA_noSkn1us	TCAAAACAGAAACAAAACATAAATG	
ByEMSA_noSkn1ls	CATTTATGTTTTGTTTCTGTTTTGA	

[a]EMSA oligos with us in the name were for the upper strand, whereas oligos with ls were for the lower strand of 25 bp dsDNA probes

at 100 V on 3 wt% TAE-agarose gels stained with Midori Green Advance DNA dye (Zenon Bio Ltd.).

Bioinformatic promoter analysis

To analyse the function of the CRM2 region and corroborate the results obtained by transactivation assays and EMSA, a detailed analysis of CRM2 was carried out using CLC Genomics Workbench 8.3, searching for TF-binding sites and regulatory regions. TF-binding sites were collected from the PlantCare and PLACE promoter motif databases.

Results

Activity of *Bx7* promoter mutants

To better understand the functionality of HMW GS gene promoters, the activity of various *Glu-1Bx7* HMW GS gene promoter mutants cloned preceding GUS reporter gene was tested in transient expression assays. The basal (177 bp length) *Bx7* promoter region was still active and showed higher activity in leaves compared to the wild-type *Bx7* promoter (Figs. 3a, 4), Deletion of the whole CRM2 from the *Bx7* promoter significantly increased GUS expression in leaves (Fig. 3a: CRM2del, Fig. 4). Mutations of the VP1 and Myb TF-binding sites within CRM2 did not affect promoter strength in leaves, but decreased it in the endosperm (Fig. 3: CRM2m). Interestingly, a double mutant version without both CRM2 and ABRE|CBF (from − 288 to − 177; another inhibitory module (Makai et al. 2014, 2015) showed no activity in leaves (Fig. 3a: double del). All four *Bx7* promoter variants exhibited a similar, more than 50% decreased activity in the endosperm (Fig. 3b, Fig. 4).

Finding CRM2-binding bZIP TFs

The leaf-based inhibition of bZIP TFs on the HMW GS gene promoters, presumably binding to the CRM2 region, was further elucidated. A one–one member with high expression in leaves and early-stage endosperm was chosen for 8 of the 12 bZIP phylogenetic groups known in wheat (Table 1), where we were able to amplify the members (Li et al. 2015). TabZIP173 and TabZIP53 (groups I1 and U1, respectively) were already found to express at high level in various wheat tissues and may take part in heat stress tolerance (Agarwal et al. 2011). The tissue-specific expression pattern of TabZIP34 (group C) and TabZIP115 (group G) are shown as examples (Fig. 5). These factors show high expression in leaves and early-stage endosperm (2 DPA) and show lower, but detectable expression during later endosperm development (Fig. 5). The eight bZIP TF genes were used in antisense transactivation experiments. For this, two constructs were co-bombarded to wheat leaves, i.e. antisense

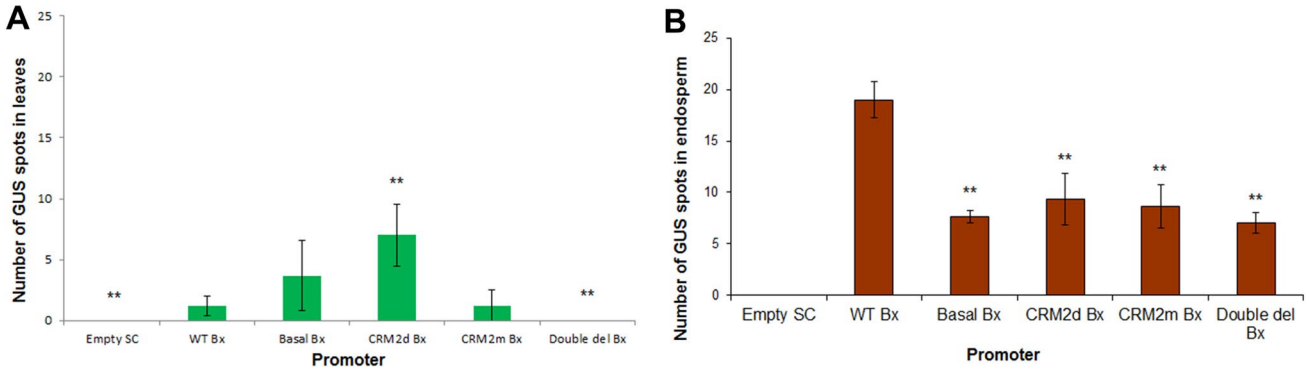

Fig. 3 Transient activity of different *Glu-1Bx7*::GUS reporter gene constructs expressed as the average number of GUS-positive blue spots in leaves (**A**) and bisected endosperm (**B**). Activity of the empty vector (termed ShortCambia, SC, a derivative of pCAMBIA1391Z with the bacterial *lac* promoter and operon removed), wt *Bx7* gene promoter, basal region of *Bx7* gene promoter (basal), and *Bx7* promoter mutants (CRM2del, CRM2m) in wheat leaves and endosperm. Both the CRM2 and the ABRE|CBF cluster were removed from double mutant *Bx* promoter. Number of independent replicates = 5. Significant differences from wt *Bx7* are indicated by *(95% level) or **(99% level)

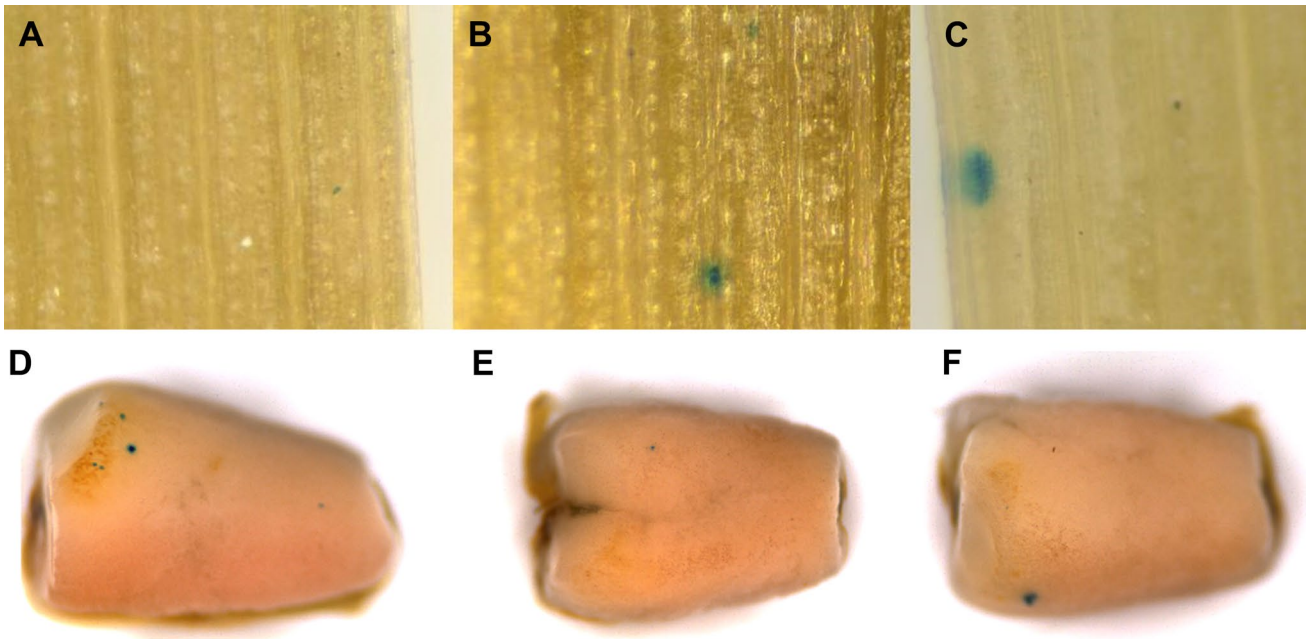

Fig. 4 Representative images of Bánkúti '1201' wheat leaves (**A**–**C**) and bisected endosperms (**D**–**F**) after transformation with GUS reporter gene constructs. Transient expression of wtBx7::GUS (**A, D**), basalBx7::GUS (**B, E**) and CRM2del Bx7::GUS (**C, F**) can be seen. Photosynthetic pigments have been removed from leaves using ethanol

half-lengths of each of the eight bZIP TF coding regions in antisense orientation and driven by the constitutive rice actin promoter, and a GUS gene driven by the two HMW GS gene promoter to be tested (Fig. 2). In our scheme, antisense suppression of true repressors would release HMW GS gene promoters from inhibition and increase their activity. The transactivation experiments yielded three and four TF candidates, which originally repressed the *Glu-1Bx7* (Fig. 6a: TabZIP4, TabZIP94, TabZIP115) and *Glu-1By8* (Fig. 7a: TabZIP34, TabZIP53, TabZIP94, TabZIP173) HMW GS genes in wheat leaves, respectively. The *Bx7*-repressing factors (TabZIP4, TabZIP94, TabZIP115) were also tested on the CRM2del *Bx7* promoter (Fig. 6b), and the *By8*-repressing factors (TabZIP34, TabZIP53, TabZIP94, TabZIP173) were tested on the CRM2del *By8* promoter (Fig. 7b). As a result, the activity of CRM2del promoters did not change significantly, which may indicate that the respective factors are able to bind to the CRM2 region.

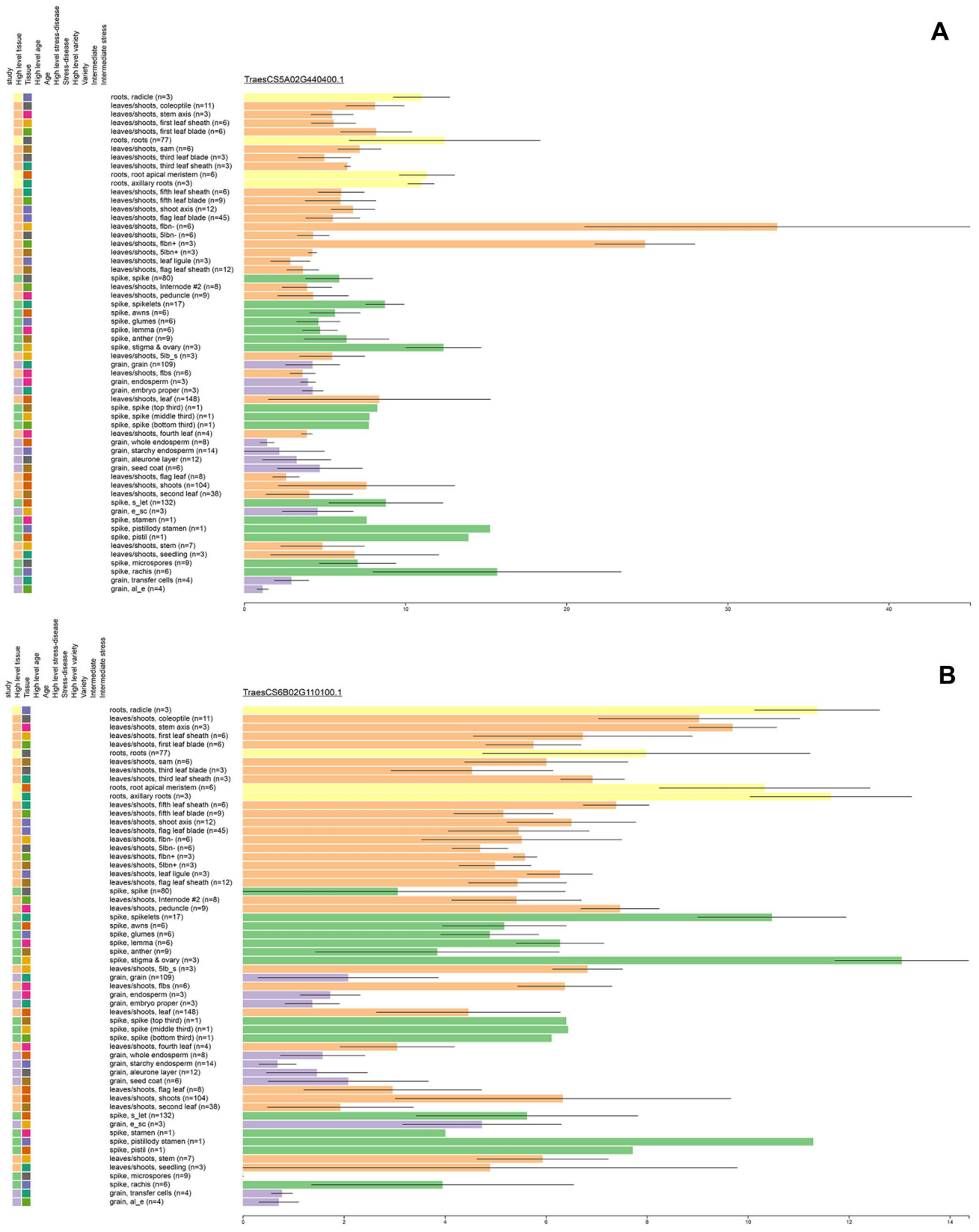

Fig. 5 Tissue-specific expression (TPM) of wheat TabZIP34 (**A**) and TabZIP115 (**B**) in the ExpVIP database (http://www.wheat-expression.com/)

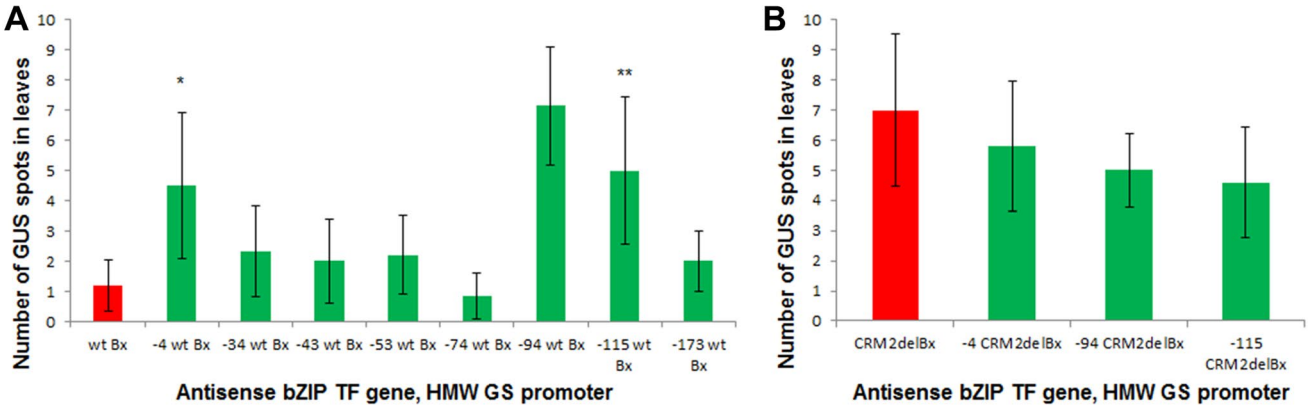

Fig. 6 Results of transactivation assays for studying CRM2-binding TFs in the *Glu-1Bx7* HMW GS promoter. Results of such transactivation assays on wt *Bx7* (**A**) and CRM2del *Bx7* (**B**) promoters in 'Bánkúti 1201' wheat leaves. bZIP TFs showing high expression in leaves and early endosperm development (based on ExVIP wheat gene expression browser, http://www.wheat-expression.com/) were used as antisense constructs. Significant differences from the wild-type gene promoter alone are indicated by *(95% level) or **(99% level)

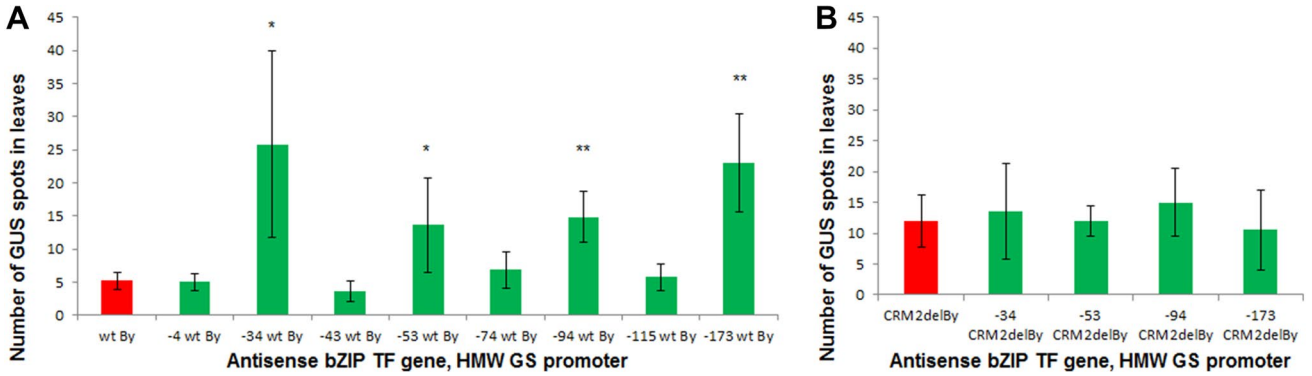

Fig. 7 Results of transactivation assays for studying CRM2-binding TFs in the *Glu-1By8* HMW GS promoter. Results of such transactivation assays on wt *By8* (**A**) and CRM2del *By8* (**B**) promoters in 'Bánkúti 1201' wheat leaves. bZIP TFs showing high expression in leaves and early endosperm development (based on ExVIP wheat gene expression browser, http://www.wheat-expression.com/) were used as antisense constructs. Significant differences from the wild-type gene promoter alone are indicated by *(95% level) or **(99% level)

Confirmation of the promoter-binding affinity using EMSA

Among the found HMW GS-repressing bZIP TFs, we considered TabZIP94, TabZIP34 and TabZIP115 as the strongest hits. TabZIP94 was the only bZIP TF gene whose antisense suppression increased the promoter strength of both *Bx* and *By*, whereas homologues of TabZP34 and TabZIP115 were previously identified within the early genetic programme to both TabZIP34 (87.8% aa. identity to Tae064063 in our earlier publications, RefSeq ID: TraesCS5D02G447500) and TabZIP115 (76.8% aa. identity to Tae057404 in our earlier publications, RefSeq ID: TraesCSU02G019200) which express at early phase (0–5 DPA) of endosperm development (Makai et al. 2015; Éva et al. 2018). HMW GS genes do not express at this developmental stage and therefore this programme may contain their suppressor factors (Makai et al. 2015; Éva et al. 2018). Indeed, both TabZIP34 and TabZIP115 show much higher expression in grains at 2 DPA than at later stages (14–35 DPA), but TabZIP94 is also highly expressed later, according to ExpVIP wheat expression browser (Fig. 8). Genuine binding of TabZIP34, TabZIP94 and TabZIP115 to the CRM2 region of HMW GS gene promoters was validated here using electrophoretic mobility shift assay (EMSA). The TFs reacted with 25 bp dsDNA probes (see Table 2) from the CRM2 region of *Glu-1Bx7* and *Glu-1By8* gene promoters. Both wild-type sequence probes (Bx+, By+) containing the predicted bZIP-binding site, the Skn-1 motif: GTCAT, and mutated probes (Bx-, By-) containing the GTTTT sequence were tested. TabZIP34 and TabZIP115 could bind to both promoters (Fig. 9a). In case of the *Glu-1By8* gene promoter,

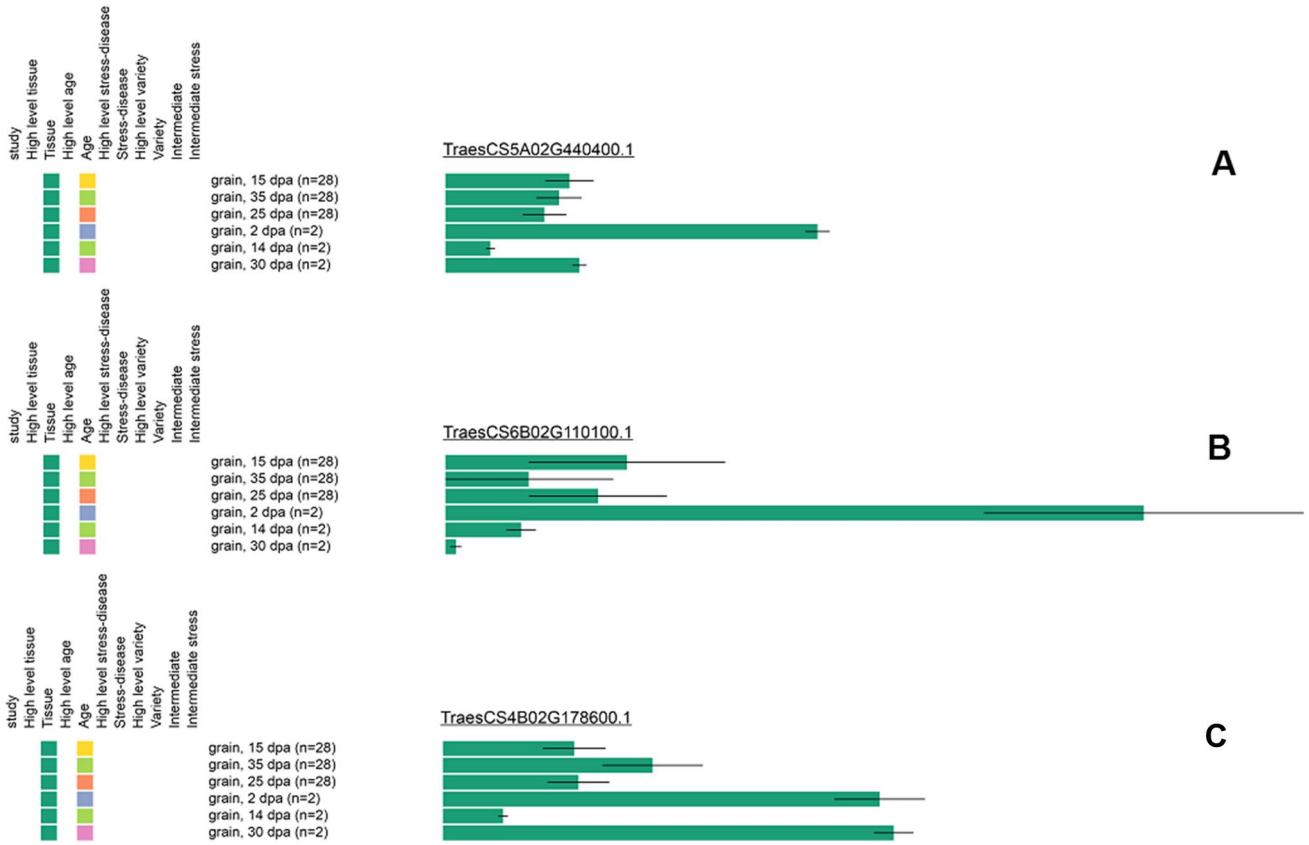

Fig. 8 Expression level (TPM) of TabZIP34 (**A**), TabZIP115 (**B**) and TabZIP94 (**C**) in developing wheat grain between 2 and 35 days post-anthesis (DPA), based on the ExpVIP wheat gene expression browser (http://www.wheat-expression.com/)

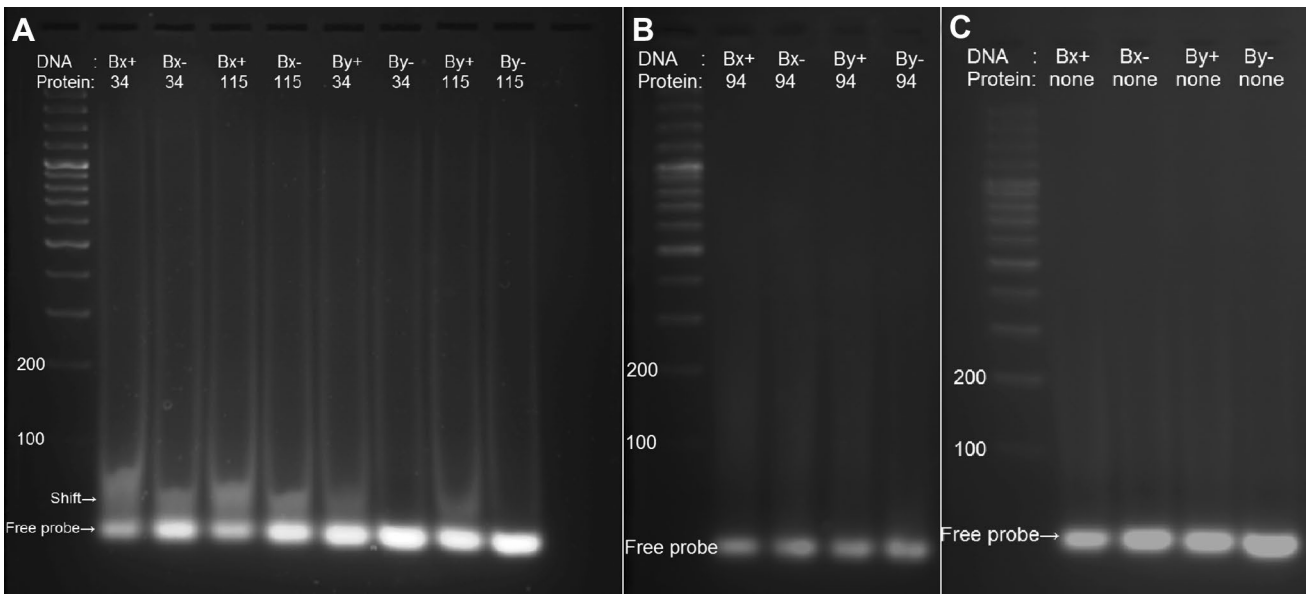

Fig. 9 EMSA validation of the binding of TabZIP34 and TabZIP115 TF (**A**) as well as TabZIP94 (*b*) proteins to 25 bp dsDNA probes from the CRM2 region of HMW GS gene promoters (*Glu-1Bx7*, *Glu-1By8*). Bx + and By + probes included the predicted bZIP-binding site GTCAT, while the mutant probes (Bx-, By-) contained the sequence GTTTT. All reactions included poly(dI-dC) competitor. Protein containing reactions can be seen on (**A**, **B**) and protein-free reactions on (**C**). Shifts indicate protein binding

they bound only the wild-type probe, whereas in case of the *Glu-1Bx7* gene promoter they also bound to the mutated probe, though with less affinity. However, TabZIP94 did not bind any of the DNA probes (Fig. 9b).

Further TF-binding sites and regulatory regions within CRM2

The second *cis*-regulatory motif (CRM2) of HMW GS gene promoters has been identified in our previous works (Makai et al. 2014, 2015) as containing an ABI/VP1, an Myb and a bZIP TF site. However the fast development of molecular biology including the description of new TF-binding sites prompted us to carry out a new analysis on this promoter region. It was also aimed to better understand the found dual activating and suppressing function of CRM2. The new detailed analysis of CRM2 uncovered many new TF-binding sites and regulatory regions within CRM2, such as WRKY, BELL, DOF, FUS3, Rav1, starch synthesis box (SBX), GATA motif and anaerobic response element (ARE) (Table 3).

Discussion

In this study, transient expression assays revealed that the second *cis*-regulatory module (CRM2) of HMW GS gene promoters contributes to their endosperm specificity by providing inhibition in vegetative tissues. CRM2 binding and inhibitory effect of TabZIP34 and TabZIP115 TFs on HMW GS expression was also demonstrated. Furthermore, new TF-binding sites (e.g. WRKY, Bell, Myb, and DOF) were identified within CRM2. Some of the cognate TF counterparts might increase HMW GS expression in late-stage endosperm as activity of the CRM2-less promoter mutant was lower in its native tissue, compared to the wild-type promoter (Fig. 3). Notably, the promoter with mutant VP1 and Myb sites in CRM2 also showed lower activity (Fig. 3). VP1 was shown to be a positive regulator of seed maturation in maize (Hoecker et al. 1995), and a VP1-binding CRM2 may have a similar function in wheat. Interaction between zinc finger TFs and bZIPs is well known. Such interactions between CRM2-binding TFs may also serve as a basis for further research. We would underline here the GATA zinc finger factors, which have been implicated in nitrate-dependent control of transcription (Reyes et al. 2004). Possible interactions of GATA TFs with the inhibiting bZIPs TabZIP34 and TabZIP115 might serve as an additional explanation for the inhibitory role of these factors in endosperm. Altogether, we consider CRM2 as another dual activating/inhibiting element just like the previously identified ABRE|CBF (Makai et al. 2015).

Our results shed new light on the function of CRM2 promoter region. In a comparable work, Li et al. (2019) described the so-called second conserved *cis*-regulatory module (CCRM2) in HMW GS gene promoters, spanning from − 650 to − 400. It is around the same region, though longer than our CRM2 (− 588 to − 519 in *Bx7*; − 521 to − 465 in *By8* promoter). Some of the same binding sites were identified during the two works (RY, Skn-1, RAV1), but different conclusions were made on the role of the promoter elements. Based on truncated (300-, 650- and 950-bp) promoters and on the fact that the 300-bp promoter was still endosperm specific, the authors (Li et al. 2019) concluded that CCRM2 might increase the expression of HMW GS genes, but does not contribute to its tissue specificity. However, regulation of a weak 300-bp promoter can be different from a 1000-bp highly active promoter, as the latter may bind more activating and inhibiting factors and its structure can be different, DNA-bending included (Makai et al. 2014, 2015). When we deleted CRM2 from the *Glu-1Bx7* HMW GS gene promoter, its activity appeared in other tissues than the endosperm target, which indicates a role of CRM2 in determining tissue specificity (cf. Figs. 2 and 3). Interestingly, a double mutant

Table 3 Promoter elements identified within the CRM2 region

Bx7 CRM2 (− 588 to − 519)			By8 CRM2 (− 521 to − 465)		
Name	Sequence	Location	Name	Sequence	Location
Skn-1	GTCAT	− 587 (−)	Skn-1	GTCAT	− 520 (−)
WRKY-binding site	TGAC	− 586 (+)	WRKY-binding site	TGAC	− 519 (+)
Bell TF-binding site	TGTCA	− 586 (−)	Bell TF-binding site	TGTCA	− 519 (−)
Rav-1	CAACA	− 579 (+)	–	–	–
Myb-binding site	AACAAA	− 577 (+)	Myb-binding site	AACAAA	− 510 (+)
SBX	AAGCCC	− 564 (−)	SBX	AAGCCC	− 497 (−)
DOF-binding site	AAAG	− 560 (−)	DOF-binding site	AAAG	− 494 (−)
DOF-binding site	TAAAG	− 560 (−)	DOF-binding site	AAAAG	− 494 (−)
ARE	TGGTTT	− 547 (+)	ARR1	TGATT	− 482 (+)
GATA motif	GATA	− 543 (−)	GATA motif	GATA	− 473 (−)
VP1-binding site	CATGCA	− 514 (+)	VP1-binding site	CATGCA	− 460 (+)

version without both CRM2 and ABRE|CBF (another inhibitory module, (Makai et al. 2014, 2015) showed no activity in leaves. Previously, it was found that ABRE|CBF deletion might considerably alter the hairpin promoter structure (Makai et al. 2015), and the resulting promoter had little activity in leaves or the endosperm. Deletion of CRM2 could not strengthen this promoter during the present work. On the other hand, the 177 bp-long (spanning from 0 to 177 bp) basal promoter was not tissue specific and it was still functional. Taken together, HMW GS promoters can be considered as long promoters with a complex structure, containing various activating and inhibiting elements as well.

We also showed here by transactivation assays and EMSA experiments that the suppressing effect of CRM2 in leaves is achieved by binding of bZIP TFs such as TabZIP34 and TabZIP115 to the single Skn-1 motif present in CRM2. In fact, antisense suppression of many bZIP TFs increased HMW GS promoter activity in leaves (Figs. 6 and 7). bZIP TFs are common among eukaryotes and due to their early evolutionary origin are highly variable: sequence identity between different bZIP TFs may be lower than 20%, so it is remarkable that various bZIP TFs could have identical effects (Nijhawan et al. 2008). Interestingly, mainly different TFs repressed here *Bx7* (TabZIP4, TabZIP94, TabZIP115) and *By8* (TabZIP34, TabZIP53, TabZIP94, TabZIP173) gene promoters, with TabZIP94 (phylogenetic group D) being the only common factor. Besides TabZIP94, TabZIP34 (phylogenetic group C) and TabZIP115 (phylogenetic group G) were further strong hits because they had been supported by previous bioinformatic analysis. Their close homologues were part of the early genetic programme, which may contain suppressor TFs (Makai et al. 2015; Éva et al. 2018). Binding of TabZIP34 and TabZIP115 to the CRM2 region of HMW glutenin gene promoters was confirmed using EMSA experiments (Fig. 9a). However, TabZIP94 did not bind CRM2 in EMSA (Fig. 9b), though its suppression boosted the *Bx7* and *By8* promoters in the transactivation assays. A plausible explanation can be that antisense constructs can suppress related genes as well; therefore in reality, not TabZIP94, but another bZIP TF from the same phylogenetic group D may have participated in the suppression of *Bx* and *By* HMW GS genes. Though TabZIP34 appeared to be a repressor of *By* and TabZIP115 a repressor of *Bx* in transactivation experiments, both of them could bind both promoter fragments in EMSA. The reverse complement of both *Bx7* and *By8* CRM2 region contain the GTCAT Skn-1 motif, but the adjacent regions are a little different (GCTTGTCATT in *Bx7* and TCTTGTCATA in *By8* promoter). Together with interactions with other TFs that in vivo bind the two different promoters, these context differences might have caused the observed discrepancy.

Earlier work showed that bZIPs like the storage protein activator (SPA) bZIP activate and not repress HMW GS gene expression in the endosperm (Albani et al. 1997; Ravel et al. 2009). However TabZIP34, also called SPA heterodimerizing protein (SHP), was already identified as a repressor of wheat HMW GS expression in endosperm (Boudet et al. 2019). Based on our results, we would add that many other bZIP factors might be responsible for this inhibition, and it is more characteristic for vegetative tissues and early-stage endosperm than during seed maturation. Boudet and co-workers (Boudet et al. 2019) identified the GLM2 (TGTGAGTCA) and G-box (TTACGTGG) as the binding sites of SHP. These are different sites than the Skn-1 motif that we identified as a binding site for TabZIP34 and TabZIP115 (GTCAT). The Skn-1 motif (G/ATCAT) is a known element needed for endosperm-specific expression, and also frequent in promoters of heat-shock proteins (Washida et al. 1999; Chandel et al. 2013), but up to now the cognate TF in plants remained obscure, to our knowledge.

Hardly any information has been available on the inhibition of HMW GS genes in vegetative tissues, although some elements of epigenetic (histone deacetylation) and transcriptional regulation-based inhibition (ABRE|CBF) were known (Guo et al. 2015; Makai et al. 2015; Éva et al. 2018). Cereal seed-storage protein genes are generally repressed by Myb TFs in vegetative tissues (Suzuki et al. 1998; Chen et al. 2005). However, the regulation of HMW GS genes is different from other storage proteins. The repression by Myb factors has not been reported on HMW GS gene promoters to date. When we mutated the Myb-binding site AACAAA in CRM2, it had no effect on the HMW GS gene promoter strength in leaves (Fig. 3). In case of HMW GS genes, bZIP TFs might replace Mybs in this suppressor function. Our present results show that the second *cis*-regulatory module (CRM2) in HMW GS gene promoters has a dual function: inhibiting HMW GS expression in vegetative tissues and early-stage endosperm, but activating it during seed maturation. Here, we focused on the inhibition aspect: TabZIP34 and TabZIP115 were the responsible TFs for spatio-temporal HMW GS gene suppression. Their lower, but detectable expression in late-stage endosperm indicates that these TFs may still have a function during later seed development, probably inhibiting HMW GS synthesis under adverse environmental conditions, like low N availability.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11738-022-03503-6.

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Author contributions statement CÉ designed the experiments with the help of KM, SM and AJ. CÉ, BM, KS-P and VK carried out most of the research. CÉ wrote the manuscript with the help of LS and AJ. All authors read and approved the manuscript.

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Code availability Not applicable.

Availability of data and material (data transparency) All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval We accept that the manuscript will become the property of Springer after publication.

Consent to participate Not applicable.

Consent for publication (include appropriate statements) All authors read and approved the manuscript.

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