The wheat HMW-glutenin *1Dy10* gene promoter controls endosperm expression in *Brachypodium distachyon*

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The grass species *Brachypodium distachyon* has emerged as a model system for the study of gene structure and function in temperate cereals. As a first demonstration of the utility of *Brachypodium* to study wheat gene promoter function, we transformed it with a T-DNA that included the *uidA* reporter gene under control of a wheat High-Molecular-Weight Glutenin Subunit (HMW-GS) gene promoter and transcription terminator. For comparison, the same expression cassette was introduced into wheat by biolistics. Histochemical staining for β-glucuronidase (GUS) activity showed that the wheat promoter was highly expressed in the endosperms of all the seeds of *Brachypodium* and wheat homozygous plants. It was not active in any other tissue of transgenic wheat, but showed variable and sporadic activity in a minority of styles of the pistils of four homozygous transgenic *Brachypodium* lines. The ease of obtaining transgenic *Brachypodium* plants and the overall faithfulness of expression of the wheat HMW-GS promoter in those plants make it likely that this model system can be used for studies of other promoters from cereal crop species that are difficult to transform.

Introduction

Heterologous production of valuable compounds in cereal grains requires the use of transcriptional control elements that can support high levels of expression in their seed storage tissues, i.e., endosperm. To avoid side effects on other plant processes, it would be ideal if the expression of such promoters were limited to the endosperm. The promoters of the wheat *Glu-1* genes are candidates for such promoters in that each of these single copy genes supports accumulation of its High-Molecular-Weight Glutenin Subunit (HMW-GS) product to 1–2% of the proteins in wheat endosperm. Tightly linked pairs of *Glu-1* genes are found on the long arms of each of the wheat group 1 chromosomes. One member of each pair encodes a y-type HMW-GS and the other an x-type subunit.[1] In addition to their allelic names, the genes can be referenced by the HMW-GS they encode (for example *Glu-D1-1b* by *1Dx5* and *Glu-D1-2b* by *1Dy10*).

Wheat genomic fragments containing intact *Glu-1* genes, including both 5′ and 3′ control regions, have been transformed into wheat, rye, and tritordeum (a wheat/barley hybrid), as well as into other cereals including rice, sorghum, and maize.[2-14] Intact *Glu-1* genes have also been introduced into tobacco.[15] The HMW-GS encoded by these genes accumulated in the seeds of each of these plants, demonstrating that native wheat *Glu-1* promoters can support endosperm expression, even in a species as distantly related as tobacco. Transgene expression in non-seed tissues was examined in only two of these studies. In the maize transgenics, no HMW-GS was detected in immunoblots of protein extracts from embryos, 14- and 58-d leaves, anthers, mature pollen or young ears from the same plants that contained readily detectable 1Dx5 subunit in their endosperm.[14] In the tobacco transgenics, no *Glu-1* mRNA was detected in leaves.[15]

To study promoter function, DNA fragments containing various *Glu-1* gene promoters have been used to express reporter genes in wheat, barley, rice, maize, oats, and tobacco. A 1251 base pair (bp) version of *1Dx5* gene promoter extending from 4 bp upstream of the translation initiation codon supported endosperm but not aleurone expression of the *uidA* reporter gene in transgenic wheat, starting at 10 d after pollination (DAP).[16] The *uidA* transcript was not detected by RT-PCR of RNA from leaves, inflorescences, florets, roots, embryos or in caryopses 5–7 DAP. The same construction supported expression of *uidA* in both endosperm and aleurone of transgenic oat plants, beginning 12 DAP.[17] No β-glucuronidase (GUS) activity was detected by the histochemical assay in oat embryos, the outer seed envelope, leaves, roots or florets.[17] Norre and colleagues studied the promoter

activity of variant versions of the *1Dx5* gene promoter in transgenic maize.[18] Versions containing a duplication or triplication of the region 225 to 136 bp upstream of the transcription start site supported higher levels of endosperm expression than the 417 bp native promoter. No GUS activity was detected in the embryo, pericarp, leaves, or roots of these maize plants.[18] A 425-bp promoter fragment (coordinates not specified) from the *1Dy12* allele supported green fluorescent protein (GFP) gene expression only in endosperm and aleurone from 7 DAP (earliest measured) through 24 DAP (latest measured) in transgenic wheat.[19] GFP fluorescence was not detected in glumes, lemma, palea, ovary, anthers, anther filament, stigma, leaf, or root tissues, or in the pericarp, embryos or vascular parenchyma of seeds at the same stages.[19] The same report noted similar results were obtained for one transgenic barley event containing the same construct.[19] In transgenic rice however, the same construction was active not only in endosperm and aleurone, but also in the pericarp, and in vascular parenchyma of seed, leaf and root tissues.[20] In contrast, a 251 bp promoter fragment from the *1Bx17* gene with a modified 5′ untranslated region that included the rice *Act1* intron exhibited tissue specificity when fused to *uidA* and transformed into rice.[21] A *1Dy12* gene promoter fragment similar to the one used by Furtado and colleagues had previously been shown to support expression of the *Chloramphenicol Acetyltransferase* (*CAT*) reporter gene specifically in the endosperm of transgenic tobacco beginning 8 DAP.[15,19,20] A larger promoter fragment consisting of 2600 bp upstream of the *1Dy12* gene also supported endosperm-specific expression of the *CAT* and *uidA* reporter genes in tobacco.[15,22] Halford and colleagues showed that 295 bp of the *1Dx5* gene promoter were sufficient to support tissue-specific expression of *uidA* in tobacco.[23]

The finding that the tissue specificity of the wheat *Glu-1* promoter was preserved in transgenic tobacco was somewhat surprising and highlights the importance of having good model systems for testing the functionality of genes from wheat and other species that are difficult to transform. For promoter functional testing, tissue specificity is a more important parameter than quantitative expression levels, but both are needed for characterization. In recent years, the grass *Brachypodium distachyon* has emerged as a model plant for the study of temperate cereals. It has a small genome that has been sequenced, a generation time of a few months, and requires much less growing space than wheat or barley.[24,25] Efficient transformation systems and resources for forward and reverse genetics have been developed.[26-28] However, there have been no published reports to date of heterologous promoter expression studies in *Brachypodium distachyon* transgenic plants.

The seed storage proteins of *Brachypodium distachyon* consist mainly of salt-soluble globulins and glutelins (salt-insoluble globulins).[29-31] Although there are several *Brachypodium* genes that encode the alcohol-soluble prolamine types of storage proteins, they accumulate to less than 5% overall of the seed proteins. In this regard, *Brachypodium* is more like oats and rice than it is like wheat, barley or maize in which prolamines are the dominant seed storage proteins. Orthologs of the wheat *Glu-1* genes have been found in the syntenic regions of the *Brachypodium* genome, but they contain stop codons that prevent the synthesis of HMW-GS-like proteins.[32] Thus, it is difficult to predict whether or not the wheat *Glu-1* gene promoters would be faithfully regulated in *Brachypodium*.

In the research reported here, we examine the expression specificity of the wheat *1Dy10* gene promoter in *Brachypodium* by documenting the activity of a *uidA* transcriptional fusion construct in transgenic plants. The expression results for the same expression cassette in transgenic wheat are included for comparison.

Results

An endosperm expression cassette for use in transgenic plants was constructed by fusing a 2936 bp wheat *1Dy10* promoter fragment (GenBank accession number X12929; **Fig. S1**) with a 2002 bp wheat *1Dx5* transcription terminator sequence that begins 14 nucleotides 3′ to the two *1Dx5* stop codons (GenBank accession number X12928). A diagram of the resulting *1Dy10-1Dx5* endosperm expression vector is shown in **Figure 1A**. The *1Dy10* promoter and *1Dx5* transcription terminator sequences are separated by 28 bp that contain four unique restriction sites (**Fig. 1A and B**). The entire expression cassette is flanked by *Eco*RI recognition sites that allow convenient subcloning into other vectors.

To examine the expression specificity conferred by the *1Dy10-1Dx5* endosperm expression cassette, the *uidA* coding sequence was inserted into the *Pme*I site in the correct orientation. The resultant plasmid, pJLDy10GUSDx5, was used for biolistic-mediated transformation of wheat immature embryos. Regenerating shoots and roots were selected for bialaphos resistance as described by Okubara et al. (2002).[33] Multiple independent transgenic events were characterized further by histochemical staining of the endosperm halves of their T_1 seeds. The embryo halves of seeds whose endosperm exhibited GUS activity were selected for further study. Homozygous progeny were identified from three independent lines and propagated through the T_4 generation. These seeds and plants grown from them were used for characterization of *uidA* reporter gene expression.

To examine the functionality of the endosperm expression cassette in the heterologous species *Brachypodium distachyon*, the *Eco*RI *1Dy10::GUS::1Dx5* fragment was subcloned into a binary vector to create the pGPro3-Dy10::GUS::Dx5 construct (**Fig. 1C**). *Agrobacterium tumefaciens* strain AGL1 carrying this construct was used to generate multiple independent hygromycin resistant T_0 transgenic *Brachypodium* plants. Nine transgenic plant lines were grown to maturity in the greenhouse to obtain T_1 seed. Genomic DNA was isolated from seven of these lines and digested with either *Bam*HI or *Nhe*I restriction enzymes. These restriction enzymes each recognize only a single site within the T-DNA and thus enable an estimation of the T-DNA insertion copy number. DNA gel blot hybridization analysis using a *uidA* gene probe illustrates that these seven lines are either single copy or contain 2–3 copies of the T-DNA (**Fig. 2**). Five of these lines were propagated through the T_3 generation. Homozygous
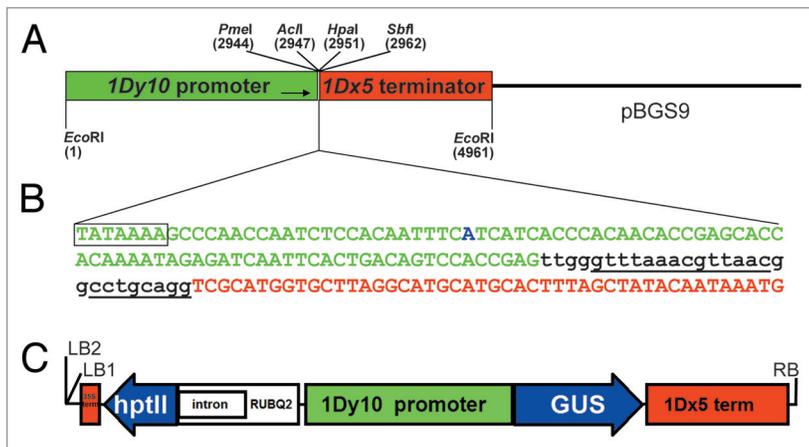
Figure 1. *1Dy10 -1Dx5* transformation constructs. (**A**) Diagrammatic representation of the *1Dy10 -1Dx5* endosperm expression vector. The green box is the 5′ flanking promoter sequence (**Fig. S1**) from the wheat *1Dy10* gene and the red box is the 3′ flanking sequence from the wheat *1Dx5* gene. An arrow shows the direction of *1Dy10* transcription. The flanking *Eco*RI sites and unique *Pme*I, *Acl*I, *Hpa*I, and *Sbf*I restriction sites and their location coordinates are shown. The pBGS9 cloning vector plasmid (drawn as a solid black line) confers kamamycin resistance in *E. coli*.[52] (**B**) The 165 bp sequence surrounding the junction site of the *1Dy10* promoter (capitalized green text) and *1Dx5* transcription terminator (capitalized red text) is shown. Unique restriction sites that can be used for insertion of transcriptional fusions are annotated as underlined lower case black text. The capitalized blue "A" denotes the start site of the *1Dy10* transcript.[62] (**C**) Diagram of the T-DNA from the pGPro3 *Dy10::GUS::Dx5* binary vector used for *Brachypodium* transformation. Blue arrows represent the *uidA* and *hptII* coding sequences, the green box is the *1Dy10* promoter, the white boxes are the *RUBQ2* promoter and 5′ intron and the red boxes are the *1Dx5* and CaMV 35S terminators. Also shown are the locations of the Right Border (RB) and Left Border (LB) sequences that mediate *Agrobacterium* T-DNA transfer. The pGPro3 vector contains two copies of the Left Border sequence (LB1 and LB2) in tandem as shown.

individuals were identified by germination on hygromycin-containing media and these were used for the characterization of *uidA* reporter gene expression.

The specificity of expression conferred by the *1Dy10::GUS::1Dx5* cassette was examined by performing histochemical detection of GUS activity in several tissues and organs of the transgenic wheat and *Brachypodium distachyon* plants. Transgenic seeds of independent lines were germinated and grown for 7–10 d and then the seedlings were stained for GUS activity using the X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) substrate. Blue staining was not visible in any of the vegetative seedling tissues of either species (**Fig. 3A** and **Fig. 4A**). Transgenic seeds from both species exhibited strong GUS staining of the endosperm of longitudinally sectioned mature seeds (**Fig. 3B** and **Fig. 4B**). GUS activity was not detected in the embryo or aleurone, consistent with the known expression pattern of the wheat *1Dy10* gene and other HMW-GS genes. To examine whether expression was detected in reproductive tissues and when it commenced during endosperm development, florets containing developing reproductive tissues and developing seeds were dissected and stained for β-glucuronidase activity. As expected, GUS activity was only detected in endosperm tissues in the transgenic wheat seeds, beginning between 7 and 10 DAP (**Fig. 3C, D and E**). Embryos of whole seeds picked up stain from

the solution at 10 and 14 DAP when expression levels were high, but showed no GUS activity when excised and incubated separately in X-gluc solution (data not shown). In *Brachypodium*, GUS activity was detected in the endosperm of mature seeds (**Fig. 4B**). The pericarp, other tissues surrounding the endosperm, and the embryo did not stain, even when incubated in X-gluc solution for as long as 16 h. GUS activity in the developing endosperm was first detected at 6–7 DAP (**Fig. 4F**).

GUS activity was not detected in any immature reproductive tissues or the lemma and palea of the transgenic wheat plants (**Fig. 3F and G**). Somewhat surprisingly, we occasionally detected GUS staining within the style of the pistil in developing *Brachypodium distachyon* florets (**Fig. 4C, D and F**). This GUS activity is unlikely to be an artifactual false positive, since it was never observed in the styles of pistils from wild type non-transgenic *Brachypodium distachyon* plants. The GUS activity was not found in all the *Brachypodium* transgenic events or even in all of the styles from the homozygous lines in which it occurred. Typically, only 10–15% of the pistils exhibited GUS-mediated staining in the style. For example, of 18 pistils stained from homozygous line #16, only 3 exhibited detectable staining in the style. Similar results were observed for line #19 (1 of 11 pistils) and line #20 (5 of 48 pistils). In transgenic line #2, approximately 1/3 of the styles exhibited staining (10 of 33 pistils). More than 25 T_4 seed from each of these homozygous transgenic plants were tested and all exhibited GUS activity within the endosperm. Staining of 20–25 wheat pistils of a similar developmental stage did not detect GUS activity in any of the three wheat transgenic lines (an example is shown in **Fig. 3G**).

Discussion

We have shown that the promoter of the wheat *1Dy10* HMW-GS gene is active in the endosperm of the grass *Brachypodium distachyon*, a model plant for temperate cereals. The HMW-GS gene promoter activity is first detected in *Brachypodium* seeds at approximately 6–7 DAP. At this stage the developing seeds have reached their final length of 6–8 mm, and endosperm development and grain filling has begun.[34] Similarly HMW-GS gene promoter activity in wheat can be detected at or just after 7 DAP and expression continues throughout the periods of grain filling and maturation. The detection of GUS activity in wheat endosperm at about 7 DAP is also in agreement with the results of Lamacchia et al. for a *1Dx5::GUS::nos* expression cassette in transgenic durum wheat "Ofanto".[16]

It is difficult to precisely compare stages of seed development in wheat and *Brachypodium* because there are several differences between them. The main storage proteins of mature wheat seeds are members of the prolamine family, while in *Brachypodium*,

the main storage proteins are globulins.[31] Starch accumulation is minimal in *Brachypodium*, and seed maturation occurs in approximately 24 d compared with 36 d for wheat.[31,34,35] However, the initial detection of *1Dy10* gene promoter activity in *Brachypodium* at 6–7 DAP coincides with an increase in metabolic activity prior to seed storage protein synthesis.[31,36] It is in these early stages of seed development that *Brachypodium* and wheat are most alike. Thus, the commencement of *1Dy10::GUS* expression just prior to the time of seed fill in *Brachypodium* is consistent with its expression profile in wheat.

The activity of the wheat *1Dy10* promoter in transgenic *Brachypodium* endosperm is not surprising, given previous results about the behavior of HMW-GS gene promoters in species as diverse as rice, corn and tobacco.[15,18,21,36] In transgenic plants of each of these species, the endosperm specificity of these promoters was preserved with the exception of the report of Furtado and colleagues, who found activity of a *1Dy12::GFP::nos* construct in the pericarp and vascular parenchyma of vegetative organs of transgenic rice.[20] In light of the specificity exhibited by the HMW-GS gene promoters in a variety of plants, it is difficult to account for the results of Furtado et al.[20] The promoter fragment they used was shorter (425 bp) than the one used here (2936 bp), but an even shorter 251 bp fragment of the *1Bx17* HMW-GS gene was found by Oszvald and colleagues to be endosperm specific in rice.[21] A unique feature of the Furtado et al. study, compared with all other published studies of HMW-GS promoter function, was the use of *GFP* as the reporter gene. However, we speculate that the ectopic expression of *1Dy12::GFP::nos* in rice could have been due to the inclusion of the CaMV *35S* enhancer in the transformation vector. This strong enhancer has been shown to activate the ectopic expression of nearby trangenes whose expression is controlled by tissue-specific promoters.[37-43] Because of these results, we have constructed transformation vectors for promoter studies that do not include the *35S* enhancer, such as the pGPro3 vector used here.[44-47]

In *Brachypodium*, we observed apparent ectopic expression of the *uidA* reporter in some of the styles in four independent homozygous transgenic lines. We did not detect this expression pattern from the same expression cassette in three independent transgenic wheat lines. Only a minority (10–33%) of the *Brachypodium* styles exhibited detectable GUS activity and the levels of staining varied, even among genetically identical tissues within the same plant spike. We have no explanation for this phenomenon, but do not believe it will compromise the ability to use *Brachypodium* as a model system to investigate the activities of promoters from wheat and other cereals that are difficult to transform. From a single experiment we obtained nine transgenic *Brachypodium* plants, several of which contained a single copy of the transgene. From these plants, we readily derived homozygous progeny in which to characterize the tissue specificity of the wheat *1Dy10* promoter.

The *1Dy10* promoter sequence contains several regulatory cis elements that are conserved with other HMW-GS gene promoters (**Fig. S1**), including an enhancer sequence 147 bp upstream of the predicted transcription start site. The same sequence is located
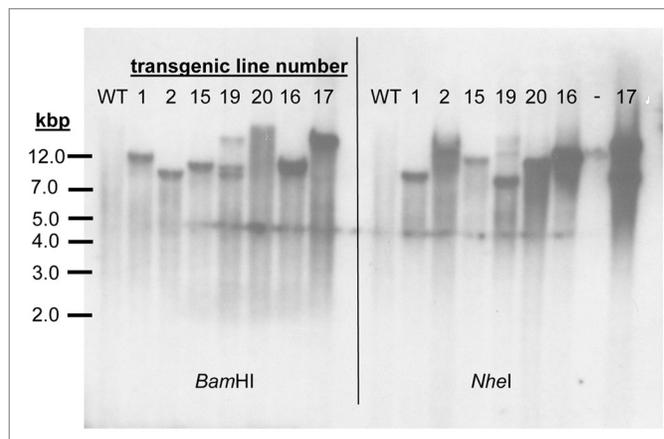

Figure 2. DNA gel blot hybridization analysis. Genomic DNA from wild type Bd21–3 (WT) and seven independent *1Dy10::GUS::1Dx5* transgenic *Brachypodium* T₂ plants (numbered lanes) was digested with either *Bam*HI or *Nhe*I (each enzyme cuts a single time within the T-DNA) and hybridized with a *uidA* probe. The sizes in kilobase pairs (kbp) of DNA marker fragments are shown on the left.

from -375 to -45 upstream of the *1Dy12* gene transcription start site and was shown to activate endosperm expression in tobacco even when located 3′ to the expression cassette.[36] The *1Dy10* promoter sequence also contains the HMW-GS "cereal box" sequence, the prolamin box, the -300 motif and several other cis elements associated with endosperm expression in various plant species (**Fig. S1**).[48] In addition, the 2936 bp *1Dy10* promoter sequence used in this report includes a predicted Matrix Attachment Region (MAR) in the region identified as having MAR activity by a chromatin binding assay.[49]

The availability of *Brachypodium* as a model system for cereal gene expression will facilitate functional characterization of the conserved *cis* elements of the *1Dy10* promoter. For example, the importance of the predicted MAR region in supporting high levels of endosperm-specific *Glu-1* gene promoter expression could be tested in multiple independent *Brachypodium* transformants. It would also be interesting to test the predicted enhancer sequence for activity in a species more closely related to wheat than tobacco.

The wheat *1Dx5* gene promoter has been used in wheat to express the heterologous coding region for the *Aspergillus niger* phytase gene *phyA*.[50] Active phytase was detected in the endosperm tissue of 10 DAP seeds and accumulated over the course of seed development.[50] The activity and endosperm specificity of this and other HMW-GS gene promoters in a variety of plants make them good choices for expressing proteins in seed storage tissues. The *1Dy10-1Dx5* endosperm expression vector described here will be useful for the expression of novel proteins in the endosperm of wheat or potentially other genetically engineered crops.

Materials and Methods

Vector construction

For wheat transformation, the plasmid pJLDy10GUSDx5 was constructed by excising the *uidA* coding region pAHC15 with *Sma*I and *Eco*RI and ligating the blunt-ended fragment
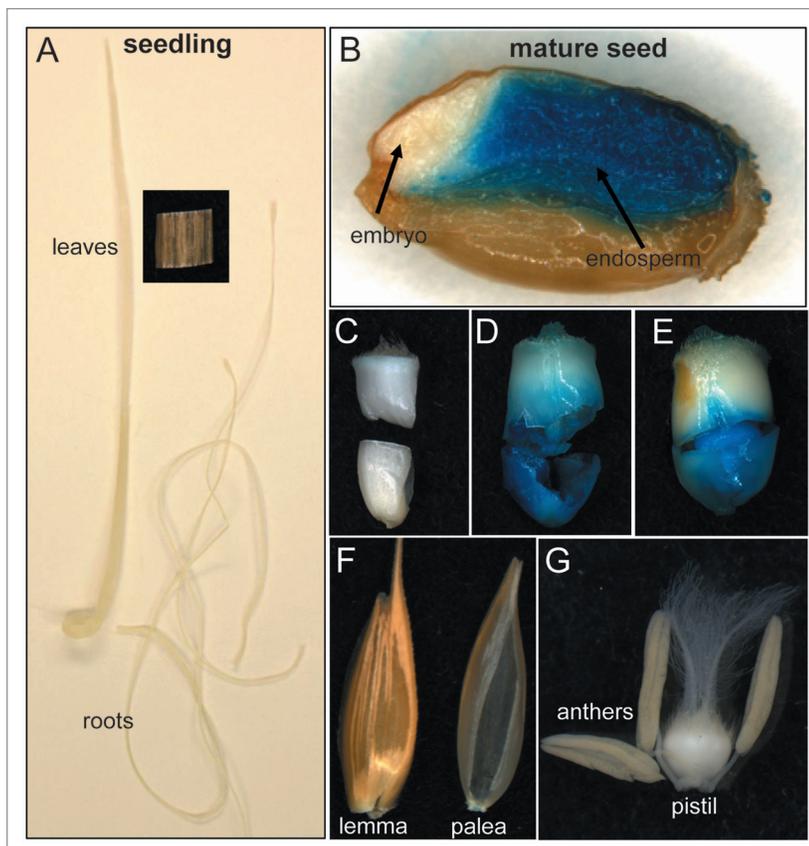
Figure 3. Spatial and temporal β-glucuronidase (GUS) activity in transgenic wheat. Homozygous transgenic *1Dy10::GUS::1Dx5* wheat plants were histochemically stained to detect *uidA* reporter gene activity. (**A**) Seedling leaves and roots (inset, segment from a flag leaf at anthesis). (**B**) Mature seed longitudinal section showing the endosperm and embryo.(**C–E**) Transverse-sectioned seeds 7, 10, and 14 DAP, respectively. (**F**) Lemma and palea from a dissected floret. (**G**) Dissected anthers and pistil.

into the *1Dy10–1Dx5* expression cassette (**Fig. 1A and B**) that had been cut by *Pme*I and dephosphorylated.[51] It contains 2936 bp upstream of the translation start site of the wheat *1Dy10* gene (GenBank accession number X12929) followed by the *uidA* coding region and 2002 bp of sequence beginning 14 nucleotides after the two stop codons in the native wheat *1Dx5* gene (GenBank accession number X12928), all cloned into the *Eco*RI site of pBGS9.[52] For selection of wheat transformants, plasmid pAHC20 or pUBK, each containing the *bar* resistance gene under control of the maize *Ubi1* promoter/first intron was co-bombarded with pJLDy10GUSDx5.[33,51] For *Brachypodium* transformation, the *1Dy10::GUS::1Dx5* expression cassette was excised from pJLDy10GUSDx5 with *Eco*RI and subcloned into a derivative of the pGPro3 binary vector that lacked the *GUS-eGFP* reporter gene and *nos* terminator. The parent pGPro3 vector (GenBank accession number JN593323) is a derivative of pGPro1, with the *rice ubiquitin2* (*RUBQ2*) promoter controlling expression of the *hptII* hygromycin resistance gene instead of the rice *actin1* (*Act1*) promoter and is specifically designed for promoter analyses.[44,47] Insertion of the *1Dy10::GUS::1Dx5* *Eco*RI fragment into the pGPro3 derivative vector generated the pGPro3 Dy10::GUS::Dx5 construct shown in **Figure 1C**. The

described plasmids were confirmed with restriction enzyme digestion and/or DNA sequencing and are available from the corresponding authors upon request.

Plant growth and transformation

Spring bread wheat "Bobwhite" was transformed via particle bombardment as described by Okubara and colleagues except that a 3:1 molar ratio of pJLDy10GUSDx5 to pAHC20 or pUBK was used to coat the gold particles.[33] After bombardment, embryos were transferred to MMS media containing 2 mg/L 2,4-D for two weeks, followed by 2 wk on MMS media containing 2 mg/L 2,4-D and 1 mg/L bialaphos, and then followed by 2 wk on MMS media containing 2 mg/L 2,4-D, and 2 mg/L bialaphos. Then the surviving calli were transferred to media for shoot and root regeneration in 3 mg/L bialaphos as previously described.[33] Plants were grown in growth chambers or in a greenhouse at approximately 23 °C under a 16 h light/8 h dark regime. *Brachypodium distachyon* Bd21-3 was transformed via *Agrobacterium*-mediated transformation as previously described.[27] Hygromcyin resistant T_0 plants were transferred to soil and grown in the greenhouse. Harvested T_1, T_2, and T_3 transgenic seed was dried, de-hulled and then surface sterilized (placed in 70% ethanol for 5 min, transferred to a solution of 30% bleach with 0.1% Triton X-100 for 20 min, and then rinsed five times with sterile water) prior to excising the embryo for germination on selective media and staining the endosperm portion to confirm *uidA* expression. Excised embryos were germinated on media containing 4.33 g/L of MS basal salts, 2.6 g/L of Phytagel, 0.5 mg/L of 6-benzylaminopurine, and 40 mg/L of hygromycin. Plants were transferred to soil mix and grown in a growth chamber or in a greenhouse at approximately 24 °C under a 16 h light/8 h dark regime.[53]

DNA gel blot analyses

Brachypodium distachyon genomic DNA was isolated from shoots of greenhouse grown plants using a procedure previously described.[54] Ten micrograms of genomic DNA was digested with either *Bam*HI or *Nhe*I (each enzyme cuts a single time within the T-DNA allowing the estimation of transgene copy number). The digested genomic DNA samples were separated on a 0.8% agarose gel and transferred onto Hybond N+ nylon membrane using a 0.4M NaOH, 0.6M NaCl transfer solution.[55] A 480 bp *uidA* gene fragment amplified with the following primers: 5′- ACTCCTACCGTACCTCGCATTACCCT-3′ and 5′-CCTTCTCTGCCGTTTCCAAATCGCC-3′ was labeled using αP[32]-dCTP and the NEBlot kit (New England Biolabs). Blot hybridizations were performed using the Sigma PerfectHyb-Plus hybridization buffer (Sigma-Aldrich) as recommended by the manufacturer. Hybridized blots were washed to 1xSSC 0.1% sodium dodecylsulphate and exposed to X-ray film.

Histochemical assays

β-glucuronidase activity was detected as described previously using a GUS staining solution (0.1 M sodium phosphate pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 1.5 g/L X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), and 0.5% v/v Triton X-100) generally for 4 to 20 h at 37 °C.[56] The incubation time was adjusted based on the strength of the staining observed. Samples that exhibit little or no staining were incubated for at least 12 h while strongly staining samples (i.e., containing endosperm) were incubated for shorter times and/or assayed at 55 °C to attenuate the strength of staining observed and to obtain clearer images. After staining, green tissues were passed through several changes of 70% and 95% ethanol to remove chlorophyll.

Microscopy and photography

Microscopic images between 2× and 10× magnification were documented using a Leica MZ16F stereomicroscope (Leica Microsystems) with an attached Retiga 2000R FAST Cooled Color 12 bit digital camera (Q Imaging).

Sequence analysis and cis element identification

Analysis of putative cis-regulatory elements within the wheat *Dy10* promoter was performed with the Plant Promoter Analysis Navigator, the Plant Cis Acting Regulatory Element (PlantCARE) search tool, and the Database of Plant Cis acting Regulatory DNA Elements.[57-59] Additional known cis elements that were not included within the above websites' databases were queried and annotated manually. The presence of a potential MAR was detected using the jEMBOSS MARscan search tool.[60,61] The transcription start site is annotated based on data from Sugiyama and colleagues.[62]
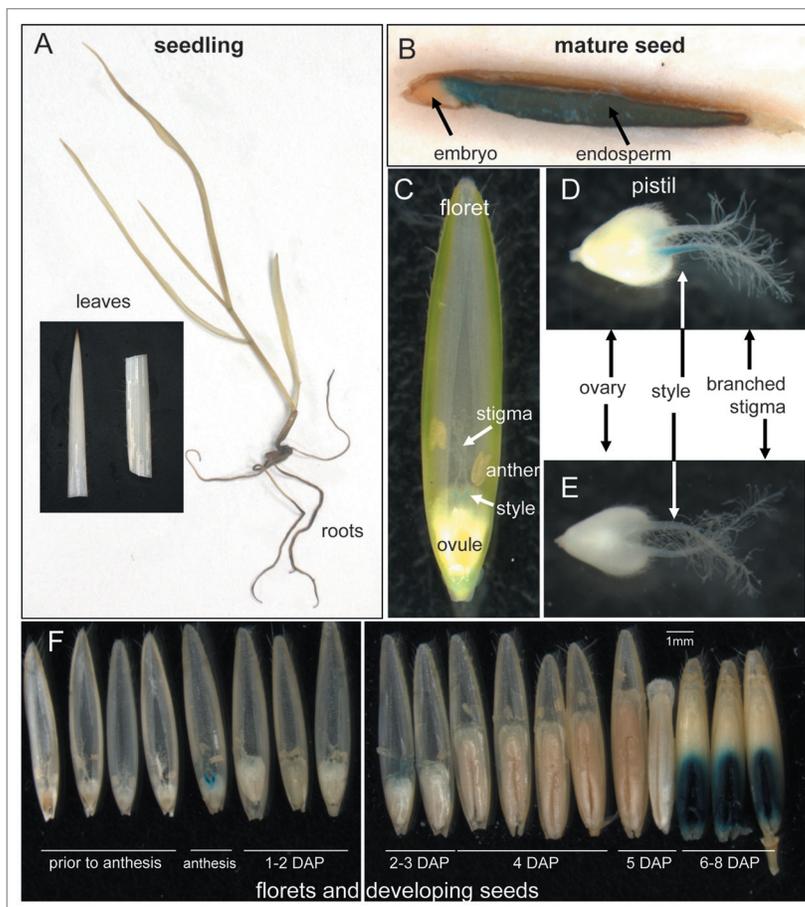

Figure 4. Spatial and temporal β-glucuronidase (GUS) activity in transgenic *Brachypodium*. Homozygous transgenic *1Dy10::GUS::1Dx5* T$_3$ plants were histochemically stained to detect *uidA* reporter gene activity. (**A**) Whole seedling and individual leaves. (**B**) Mature seed longitudinal section showing the endosperm and embryo. (**C**) Floret with lemma removed and labeled reproductive tissues. (**D**) Dissected pistil that exhibited GUS staining within the style, but not the ovary or stigma. (**E**) Dissected pistil that did not exhibit detectable staining. (**F**) Florets and developing seeds (lemmas removed) arranged in sequential order relative to pollination.

Supplemental Material

Supplemental Material may be found here: https://www.landesbioscience.com/journals/gmcrops/article/27371/

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