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

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Abbreviations: bp, base pair; CAT, chloramphenicol acetyltransferase; DAP, days after pollination; GFP, green fluorescent protein; GUS, uidA-encoded β-glucuronidase; HMW-GS, high-molecular-weight glutenin subunit; MAR, matrix attachment region; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

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. 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 1Dy). The wheat genomic fragments containing intact Glu-1 genes, including both 5’ and 3’ control regions, have been transformed into wheat, rye, and tritium (a wheat/barley hybrid), as well as into other cereals including rice, sorghum, and maize. Intact Glu-1 genes have also been introduced into tobacco. 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. In the tobacco transgenics, no Glu-1 mRNA was detected in leaves.

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 bp 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). 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. No β-glucuronidase (GUS) activity was detected by the histochemical assay in oat embryos, the outer seed envelope, leaves, roots or florets. Norrie and colleagues studied the promoter...
activity of variant versions of the 1Dx5 gene promoter in transgenic maize.23 Various containing a duplication or replication 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.15,16 A 425-bp promoter fragment (coordinates not specified) from the 1Dy12 allele supported green fluorescent protein (GFP) gene expression only in endosperm and aleurome from 7 DAP (earliest measured) through 24 DAP (latest measured) in transgenic wheat.27 GFP fluorescence was not detected in glumes, lemma, palea, ovary, anthers, anther filament, stigma, leaf, or root tissues, or in the pericarp, embryo or vascular parenchyma of seeds at the same stages.17 The same report noted similar results were obtained for one transgenic barley event containing the same construct.19 In transgenic rice however, the same construct was active not only in endosperm and aleurome, 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 Brachypodium gene with a modified 5′ untranslated region that included the rice Act5 intron exhibited tissue specificity when fused to uidA and transformed into rice.20 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.20,21,22 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.13,14 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 Gla-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 study of temperate cereals. It has a small genome that has been 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.23,24 Efficient transformation systems and resources for forward and reverse genetics have been developed.23,24 However, there have been no published reports to date of heterologous promoter expression studies in Brachypodium distachyon. The 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 EcoRI 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 Pnml site in the correct orientation. The resulting plasmid, pDy10-1Dx5, 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).23 Multiple independent transgenic events were characterized further by histochemical staining of the endosperm halves of their T1 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 T2 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 EcoRI 1Dy10–GUS:1Dx5 fragment was subcloned into a binary vector to create the pGPro3-Dy10–GUS:1Dx5 construct (Fig. 1C). Agrobacterium tumefaciens strain AGL1 carrying this construct was used to generate multiple independent hygromycin resistant T1 transgenic Brachypodium plants. Nine transgenic plant lines were grown to maturity in the greenhouse to obtain T1 seed. Genomic DNA was isolated from seven of these lines and digested with either BamHI or Ndel 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 T2 generation. Homozygous
individuals were identified by germination on bygromycin-containing media and these were used for the characterization of uidA reporter gene expression.

The specificity of expression conferred by the 1Dy10-1Dx5 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-glucuronid acid) substrate. Blue staining was not visible in any of the vegetative seedling tissues of either species (Fig. 4A). Transgenic seeds from both species exhibited strong GUS staining of the endosperm of longitudinally sectioned mature seeds (Fig. 3B and C). 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. 4C, 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 31 pistils). More than 25 T4 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. Similarly HMW-GS gene promoter 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”.* 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.\textsuperscript{30} Starch accumulation is minimal in Brachypodium, and seed maturation occurs in approximately 24 d for wheat.\textsuperscript{36,39} 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.\textsuperscript{36,39} 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.\textsuperscript{61–63,69} 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.\textsuperscript{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.\textsuperscript{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 1Dy12 HMW-GS gene was found by Ozsvárd and colleagues, who found activity of a 1Dy12-GFP::nos construct in the pericarp and vascular parenchyma of vegetative organs of transgenic rice.\textsuperscript{69} In transgenic plants, 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.\textsuperscript{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.\textsuperscript{20}

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 35\textsuperscript{S} enhancer in the transformation vector. This strong enhancer has been shown to activate the ectopic expression of nearby transgenes whose expression is controlled by tissue-specific promoters.\textsuperscript{62,64} Because of these results, we have constructed transformation vectors for promoter studies that do not include the 35\textsuperscript{S} enhancer, such as the pGPro3 vector used here.\textsuperscript{65,66}

In Brachypodium, we observed apparent ectopic expression of the uid\textsuperscript{A} reporter in some of the styles in four independent transgenic wheat 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 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.\textsuperscript{66} 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).\textsuperscript{64} 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.\textsuperscript{66}

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 phA.\textsuperscript{66} Active phytase was detected in the endosperm tissue of 10 DAP seeds and accumulated over the course of seed development.\textsuperscript{36} 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 pLDy10GUSDx5 was constructed by excising the uid\textsuperscript{A} coding region pAHC15 with Smal and EcoRI and ligating the blunt-end fragment.
into the 1Dy10–1Dx5 expression cassette (Fig. 1A and B) that had been cut by Pmec and dephosphorylated. It contains 2936 bp upstream of the translation start site of the wheat 1Dy10 gene (GenBank accession number X12929), while the 2002 bp of sequence beginning 14 nucleotides after the two stop codons in the native wheat uidA gene (GenBank accession number X12928) followed by the coding region and 2002 bp of sequence beginning 14 nucleotides after the two stop codons in the native wheat uidA gene (GenBank accession number X12928). All cloned into the EcoRI site of pBGS9.5. For selection of wheat transformants, plasmid pAHC20 or pUBK, each containing the bar resistance gene (GenBank accession number X12929), was transformed via Agrobacterium-mediated transformation as previously described. Hygromycin resistant T0 plants were transferred to soil and grown in the greenhouse. Harvested T1, T2, and T3 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-ben zalaminopur inine, and 40 mg/L of hygromycin. Plants were transferred to soil mix and grown in a growth chamber 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. Hygromycin resistant T0 plants were transferred to soil and grown in the greenhouse. Harvested T1, T2, and T3 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-ben zalaminopur inine, and 40 mg/L of hygromycin. Plants were transferred to soil mix and grown in a growth chamber or in a greenhouse at approximately 23 °C under a 16 h light/8 h dark regime. DNA gel blot analyses Brachypodium distachyon genomic DNA was isolated from shoots of greenhouse grown plants using a procedure previously described. Ten micrograms of genomic DNA was digested with either BamHI or NheI (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. A 480 bp uidA gene fragment amplified with the following primers: 5′-AGTCTATTGCCCATCGTACCGT-3′ and 5′-CTCTCTGCAGTTTCTCTGTACCG-3′ was labeled using α-32P-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 dodecyl sulphate 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. 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 2x and 10x 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. 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. The transcription start site is annotated based on data from Sugiyama and colleagues.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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