Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed

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Abstract

The characterization of the promoter of a wheat (\textit{Triticum aestivum}) cv. Cheyenne high molecular weight glutenin subunit (HMW subunit) gene, \textit{Glu-1D-1} is reported. The nucleotide sequence of the promoter from position –1191 to –650 with respect to the transcription start site was determined, to add to that already determined. Analysis of this region of the promoter revealed the presence of an additional copy of part of the primary enhancer sequence and sequences related to regulatory elements present in other wheat seed protein genes. A chimaeric gene was constructed comprising the 5′ flanking region of the \textit{Glu-1D-1} gene from position –1191 to +58, the coding region of the \textit{UidA} (Gus) gene, and the nopaline synthase (Nos) gene terminator. This chimaeric gene was introduced into wheat (\textit{Triticum durum} cv. Ofanto) by particle bombardment of inflorescence explants. Two independent transgenic lines were produced, and both showed expression of the Gus gene specifically in the endosperm during mid-development (first detected 10–12 d after anthesis). Histochemical analysis of homozygous T\textsubscript{2} seed confirmed this pattern of expression, and showed that expression was initiated first in the central lobes of the starchy endosperm, and then spread throughout the endosperm tissue, while no expression was detected in the aleurone layer. Native HMW subunit protein was detectable by Western analysis 12–14 d after anthesis, consistent with concurrent onset of activity of the native and introduced HMW subunit gene promoters.

Key words: Gene expression, Gus, high molecular weight glutenin, reporter gene, \textit{Triticum durum}.

Introduction

The cereal endosperm is of immense economic and nutritional importance, providing the major source of energy and protein for much of the human population and for domesticated animals. The prospect of using genetic modification to alter the development, structure and composition of the endosperm has therefore been the subject of much research, with attention focused particularly on the three most important cereal species, wheat, maize and rice. The development of reliable transformation systems for all three species (Vasil, 1999) means that these goals are now achievable. However, lack of suitable gene promoters for driving expression of transgenes in cereal endosperm is still a major limitation in obtaining the required level and pattern of expression. This is particularly true for wheat because detailed studies of the activity of promoters that might be suitable have only been carried out in heterologous systems such as tobacco. The aim of this study was to characterize a promoter that has great potential for the seed-specific expression of heterologous genes in wheat biotechnology and to study its activity in transgenic wheat seeds.

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Seed storage protein (prolamin) gene promoters are, in principle, ideal candidates to drive transgene expression in cereal endosperm, as high levels of prolamin synthesis occur during the linear (cell expansion) phase of seed development. However, cereal seed storage proteins are encoded by complex multigene families, whose members may vary in expression levels and include substantial numbers of pseudogenes. The only certain way of defining the activity of a promoter, therefore, is through experimentation.

The prolamins genes of the Triticeae (wheat, barley and rye) can be divided into three multigene families, which encode the sulphur (S)-poor prolamins, the S-rich prolamins and the high molecular weight (HMW) prolamins. Although the members of the different families vary widely in sequence, all contain some homologous regions and share the unusual characteristic of containing no introns. It has therefore been suggested that they arose from a single ancestral gene (Kreis et al., 1985). Their expression is controlled in a co-ordinate manner, primarily at the transcriptional level (Bartels and Thompson, 1986; Sorensen et al., 1989). They are subject to tissue-specific and developmental regulation, being expressed exclusively in the starchy endosperm during mid- and late-development, and nutritional regulation, responding sensitively to the availability of nitrogen and sulphur in the grain (Duffus and Cochran, 1992; Giese and Hopp, 1984).

Since they have a common ancestry and show similar patterns of expression, it is logical to expect prolamins genes to have regulatory sequences in common. This is true for the S-rich and S-poor families, which contain a conserved element (consensus sequence 5′-TGACATGAATAGCATG) positioned around 300 bp upstream of the transcription start site (Forde BG et al., 1985). This was first called the −300 element, subsequently the prolamin box or endosperm element. A regulatory role for the prolamin box has been established (Müller and Knudsen, 1993; Hammond-Kosack et al., 1993). The box contains two conserved motifs, TGTAAGTCGA and G(A/G)TGATGC, with a more variable region in between. The former has been called the endosperm, or E motif (Hammond-Kosack et al., 1993), the latter the GCN4-like motif (GLM), nitrogen element or N motif (Hammond-Kosack et al., 1993; Müller and Knudsen, 1993). The N motif is similar to the binding site of the GCN4 transcription factor, which is a component of the nitrogen signalling pathway in yeast. It is present in opposite orientations in S-rich and S-poor gene promoters (Shewry et al., 1999).

The prolamin box in its entirety is not present in HMW prolamin gene promoters (Shewry et al., 1999). Instead, HMW prolamin promoters contain a major regulatory element (identified by Thomas and Flavell, 1990) located in a 38 bp sequence, consensus: 5′-GTTTTGCAAA GCTCCATTG CTCTTTGCTT ATCCAGCT. The location of this sequence is highly conserved in all HMW prolamin promoters, beginning at position −185 to −189 (Shewry et al., 1999).

It is perhaps surprising that HMW prolamin genes and other prolamins genes do not contain the same regulatory elements, since they show similar patterns of temporal and spatial expression. The major difference between them is that the HMW prolamin genes are expressed at higher levels. Each active HMW glutenin subunit (wheat HMW prolamin) gene, for example, encodes a protein which accounts for approximately 2% of the total seed protein at maturity in bread wheat (Seilmeyer et al., 1991; Halford et al., 1992). Not surprisingly, therefore, high levels of expression have been obtained from additional copies of HMW subunit genes introduced into transgenic wheat (Altpeter et al., 1996; Barro et al., 1997), making a HMW subunit promoter an obvious choice for this study.

The HMW subunit gene, Glu-1D-1, which encodes the HMW glutenin subunit 1Dx5, was already available in the laboratory. Previous experiments had shown that the region from −277 to +39 of this gene was adequate for tissue-specific and developmental control of expression of a Gus reporter gene in transgenic tobacco (Halford et al., 1989). However, expression of a heterologous sequence under the control of this promoter in wheat had not been attempted previously and the possibility could not be ruled out that additional regulatory elements could be present further upstream in the promoter. Such elements could be important for expression in wheat, but not have a significant effect in the heterologous tobacco system.

For these reasons, the present study undertook to obtain more of the nucleotide sequence of the Glu-1D-1 promoter than had been reported previously and look for additional regulatory sequences. Having identified several putative elements, the choice was made to make a chimaeric gene construct comprising the Glu-1D-1 promoter up to position −1191 and the Gus reporter gene. The nucleotide sequence of the 5′ flanking region of the Glu-1D-1 gene, and histochemical analysis of the temporal and spatial patterns of activity of the chimaeric gene in transgenic wheat seeds are reported.

Materials and methods

Nucleotide sequencing and analysis

Standard DNA manipulations were performed as previously described (Sambrook et al., 1989). Nucleotide sequence of the Glu-1D-1 promoter region was obtained by Dr Len Hall at Bristol University Molecular Recognition Centre. Otherwise, DNA sequencing was performed using the Sequenase™ 2.0 T7 DNA polymerase sequencing kit (Amersham), following the manufacturer’s instructions. Searches of the Glu-1D-1 5′ flanking
region for sequences resembling regulatory elements were carried out with the FASTA program.

Plant material
Donor durum wheat (*Triticum durum*) cv. Ofanto plants were grown under greenhouse conditions with supplementary lighting provided by sodium lamps, with a day temperature of 18–20 °C and a night temperature of 14–16 °C.

Plasmids used for bombardment
Plasmids pHMW-Gus and pAH2C20 were used for bombardment. Construction of pHMW-Gus, which contains the *UidA* gene, encoding β-glucuronidase (Gus), under the control of the *Glu-1D-1* promoter, is described in the results section. Plasmid pAH2C20 (Christensen and Quail, 1996) contains the selectable *bar* gene that confers tolerance to the herbicide Basta (active ingredient phosphinotricin-glufosinato ammonium) under the control of the maize ubiquitin promoter. Plasmid DNA was prepared using the Qiagen plasmid purification system, following the manufacturer’s instructions.

Wheat transformation
Tillers containing immature inflorescences ranging in length from 0.3–0.5 cm were harvested, surface-sterilized by treatment with 70% (v/v) aqueous ethanol for 5 min and 10% (w/v) sodium hypochlorite for 20 min, and washed with sterile distilled water. The inflorescences were then isolated under sterile conditions and cut transversely into small (≤ 1 mm) pieces. For each bombardment, 30 pieces (explants) were placed in a 2 cm diameter circle in the centre of a 9 cm diameter Petri dish containing L7D4 induction medium (Rasco-Gaut and Barcelo, 1999), 10 mg l⁻¹ AgNO₃ and 9% (w/v) maltose. The explants were cultured in darkness at 26 °C overnight prior to bombardment. Plasmid DNA (a 1:1 molar ratio of pAH2C20 and pHMW-Gus) was precipitated onto gold particles (BioRad submicron 0.6 μm) following a recently described protocol (Rasco-Gaut and Barcelo, 1999). Coated particles were delivered into the plant tissues at a helium pressure of 900 psi using a PDS 1000/He gun (BioRad). Explants were then spread over the surface of the medium (10 explants per plate) and cultured at 26 °C in darkness for 6 weeks for the induction of embryogenesis. Embryogenic calli were transferred to R2 regeneration medium (Rasco-Gaut and Barcelo, 1999) supplemented with 3 mg l⁻¹ bialaphos and cultured for 2 rounds of 3 weeks each. A third round of selection was then performed by transferring surviving cultures to R medium (Rasco-Gaut and Barcelo, 1999) supplemented with 3 mg l⁻¹ bialaphos. Surviving putative transgenic plantlets were then transferred to soil and grown to maturity in the greenhouse.

PCR and RT-PCR analysis
Total genomic DNA was isolated from leaf tissues of primary transformants and their progeny using the CTAB method (Stacey and Isaac, 1994). Total RNA and mRNA from tissues (root, leaf, inflorescence, floret, embryo, and endosperm) of the T₁ generation of transformants was isolated using the Qiagen plant RNeasy and Oligotex systems. cDNA for RT-PCR was synthesized using Superscript II enzyme from Boehringer Mannheim. PCR and RT-PCR were carried out using 50–250 ng of genomic DNA or cDNA, respectively, in a 30 μl total reaction volume containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% (v/v) Triton X-100, 200 μM of each dNTP, 0.3 μM of each primer and 0.66 units Dynazyme DNA polymerase (Flowgen). PCR and RT-PCR analyses for the *UidA* gene were carried out using the primer combinations of 5’-AGTGTACGTATACCCGTTCGTTGTCGAA and 5’-ATCGCAGCTTTGGACATACCATCCTCGTA and the hybridization temperature of 62 °C. PCR products were analysed on 1% (w/v) agarose gels.

Southern analysis
Genomic DNA was digested with either *Sac*I, which cuts once within the pHMW-Gus construct, or *Sac*I and *Sal*I, which release the *UidA* gene. Digested DNA was separated by electrophoresis in a 0.8% (w/v) agarose gel and transferred by capillary blotting to Hybond-N⁺ membrane (Amersham) according to the manufacturer’s instructions. Filters were hybridized with PCR-generated digoxigenine-labelled probes produced using primers for the *UidA* gene (above). The hybridized probe DNA was detected using chemiluminescent detection (Boehringer Mannheim).

Histochemical Gus assay
Expression of the *UidA* gene was assayed in transgenic plants by incubating pieces of plant tissue (leaf, seed, root, spikelet, embryo, endosperm) overnight at 37 °C in X-Gluc buffer (Barcelo and Lazzeri, 1995) containing 1 mM X-Gluc, 100 mM sodium phosphate pH 7.0, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100.

SDS-PAGE and Western blotting
Total proteins were extracted from single half grains and separated by SDS-PAGE using TRIS-borate buffer system and 10% (w/v) acrylamide gels (Shewry et al., 1995). After separation, proteins were electroblotted onto nitrocellulose membrane and hybridized (Fido et al., 1995) with polyclonal antibody anti-R2 HMW (Denery-Papini et al., 1996).

Results
Sequencing and analysis of the Glu-1D-1 5′-flanking region
The nucleotide sequence of the 5′-flanking region of the *Glu-1D-1* gene on a 8.5 kb EcoRI restriction fragment from wheat cv. Cheyenne was obtained by Dr Len Hall, University of Bristol, UK. The sequence, which comprises the region from an *Spht* restriction site at position −1191 (taking the transcription start site as position 1) to the ATG at +62, has been deposited in the EMBL database, accession number AJ301618. It overlaps with the sequence reported previously (Anderson et al., 1989) (accession number X12928), which starts at position −649. A schematic of the region, showing the positions of regulatory elements, is shown in Fig. 1.

The major HMW prolamin regulatory element identified by Thomas and Flavell is present at position −186 to −149 (shown as complete HMW enhancer in Fig. 1) (Thomas and Flavell, 1990). The palindromic sequence TTTGAAAA within this element is repeated further
upstream at -359 (shown as partial HMW enhancer in Fig. 1). Interestingly, this sequence is within a region (−394 to −311) that is deleted in a silent HMW subunit gene, *Glu-1A-2* (J Forde et al., 1985). However, it is not required for activity of the *Glu-1D-I* promoter in tobacco (Halford et al., 1989).

A sequence corresponding to part of the N box is present at position −530, and a reverse partial N box is present at position −1045. These sequences align with the consensus N box sequence (Shewry et al., 1999) as follows:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATGAGTCA</td>
<td>−530</td>
</tr>
<tr>
<td>CTCTATGCA</td>
<td>−1045</td>
</tr>
</tbody>
</table>

The promoter also contains a sequence that matches part of the E box. This is present at position −685 and aligns with the E box consensus as follows:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGTTAAGT</td>
<td>−685</td>
</tr>
<tr>
<td>TGTTAAGc</td>
<td>−685</td>
</tr>
</tbody>
</table>

**Production of transgenic wheat expressing HMW-Gus**

The pHMW-Gus plasmid was used to bombard small pieces of immature inflorescence of durum wheat cv. Ofanto, together with the pAHC20 plasmid containing the *bar* gene conferring resistance to the herbicide Basta. Bombardment of approximately 30 explants followed by selection on medium containing Basta gave rise to three plants that were herbicide tolerant. PCR analysis of genomic DNA showed that two of them (referred to henceforth as lines 1 and 2) contained the *UidA* gene (not shown) while Southern blotting using either *SacI* to cut at the 3’ end of the *uidA* gene or *SalI/SacI* to excise the *UidA* gene showed that both lines contained multiple inserts (approximately 8–10), with differences in pattern showing
that they were independent transgenic lines (Fig. 3). The full-length Gus sequence was present in both lines, but fragments smaller than 1.8 kb were also detected, indicating the presence of truncated and/or rearranged forms of the gene as well as the full-length version.

**Tissue-specific expression of HMW-Gus**

RT-PCR analysis showed that Gus transcripts could be detected in RNA prepared from caryopses harvested 10–12 d after anthesis, but not in RNA from leaves, inflorescences, florets, roots, embryos or young developing caryopses harvested 5–7 d after anthesis. The result for line 1 is shown in Fig. 4, and that for line 2 was identical.

Homozygous T₁ plants were then grown to produce material for histochemical detection of Gus activity. Staining of leaf, seed, root, and spikelet detected no Gus activity (not shown), confirming the result of the RT-PCR analysis. Furthermore, it revealed fine detail of the pattern of Gus expression in the seeds of the transgenic plants. Transverse and longitudinal sections of caryopses of both lines harvested at 10, 12, 14, and...
21 d after anthesis showed that expression of the HMW-Gus transgene occurred in the endosperm, but not the embryo and was initiated in mid-development. This was the same for caryopses of both lines and stained sections from line 1 at different developmental stages are shown in Fig. 5a and a transverse section of a 21 d caryopsis from line 2 is shown in Fig. 5b. It was also clear that expression was restricted initially to the lobes within the central part of the starchy endosperm, then spread throughout the starchy endosperm by 21 d (Fig. 5a).

Thinner sections were stained and photographed at higher magnification (Fig. 5c, scale bars shown on figure). This showed very clearly for both transgenic lines that the cells of the endosperm stained blue while those of the aleurone remained clear. In other words no expression was detectable in aleurone cells in either line.

**Fig. 5.** Histochemical staining for Gus activity in seeds of transgenic wheat lines containing the HMW-Gus transgene: (a) Longitudinal (top) and transverse (bottom) sections of caryopses of transgenic line 1 harvested 10, 12, 14, and 21 d after anthesis. (b) Transverse section of caryopsis of transgenic line 2 harvested 21 d after anthesis. (c) Sections of caryopses from transgenic lines 1 (left) and 2 (right) harvested 21 d after anthesis, showing Gus activity in the starchy endosperm but not the aleurone.
A comparison was made between the expression pattern of the chimaeric gene and the synthesis of a HMW subunit, 1Bx7, encoded by an endogenous gene. The onset of synthesis of the native HMW subunit was detected by Western blotting of total protein from caryopses harvested 10, 12, 14, and 21 d after anthesis and reaction with the HMW glutenin subunit-specific antibody, anti-R2 HMW (Denery-Papini et al., 1996). HMW-1Bx7 protein was first detectable in endosperm harvested 12–14 d after anthesis (Fig. 6), slightly later than the earliest point at which Gus transcripts were detectable (Fig. 4), but consistent with the endogenous HMW subunit gene promoter becoming active at approximately the same time as the introduced Glu-1D-1 promoter.

Discussion

The aim of the present study was to determine the pattern of expression of a wheat HMW glutenin subunit gene (Glu-1D-1) promoter/Gus reporter gene in transgenic wheat. It made it possible to obtain the most precise information so far on HMW glutenin subunit gene promoter activity. As well as being of fundamental scientific interest, it showed the suitability of this promoter for the expression of foreign genes in the seeds of transgenic wheat, since the promoter demonstrated strict tissue-specific, temporal and spatial regulation of gene expression.

Previous studies of this promoter in transgenic tobacco had identified a primary enhancer sequence 186 bp upstream of the transcription start site. However, analysis of the nucleotide sequence further upstream revealed the presence of a copy of part of the primary enhancer sequence, and of sequences related to E and N boxes that together comprise the prolamin box that is the major regulatory element in S-poor and S-rich prolamin genes. The presence of copies of N and E boxes in addition to the prolamin box is common in S-rich and S-poor genes, but their deletion does not appear to affect the activity of the promoters in transgenic tobacco (reviewed by Shewry et al., 1999). Similarly, deletion of the N and E boxes and partial enhancer sequence does not appear to affect activity of the Glu-1D-1 promoter in tobacco (Halford et al., 1999). Nevertheless, it is possible that these additional regulatory sequences have a cumulative effect that is not readily detectable in measurements of reporter gene activity, or that they play a role in wheat that is not apparent in the heterologous tobacco system. It was not the purpose of this study to undertake a functional analysis of these elements, but a length of promoter that included all of them was chosen in order to maximize potential activity.

The Glu-1D-1 promoter/Gus reporter chimaeric gene (HMW-Gus) showed strict tissue-specificity, being expressed only in the starchy endosperm, with no expression in any other seed tissue, including the aleurone. The latter is particularly interesting because the aleurone is a layer of cells that differentiates from the starchy endosperm during seed development. The chimaeric gene also showed temporal regulation, being expressed specifically in mid-development.

Differential regulation of gene expression was also evident even within the starchy endosperm, with the cells of the lobes of the central part of the endosperm expressing the gene first, and activity then spreading outwards until all of the endosperm was expressing the gene. This raises the intriguing question of what triggers expression of the promoter. Clearly, movement of a signalling molecule through the endosperm (i.e. an intercellular mechanism) could produce this pattern of expression. However, it has been proposed that the cells of the lobes of barley endosperm arise from a different meristematic origin to those of the groove region (Bosnes et al., 1992). If this were true for wheat, these results would also be consistent with expression being triggered by an intracellular mechanism.

Studies of protein deposition in the developing wheat grain have shown that all starchy endosperm cells appear to contain similar total masses of protein, although differences in the accumulation of starch mean that protein may represent up to half of the total cell mass in subaleurone cells, but only a small proportion of the total mass of the starchy-rich central endosperm cells (Evers, 1970; Kent, 1966). The uniform expression pattern that was observed in mature endosperm is consistent with these studies.

This is the first time that this level of precision has been achieved in the analysis of wheat seed protein gene promoter activity and it highlights the usefulness of promoter/reporter gene experiments in transgenic plants.
for this sort of study, even in species such as wheat that are still difficult to transform.

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References


