Detailed Analysis of the Expression of an Alpha-gliadin Promoter and the Deposition of Alpha-gliadin Protein During Wheat Grain Development

T. W. J. M. VAN HERPEN[1,2], M. RILEY[3], C. SPARKS[3], H. D. JONES[3], C. GRITSCH[3],
E. H. DEKKING[4], R. J. HAMER[5], D. BOSCH[1], E. M. J. SALENTIJN[1], M. J. M. SMULDERS[1,2],
P. R. SHEWRY[3] and L. J. W. J. GILISSEN[1,2,*]

[1]*Plant Research International, Wageningen UR, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands,* [2]*Allergy Consortium Wageningen, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands,* [3]*Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK,* [4]*Leiden University Medical Center, Albinusdreef 2, E3-Q, P.O. Box 9600, NL-2300 RC Leiden, The Netherlands and* [5]*Laboratory for Food Chemistry, Wageningen University, Bomenweg 2, NL-6700 EV Wageningen, The Netherlands*

• *Background and Aims* Alpha-gliadin proteins are important for the industrial quality of bread wheat flour, but they also contain many epitopes that can trigger celiac (cœliac) disease (CD). The B-genome-encoded α-gliadin genes, however, contain very few epitopes. Controlling α-gliadin gene expression in wheat requires knowledge on the processes of expression and deposition of α-gliadin protein during wheat grain development.
• *Methods* A 592-bp fragment of the promotor of a B-genome-encoded α-gliadin gene driving the expression of a GUS reporter gene was transformed into wheat. A large number of transgenic lines were used for data collection. GUS staining was used to determine GUS expression during wheat kernel development, and immunogold labelling and tissue printing followed by staining with an α-gliadin-specific antibody was used to detect α-gliadin protein deposited in developing wheat kernels. The promoter sequence was screened for regulatory motifs and compared to other available α-gliadin promoter sequences.
• *Key Results* GUS expression was detected primarily in the cells of the starchy endosperm, notably in the subaleurone layer but also in the aleurone layer. The α-gliadin promoter was active from 11 days after anthesis (DAA) until maturity, with an expression similar to that of a 326-bp low molecular weight (LMW) subunit gene promoter reported previously. An α-gliadin-specific antibody detected α-gliadin protein in protein bodies in the starchy endosperm and in the subaleurone layer but, in contrast to the promoter activity, no α-gliadin was detected in the aleurone cell layer. Sequence comparison showed differences in regulatory elements between the promoters of α-gliadin genes originating from different genomes (A and B) of bread wheat both in the region used here and upstream.
• *Conclusions* The results suggest that additional regulator elements upstream of the promoter region used may specifically repress expression in the aleurone cell layer. Observed differences in expression regulator motifs between the α-gliadin genes on the different genomes (A and B) of bread wheat leads to a better understanding how α-gliadin expression can be controlled.

Key words: Alpha-gliadin, promoter, expression, deposition, wheat, *Triticum aestivum*, grain development.

INTRODUCTION

Wheat is an important staple food over much of the world, being consumed after processing into a range of food products including breads, noodles and pasta. These processing properties depend primarily on the gluten proteins, which account for about 80 % of the total proteins in white flour.

Much of the research on wheat is focused on understanding and improving its properties in bread making, including understanding the relative contributions of genetic and environmental factors to variation in these properties (Dupont *et al.*, 2006). Controlling the expression of specific gliadins is also relevant from the perspective of celiac (cœliac) disease (CD). About 0·5 to 1 % of the human population in Western Europe is affected (Koning *et al.*, 2005). The α-gliadins of gluten appear to be the most active group of wheat proteins in triggering CD (Arentz-Hansen *et al.*, 2000, 2002; Vader *et al.*, 2002, 2003; Molberg *et al.*, 2003). These proteins are encoded

by *Gli-2* loci of bread wheat, with estimates of the numbers of individual α-gliadin genes ranging from 25–35 copies (Harberd *et al.*, 1985) to 150 copies (Anderson *et al.*, 1997) per haploid genome. A considerable amount of data on the sequences of epitopes that are recognized by T-cells of CD patients (CD toxic epitopes) and their distributions within the α-gliadin family of proteins is available (Van Herpen *et al.*, 2006). This shows that they are non-randomly distributed between the sequences encoded by the different genomes: four different epitopes have been found among the D-genome-encoded α-gliadins, two of these were also in A-genome-encoded genes, but hardly any in the B-genome-encoded genes.

Modern biotechnology offers opportunities to remove the proteins containing the most toxic epitopes or even the epitopes themselves, using transgenesis or *in vivo* mutagenesis. However, such modifications may also lead to altered technological properties, as the gluten proteins are the major determinants of the functional properties. For example, wheat has been genetically engineered to add additional

* For correspondence. E-mail luud.gilissen@wur.nl

genes encoding high molecular weight (HMW) glutenin subunits using their own endosperm-specific promoters (Altpeter *et al.*, 1996; Blechl and Anderson, 1996; Barro *et al.*, 1997), leading to both positive and negative effects on the properties of the dough for mixing and bread making (Popineau *et al.*, 2001).

Becker *et al.* (2006) demonstrated that inhibition of the expression of the complete α-gliadin family can be achieved by using RNA interference. This drastic modification resulted in little effect on dough resistance and extensibility but in an increase in dough strength and a small decrease in loaf volume (Wieser *et al.*, 2006). A less drastic approach would be to reduce the expression of only A- and D-genome-encoded α-gliadin genes, which contain most of the known CD toxic epitopes (Van Herpen *et al.*, 2006), while retaining or enhancing the expression of α-gliadin genes from the B genome, which do not contain CD epitopes. However, to achieve this we require a more detailed understanding of the structure and regulation of expression of the α-gliadin gene promoters.

Previous studies revealed three important conserved *cis*-motifs in endosperm-specific promoters from wheat, barley and rice, namely the GCN4-like motif, the prolamin box and the AACA/TA motif (Dong *et al.*, 2007). Additionally, the RY repeat is described to have a key role in seed-specific gene regulation of seed storage proteins (Fujiwara and Beachy, 1994). The CGN4-like motif plays a central role in controlling endosperm-specific expression (Onodera *et al.*, 2001) and is the target of basic leucine zipper transcription factor (bZIP) proteins that belong to the Opaque2 subfamily (Albani *et al.*, 1997; Oñate *et al.*, 1999; Wu *et al.*, 2000). Deletion and point mutation experiments have revealed that the prolamin box is important for the regulation of expression of endosperm-specific genes (Mena *et al.*, 1998; Diaz *et al.*, 2005). The AACA motif was found to be conserved in the rice glutelin gene promoter and is involved in endosperm-specific expression (Takaiwa *et al.*, 1996; Wu *et al.*, 2000).

Stoger *et al.* (1999, 2001) studied an endosperm-specific low molecular weight (LMW) subunit gene promoter, showing specific expression in the outer subaleurone cells of the endosperm of transgenic bread wheat (*Triticum aestivum*). These cells are known to be rich in protein (Kent, 1966; Evers, 1970). Lamacchia *et al.* (2001) similarly characterized an endosperm-specific HMW subunit gene promoter in transgenic durum wheat (*Triticum durum*), showing that expression was higher in the central, starchy endosperm cells with no expression in the aleurone cells.

Reeves and Okita (1987) described a gene encoding an α-gliadin isolated from the bread wheat cultivar 'Yamhill' and identified a promoter region with various regulatory elements. Aryan *et al.* (1991) also showed that a segment of the α-gliadin gene promoter from −151 to −75 was required for optimum expression in a heterogeneous tobacco protoplast system. Six nuclear proteins from developing wheat kernels were found to interact with the first 165 bp upstream of the transcriptional start and this region was therefore suggested to have a role in the transcription of α-gliadin synthesis (Vellanoweth and Okita, 1993). However, to our knowledge, expression of a functional gene under control of an α-gliadin promoter in wheat has not been reported previously.

To determine the pattern of α-gliadin expression in various tissues of wheat during kernel development, we studied the expression of a GUS (beta-glucuronidase) reporter gene under control of a 592-bp α-gliadin promoter fragment derived from the B genome in stably transformed bread wheat. Using immunogold labelling and tissue printing, the deposition of α-gliadin protein was determined in developing and mature wheat kernels, and the results were compared to the deposition of the HMW glutenin subunit in developing wheat kernels.

MATERIALS AND METHODS

Sequence similarity analysis of the α-gliadin sequence

The clone of Reeves and Okita (1987; *Triticum aestivum* L. 'Yamhill', accession number M16496) contains the coding region of an α-gliadin gene including 1814 bp of 5′ upstream sequence. The coding region was translated into an amino acid sequence and aligned from the *N*-terminus of the mature protein (omitting the first 17 amino acids comprising a signal peptide) to the last conserved cysteine residue (lengths ranging from 244 to 271 amino acids) with the same set of accessions of α-gliadin genes from the three genomes of hexaploid wheat and the diploid α-gliadin genes using Clustal X 1·81, as in Van Herpen *et al.* (2006). A neighbour-joining tree was subsequently produced in TreeView 1·6·6.

Epitope screening of the α-gliadin sequence

The α-gliadin protein sequence was searched for known α-gliadin epitopes (Glia-α, Glia-α2, Glia-α9 or Glia-α20; Spaenij-Dekking *et al.*, 2004) with only full identity matches being considered in the scoring (Van Herpen *et al.*, 2006). The antibody recognition motif, QPFPQPQL was then used for full identity matching, re-analysing the results from Van Herpen *et al.* (2006; DQ002569-DQ002599) and the available database sequences assigned to chromosomes 6A, 6B and 6D.

Regulatory motif screening of database α-gliadin promoter sequence

The known α-gliadin promoter sequences were extracted from the NCBI (http://www.ncbi.nlm.nih.gov) database. This gave 30 promoter sequences from putative α-gliadin genes. Nine of these promoter sequences formed part of pseudogenes and seven were not accompanied by an open reading frame, so that it was not possible to determine their genomic origin based on gliadin sequence homology. The 14 remaining promoter sequences were accompanied by full α-gliadin open reading frames (Table 1) and were assigned to chromosomes 6A, 6B or 6D as in Van Herpen *et al.* (2006). A pattern search on the promoter regions of the 14 promoter sequences allowed us to identify the presence of various regulatory sequences, including the GCN4-like motif (TGAGTCA; Onodera *et al.*, 2001),

TABLE 1. *The presence (+) or absence (−) of RY-repeat, CGN4-like motif, prolamin box, AACA/TA motif and the percentage of identity for the 593-bp promoter region used in this study, and the region further upstream of that 593 promoter region. Positions indicated refer to sequence M16496. The genome of origin was determined based on homology of the associated gliadin gene sequence to sequences from diploid and hexaploid* Triticum *species (Van Herpen* et al., *2006)*

		Region not used		Region used in the transformation					
Database accession number*	Genome of origin	Identity % with M16496 (−594 to −895)	RY repeat (−774)	Identity % with M16496 (0 to −593)	GCN4-like motif (−492)	AACA/ TA motif (−380)	Prolamin box (−315)	RY repeat (−294)	AACA/ TA motif (−107)
K03076 (AAA34280)	A	n/a	n/a	90	−	−	+	+	+
U08287 (AAA17741)	A	43	−	89	−	+	+	+	−
U50984 (AAA96276)	A	n/a	n/a	86	−	+	+	+	+
U51304 (AAA96523)	A	n/a	n/a	86	−	+	+	−	−
U51306 (AAA96524)	A	n/a	n/a	90	−	+	+	+	+
U51307 (AAA96525)	A	n/a	n/a	90	−	−	+	+	+
X01130 (CAA25593)	A	43	−	89	−	−	+	+	+
X02539 (CAA26384)	A	n/a	n/a	90	−	+	+	+	+
M16496 (AAA34290)	B	100	+	100	+	+	−	−	+
K03074 (AAA34277)	B	n/a	n/a	99	+	+	−	−	−
X02538 (CAA26383)	B	n/a	n/a	99	+	+	−	−	−
X02540 (CAA26385)	B	n/a	n/a	89	+	−	−	−	−
K03075 (AAA34279)	B	85	−	80	+	−	+	−	−
U51303 (AAA96522)	B	92	−	81	+	−	+	−	−
Not available in database	D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

*The accession number of the DNA sequence is given, with the protein database number in brackets.

n/a = not available.

the prolamin box (TGT/CAAAG; Vicente-Carbajosa *et al.*, 1997), AACA/TA motif (Takaiwa *et al.*, 1996; Wu *et al.*, 2000) and the RY repeat (CATGCAC/T; Baumlein *et al.*, 1992; Fujiwara and Beachy, 1994). The percentage identities of the different promoter sequence regions were determined using MegAlign 6·1 from the DNASTAR package using Clustal V method. A blastn search was performed using the ncbi blast service at http://www.ncbi.nlm.nih.gov/BLAST/ using the nucleotide collection (nr/nt) database and optimized for somewhat similar sequences (blastn).

Constructs

A construct comprising 593 bp of the α-gliadin promoter, uidA reporter gene and nos (nopaline synthase) terminator was made. The α-gliadin promoter, without α-gliadin protein or α-gliadin signal peptide sequence, was obtained using PCR from the pCR-1 construct (provided by Okita, Washington State University) using forward 5′-GCCG GAATTCAAGCTTGTCTAGTTACAGTAACAAC-3′ and reverse 5′-GCCGGGATCCGGTGGATTTGTATTGACCA CTGC-3′ primers, introducing EcoRI at the 5′ side and BamHI restriction sites at the 3′ side. The PCR product was introduced in a PUC-based (PUC-M) vector using EcoRI and BamHI sites with the nos terminator at the BamHI side of the PCR product. The uidA gene was obtained using PCR on the pMDC162 construct (Curtis and Grossniklaus, 2003) using forward 5′-GCCGGGATCC ATGTTACGTCCTGTAGAAACCCC-3′ and reverse 5′-GC CGGGGATCCTCATTGTTTGCCTCCCTGCTG-3′ primers, introducing BamHI sites to 3′ and 5′side of the gene.

The uidA gene was finally introduced in the BamHI side of the previously obtained PUC-based vector.

The pAHC20 selectable marker construct (Christensen and Quail, 1996), which contains the bar gene encoding phosphinothricin acetyltransferase (PAT), was used to confer resistance to the herbicide phosphinothricin (PPT).

Transformation

Immature embryos of bread wheat 'Cadenza' were used as targets for transformation by particle bombardment using the protocol of Sparks and Jones (2004). The following modifications were made: regeneration media contained 0·05 mM $CuSO_4$ instead of 10 mg L^{-1} AgNO$_3$; selection media did not contain 2,4D; bombardments were carried out at a pressure of 900 psi ($6·21 \times 10^{-6}$ Pa). The presence of the transgenes in putative transgenic plants was confirmed by PCR using uidA gene specific primers (forward primer, 5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′; reversed primer, 5′-ATCGCCGCTTTGGACATACCATCC GTA-3′). Transformed plants were grown to maturity in a containment glasshouse and T_1 kernels harvested.

Histochemical GUS assay

Transverse sections approximately 0·5 mm thick were cut manually with a razor blade from developing or mature kernels of transgenic lines and controls. Expression of the GUS reporter enzyme was detected by incubating the sections in X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer, pH 7, 0·5 mM potassium ferricyanide, 0,5 mM

potassium ferrocyanide and 0·1 % (v/v) Triton X-100 at 37 °C for 1 h for developing kernels, or 24 h for mature kernels. Staining was visually assessed using a Zeiss stemi SVII stereomicroscope for mature kernels and a Leica MZ FLIII stereomicroscope for developing kernels.

Transformant progeny lines with homozygous segregations

A total of 62 independent T_0 lines were initially shown by PCR amplification to contain the *uidA* gene. Histochemical staining was carried out on 24 individual mature T_1 half-kernels of each of 60 different PCR-positive T_0 lines and on six and 12 half-kernels of two other PCR-positive T_0 lines, identifying 54 lines that expressed GUS in the endosperm. The other halves of the kernels were kept for sowing. Thirty-one of these 54 lines that survived and set kernels showed segregation ratios for the expression of GUS consistent with 3 : 1 segregation (χ^2 test with P-values < 0·05). Six lines out of these 31 lines that showed the highest P-values (all higher then 0·5) were selected for segregation analysis in the T_1 generation. Eight individual GUS-positive T_1 half-kernels of each of these six lines were planted and eight T_2 kernels from each plant were tested for histochemical GUS staining. Five out of the six selected lines again showed segregation ratios in the eight progeny that were consistent with the expected segregation ratios for a single insertion site (2 : 1 of homozygote : hetrozygote segregation; χ^2 test with P-value = 0·05). One progeny of each of these five lines that appeared to be homozygous in the T_2 kernels (with all eight kernels stained) was selected and again appeared homozygous in the T_3 kernels (all eight kernels again being positive for GUS staining). The T_3 kernels of these five lines were used for detailed analysis.

Cryostat sectioning (GUS-stained)

Previously GUS-stained mature kernels were frozen in cryostat embedding medium (Tissue-Tek, OCT Compound, Sakura) on a cryostat holder inside of the cryostat microtome (MicroM HM500 O). Sectioning was carried out at a temperature of −17 °C. Sections of 200 μm were cut, placed on a microscope slide and visually assessed using a Zeiss Axiophot light microscope at 100× magnification.

Cryostat tissue printing

The method was based on that of Conley and Hanson (1997). In order to soften the grain, whole mature kernels (approximately 30–35 DAA) were imbibed by placing the kernels onto moistened filter paper in Petri dishes sealed with Parafilm for 42 h at 4 °C. A small transverse portion of the kernel was removed on the embryo side and the kernel was attached cut-side down onto a cryostat holder with tissue freezing medium (Jung, Leica Microsystems) and quickly frozen in liquid nitrogen. Transverse sections of 15–20 μm thickness were cut with a Leica CM1850 cryostat at −17 °C and attached onto nitrocellulose-coated slides by gentle pressure (Oncyte Film-slides, Grace Bio-Labs

Inc.). The slides were dried overnight at room temperature before proceeding with the immunolocalization.

Immunolocalization (tissue prints)

The nitrocellulose-coated slides with tissue prints were rinsed in washing buffer [phosphate-buffered saline, PBS, pH 7·2, 0·3 % (v/v) Tween 20] twice for 15 min each with agitation, and blocked for 1 h with 5 % (w/v) skimmed milk powder in washing buffer (tissue remnants were carefully removed at this stage) and then washed twice for 10 min each with washing buffer. The slides were then incubated for 2 h in either of the two primary antibodies: the mouse monoclonal anti-α-gliadin-specific antibody (Mitea *et al.*, 2008; Glia-α9 antibody obtained from Koning, Leiden University Medical Center) diluted 1 : 7000; or the rabbit polyclonal antibody raised to oat 8S globulin (Yupsanis *et al.*, 1990) diluted 1 : 5000 in washing buffer containing 0·5 % bovine serum albumin (BSA). Following incubation, the slides were washed four times, for 8 min each, with PBS and incubated for 1 h in the secondary antibody (goat anti-mouse or goat anti-rabbit IgG alkaline phosphatase conjugate, Sigma A-3688 and A-9919) diluted 1 : 3000 in washing buffer containing 0·5 % BSA. The prints were washed twice, for 5 min each, with the following solutions: washing buffer + 0·5% BSA, washing buffer, and finally in washing buffer containing 0·05 % SDS, before developing using the Sigma BCIP/ NBT Purple liquid solution for membranes (Sigma B3679). The reaction was stopped after a red/purple colour was observed by rinsing slides with distilled water. Tissue prints were also stained with Ponceau S (Sigma P7767) for total protein after examination of the immuno-reactions. The slides were examined with a Leica MZ8 stereomicroscope and photographed with a Leica DFC 300FX digital camera. The Glia-α9 T-cell epitope is absent from most sequences originating from the B genome (Van Herpen *et al.*, 2006; Salentijn *et al.*, unpubl. res.). However, the determined minimal recognition motif of the Glia-α9 antibody (QPFPQPQ) is smaller then the minimal recognition motif of the Glia-α9 T-cell clone (PFPQPQLPY; Mitea *et al.*, 2008). By re-analysing the results of Van Herpen *et al.* (2006), we found that the minimal recognition motif of this Glia-α9 antibody is found in two out of five B genome diploid sequences and in 15 out of 20 hexaploid 6B database sequences (also found in M16496). This indicates that this antibody recognizes α-gliadin proteins originating from all three different genomes.

Fixation and embedding in LR white for immuno-assay

Approximately 0·5-mm thick sections of developing wheat kernels 11, 18, 25 and 32 DAA were manually cut with a razor blade in 0·05 M phosphate buffer. The sections were fixed for 5 h at room temperature in 4 % (v/v) paraformaldehyde, 2·5 % (v/v) glutaraldehyde in 0·05 M phosphate buffer pH 7·2. The fixed sections were washed three times for 30 min each in 0·05 M phosphate buffer pH 7·2, dehydrated in an ethanol series of ten steps from 10 % (v/v) to 100 % (v/v), and infiltrated in increasing concentrations of

LR white resin (TAAB Lab. Equipment Ltd.). Finally, the samples were embedded at 50 °C in an oxygen-free environment for 24 h. Sections of 1 μm thickness were cut using a Reichert-Jung Ultracut ultramicrotome (Reichert, Vienna, Austria) and placed on Poly-l-lysine- (Sigma P-1399) coated multi-well slides for immunogold labelling.

Immunogold labelling and silver enhancement

The samples were blocked with blocking buffer containing 3 % (w/v) BSA in 0·1 M phosphate buffered saline with Tween (PBST) at room temperature for 30 min and then washed for 30 min with 1 % (w/v) BSA in PBST. They were then incubated for 2 h with a monoclonal α-gliadin-specific (Glia-α9, 9–68, QPFPQPQ) and HMW-specific (HMW 30-29 GYYPTS) antibody (obtained from Koning, Leiden University Medical Center) diluted 1 : 100 in 1 % (w/v) BSA in PBST and washed three times for 5 min with 1 % (w/v) BSA in PBST. They were then incubated for 1 h using a secondary 10-nm gold conjugated anti-mouse antibody diluted 1 : 50 in 1 % (w/v) BSA in PBST, washed three times for 5 min with 1 % (w/v) BSA in PBST, then three times with PBST and finally three times with distilled water. The samples were developed using a silver enhancement kit (British Biocell International, UK) and observed using a Zeiss Axiophot light microscope.

Pre-fixation, GUS staining, fixation and embedding in LR white

Approximately 0·5-mm thick sections of developing wheat kernels at 11, 18, 25 and 32 DAA were manually cut with a razor blade in 0·01 M phosphate buffer. The sections were pre-fixed for 30 min in 0·1 % (v/v) glutaraldehyde in 0·05 M phosphate buffer, pH 7, and then washed five times for 10 min in 0·05 M phosphate buffer, pH 7. Histochemical GUS staining was then performed as described above and the samples washed again in 0·05 M phosphate buffer, pH 7, re-fixed in 3 % (v/v) glutaraldehyde in 0·05 M phosphate buffer, pH 7, for 2 h and washed again four times for 10 min each. The samples were then dehydrated, embedded and sectioned as described above before examination using a Zeiss Axiophot light microscope

RESULTS

The expression pattern of an α-gliadin gene promoter in developing grains of wheat was determined by transformation of bread wheat with an B-genome α-gliadin promoter−GUS reporter construct. The accumulation of α-gliadin protein was also determined in the same tissues and stages of development were examined by immunolocalization using a specific monoclonal antibody. Finally, the sequence of the α-gliadin promoter that was used was compared to those of other α-gliadin genes originating from the different genomes of hexaploid bread wheat in order to relate differences in sequence and genome origin to expression patterns.

Phylogenetic analyses, epitope- and regulatory motif screening

Alignment the α-gliadin gene (M16496) accompanying the promoter sequence as reported by Reeves and Okita (1987) showed a close relationship to α-gliadin genes encoded by chromosome 6B (bootstrap value 951/1000). The predicted α-gliadin protein encoded by the gene (M16496) was also screened for the presence of toxic celiac disease (CD) epitopes. None of the four identified CD epitopes (called Glia-α, Glia-α2, Glia-α9 or Glia-α20; Van Herpen *et al.*, 2006) were detected, which is consistent with its origin from chromosome 6B (Van Herpen *et al.*, 2006).

Other α-gliadin promoter sequences in the database were derived from chromosomes 6A and 6B (Table 1), but no sequences from the D genome were found. As shown previously (Molberg *et al.*, 2005; Van Herpen *et al.*, 2006), CD epitopes are non-randomly distributed, indicating that the three genomes make different contributions to the ability of wheat to trigger CD. To determine if there are also sequence differences in the promoter sequences of the α-gliadin genes encoded by different genomes, we compared database sequences for the presence of several regulatory motifs (Takaiwa *et al.*, 1996; Wu *et al.*, 2000; Table 1).

The thirteen promoter sequences of α-gliadin genes show a high sequence identity (<80 %; Table 1) to the 593-bp promoter region used in this study (M16496). The GCN4-like motif was present twice in the 593-bp region of M16496 (at positions −300 and −492). The −300 GCN4-like motif was present in all of the sequences from the A and B genome, but the −492 GCN4-like motif was primarily present in the genes originating from the B genome (Table 1). The prolamin box was not present in the 593-bp promoter region of M16496 (due a SNP at position two of the motif), but is present in some other α-gliadin promoters (Table 1). The AACA/TA motif was found five times (−107, −252, −380, −421, −574) in the 593-bp region of M16496. The AACA/TA motifs at −252, −421 and −574 were present in all gliadin gene promoters from the A and B genomes, but the motifs at −107 and −380 were present in some α-gliadin promoters (Table 1). Finally, the RY repeat at position −294 was present in seven out of the eight A genome sequences and in none of the B genome sequences.

Of four out of the thirteen promoter fragments, sequences are available upstream of −593 bp (Table 1). Analysis of the region upstream of −593 bp failed to identify a CGN-4-like motif or prolamin box. A RY repeat was present at −774 bp in the original promoter of which a fragment was used in this study (M16496, Table 1). When analysing for sequence identity of the region −593 to −895 bp, the two B genome sequences were highly similar to each other and to M16496, whereas the two A genome sequences were very similar to each other (data not shown) but not to the B genome sequences. From these data we observe a general difference between A and B genome promoter sequences. The M16496 sequence that was used in this study also for the promoter sequence shows consistency with its origin from chromosome 6B.

GUS expression phenotypes and segregation ratios in different transgenic lines

Segregation analysis of GUS expression in the individual T_1 kernels of each GUS expressing line showed that 31 of the 54 lines gave ratios that were consistent with a single insertion being inherited in a $3:1$ ratio (Table 2). Segregation ratios consistent with $15:1$, suggesting two transgenic insertion sites, were found in seven of the 54 lines. Finally, 16 out of 54 lines showed segregation ratios that were significantly different from $3:1$ and $15:1$ ratios (χ^2 test with P-values < 0.05; ranging from one to 12 GUS expressing kernels out of 24). The reason for this non-Mendelian pattern of expression is not known but it is possible that transgene silencing may have occurred in some lines, thus under-estimating the number of positive segregants.

A total of 1266 kernels from the 54 lines were tested, of which 742 kernels expressed GUS in the endosperm (Table 2, 'Total' column). Although considerable variation in the intensity of the GUS staining was observed, there was a consistent pattern in most lines. In 554 out of the 742 GUS-expressing kernels (about 75 %) the GUS staining was strongest in the outer part of the kernel and this pattern was therefore called 'normal staining'. Of these, 234 kernels showed strong staining only in the outer part (Fig. 1B), another 189 kernels had a strong staining in the outer part and a weak staining in the inner part of the endosperm (Fig. 1C), and 131 kernels showed weak staining only in the outer part of the endosperm (Fig. 1A). In 145 out of the 742 GUS-expressing kernels (about 20 %) showed strong staining across the whole endosperm (Fig. 1D), with no apparent spatial differences in expression. However, this may be due to limitations with the assay in strongly expressing lines resulting in loss of discrimination between peripheral and central endosperm. The remaining 43 kernels (about 6 %) showed partial, weak and irregular staining.

TABLE 2. *Analysis of segregation and phenotypes of the* T_0 *transgenic lines. Percentages quoted in the text were calculated as the fraction of the total number of* T_1 *kernels where GUS staining was observed (numbers in bold). The different* T_0 *lines showed different segregation ratios and were classified into three segregation classes (1 : 3, 1 : 15 and non-Mendelian). The* T_1 *kernels showed different patterns of staining and were classified into five groups, where the first three groups were considered as 'normal' staining*

|	Segregation class			
	15 : 1	3 : 1	Non-Mendelian	Total
Numbers of T_0 lines	7	31	16	54
Numbers of T_1 seeds tested	168	714	384	1266
Numbers of T_1 seeds with no GUS staining observed	10	217	297	524
Numbers of T_1 seeds with GUS staining observed	**158**	**497**	**87**	**742**
Numbers of T_1 seeds with weak outer staining and no inner staining (Fig. 1A)	9	76	46	131
Numbers of T_1 seeds with strong outer and no inner staining (Fig. 1B)	29	193	12	234
Numbers of T_1 seeds with strong outer and low inner staining (Fig. 1C)	48	137	4	189
Numbers of T_1 seeds with strong staining of the complete kernel (Fig. 1D)	67	75	3	145
Numbers of T_1 seeds with partial, weak and irregular staining (atypical)	5	16	22	43
Numbers of 'normal' staining (Fig. 1A − C) of all T_1 seeds with GUS staining observed	86	406	62	554
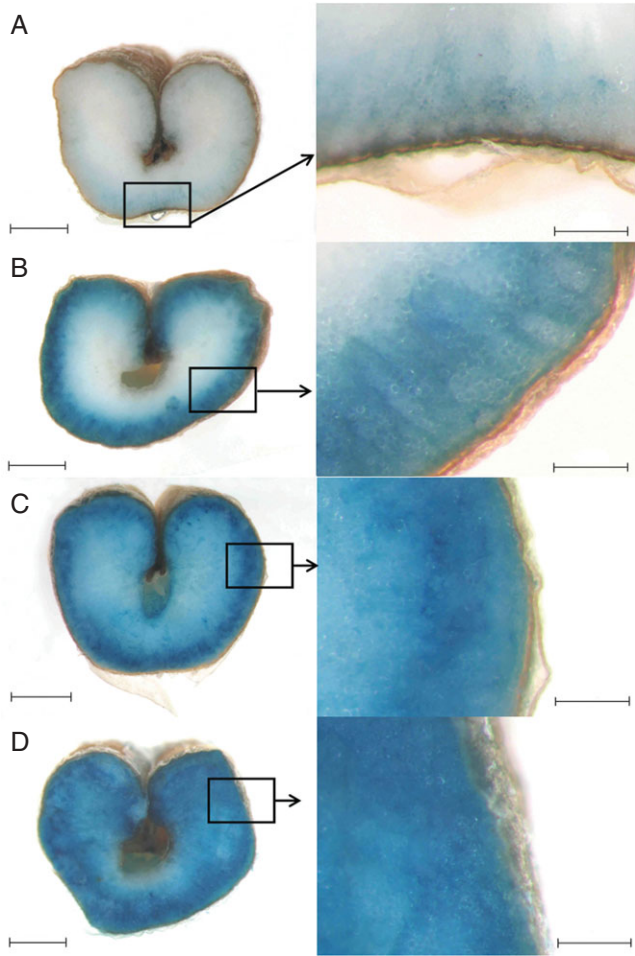
FIG. 1. Representative examples of four different phenotype classes based on differences in GUS expression levels in the mature T_1 kernels of different transgenic lines showing α-gliadin promoter activity. Left panels show an overview of the kernel (scale bars = 1 mm), right panels show a 5 × magnification of the outermost cell layers of the grain (scale bars = 0·2 mm). All samples were histochemically stained for 24 h as described in the Methods. (A) Low expression only on the outer part of the endosperm (18 % of the kernels; picture of line B2219R2P5). (B) Strong expression only in the outer part of the endosperm (31 % of the kernels; picture of line B2219R1P12F). (C) Strong expression in the outer part and a weak expression in the inner part of the endosperm (25 % of the kernels; picture of line B2252R5P1). (D) Strong staining across the whole endosperm (20 % of the kernels; picture of line B2219R9P13C).

There appeared to be a relationship between the segregation ratio and intensity of expression (Table 2). Strong staining of the whole kernel (Fig. 1D) was observed in only three of the 87 GUS-expressing kernels in the non-Mendelian group (about 3 %), but in 75 of the 497 GUS-expressing kernels segregating in a 3 : 1 ratio (about 15 %), and in 67 of the 158 GUS-expressing kernels in the 15 : 1 group (42 %). In contrast, weak and irregular staining was observed in five out of the 158 GUS-expressing kernels in the 1 : 15 group (3 %), in 16 out of the 497 GUS-expressing kernels in the 3 : 1 group (3 %), but in 22 out of the 87 GUS-expressing kernels in the non-Mendelian group (25 %). This indicates that expression pattern and intensity were related to differences in insertion patterns, with multiple insertions (15 : 1 ratio) giving more intense staining and non-Mendelian events giving weak, irregular staining.

Detailed analysis of expression patterns

In order to determine tissue specificity, six T_3 lines were tested for GUS staining in different plant tissues. One of the lines (B2219R4P5·6·1) showed staining of the embryo, flower, pollen and at the connection point of the grain to the lemma in addition to the endosperm itself, but no GUS expression was observed in root, leaf and stem tissues. This expression pattern was only observed in the one line, which was therefore excluded from further detailed analysis. The remaining five lines showed GUS staining only in endosperm tissue with no staining being observed in other tissues (stem, leaf, root, flower, pollen, embryo, lemma or testa).

In order to determine GUS expression during kernel development, developing grains were harvested from the central part of the head between 11 and 28 DAA. GUS staining was observed in half-kernels from 11 DAA until maturity. The pattern was similar in all five lines and is illustrated by Fig. 2, which shows developing grains at 14, 21 and 28 DAA at low magnification and by Fig. 3, which shows a developing grain at 11 DAA and a mature grain at higher magnification.

Although these analyses show expression across the endosperm, the staining is less intense in the central than in the outer cells. The latter include the protein-rich subaleurone cells, but also the cells of the aleurone layer, shown clearly in Fig. 3.

α-gliadin labelling on thin (1 µm) sections and tissue prints. Although the promoter studies showed clear expression in aleurone cells of GUS driven by the α-gliadin gene promoter, this is not consistent with other reported studies that show that native gluten proteins are not present in this tissue (Fincher, 1989). To confirm the latter, we carried out immunolocalization studies using the α-gliadin-specific Glia-α9 epitope antibody (Mitea *et al.*, 2008) and two different experimental approaches. Firstly, 1-µm sections of developing grains of 11, 18 and 25 DAA were fixed for conventional immunolocalization using light microscopy (Fig. 4). Secondly, proteins were transferred to a nitrocellulose membrane by tissue printing (Fig. 5). As a control, a monoclonal antibody specific for HMW subunits of glutenin (Mitea *et al.*, 2008) was also used on the thin sections
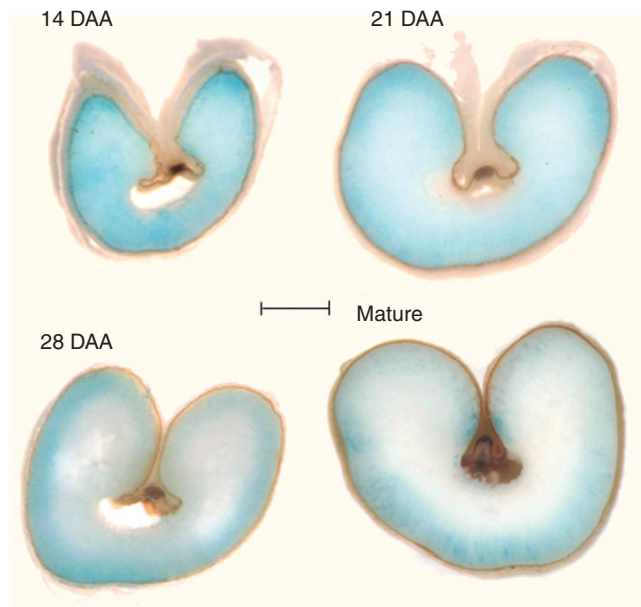

FIG. 2. GUS staining in developing kernels (14, 21, 28 DAA) and mature kernel of a representative homozygous T_3 transgenic line (B2219R1P10e.2·2). Scale bars = 1 mm.

of developing grains. Figure 4 compares immunostaining of thin sections with the α-gliadin-specific antibody (left) and HMW gluten antibody (right). This shows that α-gliadin and HMW subunits are present throughout the endosperm of the wheat kernel, including the subaleurone and central cells, and both are located in the protein bodies. However, in contrast to the GUS staining, no staining was observed in the aleurone cells. Similarly, immunostaining of tissue prints of mature grain (Fig. 5) showed the location of α-gliadin in the subaleurone and starchy endosperm cells but not in the aleurone cell layer. Post-staining with Ponceau S revealed the aleurone cells that were not labelled with the anti-α-gliadin antibody (Fig. 5C, D). To further confirm this, an antibody to 8S globulin, a protein present only in the aleurone cells and embryo of the grain was used. This stained only the outer layer of the endosperm corresponding to the aleurone (Fig. 5E), which was shown by double labelling to be outside the layer stained by the α-gliadin antibody (Fig. 5F; Wiley *et al.*, 2007).

DISCUSSION

Genome specificity and epitope content

The promoter sequence used in this study was obtained by Reeves and Okita in 1987. Based on gene sequence homology, we concluded that the accompanying α-gliadin gene, and with that this promoter sequence, is more closely related to the B genome (the Gli-2 locus on chromosome 6BS) instead of the A genome as suggested by Reeves and Okita (1987). It is not known if the promoters of α-gliadin genes originating from the A, B and D genomes differ in their expression patterns. Molberg *et al.* (2005) and Van Herpen *et al.* (2006) showed that a number of α-gliadins from the B genome do not contain any of the
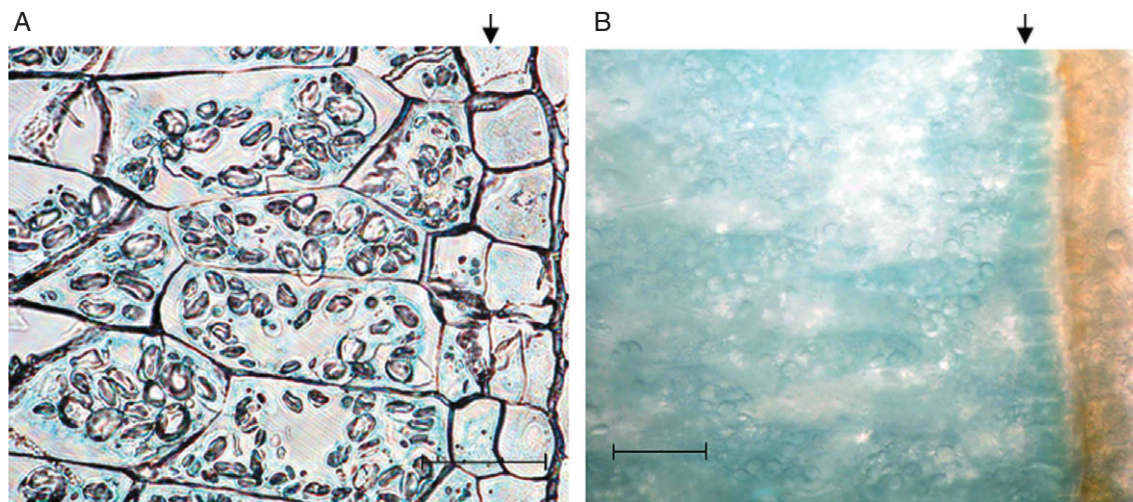
Fɪɢ. 3. GUS staining visualized at the cellular level of homozygous T_3 kernels at 11 DAA and in the mature stage. (A) B2255R9P7, 11 DAA. Microscopic observation of 1-μm thick fixed and embedded section of a kernel showing GUS staining in the subaleurone and aleurone cell layer of the kernel. (B) B2219R1P10e, mature stage. Microscopic observation of a 200 μm-thick cryostat section of a mature kernel showing clear GUS staining in the subaleurone and aleurone cell layers of the wheat grain. Arrows indicate the aleurone cell layer. Scale bars = 50 μm.

four identified α-gliadin T-cell toxic CD-epitopes (Glia-α, Glia-α2, Glia-α9 or Glia-α20; Spaenij-Dekking *et al.*, 2004), and these epitopes were also absent from the α-gliadin encoded by the gene used in this study.

Location of α-gliadin promoter activity during wheat kernel development

Expression of the GUS reporter gene driven by the α-gliadin promoter in transgenic wheat grains was observed as early as 11 DAA. This is in agreement with the previous report that gliadin transcripts began to accumulate in the endosperm by 9 DAA (Drea *et al.*, 2005). The expression became more prominent in the outer cells of the kernel as maturity progressed. The most frequently observed phenotype in the mature T_1 kernels (75 % of the GUS-positive kernels) was that GUS staining was strongest in the outer part of the kernel, and weaker in the central starch-rich endosperm cells. Previous studies by Kent and Evers (Kent, 1966; Evers, 1970) showed that all starchy endosperm cells appear to contain similar total amounts of protein but that the accumulation of starch occurs mainly in the central starchy endosperm cells. As a consequence, protein may represent up to half of the total cell mass in the subaleurone cells, but only 10 % of the total mass of the starch-rich central endosperm cells (Kent, 1966; Evers, 1970). The most frequently observed expression pattern of the α-gliadin promoter is consistent with a relatively greater accumulation in the outer layers as a consequence of dilution of the protein in the central cell by starch granules. This is similar to the reported expression pattern of a LMW glutenin promoter (Stoger *et al.*, 2001).

Correlation between phenotype and segregation

A correlation was observed between the segregation ratios and the phenotype, with a higher proportion of the T_1 kernels showing strong expression in lines segregating in a 15 : 1 ratio compared to 3 : 1 or non-Mendelian ratios. This is consistent with two transgene insertion sites and possibly a higher number of transgene copies, although transgene copy number was not measured here. Some atypical expression patterns, often with weak staining, were also observed in T_1 kernels from a few lines, particularly those showing non-Mendelian segregation ratios. The observation of non-Mendelian segregation may be due to expression levels being under the detection limit of the GUS staining procedure, leading to an under-estimation of true segregation ratios in these lines and patchy light staining. Alternatively, rearrangements of the transgene cassette during biolistic transformation may have resulted in truncation or rearrangements of functional promoter elements, resulting in loss of the typical expression pattern seen in the majority of lines.

Cellular location of the α-gliadin and HMW glutenin protein

The demonstration of GUS expression in the aleurone cells of the transgenic plants expressing the α-gliadin promoter–GUS construct was unexpected, as gluten protein gene expression has not previously been reported in this tissue. Lamacchia *et al.* (2001) and Stoger *et al.* (2001) reported similar studies, in which the GUS reporter gene was fused to glutenin subunit promoters and expressed in transgenic wheat: the HMW subunit 1Dx5 promoter in durum wheat (Lamacchia *et al.*, 2001) and a LMW subunit promoter in bread wheat (Stoger *et al.*, 2001). Both promoters drove GUS expression in the starchy endosperm cells, including the subaleurone, but not in the aleurone layer. Similarly, the *in situ* hybridization study of Drea *et al.* (2005) also showed α-gliadin transcripts in the subaleurone and central starchy endosperm but not in the aleurone.

To confirm our results we therefore used a specific monoclonal antibody to the Glia-α9 epitope of α-gliadin for
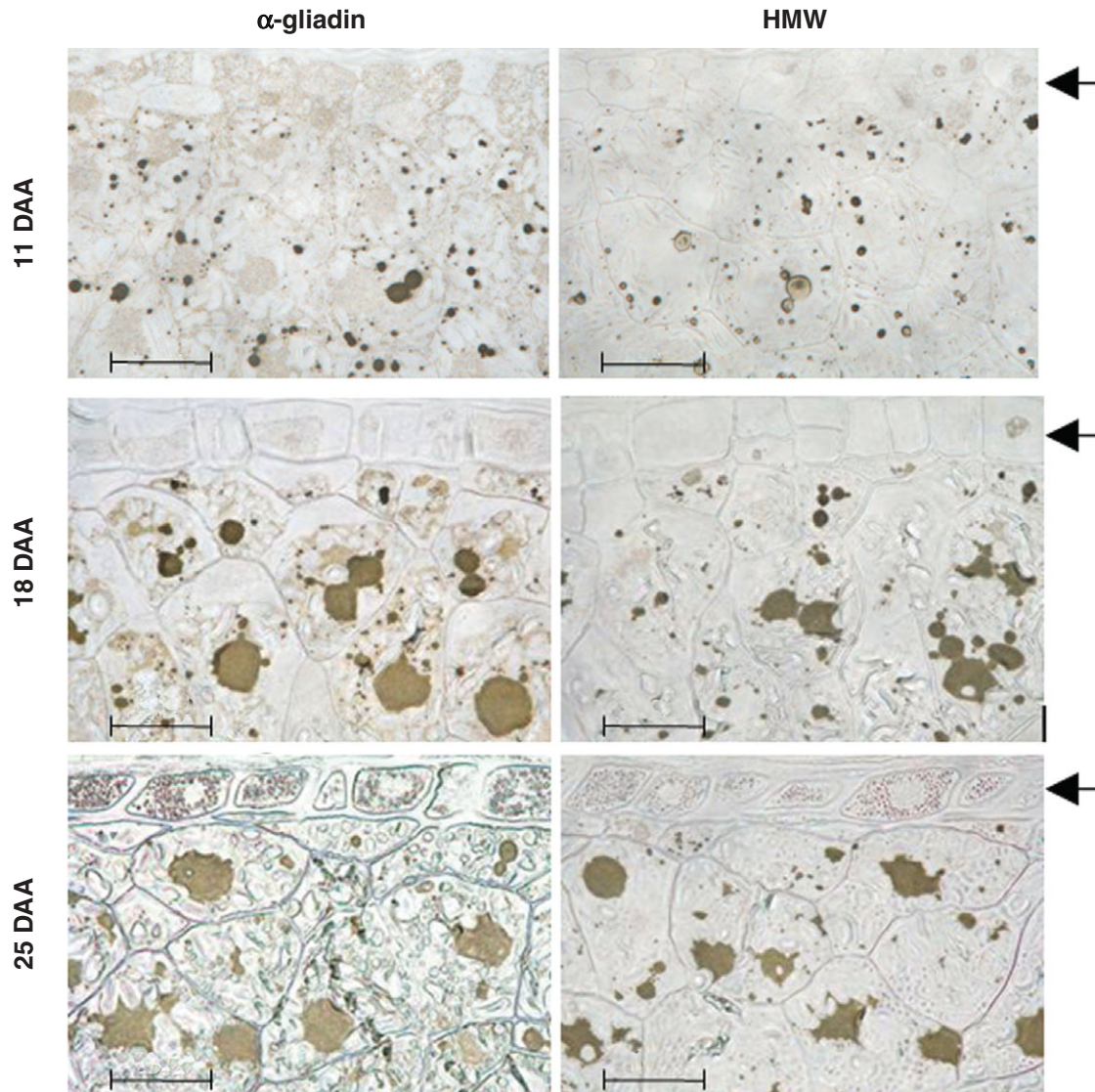
α-gliadin **HMW**

Fig. 4. Expression of α-gliadins and HMW glutenins in developing, non-transformed wheat grains at 11, 18 and 25 DAA. Immunogold labelling was carried out on 1-μm sections using (left) anti-α-gliadin-specific and (right) HMW-glutenin-specific antibodies. At all stages only the protein bodies in the subaleurone cell layer are labelled and no staining in the aleurone cells was observed (arrows indicate aleurone). Scale bars = 50 μm.

immunolocalization studies, using a specific monoclonal antibody to HMW glutenin subunits as a control (Mitea *et al.*, 2008). These antibodies showed similar labelling to the starchy endosperm cells, including the subaleurone, but not to the aleurone cells. This is consistent with the generally accepted view that aleurone cells do not contain gluten proteins, as reviewed by Fincher (1989).

The aleurone and starchy endosperm cells arise from the same cell lineage, with some of the starchy endosperm cells being derived from cell divisions in the aleurone, which acts as a meristem. It is therefore possible that (1) additional regulatory elements are present in the native α-gliadin promoter configuration that prevent expression in the aleurone cell layer, and (2) the lack of these elements in the 593-bp promoter used here resulted in aleurone misexpression. Of thirteen promoter sequences from the NCBI database (Table 1), nine start at −593 bp, probably because

of the presence of a Pst 1 restriction site that was used for cloning these genes. Analysis of the region upstream of −593 bp in the four remaining sequences failed to identify a CGN-4-like motif or prolamin box. A RY repeat (CATGCAC/T; Baumlein *et al.*, 1992; Fujiwara and Beachy, 1994; Moreno-Risueno *et al.*, 2008) was present at −774 bp in the original promoter (M16496), but not used in the 593 promoter region of this study. RY repeats have been described as playing a key role in the seed-specific regulation of seed storage protein genes in dicotyledons leguminous species. A deletion of part of the RY repeat in the *Vicia faba* legumin promoter (Baumlein *et al.*, 1992) resulted in expression in leaves of stably transformed tobacco plants, whereas a legumin promoter with an intact RY repeat was primary expressed in seed tissue. This indicates that this cis-acting element can repress transcription in certain tissues. However, Moreno-Risueno *et al.*
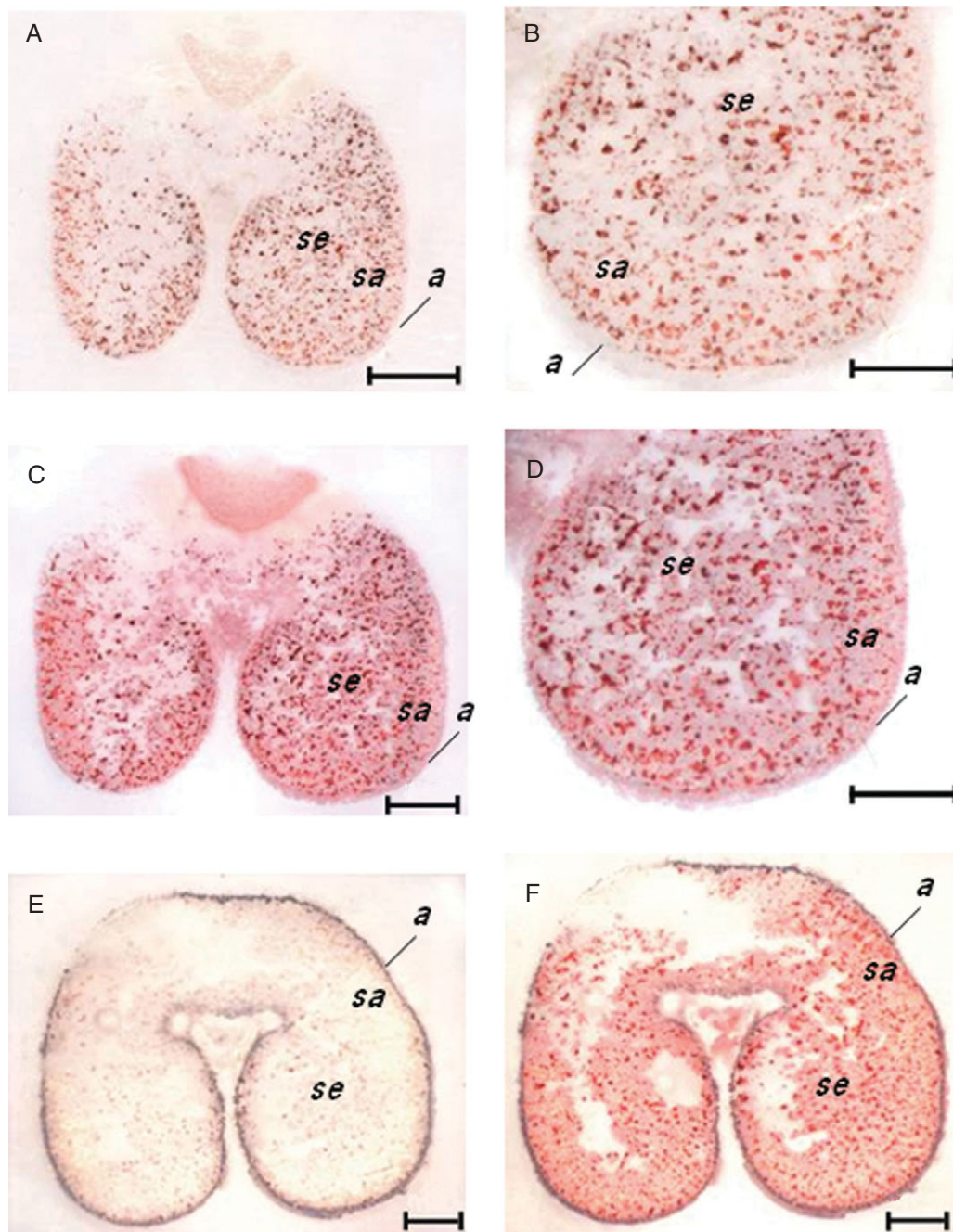
FIG. 5. Immunodetection of α-gliadin protein containing the Glia-α9 epitope and 8S globulin in tissue prints of mature wheat grains. (A) Immunodetection of α-gliadin shown by the red stain in the starchy endosperm labelling. (B) Detail of (A): note the lack of labelling in the aleurone layer. (C) Same tissue print as in (A) labelled with the anti-gliadin antibody and also post-stained with Ponceau S, which stains the protein-rich aleurone pink and changes the colour of the starchy endosperm cells. (D) Detail of (C): note the purple/pink stain in the aleurone layer not previously labelled in (A) and (B). (E) Immunodetection of 8S globulin shown by the clear purple labelling of the aleurone layer. (F) The same print as in (E) but also post-stained with Ponceau S, showing the presence of protein in the endosperm not previously labelled with the anti-8S globulin antibody. Abbreviations: *a*, aleurone; *sa*, sub-aleurone; *se*, starchy endosperm. Scale bars = 1 mm.

(2008) recently showed that the barley HvFUS3 transcription factor binds *in vitro* to the RY repeat in the promoter of *Hor2* (encoding the B-hordein storage protein). HvFUS3 was observed in the endosperm and aleurone cells as well as in the embryo (Moreno-Risueno *et al.*, 2008). Gene expression arises from the effects of various

transcription factors (Lara *et al.*, 2003), and full transactivation of Hor2 needed a combination of the transcription factors HvFUS3 and BLZ2 (Moreno-Risueno *et al.*, 2008). The RY-element would therefore be expected to be more important for activation rather than repression of transcription of the α-gliadin gene in wheat. The RY

element was also present in a number of the other α-gliadin promoter sequences, but located much closer to the transcription start site (position −294; Table 1).

Regulatory motifs in α-gliadin promoters from different genomes

We also observed an association between the genome of origin and the presence of endosperm-specific promoter motifs in the 593-bp promoter region. The GCN4-like motif at −492 bp was only present in the genes originating from the B genome and not in the sequences originating from the A genome (Table 1). Aryan *et al.* (1991) described a 20 % increase in promoter strength in the −592 to −448 region and the authors suggested the presence of an activating element in this region. The CGN4-like motif present at −492 is a candidate for such an activating element. These results indicate that differences in regulation of expression may exist between different members of the α-gliadin gene family, with the genome of origin being important. The RY repeat at position −294 was present in seven out of the eight A genome sequences and in none of the B genome sequences. The RY repeats have been proposed to have a key role in seed-specific gene regulation (Baumlein *et al.*, 1992). When analysing for sequence identity of the region upstream of −593 bp we observed an association between genome of origin and sequence identity.

The differences in regulator motifs between sequences originating from the A or B genome suggest that the regulation of expression between α-gliadin genes present on chromosomes 6A and 6B may be different. Kawaura *et al.* (2005) observed that early α-gliadins gene expression were preferentially from the D genome, and late expression from the A genome. However, further research is required to determine whether this can be exploited to manipulate the proportion of α-gliadin encoded by the *Gli 2* loci on the different genomes of wheat to decrease the toxicity in celiac disease.

CONCLUSIONS

We have studied the expression of a specific B-genome-encoded α-gliadin gene promoter fragment and the deposition of α-gliadin proteins. The promoter was active in the cells of the starchy endosperm, the subaleurone and the aleurone cell layer. No expression of GUS was found in other tissues. However, α-gliadin protein was detected together with HMW subunit protein in the protein bodies of the starchy endosperm and subaleurone cells but was not detected in the aleurone cells. The α-gliadin promoter was active from 11 DAA until maturity and showed a similar pattern of expression to the LMW subunit promoter. A large number of different transgenic plants with the same construct revealed differences in the observed phenotypes of expression. An association between segregation ratios of the transgene and the different phenotypes of expression was observed. These results emphasize the importance of testing a large number of different transgenic lines when studying the effects of transgenes. The results indicate that additional regulator elements upstream of the promoter region used may specifically repress expression in the aleurone cell layer. Observed differences in expression regulator motifs between the α-gliadin genes on the different genomes (A and B) of bread wheat leads to a better understanding how α-gliadin expression can be controlled.

LITERATURE CITED

Albani D, Hammond-Kosack MC, Smith C, Conlan S, Colot V, Holdsworth M, Bevan MW. 1997. The wheat transcriptional activator SPA: a seed-specific bZIP protein that recognizes the GCN4-like motif in the bifactorial endosperm box of prolamin genes. *The Plant Cell* **9**: 171–184.

Altpeter F, Vasil V, Srivastava V, Vasil IK. 1996. Integration and expression of the high-molecular-weight glutenin subunit 1Ax1 gene into wheat. *Nature Biotechnology* **14**: 1155–1159.

Anderson OD, Litts JC, Greene FC. 1997. The α-gliadin gene family. I. Characterization of ten new wheat α-gliadin genomic clones, evidence for limited sequence conservation of flanking DNA, and southern analysis of the gene family. *Theoretical and Applied Genetics* **95**: 50–58.

Arentz-Hansen EH, Körner R, Molberg Ø, Quarsten H, Vader W, Kooy Y, *et al.* 2000. The intestinal T-cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *The Journal of Experimental Medicine* **191**: 603–612.

Arentz-Hansen EH, McAdam S, Molberg Ø, Fleckenstein B, Lundin KEA, Jørgensen TJD, *et al.* 2002. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* **123**: 803–809.

Aryan AP, An G, Okita TW. 1991. Structural and functional analysis of promoter from gliadin, an endosperm-specific storage protein gene of *Triticum aestivum* L. *Molecular and General Genetics MGG* **225**: 65–71.

Barro F, Rooke L, Bekes F, Gras P, Tatham AS, Fido R, *et al.* 1997. Transformation of wheat with high molecular weight subunit genes results in improved functional properties. *Nature Biotechnology* **15**: 1295–1299.

Baumlein H, Nagyt l, Villarroel R, Inz D, Wobus U. 1992. Cis-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene. *The Plant Journal* **2**: 233–239.

Becker D, Folck A, Wieser H. 2006. Inhibierung der α-Gliadin-Genexpression in hexaploidem Brotweizen. *Getreidetechnologie* **60**: 153–156.

Blechl AE, Anderson OD. 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. *Nature Biotechnology* **14**: 875–879.

Christensen AH, Quail PH. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research* **5**: 213–218.

Conley CA, Hanson MR. 1997. Cryostat tissue printing: an improved method for histochemical and immunocytochemical localization in soft tissues. *Biotechniques* **22**: 491–496.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**: 462–469.

Diaz I, Martinez M, Isabel-LaMoneda I, Rubio-Somoza I, Carbonero P. 2005. The DOF protein, SAD, interacts with GAMYB in plant nuclei and activates transcription of endosperm-specific genes during barley seed development. *The Plant Journal* **42**: 652–662.

Dong G, Ni Z, Yao Y, Nie X, Sun Q. 2007. Wheat Dof transcription factor WPBF interacts with TaQM and activates transcription of an alpha-gliadin gene during wheat seed development. *Plant Molecular Biology* **63**: 73–84.

Drea S, Leader DJ, Arnold BC, Shaw P, Dolan L, Doonan JH. 2005. Systematic spatial analysis of gene expression during wheat caryopsis development. *The Plant Cell* **17**: 2172–2185.

Dupont FM, Hurkman WJ, Vensel WH, Tanaka C, Kothari KM, Chung OK, Altenbach SB. 2006. Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature. *European Journal of Agronomy* **25**: 96–107.

Evers AD. 1970. Development of the endosperm of wheat (*Triticum aestivum* L.). *Annals of Botany* **34**: 547–555.

Fincher GB. 1989. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 305–346.

Fujiwara T, Beachy RN. 1994. Tissue-specific and temporal regulation of a beta-conglycinin gene: roles of the RY repeat and other cis-acting elements. *Plant Molecular Biology* **24**: 261–272.

Harberd NP, Bartels D, Thompson RD. 1985. Analysis of the gliadin multigene locus in bread wheat using nullisomic-tetrasomic lines. *Molecular and General Genetics MGG* **198**: 234–242.

Kawaura K, Mochida K, Ogihara Y. 2005. Expression profile of two storage-protein gene families in hexaploid wheat revealed by large-scale analysis of expressed sequence tags. *Plant Physiology* **139**: 1870–1880.

Kent NL. 1966. Sub-aleurone endosperm cells of high protein content. *Cereal Chemistry* **43**: 585–601.

Koning F, Schuppan D, Cerf-Bensussan N, Sollid L. 2005. Pathomechanisms in celiac disease. *Best Practice & Research Clinical Gastroenterology* **19**: 373–387.

Lamacchia C, Shewry PR, Di Fonzo N, Forsyth JL, Harris N, Lazzeri PA, et al. 2001. Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed. *Journal of Experimental Botany* **52**: 243–250.

Lara P, Onate-Sanchez L, Abraham Z, Ferrandiz C, Dıaz I, Carbonero P, Vicente-Carbajosa J. 2003. Synergistic activation of seed storage protein gene expression in *Arabidopsis* by ABI3 and two bZIPs related to OPAQUE2. *Journal of Biological Chemistry* **278**: 21003–21011.

Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P. 1998. An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. *The Plant Journal* **16**: 53–62.

Mitea C, Havenaar R, Drijfhout JW, Edens L, Dekking L, Koning F. 2008. Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. *Gut* **57**: 25–32.

Molberg Ø, Solheim FN, Jensen T, Lundin KEA, Arentz-Hansen EH, Anderson OD, Kjersti UA, Sollid LM. 2003. Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. *Gastroenterology* **125**: 337–344.

Molberg Ø, Uhlen AK, Jensen T, Flaete NS, Fleckenstein B, Arentz-Hansen H, et al. 2005. Mapping of gluten T-cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology* **128**: 393–401.

Moreno-Risueno MÁ, González N, Díaz I, Parcy F, Carbonero P, Vicente-Carbajosa J. 2008. FUSCA3 from barley unveils a common transcriptional regulation of seed-specific genes between cereals and Arabidopsis. *The Plant Journal* **53**: 882–894.

Oñate L, Vicente-Carbajosa J, Lara P, Díaz I, Carbonero P. 1999. Barley BLZ2, a seed-specific bZIP protein that interacts with BLZ1 in *vivo* and activates transcription from the GCN4-like motif of B-hordein promoters in barley endosperm. *Journal of Biological Chemistry* **274**: 9175–9182.

Onodera Y, Suzuki A, Wu CY, Washida H, Takaiwa F. 2001. A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *Journal of Biological Chemistry* **276**: 14139–14152.

Popineau Y, Deshayes G, Lefebvre J, Fido R, Tatham AS, Shewry PR. 2001. Prolamin aggregation, gluten viscoelasticity and mixing properties of transgenic wheat lines expressing 1Ax and 1Dx high molecular weight glutenin subunit transgenes. *Journal of Agricultural and Food Chemistry* **49**: 395–401.

Reeves CD, Okita TW. 1987. Analyses of alpha/beta-type gliadin genes from diploid and hexaploid wheats. *Gene* **52**: 257–266.

Spaenij-Dekking EH, Kooy-Winkelaar EM, Nieuwenhuizen WF, Drijfhout JW, Koning F. 2004. A novel and sensitive method for the detection of T cell stimulatory epitopes of alpha/beta- and gamma gliadin. *Gut* **53**: 1267–1273.

Sparks CA, Jones HD. 2004. Transformation of wheat by biolistics. In: Curtis IP ed. *Transgenic crops of the world – essential protocols*. Dordrecht: Kluwer Academic Publishers, 19–34.

Stoger E, Williams S, Keen D, Cristou P. 1999. Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Research* **8**: 73–82.

Stoger E, Parker M, Christou P, Casey R. 2001. Pea legumin overexpressed in wheat endosperm assembles into an ordered paracrystalline matrix. *Plant Physiology* **125**: 1732–1742.

Takaiwa F, Yamanouchi U, Yoshihara T, Washida H, Tanabe F, Kato A, Yamada K. 1996. Characterization of common cis-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Molecular Biology* **30**: 1207–1221.

Vader W, Kooy Y, Van Veelen P, De Ru A, Harris D, Benckhuijsen W, et al. 2002. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* **122**: 1729–1737.

Vader LW, Stepniak DT, Bunnik EM, Kooy YM, De Haan W, Drijfhout JW, et al. 2003. Characterization of cereal toxicity for celiac disease patients based on protein homology in grains. *Gastroenterology* **125**: 1105–1113.

Van Herpen TWJM, Goryunova SV, Van der Schoot J, Mitreva M, Salentijn E, Vorst O, et al. 2006. Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. *BMC Genomics* **7**: 1. doi:10·1186/1471-2164-7-1.

Vellanoweth RL, Okita TW. 1993. Analysis of nuclear proteins interacting with a wheat α/β-gliadin seed storage protein gene. *Plant Molecular Biology* **22**: 25–41.

Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ. 1997. A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. *Proceedings of the National Academy of Sciences of the USA* **94**: 7685–7690.

Wieser H, Koehler P, Folck A, Becker D. 2006. Characterization of wheat with strongly reduced α-gliadin content. In: Lookhart GL, Ng PKW, eds. *Gluten proteins*. St Paul, MN: AACC International, Inc, 14–16.

Wiley PR, Tosi P, Evrard A, Lovegrove A, Jones HD, Shewry PR. 2007. Promoter analysis and immunolocalisation show that puroindoline genes are exclusively expressed in starchy endosperm cells of wheat grain. *Plant Molecular Biology* **64**: 125–136.

Wu C, Washida H, Onodera Y, Harada K, Takaiwa F. 2000. Quantitative nature of the prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. *The Plant Journal* **23**: 415–421.

Yupsanis T, Burgess SR, Jackson PJ, Shewry PR. 1990. Characterization of the major protein component from aleurone cells of barley (*Hordeum vulgare* L.). *Journal of Experimental Botany* **41**: 385–392.