Theor Appl Genet (1999) 98: 1253–1262

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Inheritance of tissue-specific expression of barley hordein promoter-*uidA* fusions in transgenic barley plants

Received: 28 May 1998 / Accepted: 19 December 1998

Abstract Barley (*Hordeum vulgare* L.) hordeins are alcohol-soluble redundant storage proteins that accumulate in protein bodies of the starchy endosperm during seed development. Strong endosperm-specific β-glucuronidase gene-(*uidA*; *gus*) expression driven by B_1- and D-hordein promoters was observed in stably transformed barley plants co-transformed with the selectable herbicide resistance gene, *bar*. PCR analysis using DNA from calli of 22 different lines transformed with B_1- or D-hordein promoter-*uidA* fusions showed the expected 1.8-kb *uidA* fragment after PCR amplification. DNA-blot analysis of genomic DNA from T_0 leaf tissue of 13 lines showed that 12 (11 independent) lines produced *uidA* fragments and that one line was *uidA*-negative. T_1 progeny from 6 out of 12 independent regenerable transgenic lines tested for *uidA* expression showed a 3:1 segregation pattern. Of the remaining six transgenic lines, one showed a segregation ratio of 15:1 for GUS, one expressed *bar* alone, one lacked transmission of either gene to T_1 progeny, and three were sterile. Stable GUS expression driven by the hordein promoters was observed in T_5 progeny in one line, T_4 progeny in one line, T_3 progeny in three lines and T_2 or T_1 progeny in the remaining two fertile lines tested; homozygous transgenic plants were obtained from three lines. In the homozygous lines the expression of the GUS protein, driven by either the B_1- or D-hordein promoters, was highly expressed in endosperm at early to mid-maturation stages. Expression of *bar* driven by the maize ubiquitin promoter was also stably transmitted to T_1 progeny in seven out of eight lines tested. However, in most lines PAT expression driven by the maize ubiquitin promoter was gradually lost in T_2 or later generations; one homozygous line was obtained. In contrast, six out of seven lines stably expressed GUS driven by the hordein promoters in T_2 or later generations. We conclude that the B_1- and D-hordein promoters can be used to engineer, and subsequently study, stable endosperm-specific gene expression in barley and potentially to modify barley seeds through genetic engineering.

Key words Barley (*Hordeum vulgare* L.) · Hordein promoters · Tissue-specific expression · Transgene inheritance · Transgene expression stability

Communicated by P. Langridge

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Introduction

Storage proteins account for about 8–15% of the dry weight of the mature barley grain. The major seed storage proteins in barley are alcohol-soluble prolamines, termed hordeins, which are classified into two major groups, B and C, and two minor groups, D and γ (Shewry 1993). Depending on nitrogen levels, these four groups account for about 35–55% of total protein of the seed. The B-and C-hordeins account for about 70–80% and 10–20%, respectively, of the total hordein fraction, with small amounts of D- (2–4%) and γ-hordeins (not precisely determined). The B-, D- and γ-hordeins are sulfur-rich while the C hordeins are sulfur-poor (Bright and Shewry 1983). The hordeins are coordinately synthesized in the developing starchy endosperm tissue (Giese et al. 1983; Sørensen et al. 1989). They are co-translationally transported into the lumen of the rough endoplamic reticulum, with simultaneous cleavage of the signal peptide, and are ultimately deposited into protein bodies (Cameron-Mills 1980; Cameron-Mills and von Wettstein 1980; Cameron-Mills and Madrid 1989).

Genetic analyses show that all hordeins are encoded by structural genes on chromosome 5 (1H) of barley; the *Hor1*, *Hor2*, *Hor3* and *Hor5* loci encode the C-, B-,

D-, and γ-hordein polypeptides, respectively (Jensen et al. 1980; Shewry et al. 1980; Blake et al. 1982; Shewry et al. 1983; Shewry and Parmar 1987). The genes for B-, C- and D-hordeins have been isolated and characterized (Brandt et al. 1985; Forde et al. 1985; Rasmussen and Brandt 1986; Sørensen et al. 1996). The B- and C-hordeins are encoded by multigene families comprising 10–20 members while D-hordein is encoded by a single gene (Brandt et al. 1985; Rasmussen and Brandt 1986; Sørensen et al. 1996).

The regulation and expression of these hordein promoters have been studied using transient expression assays in the barley endosperm (Entwistle et al. 1991; Knudsen and Müller 1991; Müller and Knudsen 1993; Sørensen et al. 1996; Cho and Lemaux 1997). As determined by these assays, which utilized promoter-*uidA* fusions, the D-hordein promoter is 3- to 5-fold more active than the B- or C-hordein promoters tested (Sørensen et al. 1996).

The developmental and tissue-specific expression driven by the barley B-hordein promoter has also been studied in a heterologous system using promoter-*cat* fusions in transgenic tobacco plants (Marris et al. 1988). With the development of a reproducible and efficient transformation system for barley (Wan and Lemaux 1994; Lemaux et al. 1996; Cho et al. 1998) it became possible to characterize these promoters more accurately in stably transformed barley. In this report, we use transient and stable gene expression assays to test the tissue-specificity and timing of GUS expression driven by hordein promoters.

Materials and methods

Plant materials

A two-rowed spring cultivar of barley, Golden Promise, was grown in growth chambers as described previously (Wan and Lemaux 1994; Lemaux et al. 1996).

Plasmids

p16 (Sørensen et al. 1996) contains a pUC18 backbone with the β-glucuronidase gene (*uidA*; *gus*) controlled by 550 bp of the barley endosperm-specific B_1-hordein promoter and terminated by the *Agrobacterium tumefaciens* nopaline synthase 3′ polyadenylation signal, *nos*. Plasmid pD11-Hor3 (Sørensen et al. 1996) contains *uidA* controlled by 434 bp of the D-hordein promoter and a *nos* terminator. pAHC20 (Christensen and Quail 1996) contains *bar* driven by the maize ubiquitin promoter first intron, and terminated by the *nos* 3′-end. pAHC25 (Christensen and Quail 1996) consists of *uidA* and *bar*, each under the control of the maize ubiquitin (*Ubi1*) promoter and first intron, and terminated by *nos*.

Transient gene expression of the *uidA* genes driven by the B_1- and D-hordein promoters

Spikes about 20 days after pollination were surface-sterilized for 10–15 min in 20% (v/v) bleach (5.25% sodium hypochlorite), followed by three washes with sterile water. Immature embryos and endosperm were aseptically separated and placed either scutellum-side up (embryo) or endosperm groove-side down (endosperm) on MS medium (Murashige and Skoog 1962) supplemented with 3.5 g/l of Phytagel (Sigma, St. Louis, Mo.). The tissues, bombarded using a Biolistic PDS-1000 He gun (Bio-Rad, Hercules, Calif.) at 1100 psi with 1.0 μm gold particles coated with either p16 or pD11-Hor3 (Lemaux et al. 1996), were incubated at 24 ± 1°C in the dark for 1 day and stained for activity using a histochemical GUS assay (see below).

Stable barley transformation

Stable transgenic lines of barley containing B_1-hordein-*uidA* or D-hordein-*uidA* were obtained using modifications of published protocols (Wan and Lemaux 1994; Lemaux et al. 1996). Gold particles (1.0 μm) were coated with 25 μg of pAHC25 alone or a 1:2 molar ratio of pAHC20 and either p16 or pD11-Hor3, and used in bombardment experiments as described above. Callus obtained from bombarded immature embryos was selected on callus-induction medium containing 5 mg/l of bialaphos. Bialaphos-resistant calli were regenerated on FHG (Hunter 1988) medium containing 1 mg/l of 6-benzylaminopurine (BAP) and 3 mg/l of bialaphos. Regenerated shoots were transferred to Magenta boxes with rooting medium (callus-induction medium without phytohormones) containing 3 mg/l of bialaphos. When shoots reached the top of the box, plantlets were transferred to soil and grown to maturity in the greenhouse.

Histochemical and quantitative assays of GUS activity

Histochemical staining for GUS was performed (Jefferson et al. 1987) using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc) (Gold Biotechnology, Inc., St. Louis, Mo.). Samples were incubated overnight at 37°C in GUS assay buffer.

Quantitative GUS-activity measurements were performed by the method of Jefferson et al. (1987) using a 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate (Sigma, St. Louis, Mo.). From homozygous lines a single mature or immature endosperm at 10–14, 20 and 30 days after pollination was isolated, frozen in liquid nitrogen, and ground in GUS extraction buffer; each treatment had four replicates. After centrifugation the supernatant fractions were used to determine GUS activity. Fluorescence of 4-methylumbelliferone (4-MU) (Sigma, St. Louis, Mo.) was measured on a TKO 100-dedicated mini fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Proteins were extracted as described previously (Jefferson 1987; Jefferson et al. 1987) and protein concentrations in extracts were measured according to Bradford (1976) using Bio-Rad reagent (Bio-Rad, Richmond, Calif.).

Herbicide application

To determine the herbicide sensitivity of plants, a section of leaf blade at the 4–5-leaf stage was painted using a cotton swab with a 0.25% solution (v/v) of Basta™ (starting concentration, 200 g/l phophinothricin, Hoechst AG, Frankfurt, Germany) plus 0.1% Tween 20. Plants were scored 1 week after herbicide application.

Genomic DNA isolation, polymerase chain reaction (PCR) and DNA-blot hybridization

Total genomic DNA from independent calli or leaf tissues was purified as described (Dellaporta, 1993). To test for the presence of

uidA in the genomic DNA of putatively transformed lines, 250 ng of genomic DNA was amplified by PCR using the primer set UIDA1 (5′-agcggccgcaTTACGTCCTGTAGAAACC-3′) and UID2R (5′-agagctcTCATTGTTTGCCTCCCTG-3′), each with a restriction enzyme site (small letters) for subcloning of another DNA construct containing the *uidA* gene (Cho et al. 1998). The presence of *bar* was tested using the primer set BAR5F (5′-CATCGAGACAAGCACGGTCAACTTC-3′) and BAR1R (5′-ATATCCGAGCGCCTCGTGCATGCG-3′) (Lemaux et al. 1996). Amplifications were performed with *Taq* DNA polymerase (Promega, Madison, Wis.) in a 25-µl reaction (Cho et al. 1998). Twenty five microliters of the PCR product with loading dye was electrophoresed on a 0.8% agarose gel with ethidium bromide and photographed using UV light. For DNA hybridization analysis, 10 µg of total genomic DNA from leaf tissue of each line was digested with *Eco*RI and *Bam*HI or *Eco*RI and *Eco*RV, separated on 1.0% agarose gels, transferred to a Zeta-Probe GT membrane (Bio-Rad, Hercules, Calif.) and hybridized with a radiolabeled *uidA*-specific probe following manufacturer's instructions. The *uidA*-containing 1.48-kb *Sna*BI-*Bam*HI fragment from pD11-Hor3 was purified using a QIAEX gel extraction kit (QIAGEN, Chatsworth, Calif.) and labeled with α-^{32}P-dCTP using random primers.

Cytological analysis

For cytological analysis of transgenic barley plants, healthy root meristems were collected from young plants grown in the greenhouse. After pre-treatment in a saturated 1-bromonaphthalene solution overnight at 4°C, root meristems were fixed in 1 : 3 glacial acetic acid:ethanol and stored at 4°C. Root meristems were hydrolyzed in 1 M HC1 at 60°C for 5–7 min, stained in Feulgen solution and then squashed on a glass slide in a drop of 1% acetocarmine. Chromosomes were counted from at least five root tips of plants from each T_0 line.

Results

PCR and DNA blot-hybridization analysis of transgenic plants

To characterize the B_1- and D-hordein promoters, we obtained 22 putatively independent stably transformed barley callus lines containing either B_1- or D-hordein promoter-*uidA* fusions. Thirteen lines were regenerable, seven B_1-hordein-*uidA* transformants and six D-hordein-*uidA* transformants. Genomic DNA from the callus of regenerable transformants was isolated. PCR analysis was performed using UIDA and BAR primers and resulted in the generation of a 1.8-kb intact *uidA* and 0.34-kb internal *bar* fragments from all 13 lines (Fig. 1 A and B, respectively). Of these lines, however, one (GPDhGN-22) did not produce a PCR-amplified fragment for *uidA* from T_0 leaf tissue (see Table 1). Figure 2 shows a DNA-hybridization blot of genomic DNA from T_0 leaf tissue of the seven B_1-hordein-*uidA* and the six D-hordein-*uidA* transformants. Twelve of thirteen transformed lines produced the expected 2.35-kb or 2.25-kb hordein-*uidA* fusion fragments after digestion with *Eco*RI and *Bam*HI, but a clear size difference in the hybridizing fragments could not be observed between the B_1-hordein-*uidA* and D-hordein-*uidA* transformants (Fig. 2). A size difference after digestion with *Eco*RI and *Eco*RV was observed between the two classes of transformants, 1.12-kb and

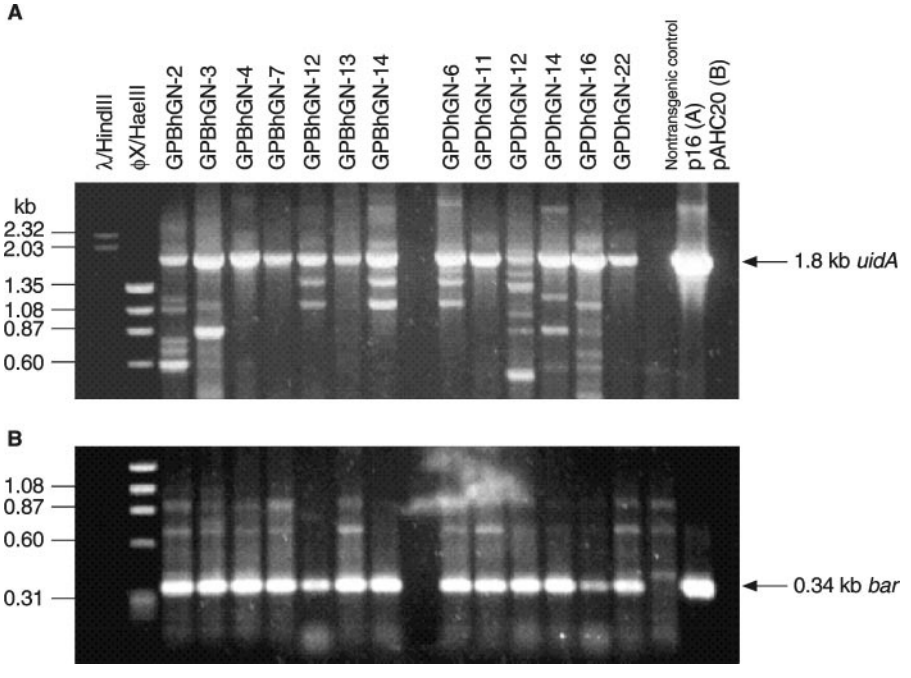

Fig. 1A, B PCR analysis of genomic DNA from control and transgenic callus tissues. Genomic DNA from non-transgenic control and independently transformed callus lines was used in PCR reactions with (**A**) the primer set UIDA1 and UIDA2R to amplify the 1.8-kb intact *uidA* fragment (*arrow*) or with (**B**) the primer set BAR5F and BAR1R to amplify the 0.34-kb internal *bar* fragment (*arrow*). The markers are λ/*Hin*dIII and ϕX174/*Hae*III; the plasmids p16 and pAHC20 were used in control reactions in **panels A and B,** respectively

Table 1 Analysis of T_0 barley plants and their progeny transformed with B_1- and D-hordein-*uidA* fusions. Expression of *bar* and *uidA* was tested by Basta painting and histochemical GUS assay, respectively, except for confirmation of *uidA* in T_0 plants by PCR

Plasmids used for bombardment	Transgenic barley lines	T_0		T_1		T_2		T_3		T_4		T_5	
		bar	*uidA* (PCR)	*uidA* (+/−)	*bar* (+/−)	*uidA* (+/−)	*bar* (+/−)	*uidA* (+/−)	*bar* (+/−)	*uidA* (+/−)	*bar* (+/−)	*uidA* (+/−)	*bar* (+/−)
p16 + pAHC20	B2 (GPBhGN-2)*[a]	+	+										
	B3 (GPBhGN-3)	+	+	11/3†									
	B4 (GPBhGN-4)[b]	+	+	70/20†	25/10								
	B4-34					17/0	0/14						
	B4-34-6-3									41/0			
	B4-34-6-4									51/0			
	B4-34-6-5									39/0			
	B4-34-6-6									35/0			
	B4-34-7							48/0	0/9				
	B4-34-7-1									38/0	0/8		
	B4-34-7-2									26/0			
	B4-34-7-3									23/0			
	B4-34-7-4									25/0			
	B4-34-7-1-1											22/0	
	B4-34-7-1-2											73/0	
	B7 (GPBhGN-7)[b]	+	+	45/18†	18/6								
	B7-2					25/0	13/1						
	B7-2-1							32/0	14/4				
	B7-2-1-1									20/0			
	B7-2-1-2									59/0			
	B7-2-1-3									38/0			
	B7-2-2-4									24/0			
	B7-2-2							n.t.	6/7				
	B12 (GPBhGN-12)**	+	+	21/12†	15/2								
	B12-2					9/11	5/2						
	B12-2-2							4/9					
	B12-2-3							1/2					
	B12-4					2/0	6/4						
	B13 (GPBhGN-13)**	+	+	0/21	0/46								
	B13-1					0/26	0/14						
	B13-1-8							0/20					
	B13-1-12							0/27					
	B13-2					0/17	0/15						
	B14 (GPBhGN-14)**	+	+	19/12†	20/9								
	B14-1					10/1	11/1						

pD11-Hor3 + pAHC20	D6 (GPDhGN-6)**	+	+	43/2‡	8/7					
	D6-3					40/1	2/10			
	D6-3-3							18/1		
	D6-3-4							51/0		
	D6-3-5							60/1		
	D6-9					24/1	1/7			
	D6-9-1							24/0		
	D6-9-6							81/0		
	D6-9-7							39/1		
	D11 (GPDhGN-11)**	+	+	17/10†	2/1					
	D11-2					1/0	9/1			
	D11-2-3							2/1		
	D12 (GPDhGN-12)*[a]	+	+							
	D14 (GPDhGN-14)*[c]	+	+	n.t.[d]	1/2					
	D14-1					0/20	12/5			
	D14-1-9							0/28		
	D14-1-10							0/29		
	D14-1-13							0/25		
	D14-1-15							0/14		
	D16 (GPDhGN-16)**	+	+	7/2†	7/1					
	D16-5					25/18	4/0			
	D16-5-2							4/0	15/0	
	D16-5-2-3									19/0
	D16-5-2-7									9/0
	D16-5-2-8									10/0
	D16-5-2-10									5/0
	D22 (GPDhGN-22)*[a]	+	−							

* Sterile
** Tetraploid
†, ‡, analyses using the χ^2-test indicate that the segregation ratios of T_1 progeny for GUS were not significantly different from 3 : 1 and 15 : 1, respectively (at $\alpha = 0.05$)
[a] Chromosomes were not counted
[b] GPBhGN-4 and -7 had identical DNA hybridization patterns for *uidA*
[c] Outcrossed with non-transgenic plants
[d] n.t.; not tested

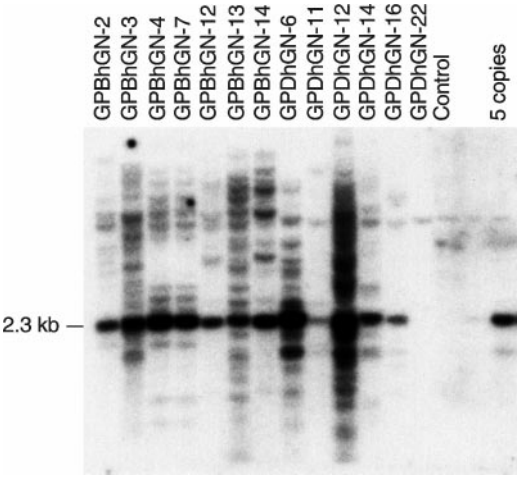

Fig. 2 DNA blot-hybridization analysis of genomic DNA from control and transgenic leaf tissues. Genomic DNA was isolated from leaf tissues of non-transformed control, seven B_1-hordein-*uidA* T_0 transformed plants and six D-hordein-*uidA* T_0 transformed plants. Ten micrograms of genomic DNA per lane were digested with *Eco*RI and *Bam*HI; gel blots were hybridized with a radiolabeled *uidA* probe. The *lane designated 5 copies* represent five copies of plasmid (pD11-Hor3) per barley genome

1.02-kb fragments for B_1-hordein-*uidA* and D-hordein-*uidA* transformants, respectively (data not shown). Consistent with the PCR results, the remaining line (GPDhGN-22) did not produce a *uidA*-hybridizing fragment (Fig. 2), although this line did contain the appropriately sized *bar*-hybridizing bands (data not shown). GPBhGN-4 and -7 had an identical hybridization pattern for *uidA* (Fig. 2) indicating that these two lines most likely originated from the same original transformed cell.

Hordein-*uidA* expression in barley endosperm

To study the endosperm-specificity of the B_1- and D-hordein promoters, plasmids p16 and pD11-Hor3 were used in transient assays involving microprojectile bombardment of immature barley endosperm and embryos. GUS driven by B_1- and D-hordein promoters was strongly expressed in endosperm, but not embryo, tissue. Expression driven by the D-hordein promoter resulted in greater numbers of GUS spots in endosperm tissue than that driven by the B_1-hordein promoter (data not shown). As expected, negative controls bombarded with $1 \times$ TE buffer did not yield GUS expression.

Different tissues from stably transformed T_1 plants and their progeny were tested for histochemical GUS activity (Fig. 3). Strong GUS expression was seen in endosperm tissues derived from transformants having both B_1- and D-hordein-*uid*A constructs (Fig. 3 A); no expression was observed in the embryo (Fig. 3 A), ovary, stigma, anther (Fig. 3 B) or leaf tissues (Fig. 3 C). GUS expression was observed in all transformed tissues that contained *uid*A under the control of the maize ubiquitin (*Ubi1*) promoter; no GUS expression was observed in the non-transformed control tissues (Fig. 3 A–C). No histochemical GUS activity was observed in roots and shoots from germinating T_1 seed of transformants containing either B_1- (Fig. 3 D) or D-hordein (data not shown) promoter-*uidA* fusions.

Relative activities of the B_1- and D-hordein-*uidA* constructs were determined by fluorometric analyses of GUS in extracts of developing and mature seeds from the homozygous lines (Table 2). Of the time-points tested, the specific acitivities of GUS driven by the B_1-promoter had maximum levels expression at 10–20 days post-pollination. The D-hordein promoter showed a developmental pattern with peak specific activities at 20–30 days post-pollination.

Analysis of T_0–T_5 progeny

Enzyme activity in T_0 plants and their progeny was tested by painting leaves with Basta for PAT (phosphinothricin acetyltransferase, product of *bar*) and by testing by histochemical assay for GUS. Leaf tissue from T_0 plants of all 13 (12 independent) lines exhibited Basta resistance (Table 1). In T_1 progeny 7 out of the 13 lines tested showed a 3 : 1 segregation pattern for the expression of GUS (Table 1). Of the remaining six lines, one line (GPDhGN-6) had a 15 : 1 segregation ratio for GUS expression; one line (GPDhGN-22) expressed PAT but did not contain *uidA*; one line (GPBhGN-13) contained neither *uidA* nor *bar*; and three lines (GPBhGN-2, GPDhGN-12 and GPDhGN-14) were sterile. T_1 endosperm from all fertile T_0 transgenic lines having positive DNA hybridization signals for *uidA* [2.35-kb and 2.25-kb fragments for the B_1-hordein-*uidA* and D-hordein-*uidA* genes, respectively (Fig. 2)], exhibited strong GUS activity (Table 1; Fig. 3 A) except for GPBhGN-13 and GPDhGN-14. The *bar* gene was stably transmitted to T_1 progeny of all fertile lines except GPBhGN-13, but in most lines loss of *bar* expression was observed in T_2 or later generations; one stably expressing homozygous line (GPDhGN-16) was obtained (Table 1). Expression of *uidA* driven by either the B_1- or D-hordein promoter was much more stable in its inheritance pattern; for example, in T_2 progeny of all seven independent lines tested (GPBhGN-4, -7, -12, -14, GPDhGN-6, -11 and -16) (Table 1; Fig. 3F). Expression of the *uidA* gene was stably transmitted in the one line tested at the T_5 generation (GPBhGN-4), in the two lines tested at T_4 (GPBhGN-7 and GPDhGN-16), in the three lines tested at T_3 (GPBhGN-12, GPDhGN-6 and -11), one line at T_2 (GPBhGN-14) and one line at T_1 (GPBhGN-3)

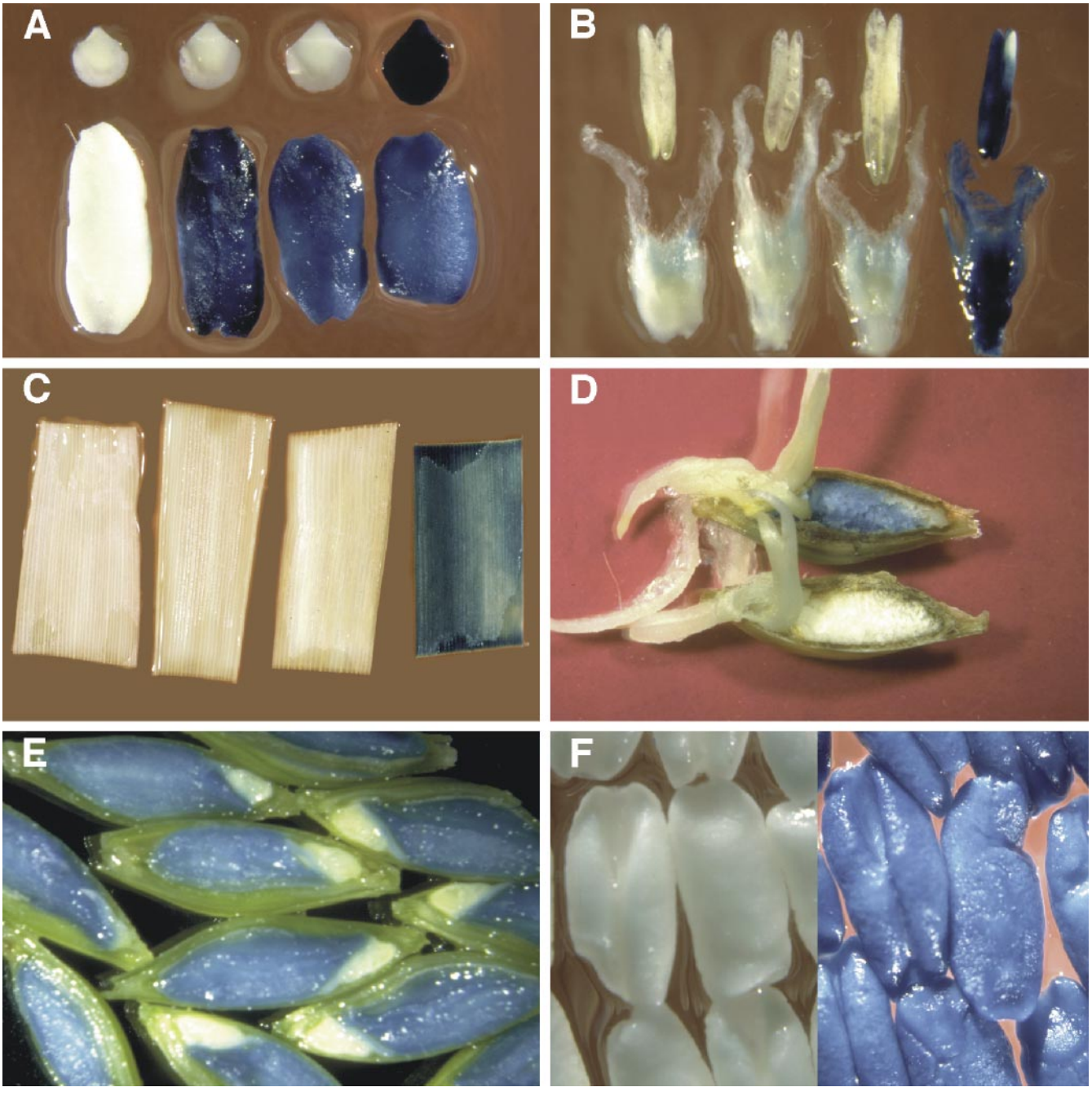

Fig. 3A–F Histochemical GUS assay in different tissues of stably transformed barley. (**A–C**) GUS activity in T_1 endosperm and embryo tissues (**A**), ovary, stigma and anthers (**B**), and leaf (**C**), in (from left to right) non-transformed control plants and those transformed with B_1-hordein-*uidA* (p16), D-hordein-*uidA* (pD11-Hor3), and ubiquitin-*uidA* (pAHC25). (**D**) A germinating non-transgenic seed (bottom) and a transgenic T_1 seed (top) transformed with p16, showing endosperm-specificity of the B_1-hordein promoter. (**E**) Homozygous T_4 seeds stably transformed with p16 showing endosperm-specificity of the B_1-hordein promoter. (**F**) Non-transformed (left) and p16-transformed (right) T_2 endosperm tissue showing endosperm-specific GUS expression driven by the B_1-hordein promoter

(Table 1). Homozygous transgenic lines stably expressing GUS were obtained from GPBhGN-4, -7, GPDhGN-6 and -16 (Figs. 3 E and F; Tables 1 and 2).

Cytological analysis of transgenic plants

Chromosome numbers were counted in root meristem cells of independently transformed T_0 barley plants. Out of ten independent transgenic lines examined, four lines [GPBhGN-3, -4 (or -7), GPDhGN-14 and -22] had the normal diploid chromosome complement ($2n = 2x = 14$), while the remaining six lines

Table 2 GUS specific activities in developing and mature transgenic barley endosperm. GUS activity was determined by fluorometric assays on the protein extracts from developing and mature endosperm of homozygous lines. Values for GUS activity represent mean ± standard deviation of four replicates for each treatment. GPBhGN-4-34-7-1-2 transformed with B_1-hordein-*uidA* (p16) and GPDhGN-6-9-6 transformed with D-hordein-*uidA* (pD11-Hor3) are T_4 and T_2 homozygous plants, respectively

Transgenic line	GUS activity (pmol/min per mg protein)			
Days after pollination	10–14	20	30	Mature
GPBhGN-4-34-7-1-2	3514 ± 1326	2309 ± 454	1313 ± 340	106 ± 37
GPDhGN-6-9-6	402 ± 163	3812 ± 969	2806 ± 949	281 ± 52
Non-transgenic control	80 + 31	81 ± 23	36 ± 13	43 ± 9

(GPBhGN-12, -13, -14, GPDhGN-6, -11, and -16) were tetraploid ($2n = 4x = 28$) (Table 1).

Discussion

The study of the B- and D-hordein promoters using molecular approaches has been carried out previously using transient expression assays in barley (Knudsen and Müller 1991; Müller and Knudsen 1993; Sørensen et al. 1996; Cho and Lemaux 1997) and stable transformation of tobacco (Marris et al. 1988). In this report, we tested the functionality of the barley B_1- or D-hordein promoters using both transient and stable expression assays in barley. Consistent with earlier studies (Müller and Knudsen 1993; Sørensen et al. 1996), transient expression of GUS under the control of the B_1- and D-hordein promoters was not observed in embryos but was observed in developing endosperm.

PCR analysis confirmed the presence of *uidA* and *bar* in genomic DNA from T_0 plants of 13 lines stably transformed with B_1- or D-hordein promoter-*uidA* fusions. Transgenic T_0 barley plants and their progeny were further analyzed by DNA-blot analysis, Basta painting, and GUS assay. T_0 plants from all 13 (12 independent) lines examined contained *bar* and were Basta-resistant; 12 (11 independent) lines had *uidA*-hybridizing sequences, giving a 92% co-transformation frequency, similar to the frequencies reported in earlier studies in barley (Wan and Lemaux 1994; Lemaux et al. 1996). DNA-blot analysis of the T_0 plant tissue of 12 lines showed that multiple (2 to >10) copies of *uidA* were integrated into the genome; GPBhGN-4 and -7 were identical in hybridization patterns indicating that these two lines most likely originated from a single original transformed cell. Leaf tissue from a T_0 plant, derived from one callus line (GPDhGN-22) that contained the expected *uidA* fragment by PCR, was positive for *bar* but negative for *uid*A in DNA-blot analyses. The loss of *uidA* in DNA-blot analyses is possibly due to the presence of both *uidA*-transformed and non-transformed cells in the callus tissue used for DNA extraction.

Stably transformed, developing and germinating barley seeds were characterized in terms of the tissue-specificity of hordein promoter-driven GUS expression. GUS driven by B_1- and D-hordein promoters was expressed exclusively in the endosperm tissue of stably transformed plants, but not in other tissues. These results are consistent with the conclusions of Brandt (1976) that B_1- and D-hordein polypeptides are found only in endosperm tissues of barley. They are also consistent with those of Marris et al. (1988), who showed that chloramphenicol acetyl transferase (CAT) activity, driven by a B_1-hordein promoter, only occurred in the endosperm tissues of transgenic tobacco seeds.

We also found that, based on fluorometric GUS assay results, GUS expression driven by the B_1-hordein promoter reaches maximum levels at the early to mid-maturation stage, 10–20 days post-pollination, although it appears to drop at later stages (30 days post-pollination). Measurements of expression at times earlier than 10 days were not technically feasible and, therefore, the possibility that peak expression occurred at an earlier time point cannot be eliminated. Given the error in our measurements at 10–14 and 20-days post-pollination, the peak expression time we observed is consistent with the observations of Marris et al. (1988), who found that CAT activity peaked at 19 days post-pollination in transgenic tobacco seed. In their study, however, activity remained constant until maturity at 31 days post-pollination. The fact that we found GUS levels dropped at 30 days post-pollination and in the mature grain might reflect either a difference in the plant species in the stability of expression or differences in the relative stability of the marker gene product (CAT vs GUS). Peak expression of GUS driven by the D-hordein promoter occurred at a mid-maturation stage, 20–30-days post-pollination, also dropping dramatically in the mature grain. Our results on the timing of expression are also in general agreement with those of Brandt (1976) that the total hordein protein fraction reaches maximal levels at 20 days post-pollination. Since the total hordein fraction represents a summation of the expression driven by all hordein promoters, our data with the B- and D-hordein promoters falls within the appropriate time frame.

Of the nine fertile lines, seven expressed GUS in the endosperm and had segregation ratios for expression in

T_1 seed that were not significantly different from 3:1. One fertile line (GPDhGN-6) had a segregation ratio for GUS expression in T_1 seed (43:2) that was not significantly different from 15:1. This line had a 1:1 segregation ratio for PAT expression, most likely reflecting the transgene expression instability of PAT driven by the maize ubiquitin promoter. The one remaining fertile line (GPBhGN-13) did not express either GUS or PAT in T_1, T_2 or T_3 progeny, and the presence of *uidA* and *bar* in T_1 plants was also not detected by PCR.

Of the 12 independent transgenic lines, at least six ($\geq 50\%$) were tetraploid. Most tetraploid lines (GPBhGN-12, -13, -14, GPDhGN-11 and -16) gave a segregation ratio of 3:1 for GUS. This indicates that some callus cells were already tetraploid at the time of DNA integration. Analysis of preliminary results indicates that some tetraploid cells can be detected in immature embryo-derived callus tissue 1 day after callus induction and that the proportion of tetraploid cells increases with time in culture from 2–5% at 2 weeks to 20% at 6 weeks post-induction (H. W. Choi and M.-J. Cho, unpublished results). Since DNA introduced by bombardment can remain in the cultured cells for at least 2 weeks post-bombardment, this leads to an increasing probability of the transgene integrating into cells that are already tetraploid. Only one line (GPDhGN-6) gave a 15:1 ratio for GUS expression and its 15:1 segregation ratio indicates that the original transformed cell might have become tetraploid after the time of DNA integration. This ratio could also be explained by the fact that the transgene integrated into two different chromosomes.

Expression of the maize ubiquitin promoter-driven PAT was stably inherited in T_1 progeny of all nine (eight independent) lines tested. In T_2 progeny, however, some lines (GPBhGN-4 and GPDhGN-6) exhibited gene inactivation, which was indicated by the fact that a proportion of Basta-sensitive plants in these two lines (T_3 progeny of GPBhGN-4 and T_2 progeny of GPDhGN-6) contained the 0.34-kb PCR *bar* fragment, confirming that *bar* was present but not expressed (data not shown). Wan and Lemaux (1994) also reported gene inactivation in barley transformed with *uidA* and *bar* driven by the maize ubiquitin promoter. One homozygous line that stably expressed PAT in the T_3 generation was obtained, from line GPDhGN-16.

The T_0 plants of another line, GPDhGN-14, were GUS-positive by PCR and DNA-blot analysis; however, this line was male-sterile. It was outcrossed with wild-type pollen and the progeny were Basta-positive but GUS-negative; PCR analysis confirmed the expected absence of *uidA* in T_2 plants. In this line the two transgenes could have been integrated into different chromosomes, causing them to segregate to different progeny, or *uidA* could have been preferentially deleted.

Stable GUS expression was inherited in the progeny of all seven lines tested, one line each in the T_5, T_4, T_2 and T_1 generations and three lines in the T_3 generation. Homozygous transgenic lines stably expressing GUS were obtained from four (three independent) lines [GPBhGN-4 (or -7), GPDhGN-6 and -16]; the other lines (except GPBhGN-3) are currently being advanced to homozygosity. In contrast, only one line stably expressing PAT (T_3 generation) was identified among the nine independent fertile transformants containing *bar*. Interestingly GPBhGN-7, which has an identical *uidA* hybridization pattern to the Basta-susceptible line GPBhGN-4, was Basta-resistant in T_2 progeny. These data suggest that the barley B_1- and D-hordein promoters might support more stable expression of transgenes in the endosperm during generation advance than ubiquitin-driven genes and might not be as susceptible to the mechanisms of transgene expression inactivation.

The results presented here suggest that the B_1- and D-hordein promoters can be used to develop a system for limiting foreign gene expression exclusively to the endosperm of barley seed. The use of plants transformed with develomentally and spatially regulated promoters driving the expression of transgenes allows us to study in more detail aspects of gene regulation and expression using genetic-engineering technologies. These promoters can also be used to create new barley varieties that stably express transgenes specifically in the endosperm with peak expression at defined times during development. In addition, the expression of transgenes driven by these promoters might be less susceptible to mechanisms of transgene inactivation, and therefore fewer transformants will have to be screened in order to obtain lines, which in advanced generations stably express the transgene driven by these promoters.

Acknowledgments The authors thank Dr. P. Bregitzer (USDA-ARS Small Grains Germplasm Center, Aberdeen, Idaho) for Golden Promise seeds, Dr. M.B. Sørensen (Carlsberg Research Laboratory, Copenhagen, Denmark) for the gift of p16 and pD11-Hor3, and Dr. P. Quail (Plant Gene Expression Center, USDA-ARS, Albany, Calif.) for providing plasmids pAHC20 and pAHC25. We are also grateful to Ms. R. Williams-Carrier and Ms. B. Alonso for assistance in the preparation of the manuscript. M.-J. Cho was supported by funds from the USDA NRI (#93-37500-9586) and the BioSTAR Program of the University of California and APCoor, a business alliance between the Coors Brewing Company and Applied Phytologics, Inc., and H.W. Choi from the Korea Science and Engineering Foundation (KOSEF). P.G. Lemaux was supported by the USDA Cooperative Extension Service through the University of California.

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