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ORIGINAL PAPER

Stability and inheritance of endosperm-specific expression of two transgenes in progeny from crossing independently transformed barley plants

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Abstract To study stability and inheritance of two different transgenes in barley, we crossed a homozygous T_8 plant, having *uidA* (or *gus*) driven by the barley endosperm-specific B_1-hordein promoter (localized in the near centromeric region of chromosome 7H) with a second homozygous T_4 plant, having *sgfp*(S65T) driven by the barley endosperm-specific D-hordein promoter (localized on the subtelomeric region of chromosome 2H). Both lines stably expressed the two transgenes in the generations prior to the cross. Three independently crossed F_1 progeny were analyzed by PCR for both *uidA* and *sgfp*(S65T) in each plant and functional expression of GUS and GFP in F_2 seeds followed a 3:1 Mendelian segregation ratio and transgenes were localized by FISH to the same location as in the parental plants. FISH was used to screen F_2 plants for homozygosity of both transgenes; four homozygous plants were identified from the two crossed lines tested. FISH results showing presence of transgenes were consistent with segregation ratios of expression of both transgenes, indicating that the two transgenes were expressed without transgene silencing in homozygous progeny advanced to the F_3 and F_4 generations. Thus, even after crossing independently transformed, homozygous parental plants containing a single, stably expressed transgene, progeny were obtained that continued to express multiple transgenes through generation advance. Such stability of transgenes, following outcrossing, is an important attribute for trait modification and for gene flow studies.

Keywords Barley · Crossing · FISH · Hordein promoter · Transgene expression stability · Transgene inheritance

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence in situ hybridization
GFP	Green fluorescent protein
GUS	β-Glucuronidase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSC	Sodium chloride-sodium citrate

Introduction

Rapid progress in tissue culture and transformation technologies has allowed successful production of transgenic plants in most cereal crops (Lemaux et al. 1999; Vasil 2007; Ganeshan et al. 2008). For practical application of these technologies, such as trait improvement and gene flow studies, it is essential that introduced genes of interest

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be stably expressed and transmitted intact during generation advance and following outcrossing. Transgene instability (gene silencing and/or loss) can be the result of a number of factors, e.g., methylation, copy number, chromosomal insertion site, genome rearrangement and homology of the transgene with endogenous genes (Finnegan and McElroy 1994; Flavell 1994; Stam et al. 1997; Meng et al. 2006).

Numerous studies of transgene behavior have revealed that instability of expression and inheritance is common in transgenic plants (for review, Iyer et al. 2000). For example, in one study in wheat, expression of transgenes driven by a constitutive promoter showed frequent gene silencing (Demeke et al. 1999). That is, in F_2 progeny from a cross of the transgenic parent with the nontransgenic parental plant, expression from *uidA* and *nptII*, both controlled by a rice *actin1* promoter, was unstable, the result of methylation. In another study, wheat transgenes, driven by the constitutive maize *ubiquitin1* promoter, were silenced in most (20/24) wheat lines in the T_1 or T_2 generations (Anand et al. 2003). In a study in barley one of two transgenic sublines, homozygous for both *bar* and *uidA* under the control of the maize ubiquitin promoter and expressing in T_3, experienced silencing of both transgenes in all T_6 progeny (Meng et al. 2003).

In contrast to these studies, stable expression and inheritance of transgenes driven by a seed-specific promoter in transgenic barley were observed up to the T_5 generation (Cho et al. 1999; Horvath et al. 2001). In a more recent study, expression of both *uidA* and *sgfp*(S65T), driven by barley endosperm-specific promoters, B_1- and D-hordein, respectively, was more stable than expression from *bar* driven by the maize *ubi1* promoter (Choi et al. 2003). Transgene expression under the control of seed-specific promoters was stable in nearly all (93%; 14/15) transgenic barley lines in T_4 and later generations, while only 60% (9/15) of lines with *bar* under control of the maize *ubi1* promoter had stable transgene expression.

Although two or more genes can be introduced into plant cells during transformation, expression levels of each gene may not be equally stable or at the levels needed to achieve the desired result(s). One approach to avoid such undesirable outcomes is to select transgenic plants stably expressing individual transgenes at desired levels in an advanced generation and cross them to obtain plants expressing multiple transgenes. In the present study, two different transgenic barley plants, each of which stably expressed either *uidA* or *sgfp*(S65T) driven by different barley endosperm-specific promoters, were crossed and functional expression of both transgenes were analyzed in progeny up to the F_4 generation. Plants were screened in F_2 for homozygosity of both *uidA* and *sgfp*(S65T) using fluorescence in situ hybridization (FISH).

Materials and methods

Crossing of transgenic barley plants

Transgenic lines, GPBhGN-7 and GPDhGFP-12, were obtained via microprojectile bombardment of immature embryos of a spring cultivar, Golden Promise, of barley (*Hordeum vulgare* L.; $2n = 2x = 14$) (Cho et al. 1999, 2002). p16 (Sørensen et al. 1996) was used for production of transgenic line GPBhGN-7 (Cho et al. 1999), while pDhsGFP-1 was used for production of transgenic line GPDhGFP-12 (Cho et al. 2002). p16 contains *uidA* under the control of the barley endosperm-specific B_1-hordein promoter. pDhsGFP-1 contains the synthetic gene [*sgfp*(S65T)] encoding the green fluorescent protein (GFP) under control of the barley endosperm-specific D-hordein promoter. Homozygous transgenic plants derived from GPBhGN-7 (T_8) and GPDhGFP-12 (T_4) were grown in the greenhouse and used for crosses (Fig. 1). Homozygous plants were identified using PCR, FISH and segregation ratios of transgene expression (Choi et al. 2002).

Genomic DNA isolation and polymerase chain reaction (PCR)

To test the presence of *uidA* in F_1 plants and their progeny, 500 ng of genomic DNA purified from leaf tissues was used in PCR amplifications using the primer set, UIDA1 (5′-agcggccgcaTTACGTCCTGTAGAAACC-3′) and UID2R (5′-agagctcTCATTGTTTGCCTCCCTG-3′) (Lemaux et al. 1996); small underlined letters indicate restriction enzyme sites used for subcloning. Presence of *sgfp*(S65T) in pGPDhGFP-12-derived transformants was determined using the primer set, DhorsGFP1 (5′-ACGAGTCTAGACCATGGTGA-3′) and sGFP4R (5′-agaggtaccTTACTTGTACAGCTCGTC-3′) (Cho et al. 2002). Amplifications were performed in a 25-µl reaction as described (Cho et al. 1998) with modifications, i.e., *Taq* DNA polymerase (Qiagen, Valencia, CA, USA) with Q-solution was used.

Fluorescence in situ hybridization

The Fluorescence in situ hybridization (FISH) procedure used was as described previously (Choi et al. 2002, 2003) with modifications. The 1.8-kb *uidA* fragment from p16 and the 0.72-kb *sgfp*(S65T) fragment from pDhsGFP-1 were labeled with biotin-16-dUTP by nick translation according to manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA). In some cases, the entire plasmid was used for probe labeling. After detection and photography of the first probing (*uidA*/FITC-avidin D), coverslips were carefully removed and the slides washed with 2× SSC three times for 5 min, and further washed

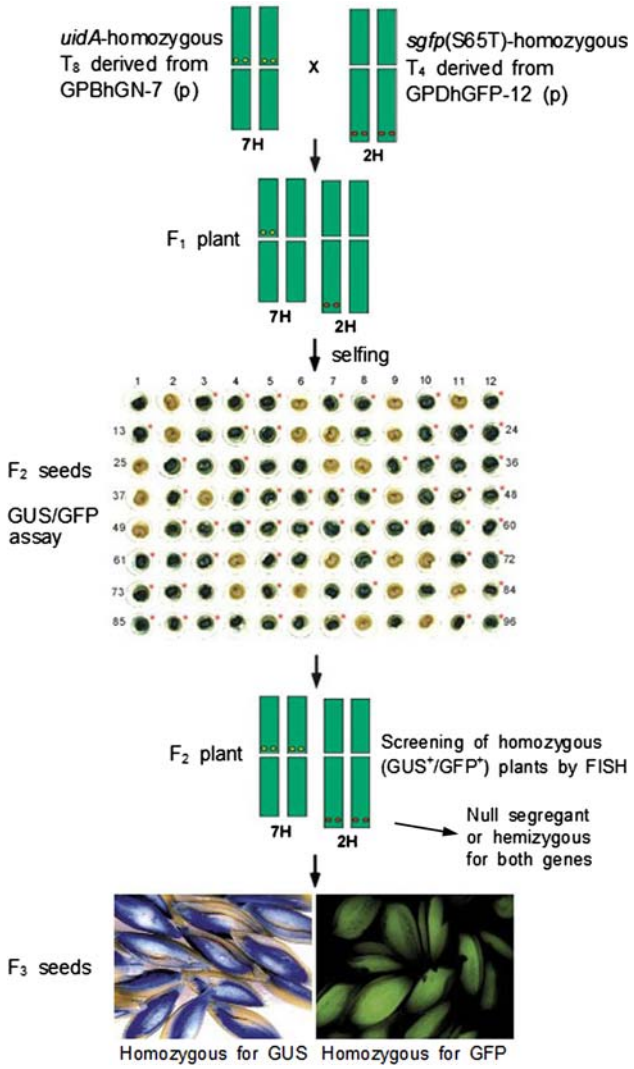

Fig. 1 Screening of homozygous plants using GUS/GFP assay and FISH from crossed transgenic barley plants. F_1 seeds were obtained from the cross of two parental plants, a homozygous T_8 plant derived from GPBhGN-7 and a homozygous T_4 plant derived from GPDhGFP-12; three F_1 plants were tested for GUS/GFP activities. GFP expression in F_2 seeds was performed using cross-sectioned half-seeds without immature embryos; GUS assay was then performed using the same materials. Expression of *sgfp*(S65T) is marked by an asterisk. Half-seeds with embryos expressing both GFP and GUS were saved and grown for next generations. *Numbers* indicate the seed number examined (Table 1). FISH technique was employed to screen the homozygous [*uidA* and *sgfp*(S65T)] F_2 plants by direct mapping of transgenes on the chromosomes (Table 2; Fig. 3). Inserted *uidA* and *sgfp*(S65T) genes were localized on the centromeric region of chromosome 7H and on the subtelomeric region of chromosome 2H, respectively. Homozygous F_3 generation seeds were obtained by analyzing segregation ratios of transgenes

with detection buffer (4× SSC/0.2% Tween 20) three times for 60 min at room temperature. Slides were dehydrated in an alcohol series and the second probe for *sgfp*(S65T), Cy3-avidin, was applied. Slides were examined with a Zeiss 510 confocal laser-scanning microscope with filter sets 02, 10 and 15. Representative FISH images were captured using Adobe Photoshop version 5.0.

Functional GFP and GUS assays of individual immature seeds

Functional assays of GFP and GUS in immature seeds were performed using cross-sectioned F_1 half-seeds without embryos (Fig. 1). Individual half-seeds were placed in 96-well ELISA plates and corresponding half-seeds with immature embryos were labeled and saved in another ELISA plate for germination. GFP expression was observed using a Zeiss Axiophot epifluorescence microscope equipped with a Chroma filter containing a 450-490 excitation filter and an LP520 emission barrier filter (Cho et al. 2002). After the nondestructive GFP activity assay, samples were used for histochemical GUS assays (Jefferson et al. 1987) using 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-gluc). Saved half-seeds with immature embryos, positive for both GFP and GUS expression, were germinated on hormone-free rooting medium (BCI-DM$^-$) and a week after germination, F_2 plantlets were transferred to soil and used for further analyses.

Quantitative assays of GUS activity and western blotting of GFP

Quantitative measurements of GUS activity were performed (Jefferson et al. 1987) using a 4-methylumbellifery-β-D-glucuronide (MUG) substrate (Sigma, St. Louis, MO, USA). From each line, ten, single mature seeds were ground and GUS extraction buffer added. After centrifugation supernatant fractions were used to determine GUS activity. Protein concentrations in extracts were measured (Bradford 1976) using Bio-Rad reagent (Bio-Rad, Richmond, CA, USA). Fluorescence of 4-methylumbelliferone (4-MU) was measured on a TKO 100-dedicated min fluorometer (Hoefer Scientific Instruments, San Francisco, CA, USA) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm (Cho et al. 1999).

For immunological detection of GFP expression, five mature seeds from each transgenic line were ground with a mortar and pestle, mixed with 0.4 ml protein extraction buffer (50 mM Tris, 500 mM NaCl, pH 7.5) containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and incubated on ice for 30 min. After centrifugation (10,000×*g* for 10 min, 4°C), the supernatant was used for immunoblot analyses. Twenty micrograms of total soluble protein from each line and 20 ng of purified GFP protein (Clontech, Mountain View, CA, USA) as a positive control were separated on SDS-PAGE using 10–20% Tris-glycine gel (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane (Molecular Probe, Eugene, OR,

USA). After transfer, the membrane was blocked in TBS-T (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) + 5% nonfat dried milk for 1 h. After washing (2 × 15 min) in TBS-T, rabbit polyclonal GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (1:2,000 dilution in blocking buffer) was added and incubated for 2.5–3 h. After washing in TBS-T, the membrane was incubated in goat anti-rabbit HRP conjugate IgG (Santa Cruz Biotechnology, Inc.) at 1:5,000 dilution for 1 h at room temperature and washed as indicated above. Labeling was monitored by chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA) according to manufacturer's instructions. GFP signal was quantified using Quantity One Quantitation Software (Bio-Rad, Hercules, CA, USA). This experiment was repeated twice.

Results

Production of F_1 plants by crossing and segregation of *uidA* and *sgfp*(S65T) in F_2 progeny

To obtain homozygous transgenic plants containing both *uidA* and *sgfp*(S65T) genes, homozygous transgenic plants derived from GPBhGN-7 and GPDhGFP-12 (Fig. 1) were crossed. GPBhGN-7 is a line with *uidA* driven by the barley endosperm-specific B_1-hordein promoter (Cho et al. 1999); a homozygous T_8 progeny plant was used for crossing. This transgenic line has both *uidA* and *bar* genes, but only *uidA* was stably expressed to the T_9 generation (Choi et al. 2003). GPDhGFP-12 is a line with *sgfp*(S65T) driven by the barley endosperm-specific D-hordein promoter (Cho et al. 2002); a homozygous T_4 plant was used for crossing. This transgenic line has both *sgfp*(S65T) and *bar*, but only *sgfp*(S65T) expression was stable to the T_6 generation (Choi et al. 2003).

After crossing the transgenic plants, three F_1 plants (GPBhGN/DhGFP-2, GPBhGN/DhGFP-5 and GPBhGN/DhGFP-6) were tested for both *uidA* and *sgfp*(S65T) by PCR (Fig. 2); the three F_1 plants were positive for both transgenes (Table 1). Functional assays of GUS and GFP in F_2 immature seeds, using cross-sectioned half-seeds without embryos, were performed to screen for seeds expressing from both *uidA* and *sgfp*(S65T) (Fig. 1). All three crossed lines showed a 3:1 Mendelian segregation ratio for both GUS and GFP expression (Table 1).

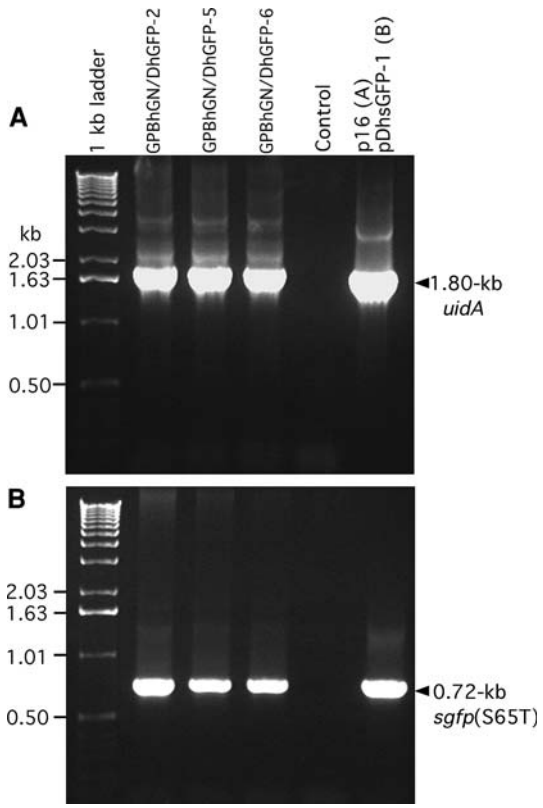

Fig. 2 PCR analysis of genomic DNA from nontransgenic control and from three F_1 lines from crosses. **a** 1.8-kb *uidA* fragment. **b** 0.72-kb *sgfp*(S65T) fragment. Plasmids, p16 (**a**) and pDhsGFP-1 (**b**) were used in positive control reactions; water was used in negative control reactions (*Control*). Molecular weights in kb are indicated on *left*

Screening of homozygous F_2 plants by FISH

The FISH technique was applied to screen for F_2 progeny homozygous for both *uidA* and *sgfp*(S65T), in two out of the three crossed F_1 plants (Table 2; Figs. 3, 4). Both transgenes were localized on metaphase chromosomes in GPBhGN/DhGFP-2 and GPBhGN/DhGFP-5. As expected, *uidA* was localized in F_1 plants near the centromeric region

Table 1 Analysis of expression and inheritance of two endosperm-specific transgenes [*uidA* and *sgfp*(S65T)] after crossing between transgenic barley plants, GPBhGN-7 (T_8) and GPDhGFP-12 (T_4)

Crossed line	F_1 plant	Expression in F_2 seed		
	uidA/*sgfp*(S65T) PCR (±)	# of seeds examined	GUS (+/−)	GFP (+/−)
GPBhGN/DhGFP-2	+/+	96	71/25[†]	77/19[†]
GPBhGN/DhGFP-5	+/+	96	80/16[†]	78/18[†]
GPBhGN/DhGFP-6	+/+	96	70/26[†]	70/26[†]

[†] Analyses of transgene segregation ratio of F_2 seed using χ^2-test were not significantly different from 3:1 (at $a = 0.05$)

Table 2 Screening of homozygous plant using FISH in F_2 plants and expression of transgenes in F_3/F_4 seeds

Crossed plant line	# of FISH signals on the homologous chromosome in F_2 plant		Expression in F_3 seed			Expression in F_4 seed		
	# of seeds examined	GUS (+/−)	GFP (+/−)	# of seeds examined	GUS (+/−)	GFP (+/−)		
	uidA	*sgfp*(S65T)						
GPBhGN/DhGFP-2-3	1	2	50	38/12[†]	50/0			
GPBhGN/DhGFP-2-4	1	1	34	21/13[†]	27/7[†]			
GPBhGN/DhGFP-2-7[a]	2	2	37	37/0	37/0			
GPBhGN/DhGFP-2-7-1[a]						58	58/0	58/0
GPBhGN/DhGFP-2-7-2[a]						53	53/0	53/0
GPBhGN/DhGFP-2-7-3[a]						46	46/0	46/0
GPBhGN/DhGFP-2-10[a]	2	2	76	76/0	76/0			
GPBhGN/DhGFP-2-12	2	1	54	54/0	43/11[†]			
GPBhGN/DhGFP-2-16	1	1	40	31/9[†]	27/13[†]			
GPBhGN/DhGFP-2-22	1	1	32	22/10[†]	27/5[†]			
GPBhGN/DhGFP-5-1	2	1	44	44/0	32/12[†]			
GPBhGN/DhGFP-5-3	1	2	33	24/9[†]	33/0			
GPBhGN/DhGFP-5-4[a]	2	2	39	39/0	39/0			
GPBhGN/DhGFP-5-5	1	1	54	39/15[†]	44/10[†]			
GPBhGN/DhGFP-5-6	1	2	44	38/6[†]	44/0			
GPBhGN/DhGFP-5-7	1	1	38	25/13[†]	29/9[†]			
GPBhGN/DhGFP-5-11[a]	2	2	33	33/0	33/0			
GPBhGN/DhGFP-5-11-1[a]						53	53/0	53/0
GPBhGN/DhGFP-5-11-2[a]						53	53/0	53/0
GPBhGN/DhGFP-5-11-3[a]						45	45/0	45/0

[a] Homozygous for both *uidA* and *sgfp*(S65T) genes

[†] Analyses of transgene segregation ratio of F_3 heterozygous seed using χ^2-test were not significantly different from 3:1 (at $a = 0.05$)

of chromosome 7H while *sgfp*(65T) was localized on the subtelomeric region of chromosome 2H, the same localization observed in parental plants (Choi et al. 2002). Hemizygous plants for both *uidA* and *sgfp*(S65T) had only a single signal on one of the homologous chromosomes (Fig. 3a, b), while homozygous plants had doublet signals on both homologous chromosomes (Fig. 3c, d). Based on FISH analysis of seven F_2 plants derived from GPBhGN/DhGFP-2, two (GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-2-10) were homozygous for both *uidA* and *sgfp*(s65T) (Table 2). The remaining plants were either hemizygous for both *uidA* and *sgfp*(s65T) or homozygous for only one of the two transgenes. In another F_1 plant (GPBhGN/DhGFP-5), two (GPBhGN/DhGFP-5-4 and GPBhGN/DhGFP-5-11) out of seven F_2 plants tested, were homozygous for both *uidA and sgfp(S65T)* (Table 2).

Stable expression of both *uidA* and *sgfp*(S65T) genes in F_3 and F_4 progeny

Homozygous plants for both *uidA* and *sgfp*(S65T) were obtained in F_2 progeny from the cross of two parental homozygous plants; FISH results showed physical presence of both transgenes in transgenic metaphase chromosomes (Fig. 3). Both transgenes were stably expressed in F_3 and F_4 seeds and segregation ratios based on expression of both transgenes were in agreement with FISH results in F_2 plants (Table 2). All four putative homozygous plants (GPBhGN/DhGFP-2-7, GPBhGN/DhGFP-2-10, GPBhGN/DhGFP-5-4 and GPBhGN/DhGFP-5-11) were also confirmed to be homozygous by segregation ratios and functional expression of transgenes.

Expression levels of transgenes in progeny from crosses

Quantitative GUS-activity measurements were performed using ten, single mature seeds from each line. As shown in Fig. 4, seeds from transgenic plants, GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-5-11, showed GUS activities similar to T_9 seeds from a homozygous parental GUS line derived from GPBhGN-7.

GFP expression in transgenic plants was determined by western analysis using five mature seeds from each line. GPBhGN/DhGFP-2-7 (3.09 ng) and GPBhGN/DhGFP-5-11 (1.79 ng) showed similar or slightly lower levels of GFP expression, compared with that of T_5 seeds from parental

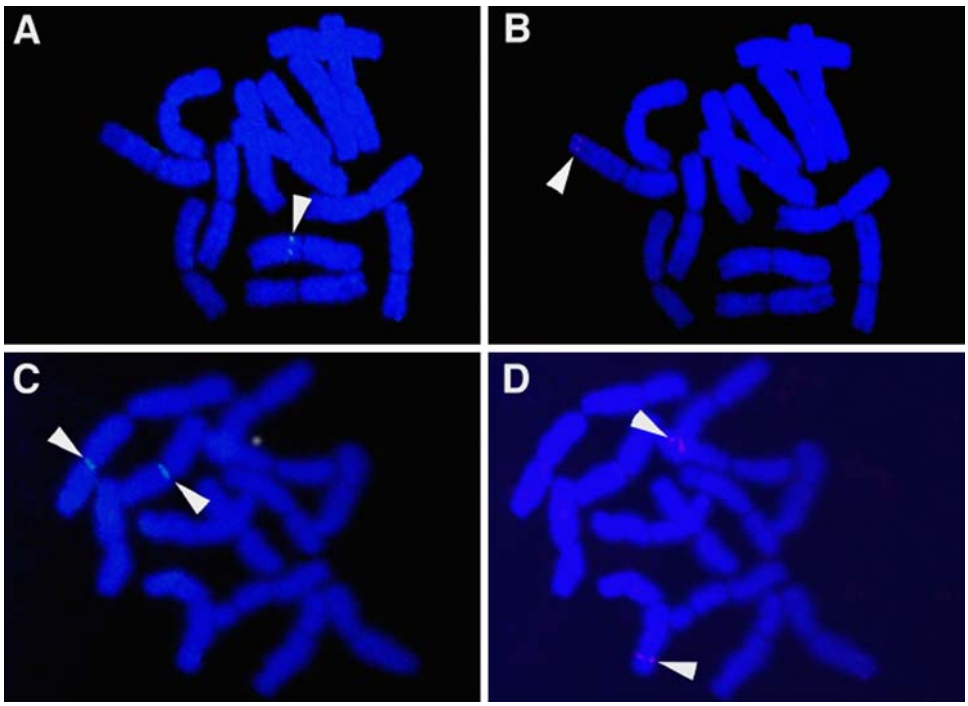

Fig. 3 FISH of transgenes [*uidA* and *sgfp*(S65T)] in F_2 plants. **a**, **b**. Hemizygous plant with both (**a**) a single signal of *uidA* (*arrow*) inserted on the centromeric region of chromosome 7H and (**b**) a single signal of *sgfp*(S65T) (*arrow*) inserted on the subtelomeric region of chromosome 2H. **c**, **d** Homozygous plant with both (**c**) doublet signals of *uidA* and (**d**) *sgfp*(S65T) on homologous chromosomes (Fig. 4)

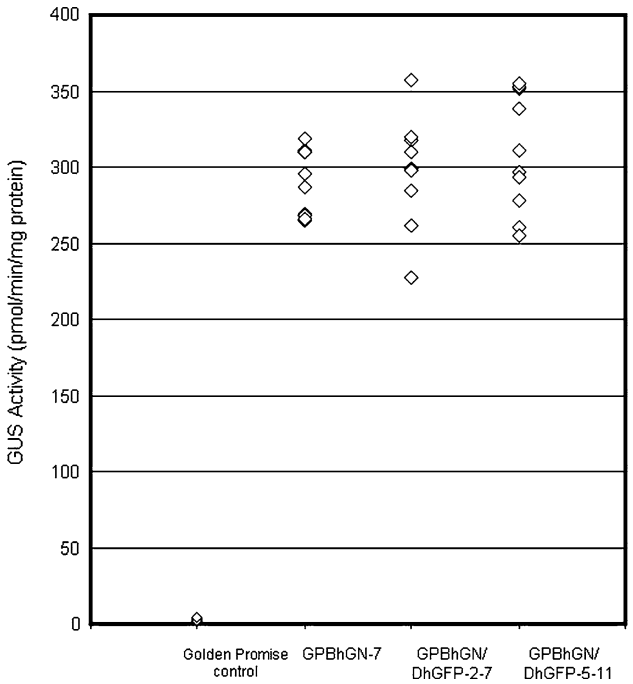

Fig. 4 GUS activities in mature transgenic seeds. GUS activity was determined by fluorometric assays on protein extracts from ten, single mature seeds derived from each homozygous plant. T_9 seeds derived from a parental GUS homozygote, GPBhGN-7, and F_3 seeds from two homozygotes for both GUS and GFP, GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-5-11, were used for GUS activity measurements

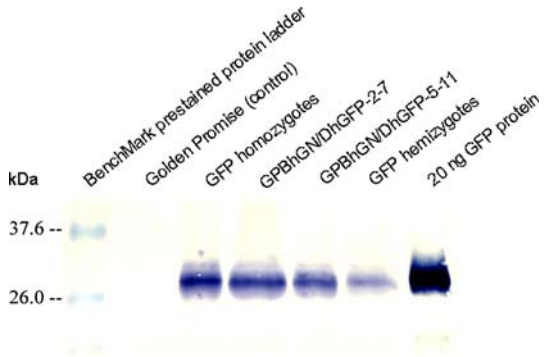

Fig. 5 GFP expression levels in mature transgenic seeds. GFP quantification was determined with western blot hybridization analysis using protein extracts from five mature seeds derived from each line. T_5 seeds derived from a parental GFP homozygote, GPDhGFP-12, and F_3 seeds from two homozygotes for both GUS and GFP, GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-5-11, were used for GFP expression level measurements. *Lane 1* BenchMark™ prestained protein ladder, *lane 2* Golden Promise, *lane 3* GFP homozygotes, *lane 4* GPBhGN/DhGFP-2-7, *lane 5*: GPBhGN/DhGFP-5-11, *lane 6* GFP hemizygotes, *lane 7* 20 ng GFP protein

transgenic GFP homozygotes (3.23 ng) derived from GPDhGFP-12 (Fig. 5). GPBhGN/DhGFP-2-7 (3.09 ng) and GPBhGN/DhGFP-5-11 (1.79 ng) showed much higher levels of GFP expression than the GFP hemizygotes (0.57 ng). Thus, the GPBhGN/DhGFP-2-7 line had similar expression levels of both GUS and GFP, compared with their homozygous parental plants containing a single transgene (Figs. 4, 5).

Discussion

Expression of two or more genes is sometimes needed to enable, for example, an entire metabolic pathway to

function properly, as with the cytosolic isoprenoid pathway leading to production of the antimalarial agent, artemisinin (for review, Liu et al. 2005), or to achieve desired levels of a trait, like vitamin A (Paine et al. 2005). Even though multiple genes can be transformed into plant cells, instability of expression of one or more transgenes frequently occurs in transgenic plants (Chen et al. 1998; Melander et al. 2006; Tobias et al. 2007) resulting in one transgene in the plant not reaching and/or maintaining levels needed to achieve the trait phenotype. One approach to avoiding this situation is to select transgenic plants at an advanced generation that are each stably expressing an individual transgene at the desired level and to cross them to obtain progeny expressing multiple transgenes.

The question then arises as to the stability of transgene expression following outcrossing. To address this question, a homozygous T_8 plant from GPBhGN-7 (Cho et al. 1999) was crossed in this study with another homozygous plant (T_4) from GPDhGFP-12 (Cho et al. 2002). These lines were chosen because the transgenes in the two lines were localized on different chromosomes and could be screened with FISH for homozygosity of both transgenes in the same plant. Progeny from the crosses of the two homozygous barley lines that individually expressed either *uidA* or s*gfp*(S65T) in late-generation plants, were analyzed for physical and expression stability up to the F_4 generation. After crossing the two homozygous plants, three F_1 plants (GPBhGN/DhGFP-2, GPBhGN/DhGFP-5 and GPBhGN/DhGFP-6) were positive for both *uidA* and *sgfp*(S65T) (Table 1). Expression of both genes in progeny of crosses showed a 3:1 Mendelian segregation ratio in F_2 seeds (Table 1).

Physical presence of inserted genes was observable by FISH and was used to establish homozygosity in early generations without the necessity of establishing segregation ratios or conducting further molecular analyses (Pedersen et al. 1997; Salvo-Garrido et al. 2001; Svitashev et al. 2000; Carlson et al. 2001; Bourdon et al. 2002; Choi et al. 2002, 2003). In the present study F_2 progeny plants, positive for both *uidA* and s*gfp*(S65T) by PCR (Fig. 2), were screened by FISH for homozygous plants (Fig. 3). Four plants (GPBhGN/DhGFP-2-7, GPBhGN/DhGFP-2-10, GPBhGN/DhGFP-5-4 and GPBhGN/DhGFP-5-11) homozygous for both *uidA* and *sgfp*(S65T) were obtained using FISH analysis from the two crossed F_2 progeny populations (GPBhGN/DhGFP-2 and GPBhGN/DhGFP-5) examined. All four plants were confirmed as homozygous based on DNA segregation and functional transgene expression ratios in F_3 seeds (Table 2; Figs. 4, 5). Thus, FISH was useful for early screening to obtain homozygous plants (Choi et al. 2002, 2003). Expression of both *uidA* and *sgfp*(S65T) was stably inherited in F_2, F_3 and F_4 progenies (Table 2), with no evidence of gene silencing.

Expression levels of GUS and GFP were measured in F_3 seeds derived from two F_2 progeny plants homozygous for both *uidA* and *sgfp*(S65T), GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-5-11. GUS expression in both was similar to that of the homozygous parental GUS line derived from GPBhGN-7 (Fig. 4). GPBhGN/DhGFP-2-7 and GPBhGN/DhGFP-5-11 showed similar and slightly lower levels of GFP expression, respectively, compared to the parental, transgenic GFP homozygotes derived from GPDhGFP-12 (Fig. 5). The GPBhGN/DhGFP-2-7 line had similar expression levels of both GUS and GFP, comparable to their homozygous parental plants containing a single transgene (Figs. 4, 5), confirming transgene expression stability following outcrossing.

The results presented in this study suggest that transgenic plants stably expressing two or more transgenes at the desired levels can be generated by crossing independently transformed plants. Further, homozygous plants to be used for such crosses can be identified in early generations using FISH. This approach can be used when expression of multiple genes is needed in a plant to realize the desired phenotype, like nutritional improvement of abiotic and biotic stress tolerance, or to increase expression levels of the same transgene driven by the same or different promoters by mimicking a gene dosage effect. Increasingly important is the identification of a reliable means to quantify transgene flow, for example the utilization of visual markers has been proposed (Shen and Petolino 2006). Irrespective of the marker used, however, it is important that transgene expression is stable following outcrossing in order to accurately quantitate gene flow. In this study demonstration of stability of expression of two marker genes driven by endosperm-specific promoters following crossing in a primarily self-pollinated species provides important information for the design of gene flow studies that insure stability of transgene expression following crossing and accurate assessments of gene flow frequencies.

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