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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Green fluorescent protein as a visual marker for wheat transformation

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Abstract Wheat (*Triticum aestivum L.)* transformation via particle bombardment is now established in many laboratories, but transformation efficiencies are still largely low and the highest efficiencies can only be obtained with certain genotypes. For rapid optimization and improvement of wheat transformation protocols, a non-destructive marker which permits early detection of transformed cells is needed. We have assessed the ability of a modified version of the *Aequorea victoria* green fluorescent protein (GFP) to act as a marker for detecting transformed cells and tissues of wheat. Multicellular clusters emitting green fluorescence were observed 14 days after particle bombardment with a *sGFPS65T* gene construct, and *gfp*-expressing shoots (often with expressing roots) could be observed as early as 21 days after bombardment. These shoots can be removed from the callus and grown further until they are ready to transfer to soil. Transgenic wheat plants could be selected on the basis of *gfp* expression alone although the inclusion of antibiotic resistance as a selectable marker could improve the efficiency. Using *sgfpS65T* as a marker gene in an experiment comparing bombardment parameters allowed the rapid identification of variables that could be targeted for optimization.

Key words Green fluorescent protein · Transformation · Wheat

Abbreviations *GFP* Green fluorescent protein · *2,4-D* 2,4 Dichorophenoxyacetic acid

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Introduction

Transformation of wheat (*Triticum aestivum L.)* via particle bombardment of immature embryo-derived material has become standard practice in many laboratories at a reported frequency of 1–5% of bombarded embryos yielding transformed plants (Altpeter et al. 1996a,b; Barro et al. 1997; Becker et al. 1994; Blechl and Anderson 1996; De Block et al. 1997; Nehra et al. 1994; Vasil et al. 1992; Weeks et al. 1993; Zhou et al. 1995). To generate the number of transgenic events necessary to be sure of getting high-expressing stable lines, a large amount of material must be carried through the system. This process is time-consuming and involves significant labor in culturing embryos, bombarding embryos, transferring callus to selection and regeneration media and then growing putatively transformed shoots to maturity and confirming transgenic status. The process could be streamlined, the efficiency increased and the labor needed to produce a specified number of transgenic lines reduced if confirmation of transformation could be achieved much earlier in the process. Optimization of the many parameters affecting biolistic-mediated transformation frequency [target distance, rupture disk pressure, gap distance, tissue preculture treatments, choice of genotype (Casas et al. 1995)] would also be more easily and effectively performed if transformed shoots could be identified and recovered as soon as possible after bombardment.

Reporter proteins have played an important role in developing and optimizing transformation protocols for plant species. The two most widely used reporter proteins have been β-glucuronidase and luciferase (Jefferson 1987; Ow et al. 1986). The analysis of expression of these proteins requires the addition of an added substrate which precludes non-invasive analysis. The green fluorescent protein from the jellyfish *Aequorea victoria* has the advantage over other reporter proteins in that the formation of a fluorescent chromophore is self-catalyzed and requires only excitation under ultrav-

iolet or blue light to emit a bright green fluorescence which can be observed with a standard fluorescent microscope equipped with the appropriate filters (Chalfie et al. 1994).

The wild-type *gfp* gene has shown such disadvantages as aberrant splicing in plant cells, a tendency to form cytotoxic and non-functional aggregates (Haseloff et al. 1997) or low expression efficiency and quantum yield (Sheen et al. 1995). These problems have been addressed by the development of a series of modified GFP proteins with improved solubility (Davis and Vierstra 1998) and improved apoprotein folding and targeting into the endoplasmic reticulum (Haseloff et al. 1997) or with higher expression efficiency and fluorescent intensity (Chiu et al. 1996). These genes have been used to observe transient GFP fluorescence after particle bombardment in a variety of plant cells such as *Arabidopsis* (Sheen et al. 1995; Chiu et al. 1996; Davis and Vierstra 1998), onion epidermis (Chiu et al. 1996) and conifer cells (Tian et al. 1997). GFP fluorescence has also been observed in transformed plants of tobacco (Chiu et al. 1996; Pang et al. 1996; Molinier et al. 2000), *Arabidopsis* (Pang et al. 1996; Haseloff et al. 1997), wheat (Pang et al. 1996) and maize (Pang et al. 1996). In all cases, the transformed plants were obtained using existing transformation and selection protocols, and the transgenic plants were then examined for GFP expression.

One of the new *gfp* genes is a synthetic gene (*sgfpS65T)* designed with optimal codons for higher organisms and with a mutation of the serine to threonine at position 65 for a protein (sGFPS65T) with improved brightness and slower photobleaching when excited by blue light (490 nm) (Chiu et al. 1996). This increased brightness allows detection of individual fluorescing cells or small multicellular structures. Recently, Elliott et al. (1999) used *sgfpS65T* to observe early transformation events and to improve the efficiency of sugarcane transformation when coupled with antibiotic selection. The objective of the study reported here was to develop a system to identify stable transformation events within 14 days after particle bombardment of wheat immature embryos and to determine if the gene would be useful not only as a scorable marker for optimizing transformation protocols but also as a selectable marker which could be used in the absence of any other marker gene for wheat transformation. The use of GFP as a selectable marker would allow the identification and isolation of transgenic wheat plants beginning as early as 21 days after bombardment.

Materials and methods

Plant material

Immature embryos of wheat cv. Fielder (six plates of 20 embryos per treatment) were cultured on RG5-N medium [MS medium (Murashige and Skoog 1962)] supplemented to a final concentration of 0.5 mg l^{-1} thiamine, 100 mg l^{-1} glutamine, 1 mM niacinamide, 2 mg l^{-1} 2,4-D and solidified with 2.5 g l^{-1} phytagel (Sigma, St. Louis, Mo.). Embryos were 1.0–1.5 mm in size. After a 5-day preculture period at 24 °C in the dark the embryos were transferred to RG5-O medium consisting of RG5-N medium without niacinamide and supplemented with 0.2 *M* sorbitol and 0.2 *M* mannitol for 4 h prior to bombardment.

DNA preparation

DNA was a 1:1 mixture of pAct1IsGFP-1 (5.2 kb) (M-J Cho and P Lemaux, unpublished results) and pH24 (Fromm et al. 1986). The structures of the selectable marker genes in these plasmids are shown in Fig. 1. The GFP gene in the pAct1IsGFP-1 construct encodes a synthetic codon optimized red-shifted GFP (*sgfpS65T*). Gold particles (0.6 or 1.0 μm) were coated with a 1:1 mixture of the two plasmids using a slightly modified version of the procedure of Sanford et al. (1991). A 5-μl aliquot of each DNA (at 1 μg/μl) was added to 2.5 mg of prewashed gold particles (either 0.6 or 1.0 μm in a 25-μl volume). A 35-μl aliquot of a solution consisting of a 5:2 ratio of 2.5 *M* $CaCl_2$ and 0.1 *M* spermidine was added and the tube immediately finger-vortexed. The tube was incubated at room temperature for 20 min followed by a brief centrifugation. The supernatant was removed and the DNA-coated particles resuspended by finger-vortexing in 200 μl of 70% ethanol. The particles were briefly centrifuged, washed in 200 μl of 95% ethanol, centrifuged again and resuspended in 40 μl of 95% ethanol after removal of the supernatant. Five microliters of the suspension was loaded onto a macrocarrier for bombardment.

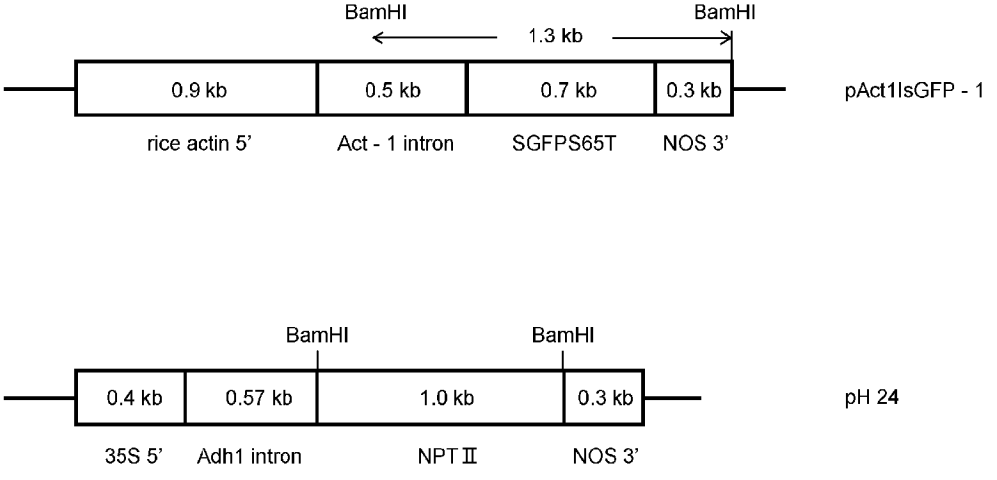

Fig. 1 Maps of pAct1IsGFP-1 and pH24 showing core features and location of *Bam*HI sites

Particle bombardment and selection

Embryos were bombarded with the PDS-1000He Particle Delivery System (Bio-Rad) using 1,100 psi rupture disks, 71.12 cm of Hg vacuum, a gap distance of 0.32 cm and a target distance of either 3, 6 or 9 cm. Sixteen hours following bombardment the embryos were transferred to RG5 medium (RG5-N without niacinamide) and kept in the dark for 14 days for callus development. The calli were then transferred to half strength basal MS medium solidified with 2.5 g l^{-1} phytagel and cultured for 14 days under an 8/16-h dark/light period at 24 °C (no antibiotic selection) followed by 14 days on half strength MS plus 35 mg l^{-1} paromomycin solidified with 3.5 g l^{-1} agarose. Expression of the green fluorescent protein was monitored regularly using a Leica stereo-fluorescent module attached to a Leica MS5 stereo microscope. The filter set used was the Leica GFP-Plant filter set (excitation filter: 470/40 nm, barrier filter: 525/50 nm). Two weeks after placement of calli on half strength MS medium (no antibiotic), identifiable somatic embryos and shoots/roots with GFP expression were physically separated and placed on fresh half strength MS medium until large rooted shoots were obtained. The plants were then transferred to soil and placed in a greenhouse. Two weeks after placement of the calli on half strength MS medium containing paromomycin, shoots which were actively growing were observed for GFP expression, removed and placed on half strength MS medium (no antibiotic) as above. After transfer to soil, developing progeny embryos were removed at approximately 20 days after anthesis and observed for GFP expression.

Southern blot analyses

Genomic DNA was extracted from freeze-dried leaf tissue by the procedure of Doyle and Doyle (1990). The DNA was digested by restriction enzymes according to the manufacturer's recommended conditions (Life Technologies) and electrophoresed on a 1% agarose gel. It was then transferred to nylon membranes (HyBond N^+) using a PosiBlot pressure blotter (Stratagene) under alkaline conditions (as per the manufacturer's instructions). Membranes were prehybridized for 4–6 h at 42 °C and then hybridized overnight. Prehybridization, hybridization with a radiolabeled probe and subsequent washing of the membrane were performed according to the instructions provided with the HyBond N^+ membranes. The probes consisted of the *gfp* coding sequence from pAct1IsGFP-1 (0.7-kb *Pst*I-*Nco*I fragment) and the *nptII* coding sequence from pH24 (1.0-kb *Bam*HI fragment). The probes were labeled with [^{32}P] by random primer labeling (Amersham Pharmacia kit). Hybridized and washed membranes were analyzed by autoradiography.

Results

An experiment to determine the usefulness of green fluorescent protein as a visual selectable marker in the early stages of a wheat transformation protocol (within 4 weeks after bombardment) as well as to rapidly optimize transformation protocols was performed. The treatments and numbers of explants used are outlined in Table 1. The two parameters which varied were target distance from the stopping plate and the size of the gold particles used.

Expression of GFP could be observed transiently beginning 24 h post- bombardment (Fig. 2a). After 2 weeks of culture on callus induction medium in the dark, embryogenic areas expressing GFP could be observed (Fig. 2b); as little as 3 weeks following bombardment, shoots (Figs. 2c, 2d) and entire plantlets (shoots plus roots) (Fig. 2e) expressing GFP could be observed. The use of the GFP-Plant (Leica) filter set eliminated background chlorophyll fluorescence that has been observed with other filter sets (Elliott et al. 1999) and allowed GFP-expressing green leaf tissue to fluoresce bright green. Non- expressing leaf tissue appeared dark and did not fluoresce. Plantlets expressing GFP can be separated at this point and grown until they are large enough for establishment in soil. No selection aside from visual GFP-based selection was used up to 4 weeks post-bombardment. Application of antibiotic selection after 4 weeks post-bombardment increased the number of transgenic shoots obtained (Table 1). The selected T0 plants were grown to maturity, and in some cases immature seeds were collected 20 days after anthesis. Southern blots confirmed transformation in the T0 plants (Fig. 3) and showed that all shoots which were selected solely on the basis of GFP expression carried both the *gfp* and *nptII* genes. Integration into high-molecular-weight DNA was shown by Southern analysis where the plant DNA was digested using an enzyme which only cuts once in the 5.2 kb pAct1IsGFP-1 construct (Fig. 3). Segregation could be observed for expression of GFP in the progeny embryos (Fig. 2f, Table 2) providing further evidence of stable transformation. Dissection of the embryos was necessary to observe segregation as embryo expression (or lack of it) was masked in whole seeds by GFP expression from maternal endosperm tissue (data not shown). Segregation data on the T1 progeny of four plants showed that for three of the four T0 plants, GFP expression was inherited in a 3:1 ratio consistent with a single transgene locus (Table 2).

Table 1 The use of GFP visual selection tooptimize bombardment parameters for wheat immature embryo-derived explants and to select transgenic shoots

Gold particle size and target distance	Number of embryos bombarded	Number of transgenic plants – GFP selection only	Number of transgenic plants – GFP plus paromomycin selection	Total number of transgenic plants (% transformation frequency)
0.6 μm, 3 cm	120	0	0	0
0.6 μm, 6 cm	120	1	3	4 (3.3)
0.6 μm, 9 cm	120	4	2	6 (5)
1.0 μm, 3 cm	120	0	0	0
1.0 μm, 6 cm	120	0	0	0
1.0 μm, 9 cm	120	0	1	1 (0.8)

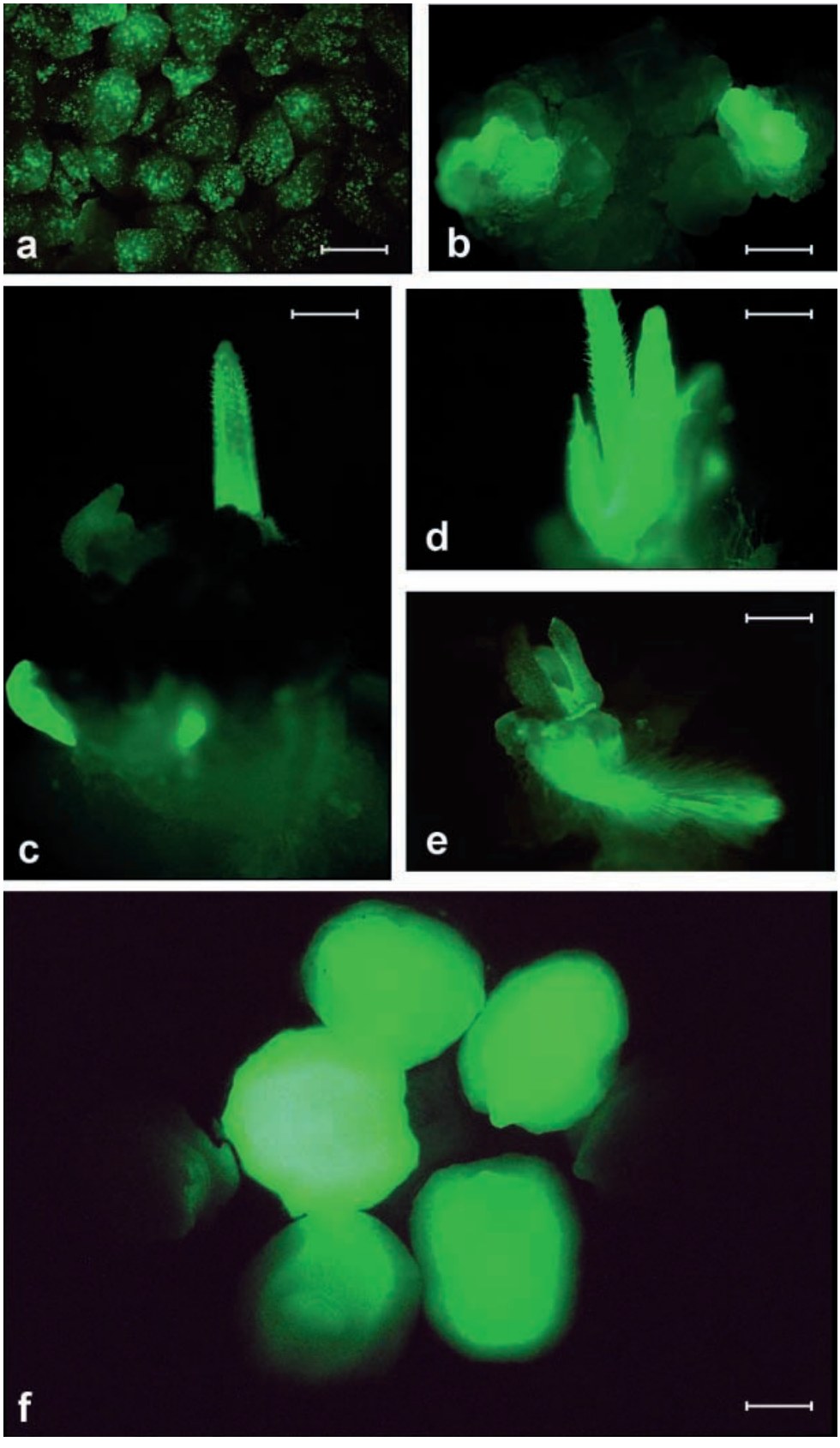

Fig. 2a–f GFP expression in tissues and shoots following particle bombardment with the pAct1IsGFP-1 vector. **a** Transient expression in wheat immature embryos 2 days following bombardment. *Bar:* 1 mm. **b** Embryogenic regions with GFP expression observed 14 days after bombardment. *Bar*: 0.63 mm. **c** Elongating leaf with GFP expression observed 21 days after bombardment. GFP selection only applied. *Bar*: 0.63 mm. **d** Shoot with a high level of GFP expression observed 23 days after bombardment. GFP selection only applied. *Bar*: 0.4 mm. **e** Plantlet with leaves and developing roots expressing GFP observed 21 days after bombardment. GFP selection only applied. *Bar*: 0.63 mm. **f** Seven progeny embryos from a transgenic plant selected on the basis of GFP expression only. Five of the seven embryos expressed GFP. Variation in the intensity of fluorescence among them was observed. *Bar*: 1 mm

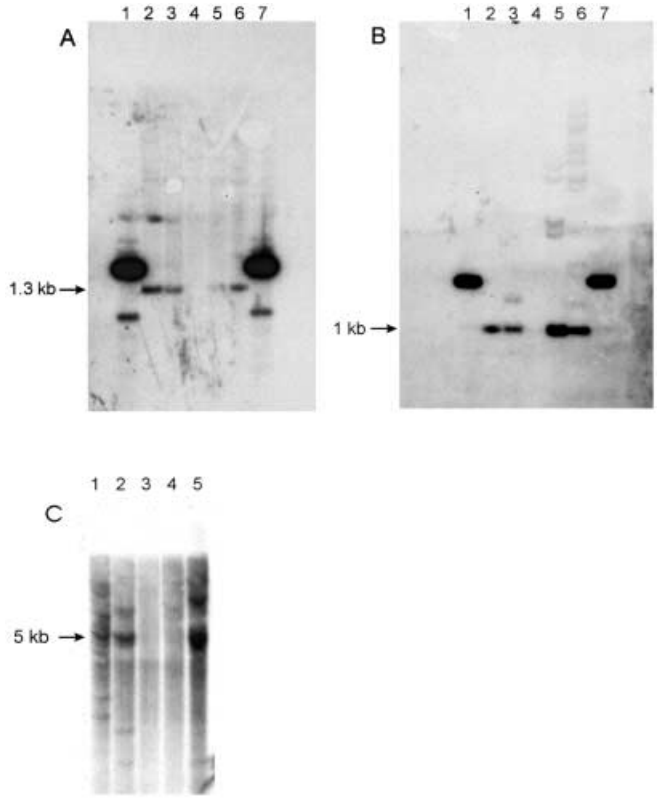

Fig. 3A–C Southern blots of 4 GFP-expressing T0 plants which were selected solely on the basis of GFP expression. All plant DNA was digested with *Bam*HI. *Lanes 1* and 7 DNA molecular-weight markers, *lanes 2,3,5,6* plants nos. 3, 4, 1 and 2, respectively; *lane 4* Fielder untransformed control. **A** Hybridization with a probe consisting of the coding region of *sgfpS65T*. All four GFP-expressing plants have the diagnostic 1.3-kb fragment released from pAct1IsGFP-1 after digestion with *Bam*HI. **B** The same blot stripped and re-hybridized with a probe consisting of the coding region of *nptII*. All four GFP-expressing plants have the diagnostic 1-kb fragment released from pH24 after digestion with *Bam*HI. **C** Southern blot of the same four GFP-expressing T0 plants. Plant DNA was digested with *Xba*I, which cuts only once in the 5.2-kb plasmid. *Lane 1* Plant no.3, *lane 2* plant no.4, *lane 3* Fielder untransformed control, *lane 4* plant no. 1, *lane 5* no. 2

Out of 120 embryos bombarded at a 6-cm target distance and using 0.6-μm gold particles, only 1 transformed plant was recovered with no antibiotic selection (within 4 weeks of bombardment) and an additional 3 GFP expressing plantlets were recovered after antibiotic selection (6 weeks after bombardment). With a 9-cm target distance and using 0.6-μm gold particles, 4 transformed shoots were recovered with GFP selection only and an additional 2 after selection was applied (Table 1). Only 1 transformed plant was recovered from treatments using 1.0-μm gold particles (9-cm target distance). From this initial experiment it could be determined within 4 weeks that 0.6-μm gold particles were probably better than 1.0-μm gold particles under the conditions used and that while either a 6-cm or 9-cm target distance could be used with 0.6-μm gold particles, a 3-cm target distance appeared to be detrimental to the recovery of transformed shoots.

Table 2 Segregation for GFP expression in the progeny of 4 transgenic plants selected solely on the basis of GFP expression

Plant	Number of seeds	Number GFP positive	Number GFP negative	χ^2
1	110	73	37	4.4*
2	39	31	8	0.4*
3	58	3	55	150.8
4	38	35	3	5.9*

* Not significantly different from a 3:1 ratio at the 0.01 level of significance

Discussion

From the results it can be seen that GFP has utility in optimizing wheat transformation protocols. There are many variables which affect transformation frequency (genotype, donor plant growth conditions, pre-culture length, bombardment conditions and type and size of particles used (Casas et al. 1995). The ability to determine the effectiveness of particular treatments quickly will allow for a more rapid improvement of transformation protocols. Using traditional selection methods it takes 6–8 weeks to identify putatively transformed shoots (Altpeter et al. 1996a) and even longer to confirm transformed status if using selectable markers which can lead to escapes (for example using the *bar* gene (Altpeter et al. 1996a; Nehra et al. 1994)). In the present study, whole expressing plants can be isolated as early as 3 weeks after bombardment, and all shoots which expressed GFP were confirmed as being transformed by Southern analysis, expression in progeny, or both. The rapid identification of transformed shoots removes the necessity to bring large numbers of calli through the entire culture/regeneration process, thereby freeing time to carry out additional bombardment experiments. The optimum time for GFP visual selection is shortly after the somatic embryos have germinated (approximately 3 weeks after bombardment). These shoots can be followed through root formation and then separated. Applying antibiotic selection can increase the number of transgenics obtained. This is likely due to being able to easily identify later-germinating transgenic embryos which otherwise are masked due to massive overgrowth of surrounding non-transgenic shoots. Elliott et al. (1999) used GFP expression for the early identification of transformed sugar cane cells under selection. This enabled the removal of untransformed tissue at an early age and facilitated the identification of transformed cell clusters. They also reported the identification of transformed callus clusters using visual selection alone but concluded that this was more laborious than combining visual and antibiotic selection. In the wheat transformation protocol used in the present study, the

continuous selection and isolation of transformed cell clusters over an extended period of time used for sugar cane is not necessary; selection can simply be applied to screen germinating somatic embryos and small shoots over a 2-week period (2–4 weeks after bombardment), thus reducing labor input. However, combining antibiotic selection with GFP selection can result in the production of more transformed plants than GFP alone because large masses of shoots make identification of GFP-expressing plants difficult after 4 weeks post-bombardment.

Wirtzens et al. (1998) reported a transformation frequency of 1.2% using paromomycin alone for selection with an escape frequency of 11% as opposed to a transformation frequency of 0.8–5% (Table 1) and an escape frequency of 0% in the present study using GFP coupled with paromomycin selection. While the use of GFP may not directly improve transformation frequency (which depends on many factors) its use would allow for the production of greater numbers of transformed plants in a given time period with a given amount of labor. This would be due to the elimination of escapes by transferring to soil only healthy shoots growing on selection medium and also expressing GFP as well as to the rapid optimization of experimental conditions which the use of GFP allows.

Of the four plants examined for inheritance of the trangenes (Table 2) three exhibited a segregation ratio consistent with a single locus (3:1), while another plant appeared to be chimeric. Chimeric shoots were also observed using the described transformation protocol but using antibiotic selection alone (data not presented). This is likely due to the timing of bombardment (if bombardment is carried out when embryogenic cell clusters have already been formed) and/or the timing of selection (selection only after somatic embryos have been formed, thus allowing chimeric cell clusters to develop into embryos). Using GFP and observing expression very early on in the transformation process may reduce chimerics by allowing the identification and manual elimination of chimeric cell clusters, embryos and shoots as they develop. GFP would also assist in optimizing conditions to reduce the frequency of chimerics.

Some forms of GFP have been reported to be toxic to plant cells either by accumulation in the nucleus (Haseloff et al. 1997) or by protein insolubility (Davis and Vierstra 1998). Various modified forms of GFP – targeted to the endoplasmic reticulum (Haseloff et al. 1997) or with increased solubility (Davis and Vierstra 1998) – have been constructed, and these showed increased brightness and lower toxicity in *Arabidopsis*. Using a non-targeted form with normal solubility, we have seen no evidence of toxicity, with highly fluorescing shoots developing into normal, fertile plants. A lack of toxicity of the *sgfpS65T* form of the gene has also been observed in sugar cane by Elliott et al. (1999). This may indicate a difference between monocots and dicots in their ability to tolerate the GFP protein.

The reason for the variation in fluorescence intensity between progeny embryos (Fig. 2f) is not known, and experiments are underway to determine if high levels of intensity are a result of homozygosity. If so, this could be a method to rapidly identify homozygous lines. Indeed, Molinier et al. (2000) have shown that homozygous lines of transgenic tobacco can be identified based on differences in the intensity of GFP fluorescence.

The use of GFP to identify transformed shoots and stably transformed cells has resulted in the rapid investigation of the possible importance of two parameters (particle size and target distance) in protocol optimization. GFP promises to be an important tool in the development of efficient transformation protocols for a wide range of elite wheat genotypes.

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