Plant Cell Reports (2000) 19:661–666 

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# Transgenic oat plants via visual selection of cells expressing green fluorescent protein

Received: 11 May 1999 / Revision received: 31 August 1999 / Accepted: 2 September 1999

**Abstract** New selectable markers and selection systems are needed to increase the efficiency and flexibility of plant transformation. The objective of this research was to determine if the green fluorescent protein (*gfp*) gene could be utilized as a visual selectable marker for transformation of oat (*Avena sativa* L.). A modified *gfp* gene was delivered into oat cells by microprojectile bombardment. Cell clusters expressing *gfp* were visually identified using fluorescence microscopy and physically isolated at each subculture. Eleven independent transgenic cell lines were obtained, and fertile plants regenerated from all lines. Transgene integration and expression were confirmed in transgenic plants and progeny. Transgene expression segregated in a 3:1 ratio in progeny of the majority of the transgenic lines.



Communicated by J.M. Widholm

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**Key words** Genetic engineering · Green fluorescent protein · Oat · Transformation

**Abbreviations** *CTAB*: alkyltrimethylammonium bromide · *gfp*: Green fluorescent protein · *GUS*: β-Glucuronidase · *MS medium*: Murashige and Skoog medium · *NOS*: Nopaline synthase · *PCR*: Polymerase chain reaction

## Introduction

Antibiotic and herbicide resistance genes have been the most widely utilized selectable markers for transformation of the important cereal crops barley, maize, oat, rice, sorghum and wheat (Cao et al. 1992; Casas et al. 1993; Gordon-Kamm et al. 1991; Songstad et al. 1996; Torbert et al. 1995; Wan and Lemaux 1994; Weeks et al. 1993). Despite the successful application of these genes, new selectable markers need to be developed to increase the efficiency and flexibility of cereal transformation. Currently, few selectable markers exist, limiting selection system options.

An optimal selectable marker gene for plant transformation would be one that is visually detectable at any time, without sample disruption, and without the addition of substrates, cofactors or selective agents. In addition, the gene product should not adversely affect cell growth, regeneration, or fertility, or impart any selective advantage if transferred to weedy relatives of the transformed crop.

Transgenes encoding luciferase, β-glucuronidase (GUS), and anthocyanins have been the most widely utilized visual reporters in cereal transformation systems (McElroy and Brettell 1994; Wilmink and Dons 1993). However, both luciferase and GUS assays require addition of a substrate at optimized levels at particular times to detect the enzymes visually. Also, GUS expression assays are usually toxic to plant cells, and some cereal monocot cultures express an endogenous GUS-like activity, interfering with detection of

transgene encoded GUS (Hansch et al. 1995; Hodal et al. 1992). Selection for anthocyanin production overcomes the disadvantages of substrate addition, but expression of anthocyanins in the regenerated plants may have detrimental effects on plant growth or may reduce the marketability of plant products because they are pigmented (McElroy and Brettell 1994).

The green fluorescent protein (*gfp*) gene possesses unique qualities that make it an ideal selectable marker and reporter for gene expression analysis (Chalfie et al. 1994). The *gfp* gene, isolated from the jellyfish *Aqueoria victoria*, encodes a small, barrel-shaped protein surrounding a fluorescent chromophore which immediately emits green fluorescent light when exposed to light in the blue to ultraviolet range (Ormo et al. 1996). Because no substrates or cofactors are required for *gfp* expression, observations can be performed repeatedly, at any time, on living cells without cell disruption. Also, modified forms of the *gfp* gene have been shown to be highly expressed in plants (Chiu et al. 1996; Haseloff et al. 1997; Pang et al. 1996; Rouwendal et al. 1997; Tian et al. 1997). Finally, the availability of mutant forms of *gfp* differing in solubilities and emission spectra make it possible to simultaneously monitor multiple transformation events within an individual transformant (Davis and Vierstra 1998; Heim and Tsien 1996; Stauber et al. 1998).

Applications of *gfp* in plant transformation include its use in monitoring the location, level and timing of gene expression (Leffel et al. 1997; Misteli and Spector 1997). High level expression of *gfp* in maize and wheat tissue cultures and transformants selected in cotransformation experiments using the herbicide resistance gene, *bar*, was also reported (Pang et al. 1996). Expression of *gfp* was also used in combination with low levels of antibiotics to increase the efficiency of selection for transgenic rice (Vain et al. 1998).

We report the successful generation of fertile, transgenic oat plants using a system based solely on visual selection for expression of the *gfp* gene. We also demonstrate stable transmission and expression of the *gfp* gene in seedling progeny of the primary regenerants. To the best of our knowledge, this is the first published report in any cereal crop of the generation of fertile, transgenic plants and progeny using a system based solely on visual selection of embryogenic tissue culture cells expressing a marker gene.

## Materials and Methods

### Initiation of friable, embryogenic, regenerable oat callus cultures

Seed from inbred oat genotype GP-1 (Torbert et al 1998), was generously provided by Dr. D.A. Somers, University of Minnesota. New cultures of GP-1 were initiated using a modification of the procedure of Torbert et al. (1998). The culture medium MS2D (Bregitzer et al. 1989; Torbert et al. 1995) was solidified with 0.2% (w/v) Phytagel (Sigma). Friable, embryogenic callus was visually selected at each 2-week subculture using a dissecting microscope (Bregitzer et al. 1989).

### Plasmid Constructs

The synthetic *gfp* gene of *A. victorea* (Chiu et al. 1996; Haas et al. 1996, generously provided by Dr. J. Sheen, Harvard Medical School) was ligated between the *Eco*RI and *Pst*I sites of pBluescript, producing pBlue-S*Gfp*-TYG-nos SK. A *gfp* expression plasmid pAHCS *GFP* was constructed by first excising *gfp* from the above plasmid, using a *Bam*HI restriction cut at the 5′ end of *gfp* and a *Not*I cut at the 3′ end. The *gfp* sequence was then ligated into the pAHC17 plasmid (Christensen and Quail 1996, generously provided by Dr. Peter Quail, USDA/ARS, Albany, Calif.), which contains the maize *ubi*1 ubiquitin promoter and first intron, followed by a multiple cloning site and a nopaline synthase (NOS) termination sequence and a portion of pUC8. The vector was opened after the *ubi*1 intron by a *Bam*HI restriction cut, and the *gfp* gene was ligated into the *Bam*HI site. The sticky end of the vector's unligated *Bam*HI site and the unligated *Not*I site on the *gfp* gene were made blunt-ended with T4 DNA polymerase and ligated together. Restriction mapping of the final recombinant clones was performed to determine that *gfp* was inserted in the sense orientation.

### Microprojectile bombardment of oat callus

Ten microliters of plasmid-coated, suspended, 1.6 μm gold particles (Kikkert 1993) were applied to macrocarriers of the PDS1000/he Biolistic particle delivery system (BioRad Laboratories). The stopping screen to sample plate distance was 12 cm, and 1300 psi rupture discs were used. The vacuum was drawn to 27 mm Hg prior to bombardment. Friable, embryogenic 3 to 4 month-old oat callus (approximately 0.4 g) was evenly spread over sterile, 7 cm Whatman No.1 filter paper discs. The discs with overlaying cells were placed on osmoticum (MS2D containing 0.2 M mannitol plus 0.2 M sorbitol) at least 4 h. prior to bombardment. Each sample was then bombarded once with particles coated with plasmid DNA, or with naked particles (controls) and then transferred to fresh, semi-solid MS2D medium. Cultures were incubated in the dark for 7 days at 25 °C.

### Selection of transgenic cultures

Expression of *gfp* in oat cell cultures was visualized using a Stemi-2000C dissecting microscope equipped with a Superlux 175 xenon blue light source (Zeiss) and dichromatic filters (Chroma Technology). The filter system consisted of a filter for detection of *gfp* expression (excitation 450–490 nm, emission 515 nm) and for blocking of chlorophyll fluorescence (emission 515–555 nm). Transient expression of *gfp* was observed 1–7 days after bombardment of oat cultures. Visual selection for *gfp* expressing sectors began 1–2 weeks after bombardment, and was performed every 2 weeks under the fluorescence viewing system. Fine-tipped forceps were used to separate and transfer glowing sectors (approximately 0.25–1.0 mm in diameter) to new culture plates. Sectors were subcultured to fresh medium every 2 weeks. Additional selection for glowing cells, and removal of nonglowing cells (if present) from sectors, was conducted using fine-tipped forceps at each subculture. After reaching a size of approximately 100–300 mg, subsamples of uniformly glowing sectors were placed on regeneration medium. The time from initiation of selection until transfer of cells onto regeneration medium was typically 5–7 weeks.

### Plant regeneration

*Gfp*-selected colonies and nonexpressing control colonies were placed on regeneration medium (Bregitzer et al. 1989). Plates containing cells on regeneration medium were placed 15 cm below Gro-Lux fluorescent lights (Sylvania, 40 W) set on 12 h light: 12 h dark cycles. After 3–4 weeks, regenerated, glowing shoots (1–2 cm) were transferred to magenta boxes containing

hormone-free MS medium to induce root formation. Rooting regenerants were placed under the same lighting regimen as described above. Rooted regenerants were transferred to peat pots containing Metromix 360 potting medium and placed in growth chambers set at 20 °C day, 15 °C night with a 12 h daylength of 300–400 μmol $m^{-2}s^{-1}$ light intensity. The distance from the lights to the plant canopy was 0.9 m. After plants were grown for 2–3 weeks, they were transplanted to 30-cm-diameter plastic pots containing a 1:1:1 mixture of soil: Metromix 360:sand, and placed in a glasshouse. Supplemental lighting was provided by 400 W metal halide lights and sodium high pressure lights set at 13 h daylength. Regenerated transgenic ($T_0$) plants were allowed to self pollinate without bagging to produce $T_1$ seed for progeny analysis.

### PCR and Southern blot analyses

Nuclear DNA was extracted from leaves using the CTAB procedure (Sambrook et al. 1989). To confirm integration of the *gfp* gene, polymerase chain reaction (PCR) detection of *gfp* was carried out with primers 5′GCGGATCCATGGTGAGCAAG (forward) and 5′GGGCGGCCGCTTTACTTGTA (reverse). The 50 μl reaction mixtures contained 250 μm of each dNTP, 0.5 μm of each primer, 1x Taq polymerase buffer (Promega), and 2.5 U Taq DNA polymerase (Promega). Initial denaturation was at 94 °C for 2 min followed by 35 cycles of melting (94 °C/30 s), annealing (60 °C/1 min), and extension (72 °C/2 min). Final extension was for 5 min at 72 °C. Positive and negative controls were included in all PCR analyses. For Southern blot analyses (Sambrook et al. 1989; Southern, 1975), 20 μg of genomic DNA was completely digested with 60 U of *Eco*RI and *Bam*HI each, electrophoresed in a 0.8% (w/v) agarose gel at 30 V, and transferred to a nylon membrane (Immobilon, Millipore). An 804 bp *gfp* fragment was liberated from pAHCSGFP by *Pst*1 digestion. This was purified by agarose gel electrophoresis and radiolabelled with $^{32}$P dCTP by hexamer priming. The membranes were washed four times with 2 × SSPE/0.1% SDS for 5 min each at 22 °C and four times with 0.2 × SSPE/0.1% SDS for 15 min each at 65 °C. The filters were exposed to Kodak BioMax MS film between Kodak Biomax MS intensifying screens for 3–4 days at minus 80 °C.

## Results and discussion

### GFP-based selection of oat tissue cultures and regeneration of transgenic plants

The synthetic *gfp* gene was delivered into friable, embryogenic oat callus (Fig. 1A) by microprojectile bombardment and 11 independent oat cell lines were obtained from a total of 28 bombarded samples following *gfp*-based selection (Table 1). The mean number of fertile plant-producing, *gfp*-expressing cell lines selected per bombardment was 0.43. Efficiency of *gfp*-based selection was comparable to that reported for an antibiotic (paromomycin)-based selection system in which the genotype, target tissues, and bombardment parameters were the same as those used in this research (Torbert et al. 1998). The level of *gfp* expression in the 11 lines was high (Fig. 1B, 1C), and colonies were easily distinguished from nontransformed, control cell lines.

The time between initiation of visual selection and transfer of selected cell lines to regeneration medium was typically 5–7 weeks. Plants were regenerated from all *gfp*-selected lines. Expression of *gfp* in regenerants was visually confirmed at the time of shoot initiation, rooting and flowering (Fig. 1E, 1F, 1G, 1H). Although *gfp* expression could be visually detected in all tissues, it was strongest in tissues containing only low amounts of chlorophyll such as cell cultures, meristematic regions, young plant tissues, reproductive structures and germinating seedlings. A slight autoflourescence was present in some cell cultures and plant tissues, such as anthers (Fig. 1H). Fluorescence of *gfp* was still easily distinguished from autofluorescence, however. Autofluorescence of cell cultures and tissues of other species that we have examined has ranged from low to high (data not shown). In cases where autofluorescence is high, optimization of the *gfp* filter set and lighting system may be necessary in order to separate *gfp*-based fluorescence from autofluorescence.

Regeneration of plants from tissue cultures and the growth of regenerants in the greenhouse was not reduced in *gfp*-expressing cell lines and plants, compared with nonexpressing controls. The number of seeds produced per transgenic $T_0$ plant varied from 0 to 169. This is comparable to the number of seeds produced on nontransformed control plants regenerated after bombardment (0–150). When compared with GP-1 oat plants grown from seed, seed yield from regenerated plants (transgenic and controls) was lower. The lower yield from the primary transformants was, therefore, attributed to the effect of growth in culture and not to expression of *gfp*. Finally, morphology and growth of *gfp*-expressing seedlings was not different from that of nonexpressing seedlings derived from the same $T_0$ plant.

### Molecular analysis of regenerated plants

Sixty regenerants, consisting of five or more plants from each of the 11 selected cell lines, were analyzed by

**Table 1** Transformation efficiency utilizing gfp-based selection. Number of transgenic callus lines regenerating fertile plants, following delivery of the green fluorescent protein (*gfp*) gene into oat tissue cultures and visual selection of transgenic cultures based on expression of *gfp*

| | No. of samples bombarded | No. of lines selected | No. of lines regenerated | No. of fertile lines | No. of plants regenerated |
|---|---|---|---|---|---|
| | 7 | 2 | 2 | 2 | 43 |
| | 5 | 2 | 2 | 2 | 10 |
| | 8 | 3 | 3 | 3 | 21 |
| | 8 | 4 | 4 | 4 | 41 |
| Total | 28 | 11 | 11 | 11 | 115 |

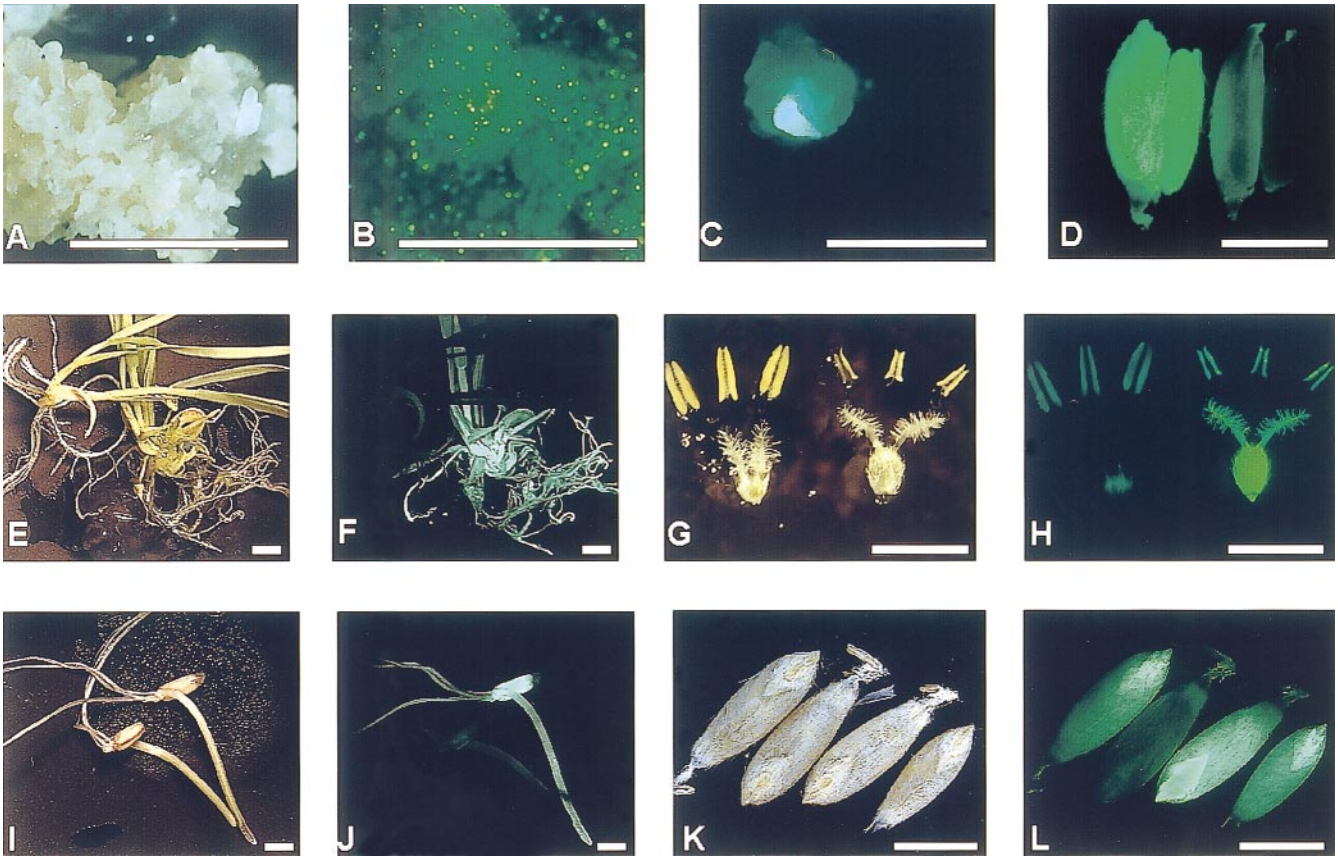


**Fig. 1** **A** Friable, embryogenic oat callus. **B** Transient expression of *gfp* in oat callus following microprojectile bombardment. **C** Sector of oat callus expressing *gfp*. **D** *Gfp* expression in bisected, immature seed from transgenic plant (*left*) and nontransgenic sibling plant (*right*). **E** Nontransformed, regenerated oat plantlet (*top*) and transformed regenerated oat plantlet (*bottom*) viewed under white light. **F** Same plantlets as **E** viewed under blue light (transgenic plantlet is expressing *gfp*). **G** Reproductive structures of non-transgenic (*left*) and transgenic (*right*) oat plants viewed under white light. **H** Same structures as in **G**, showing expression of *gfp* in reproductive structures of transgenic oat (*right*). **I** Germinating transgenic (*top*) and nontransgenic (bottom) oat seedlings viewed under white light. **J** Same seedlings as in **I** viewed under blue light (transgenic seedling is expressing gfp). **K** Dehulled seeds (caryopses) from transgenic oat plant viewed under white light. **L** Same seeds as in **K** showing segregation of *gfp* expression when viewed under blue light. **A–L** *Bar* 0.5 cm

PCR. The *gfp* sequence was amplified from nuclear DNA samples of all 60 regenerants (Fig. 2). No products were detected in control reactions where template DNA was omitted, or in DNA from regenerated, nontransformed control plants. To date, 56 of the PCR-positive regenerants, consisting of 3–8 plants from all 11 of the selected cell lines have been analyzed by Southern blotting using the *gfp* gene as a probe (Fig. 3). All 56 plants contained the *gfp* gene integrated into high molecular weight DNA, indicating transformation of plant cells. Variation in transgene rearrangements and integration patterns were evident among the transformants. This is consistent with variation in integration patterns observed in other plants transformed via microprojectile bombardment (Cao et al. 1992; Casas et al. 1993; Songstad et al. 1996; Torbert et al. 1995; Wan and Lemaux 1994; Weeks et al. 1993). The *gfp* integration patterns varied between the 11 regenerated lines. The patterns were identical among plants derived from the same line in 10 of the 11 lines (Fig. 3A). The differing integration patterns observed among plants within one transgenic line may be due to genetic chimerism of the cell line, tissue culture induced changes in DNA methylation, sample error, or other factors. Further analysis of the transgenic line and progeny will be required to determine the exact cause of the variation in transgene integration pattern. Overall, the data indicate that the 11 cell lines represent unique transformation events, and that at least 10 of the lines were derived from single events.

## Molecular and expression analysis of transgenic progeny

Seedling expression analysis was conducted using $T_1$ seed from four $T_0$ plants that were derived from independent events. Criteria for selection for segregation analysis included that the seeds were mature enough to germinate, and that they totalled at least 50 seeds per $T_0$ plant. Expression of *gfp* was easily detected visually in immature seed (Fig. 1D), mature seeds (Fig. 1K, 1L) and during seedling germination and coleoptile extension (Fig. 1I, 1J). Expression data for segregation anal-

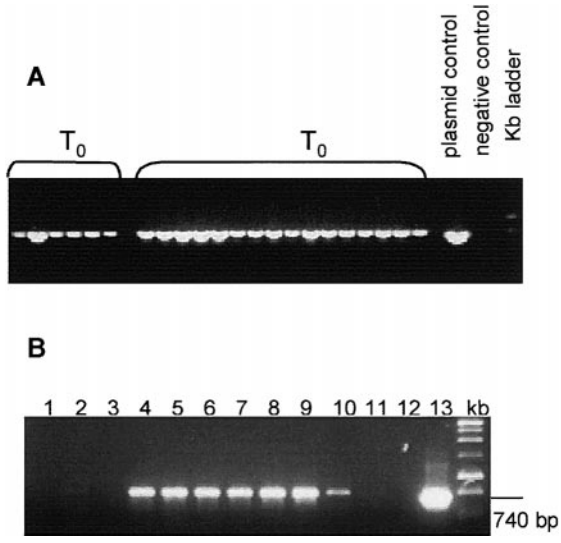


**Fig. 2** **A** Detection of *gfp* gene in transformed oat plants ($T_0$ generation) from two independent events. PCR products were electrophoretically separated on an agarose gel stained with EtBr. The predicted 740 bp product was amplified in all putative transformants. *Negative control* Non-transformed oat genomic DNA. *Plasmid control* pAHCSGFP. **B** Transmission of *gfp* gene to $T_1$ progeny of a $T_0$ transformant. Agarose gel electrophoresis of PCR amplification products of *gfp* gene (740 bp) in $T_1$ progeny showing segregation for *gfp* expression. *Lane 1* PCR reaction mixture, not amplified. *Lanes 2 and 3* nonexpressing $T_1$ plants. *Lanes 4–9* $T_1$ plants expressing *gfp*. *Lane 10* $T_0$ positive control. *Lanes 11 and 12* Nontransformed genomic DNA controls. *Lane 13* pAHCSGFP plasmid

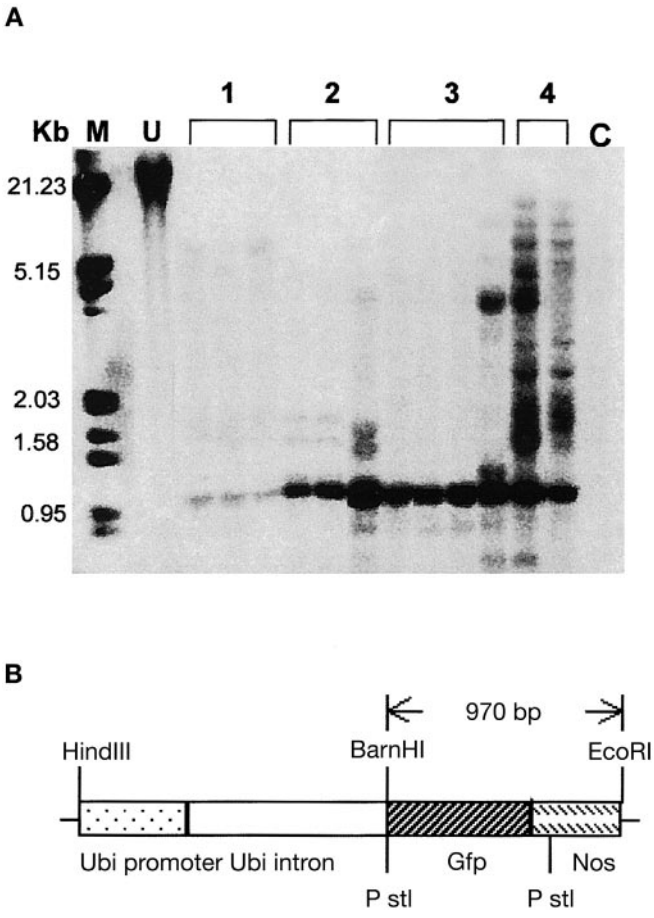


**Fig. 3** **A** Southern blot of $T_0$ oat plants regenerated from four different transgenic cell lines (*1–4*). Twenty microgramms genomic DNA was digested with *Bam*HI and *Eco*RI, and hybridized with *gfp*. The labeled probe hybridized to a 970 bp band representing *gfp* and the Nos terminator sequence. *M* DNA fragment size marker consisting of Lambda DNA digested with *Eco*R1 and *Hind*III, *U* Uncut DNA from $T_0$ line number *2*, *C* DNA from regenerated control plant digested with *Bam*HI and *Eco*RI. **B** Section of plasmid pAHCS *GFP* showing the Ubiquitin promoter and intron and the 970 bp sequence between *Bam*HI and *Eco*RI restriction sites containing *gfp* and Nos terminator

ysis were collected on germinating seedlings. PCR analysis of genomic DNA from the seedling progenies gave products for all seedlings expressing *gfp*, but none in negative controls (Fig. 2B). Observed ratios of *gfp* expressing to nonexpressing seedlings for four events were: 60:21, 71:29, 38:12, and 61:39. Segregation ratios for *gfp* expression in seedlings from three of the events closely fit a 3:1 Mendelian segregation ratio ($P<0.05$) for a dominant gene at a single locus. The segregation ratio for *gfp* expression in seedlings from the fourth event did not fit a simple Mendelian ratio. The lack of fit to expected ratios may be due to silencing of the *gfp* transgene, position effects, association of the transgene with chromosomal abnormalities induced through tissue culture, or genetic chimerism of the cell line. Distorted segregation ratios have also been observed in other cereal monocot transformation studies (Funatsuki et al. 1995; Pawlowski et al. 1998; Spencer et al. 1992). Further analysis will be required to determine the underlying cause of the distorted segregation for expression of *gfp*.

In conclusion, we have demonstrated that a new selection system based solely on *gfp* expression can be utilized to efficiently produce fertile, transgenic plants of a cereal monocot, oat. Transmission of both the *gfp* phenotype and *gfp* transgene to $T_1$ progeny verified that germline transformation had occurred. The majority of transgenic lines arose from single, unique transformation events, and demonstrated Mendelian inheritance ratios for transgene expression in progeny; however, exceptions were observed. We have recently utilized *gfp*-based selection to isolate over 70 new transgenic cell lines, which are undergoing regeneration (data not shown). The new lines will be useful to further investigate the frequency and basis of the types of exceptions described above. Additionally, it would be interesting to compare, within the same experiment, the efficiency of *gfp*-based selection with that obtained using antibiotic- and herbicide resistance-based selection systems. We are initiating an experiment to make such comparisons.

Successful application of *gfp*-based selection in the production of transgenic oat lends support to the hypothesis that the selection system may also be applicable for transformation of other agronomically important

cereal crops. Refinement of the technique may be necessary, however, depending on culture phenotype and growth. In preliminary experiments in barley and maize, we found it necessary to initiate selection for glowing sectors sooner, and to perform selective subcultures at a greater frequency, in order to maintain nonchimeric glowing sectors. This may be due, in part, to the observed faster growth rate and higher friability of the barley and maize cultures compared to that of oat. The pattern of cell division and culture growth could also differ among plant species, and may affect one's ability to select cohesive sectors. In cases where *gfp*-based selection can be applied, availability of this additional selection system could increase the efficiency, flexibility and potential applications of cereal crop transformation.

**Acknowledgements** We wish to thank Ronald Duerst for assistance in glasshouse growth of oat plants and fertility analysis, Kathy Canada for aid in molecular analysis of transgenic plants, and Michael O'Connor and John Herbst for technical assistance in the construction and sequencing of the pAHC/S *Gfp* expression plasmid. Research supported in part by USDA-HATCH and North Central Biotechnology Initiative funding.

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