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

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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.

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 *Aequoria 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 1998). 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. victoria* (Chiu et al. 1996; Haas et al. 1996, generously provided by Dr. J. Sheen, Harvard Medical School) was ligated between the EcoRI and PsI sites of pBluescript, producing pBlue-Sgfp-TYG-nos SK. A gfp expression plasmid pAHCS gfp was constructed by first excising gfp from the above plasmid, using a BamH1 restriction cut at the 5’ end of gfp and a NotI cut at the 3’ end. The gfp sequence was then ligated into the pAHCS17 plasmid (Christensen and Quail 1996, generously provided by Dr. Peter Quail, USDA/ARS, Albany, Calif.), which contains the maize ubi1 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 ubi1 intron by a BamH1 restriction cut, and the gfp gene was ligated into the BamHI site. The sticky end of the vector’s unligated BamHI site and the unligated NotI 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 DSI100/hec 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 Stemiview 2000C dissecting microscope equipped with a Superlux 175 xenon 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, subcultures 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