



Using GFP as a Scorable Marker in Walnut Somatic Embryo Transformation

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Somatic embryogenesis is the foundation of genetic transformation in several economically important tree species. In the *Juglans regia* L. (Persian walnut) somatic embryogenesis-based transformation system, a major limiting factor is the selection of non-chimeric transgenic embryos in tissue culture. We transformed Persian walnut somatic embryos with the S65T synthetic green fluorescent protein (GFP) gene in order to assess the effect of this visual marker gene on embryo viability and the selection of transgenic embryos. Following a 10 d period of transient GFP expression in all inoculated embryos, stable fluorescent sectors were apparent in several embryos, allowing efficient and rapid visual selection of primary transgenic embryos. Two chimeric embryos were selected 40 d after transformation, and these two embryos gave rise to 13 stable transgenic embryo lines and 44 whole plants. GFP-expressing walnut plants and embryos developed normally and transformation was verified by GUS analysis. Our analysis suggests that the use of GFP as a selectable marker can significantly reduce labour, cost, and time in the walnut somatic embryogenesis-based transformation system. © 2000 Annals of Botany Company

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INTRODUCTION

Progress in breeding tree species is hampered by their long generation intervals, limitations on breeding population size, and limited genome characterization. Application of plant biotechnology to woody plant improvement has the potential to significantly accelerate breeding efforts and has provided novel solutions in the areas of disease resistance, insect resistance, and fruit quality (Dandekar *et al.*, 1994; Ozdemir *et al.*, 1998). For several important agricultural and timber tree species, the foundation of biotechnological plant improvement is the *Agrobacterium*-mediated transformation of somatic embryos (McGranahan *et al.*, 1988; Jain *et al.*, 1989). A well established woody plant somatic embryo transformation system has been developed in walnut (*Juglans regia* L.), with several transgenic walnut lines already fruiting in field trials.

A major limiting factor in the generation of transgenic plants from somatic embryo transformation systems is the selection and identification of transformants in culture. Selectable marker genes, especially the kanamycin resistance gene *nptII*, are widely used to select for transformed cells, but survival of non-transformed tissue is common in somatic embryo cultures (McGranahan *et al.*, 1990; El Euch *et al.*, 1998). Certain walnut genotypes have shown low sensitivity to kanamycin, and even under stringent media selection conditions (500 mg l⁻¹ kanamycin) less than 25% of surviving embryos may be genetically transformed (El Euch *et al.*, 1998). The process of generating transgenic plants from somatic embryos is further hindered

by the cytotoxic effects of the assay for β -glucuronidase (GUS) gene activity that is commonly used to confirm transformation (Jefferson *et al.*, 1987). In walnuts, this assay requires dissection of E₁ and E₂ generation embryos, with part of the embryo utilized for colorimetric GUS assay while the remainder is returned to tissue culture to proliferate E₃–E₄ generation embryos for germination (McGranahan *et al.*, 1990).

We have transformed walnut somatic embryos from the culture line Chandler 1 (CR1) with the S65T synthetic green fluorescent protein (GFP) gene driven by the strong, constitutive cauliflower mosaic virus (CaMV) 35S promoter. GFP is a stable, cell-autonomous fluorescent protein derived from the jellyfish *Aequorea victoria* that has been used extensively as a non-destructive scorable marker gene for plant transformation (Chiu *et al.*, 1996). The S65T synthetic GFP gene construct contains optimized codon usage for plant expression, a mutation at amino acid position 65 that converts a serine residue to a threonine residue, and a deletion of the cryptic intron site found in wild type GFP (Heim *et al.*, 1995). These modifications have resulted in a 120-fold increase in fluorescence and a single 490 nm fluorescence emission peak (Maximova *et al.*, 1998). Corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), tobacco (*Nicotiana tabacum* L.), and *Arabidopsis thaliana* cells expressing S65T synthetic GFP emit a bright fluorescence that is visible to the naked eye when illuminated by ultraviolet light (Pang *et al.*, 1996). Our goals in this experiment were to assess the practicality of GFP as a scorable marker in the walnut somatic embryo transformation system and to determine whether GFP can

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improve the selection efficiency of transgenic embryos in culture.

MATERIALS AND METHODS

The *Agrobacterium* binary vector pDM96.0501 contains GUS (β -glucuronidase), S65T synthetic GFP, and *nptII* driven by CaMV 35S promoters, and has been described previously by Maximova *et al.* (1998). The pDM96.0501 plasmid was electroporated into the disarmed *A. tumefaciens* strain EHA101. *A. tumefaciens* EHA101/pDM96.0501 was cultured in liquid 523 medium (Moore *et al.*, 1988) at ambient temperature for 48 h, pelleted, and resuspended to a density of 2.5×10^8 cells ml⁻¹ in liquid Driver–Kuniyuki walnut (DKW) basal medium (Driver and Kuniyuki, 1984). The plant material used for transformation was the walnut somatic embryo line CR 1, derived from anther tissue of the *J. regia* cultivar Chandler. Forty *J. regia* CR 1 embryos were soaked for 10 min in the *Agrobacterium* suspension and blotted dry on sterile filter paper. Inoculated embryos (generation E₀) were plated on solid DKW basal medium containing 100 μ M acetosyringone and were co-cultivated at 27°C in the dark for 48 h. Following co-cultivation, the embryos were transferred to solid DKW medium containing 500 mg l⁻¹ cefotaxime and 100 mg l⁻¹ kanamycin. The embryos were maintained in the dark without transfer to fresh medium for 40 d at ambient temperature and were observed for fluorescence.

Following the initial 40 d observation period, two chimeric (partially fluorescent) E₀ generation embryos were transferred to fresh DKW basal medium containing 500 mg l⁻¹ cefotaxime and 100 mg l⁻¹ kanamycin and maintained in the dark at ambient temperature. These chimeric embryos and the wholly fluorescent E₁ generation embryos arising from them were transferred to fresh media every 7–10 d. In order to halt the proliferation of secondary embryos and to encourage germination, wholly fluorescent E₂–E₄ embryos with well-developed cotyledons were desiccated in dry microtitre plates sealed with parafilm for 2 weeks at 4°C. The desiccated embryos were rehydrated on solid DKW basal medium containing 5 g l⁻¹ charcoal in the dark for 2 weeks at ambient temperature. Embryos that did not develop roots were designated for chip budding and maintained in the dark until shoots were 3–5 cm. These embryonic shoots were excised and micropropagated in magenta boxes containing solid DKW shooting medium under 24 h light (Driver and Kuniyuki, 1984). Axillary buds from 4–8 cm long micropropagated shoots were chip budded (Hartmann *et al.*, 1997) onto seedling rootstock and maintained in a greenhouse. Embryos displaying root and shoot development were transferred to solid DKW basal medium under 24 h light at ambient temperature. Those with a well developed root system and a 2–4 cm shoot were transferred to sterile rooting plugs saturated with half-strength liquid DKW medium in test tubes. Seedlings in rooting plugs were transferred to sterile soil and acclimatized to greenhouse conditions (McGranahan *et al.*, 1988).

Fluorometric GUS analysis of somatic embryos was performed by 4-methyl umbelliferyl glucuronide (MUG) assay. Ten to 100 mg of embryo tissue was homogenized in

protein extraction buffer and the fluorescence of the protein extract in the presence of MUG substrate was quantitated using a Hoefer Scientific Instruments fluorometer as described by Jefferson *et al.* (1987).

In-culture observations of walnut somatic embryos were made using a Leica MZ12 dissecting microscope equipped with a 100 W mercury UV lamp and a GFP PLUS Fluorescence filter kit. Transformed and untransformed control plant material was observed using a Nikon fluorescence microscope equipped with illumination from a 100 W mercury lamp passed through a Nikon filter set with a 465–495 nm bandpass excitation filter, a 505 nm dichroic mirror and a 515–555 nm bandpass barrier filter. Somatic embryos and entire roots were mounted directly on slides in glycerol. Stem internode samples were sectioned by hand and mounted in glycerol. Images were recorded with an Optronics digital camera system using manual exposure settings. For whole mounts and stem hand-sections, the images of the controls were recorded using the same exposure times as for the comparable transformed material.

RESULTS AND DISCUSSION

Faint green fluorescence was visible in epidermal cells of walnut somatic embryos as early as 22 h after inoculation with *A. tumefaciens* EHA101/pDM96.0501. Uninoculated embryos and cultures of *A. tumefaciens* EHA101/pDM96.0501 were non-fluorescent. Transient GFP expression was apparent on the surface of all inoculated embryos, with maximum brightness at 4 d after inoculation. Transient expression was completely extinguished after 10 d, leaving small, dimly fluorescent sectors of cells on a small number of embryos. These results suggest a highly efficient delivery of T-DNA into the embryo cell nuclei, but a low efficiency of stable T-DNA integration into the genome. There were no observable developmental differences between the chimeric (partially fluorescent) embryos and the non-transformed embryos during the 40 d observation phase. All inoculated embryos remained alive during 40 d of selection on kanamycin-containing media, but embryos expressing the GFP transgene were readily identifiable when examined in culture with a dissecting fluorescence microscope. Thus, GFP expression allowed rapid, non-invasive visual selection of chimeric embryos with 100% efficiency as compared to selection by kanamycin resistance alone (Fig. 1). Two chimeric E₀ generation embryos were transferred to fresh media for further study.

Secondary somatic embryogenesis in walnut is known to occur by the division of a single epidermal cell (Polito *et al.*, 1989). If such an 'embryogenically fated' epidermal cell was transformed, a wholly fluorescent secondary embryo would be expected. Alternatively, if a single cell of an already multicellular secondary embryo was transformed, a chimeric secondary embryo would be expected. Under kanamycin selection the two chimeric E₀ embryos proliferated wholly fluorescent, partially fluorescent, and non-fluorescent secondary embryos. Because GFP expression could be detected in globular-stage secondary embryos, selection of transgenic E₁ generation embryos could be made by visual assay several weeks earlier than is possible

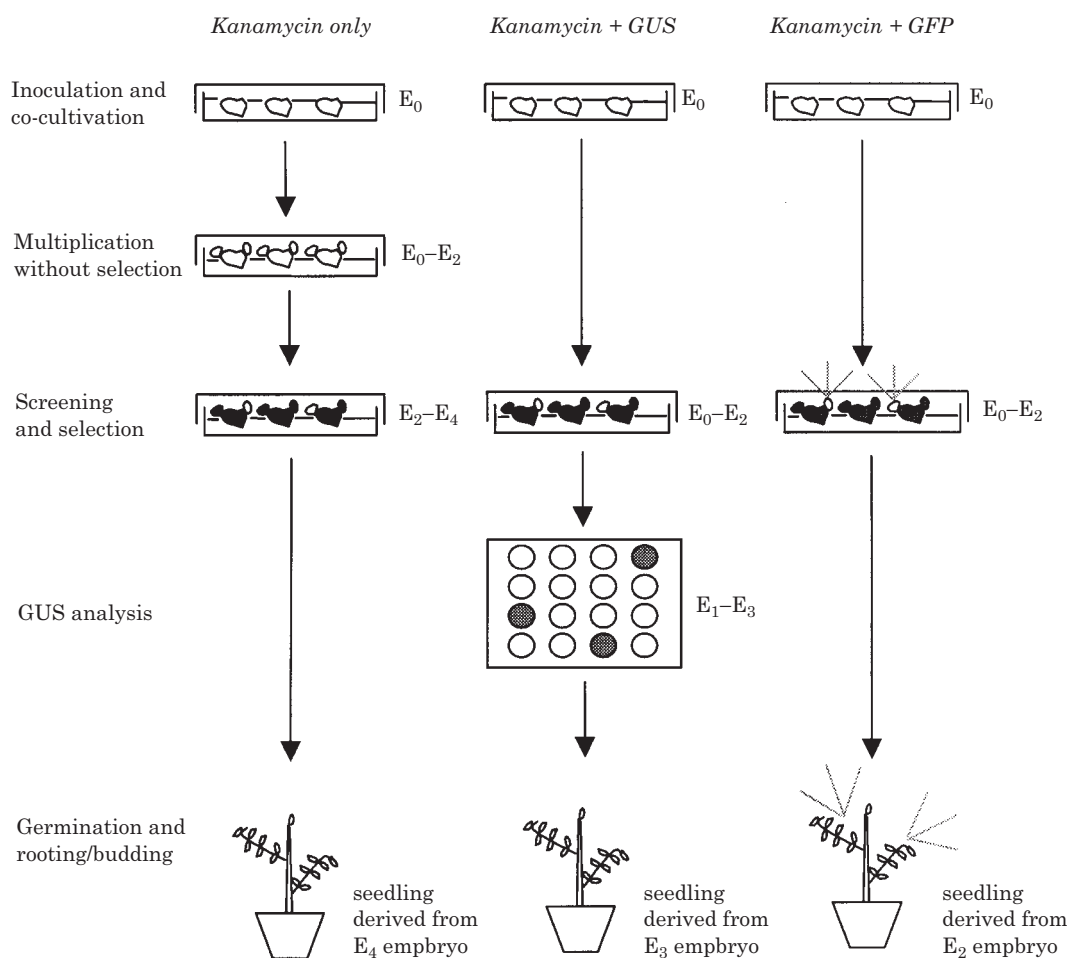


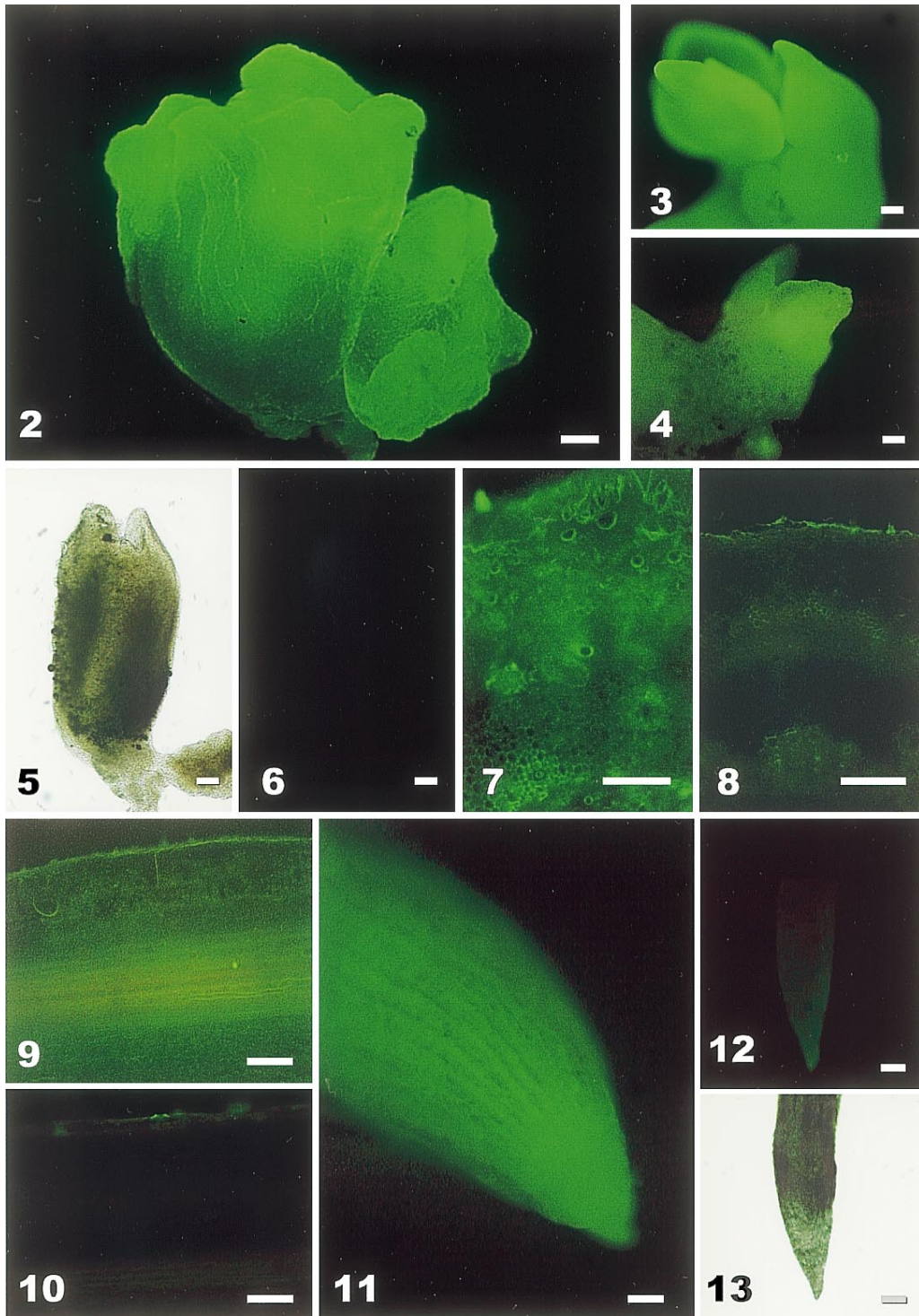
FIG. 1. Methods used for the selection of transgenic walnut somatic embryos. Selection by kanamycin only was described by McGranahan *et al.* (1988), selection by kanamycin and GUS was described by McGranahan *et al.* (1990), and selection by kanamycin and GFP is described in this paper. In all three selection systems *Agrobacterium tumefaciens*-inoculated somatic embryos (generation E₀) proliferate secondary somatic embryos (generation E₁), which in turn proliferate successive generations of embryos (E₂-E_x). Selection using kanamycin and GFP allows germination of E₂ generation embryos, reducing the time and labour required to produce transgenic walnut plants.

by embryo dissection and GUS assay. In addition, chimeric embryos could be readily distinguished from full transgenics, making germination of transgenic plants possible in the E₁ generation, if desired. Overall, we found that transformation of walnut somatic embryos with S65T synthetic GFP decreased the time and in-culture manipulation required for the selection of transgenic embryos without observable effects on embryo viability in culture (Fig. 1).

Thirteen wholly fluorescent E₂ generation embryos were excised from fluorescent E₁ embryos and cultured separately, giving rise to 13 stable transgenic lines (Figs 2-13). Fluorometric GUS assay results verified that 12 of the 13 embryo lines were concomitantly expressing β -glucuronidase, providing further evidence of transformation (data not shown). Embryos from these lines were germinated with greater than 50% efficiency and gave rise to 44 fluorescent transgenic walnut plants that are currently being grown in a greenhouse (37 produced by chip budding, seven produced by direct rooting). Whole mounts of transgenic embryos and roots demonstrate a global pattern of GFP expression

with no discernible tissue specificity (Figs 2-4), as is expected from a transgene driven by the constitutive CaMV 35S promoter (Pang *et al.*, 1996). Likewise, a non-tissue specific pattern of fluorescence was observed in stem and root tissues in longitudinal and transverse sections (Figs 7-13).

Our results suggest that S65T synthetic GFP is an effective scorable marker in walnut somatic embryo culture and does not interfere with subsequent walnut development in culture or in the greenhouse. GFP has the potential to significantly decrease labour, time, and cost constraints in walnut somatic embryo culture and represents a significant improvement over existing kanamycin and β -glucuronidase-based selection systems (Fig. 1). Future studies will expand research to other economically significant woody perennials with a somatic embryogenesis-based regeneration system, such as pecan (*Carya illinoensis*) and white pine (*Pinus strobus* L.), as well as follow the development of GFP-expressing walnuts as they are transplanted into the field.



FIGS 2–13. Green fluorescent protein (GFP)-expressing walnut embryos and organs from whole plants. Fluorescence micrographs were taken with an Optronics digital camera using manual exposure settings. Sample illumination was from a 100 W mercury lamp passed through a Nikon filter set with 465–495 nm excitation, a 505 nm dichroic mirror, and 515–555 nm barrier filtration. The bright-field micrographs in Figs 5 and 13 show the same field as for the accompanying fluorescence micrographs. Figs 2–4: GFP fluorescence in whole mounts of three walnut embryos. Figs 5 and 6: Bright-field (Fig. 5) and fluorescence (Fig. 6) micrographs of untransformed control walnut embryo. Figure 6 was taken using identical conditions of illumination and exposure as that for the micrographs in Figs 2–4. Figs 7 and 8: Fluorescence micrographs of stem cross sections from GFP-transformed (Fig. 7) and control (Fig. 8) walnut shoots taken under identical conditions of illumination and exposure. Autofluorescence is seen at the epidermis and in lignified tissues in Fig. 8. Figs 9 and 10: Fluorescence micrographs of root longitudinal sections from GFP-transformed (Fig. 9) and control (Fig. 10) walnuts taken at identical conditions of illumination and exposure. Figs 11–13: Fluorescence (Figs 11 and 12) and bright-field (Fig. 13) micrographs of whole mounts of roots from GFP-transformed (Fig. 11) and control (Figs 12 and 13) walnuts. Bars = 0.1 mm.

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