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Transient expression and stable transformation of soybean using the jellyfish green fluorescent protein

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Abstract Embryogenic soybean [*Glycine max* (L.) Merrill.] suspension cultures were bombarded with five different gene constructions encoding the jellyfish (*Aequorea victoria*) green fluorescent protein (GFP). These constructions had altered codon usage compared to the native GFP gene and mutations that increased the solubility of the protein and/or altered the native chromophore. All of the constructions produced green fluorescence in soybean cultures upon blue light excitation, although a soluble modified red-shifted GFP (smRS-GFP) was the easiest to detect based on the brightness and number of foci produced. Expression of smRS-GFP was visible as early as 1.5 h after bombardment, with peak expression at approximately 6.5 h. Large numbers of smRS-GFP-expressing areas were visible for 48 h postbombardment and declined rapidly thereafter. Stably transformed cultures and plants exhibited variation in the intensity and location of GFP expression. PCR and Southern hybridization analyses confirmed the presence of introduced GFP genes in stably transformed cultures.

Key words Genetic transformation · Green fluorescent protein · Soybean *Glycine max* · Transient expression

Abbreviations *ER* Endoplasmic reticulum · *GFP* Green fluorescent protein · *GUS* β-Glucuronidase · *smgfp* Soluble modified *gfp* · *smRS-gfp* Soluble modified red-shifted *gfp*

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Introduction

The green fluorescent protein (GFP) of jellyfish (*Aequorea victoria*) is a potentially useful reporter in heterologous systems (Chalfie et al. 1994). Native GFP is a 238-amino-acid protein that produces green fluorescence upon excitation by UV or blue light (Cubitt et al. 1995). GFP contains three amino acids (Ser65, Tyr66, and Gly67) that undergo posttranslational modification to form a *p*-hydroxybenzylideneimidazolinone chromophore. Mutations in the chromophore and at other specific sites in GFP can be introduced to alter its properties. In contrast to the widely used β-glucuronidase (GUS) reporter gene system, which requires an exogenous substrate for histochemical visualization, GFP expression can be monitored directly in living tissue. This property of GFP holds tremendous promise not only in studies involving the use of fusion proteins, but also in the optimization of transformation protocols for various plant species. In plant systems with low transformation efficiencies, non-destructive testing of putative transformed cells and tissues could significantly reduce the time and effort involved in optimizing transformation protocols and generating transgenic plant material.

Since GFP is not native to plants, modifications to the gene may be required to make it more suitable for introduction into various plant species. For instance, because *gfp* mRNA is mis-spliced in *Arabidopsis thaliana*, the native *gfp* sequence was modified to eliminate a cryptic intron (Haseloff et al. 1997). Modifications of the *gfp* cDNA include alterations in codon usage, mutations to enhance solubility of the protein, and changes in the chromophore that alter the spectral properties of the protein. Several of these versions of the *gfp* gene have been successfully expressed in *Arabidopsis* (Sheen et al. 1995; Haseloff et al. 1997; Davis and Vierstra 1998), corn (van der Geest and Petolino 1998), rice (Vain et al. 1998), wheat (Pang et al. 1996), and apple (Maximova et al. 1998). Since processing of

gfp RNA may vary depending on the species, and visualization of the protein may be complicated by background fluorescence in certain tissues, selection of the appropriate version of this reporter gene is critical.

GFP is still a relatively new scorable marker in plants and very little is known about the timing of expression and other factors that could influence its detection. This study evaluates GFP with the goal of improving and developing transformation and regeneration strategies for soybean. To this end, several available gene constructions were introduced into embryogenic soybean cultures via particle bombardment, and factors influencing transient GFP expression in these cultures were investigated. In addition, the feasibility of generating stably transformed soybean cultures expressing GFP was determined.

Materials and methods

Plasmid constructions

Several variations of the native *gfp* gene (*SGFP-TYG*, *mgfp4*, *mgfp5-ER*, *smgfp and smRS-gfp*) were tested in this study. The specific DNA constructions used to transform soybean are described below.

The plasmids *35S-SGFP-TYG-nos* (pUC18) and *HBT-SGFP-TYG-nos* (pUC18) were obtained from Sheen and Jang (Harvard Medical School, Boston, Mass.). The *gfp* gene in these constructions encodes a synthetic GFP (SGFP-TYG) which was synthesized based on optimal codon usage for humans, maize, and *Arabidopsis* (Chiu et al. 1996; J.-C. Jang, personal communication). The chromophore of the native protein (SYG) was mutated to TYG, to yield a protein with a single excitation peak by blue light. GFP expression in *35S-SGFP-TYG-nos* is driven by the CaMV 35 S promoter. The *HBT-SGFP-TYG-nos* construction has a strong, constitutive, chimeric promoter consisting of the 35 S enhancer fused to the basal promoter and 5′ untranslated region of the maize C4PPDK gene (Sheen et al. 1995; Chiu et al. 1996). Both of the constructions have the 3′ *NOS* terminator.

pBIN 35S-*mgfp4* and pBIN 35S-*mgfp5-ER* were obtained from J. Haseloff (MRC, Cambridge, UK). Both of these modified *gfp*-coding regions were cloned into binary vectors that bear the gene for kanamycin resistance. Expression of the modified *gfp* genes is driven by the 35 S promoter and both constructions have the 3 *NOS* terminator. In *mgfp4*, the sequence of the native *gfp* was altered to prevent aberrant mRNA processing in *Arabidopsis* yet result in no change in the amino acid sequence (Haseloff et al. 1997). For *mgfp5-ER*, in addition to removal of a cryptic intron, mutations (V163 A and S175G) were introduced to enhance fluorescence by improving folding of the protein. An additional substitution (I167 T), in combination with enhanced folding characteristics, yielded a protein with dual excitation peaks of similar amplitude that can be visualized upon UV or blue light excitation (J. Haseloff, personal communication). The *mgfp5-ER* construction also has sequences 5′ and 3′ to the *gfp* coding region that permit compartmentalization of the protein product in the lumen of the endoplasmic reticulum (ER). To generate a plasmid construction more suitable for particle bombardment, a *Hin*dIII-*Sac*I fragment from pBIN 35S-*mgfp5-ER* consisting of the promoter-*mgfp5–ER* sequence was used to replace the promoter-GUS coding region from pUCGUS (Finer and McMullen 1990) to give pUC-*mgfp5-ER* (CaMV 35S-*mgfp5-ER–NOS*).

A soluble modified *gfp* (*smgfp*) was generated by the introduction of three site-directed mutations (F99 S, M153 T, and V163 A) into the *mgfp4* sequence (Davis and Vierstra 1998). The *smgfp* sequence was further mutated (S65 T) to generate a soluble modified red-shifted *gfp (smRS-gfp)* with a modified chromophore (TYG). These *gfp* genes, each driven by the 35 S promoter with the 3′ *NOS* terminator are in a pUC118 background, and were obtained from the Arabidopsis Biological Resource Center at The Ohio State University, Columbus, Ohio (CD3–326 = *smgfp*, and CD3–327 = *smRS-gfp*).

The plasmid pUCGUS (Finer and McMullen 1990), which encodes GUS, was used as a negative control while monitoring transient GFP expression. For generation of stable transformants, either pHG1 (Finer and McMullen 1991) or pHygr (Finer et al. 1992), which confer resistance to hygromycin B were used. In addition, a *Hin*dIII fragment of pHG1 encompassing the hygromycin expression unit was cloned into the *Hin*dIII site of the *smRS-gfp* plasmid to yield a construction (pHOG; hygromycin opposite GFP) with expression of the selectable and scorable markers being driven in opposite directions.

Target tissue and particle bombardment

Soybean [*Glycine max* (L.) Merrill, cv. Chapman] embryogenic suspension cultures were initiated and maintained by weekly transfers to fresh FN medium as described previously (Finer and Nagasawa 1988). Plasmid DNAs for particle bombardment were isolated using a Qiagen Plasmid Maxi kit according to the manufacturer's directions (Qiagen, Santa Clarita, Calif.). Plasmid DNA (5 μg) was precipitated onto tungsten particles and delivered to embryogenic suspension culture tissue (about ten clumps/bombardment) with a particle inflow gun as described elsewhere (Finer et al. 1992; Hadi et al. 1996). To monitor transient GFP expression over time, embryogenic tissue was maintained on semisolid D20 embryo proliferation medium (Santarem et al. 1997).

For generation of stably transformed tissue, cotransformation was carried out following precipitation of 2.5 μg of GFP plasmid along with 2.5 μg of a plasmid that confers resistance to hygromycin (pHygr or pHG1) prior to bombardment. When pHOG was used for isolation of stable transformants, 5 μg of this plasmid was bombarded alone. One week after bombardment, tissue was transferred to FN liquid medium supplemented with 7.5 mg/l hygromycin B. Bombarded tissue was maintained by replacing the selective medium every week. After 6 or more weeks of selection, hygromycin-resistant green clumps of tissue were isolated, and transferred to fresh flasks of selective medium. Embryo development and germination were carried out as described previously (Finer and McMullen 1991).

To determine if GFP expression was detrimental to the growth of transformed cells, embryogenic suspension culture tissue (100 mg) was weighed aseptically and transferred to fresh flasks of FN medium. The fresh weight of tissue was determined at weekly intervals over a 21-day period.

Visualization of GFP expression

GFP expression was observed in embryogenic cultures and developing embryos with a stereo dissecting microscope equipped with a fluorescence module consisting of a 100-W mercury lamp and GFP Plus excitation and emission filters (Leica, Heerbrugg, Switzerland). This system (excitation filter 480/40 nm; dichroic mirror 505 nm LP; barrier filter 510 nm LP) permits visualization of GFP following excitation by blue light. To determine the effect of prolonged exposure to blue light on transient GFP expression, tissue was bombarded with the *smRS-gfp* plasmid and incubated on D20 medium for approximately 5.5 h. Half of the bombarded tissue was exposed to the high-intensity blue light source (750 μmol m^{-2} s^{-1}) for periods of 1 h each at various times after bombardment. The number of transient events was determined immediately preceding each of the 1-h exposures. GFP expression in the remainder of the bombarded tissue (control) was monitored at the same intervals, but this tissue was only exposed to the blue light source for brief periods (1–3 min) in order to ascertain the number of transient events.

Molecular analysis

As an initial step to determine the presence of *smgfp* in hygromycin-resistant suspension-cultured tissue, DNA was extracted from putative transformed clones or untransformed tissue (100 mg fresh weight) using a Qiagen DNeasy kit according to the manufacturer's directions (Qiagen) and subjected to PCR using forward (5′-GCA CAA TCC CAC TAT CCT TCG CAA-3′) and reverse (5′-GTC TTG TAG TTC CCG TCG TCC TTG A-3′) primers complimentary to the 35 S promoter and the *smgfp* coding region, respectively. Conditions for the thermal cycler were: step 1:94 °C (5 min), 55 °C (2 min), 72 °C (3 min); step 2 (31 cycles): 94 °C (1 min), 55 °C (2 min), 72 °C (3 min); step 3:94 °C (1 min), 55 °C (2 min), 72 °C (10 min).

For Southern analysis, DNA was extracted from tissue according to Saghai-Maroof et al. (1984). DNA was digested with *Hin*dIII and *Eco*RI to release the smGFP expression unit (CaMV 35S-*smgfp–NOS*), or with *Sac*I which cleaves the plasmid once between the *smgfp* coding sequence and the *NOS* 3′ end (Davis and Vierstra 1998). Following restriction digestion, 10 μg of DNA was electrophoresed through a 0.8% agarose gel and blotted onto a nylon membrane (GeneScreen Plus, DuPont NEN, Boston, Mass.) according to Kempter et al. (1991). A *Bam*H1-*Sac*I fragment consisting of the coding region of *smgfp* was labeled according to Feinberg and Vogelstein (1983). Hybridization of the probe to the membrane, washing of the membrane, and autoradiography were conducted as described previously (Hadi et al. 1996).

Results and discussion

Transient expression of GFP

All of the pUC-based GFP constructions tested produced visible GFP expression in embryogenic soybean cultures (Table 1). Use of a 100-W mercury lamp as the light source facilitated clear visualization of GFP expression in comparison with a 50-W bulb, which produced a much weaker fluorescent signal (data not shown). No GFP activity was observed with tungsten particles lacking DNA or with pUCGUS (data not shown), indicating that the green-fluorescent areas were specific to GFP. GFP expression remained high at the early stages following particle bombardment, but declined to low levels 72 h after bombardment (Table 1). The most intense green fluorescence (Fig. 1) in soybean tissue and the highest number of GFP-expressing areas (Table 1) were observed following bombardment with *smRS-gfp*. smRS-GFP is identical to smGFP except for a single amino acid chromophore mutation (S65T). In *Escherichia coli*, the S65T chromophore mutation of GFP resulted in increased brightness, more rapid chromophore formation and a reduction in photobleaching (Heim et al. 1995). Given equal particle delivery, a threshold detection level apparently exists for GFP with the optical system used in the present study. A more sensitive detection system might show similar spot counts while revealing differences in intensity between GFPs that contain either the native or the red-shifted chromophore, but are otherwise identical. The S65T mutation apparently increases the sensitivity of this protein to detection in both *E.coli* and soybean cells. However, *Arabidopsis* cells expressing either smGFP or smRS-GFP produced similar fluorescence yields when observed with a FITC filter set (Davis and Vierstra 1998).

Table 1 Transient GFP expression following particle bomdardment of embryogenic soybean suspension culture tissue with different pUC-based plasmids. Following bombardment (about ten clumps of tissue/bombardment), tissue was incubated on D20 semisolid medium and the number of GFP-expressing spots/bombardment was determined at various times. Values represent the mean (±SE) of four bombardments

Plasmid	Number of GFP-expressing spots/bombardment		
	6.5 h	24 h	72 h
smgfp	370± 99	251± 59	119±18
smRS-gfp	587±188	402±148	203±86
HBT-SGFP-TYP-nos	187± 60	116± 35	36±13
35S-SGFP-TYG-nos	453±119	302± 73	187±31

Because of its high level of transient expression and ease of detection, the smRS-GFP was further used to characterize the timing of gene expression in soybean. Transient smRS-GFP expression in soybean was detectable as early as 1.5 h postbombardment, and reached a peak of more than 40 spots per tissue clump after approximately 6.5 h (Fig. 2). One week after bombardment, the number of visible areas expressing GFP had declined considerably to less than four spots per clump of tissue. Although such a detailed time course was not conducted with all of the available plasmids, the trend was similar insofar as the number of transient events was high after 6.5–24 h, but declined 3 days after bombardment (Table 1). Peak transient expression of CAT in electroporated protoplasts varies from 4–24 h (Pröls et al. 1988) to 24–48 h (Fromm et al. 1985), whereas GUS activity in bombarded plant tissue is most often evaluated 48 h postbombardment (Klein

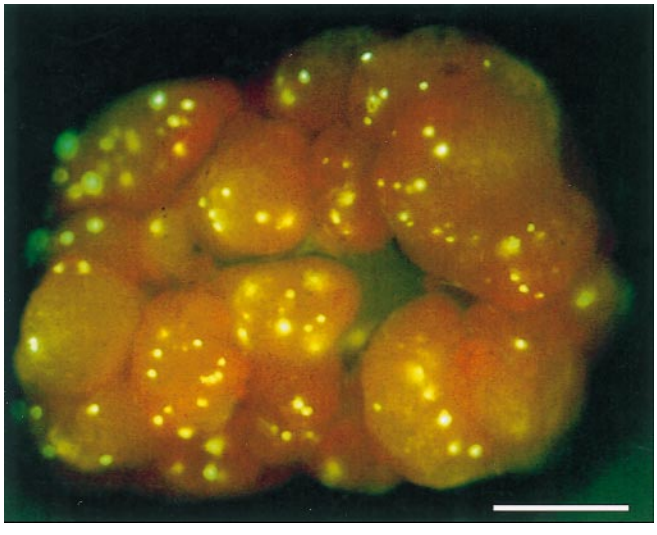

Fig. 1 Expression of smRS-GFP in soybean embryogenic suspension cultures 6.5 h after particle bombardment (*bar* 0.5 mm)

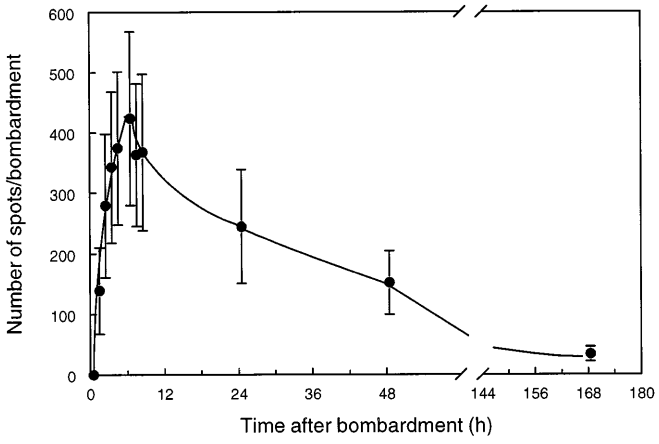

Fig. 2 Time course of smRS-GFP expression in soybean cultures. Embryogenic tissue was bombarded and maintained on D20 medium. Each point represents the mean ± SE for $n = 3$ bombardments with ten clumps of tissue/bombardment

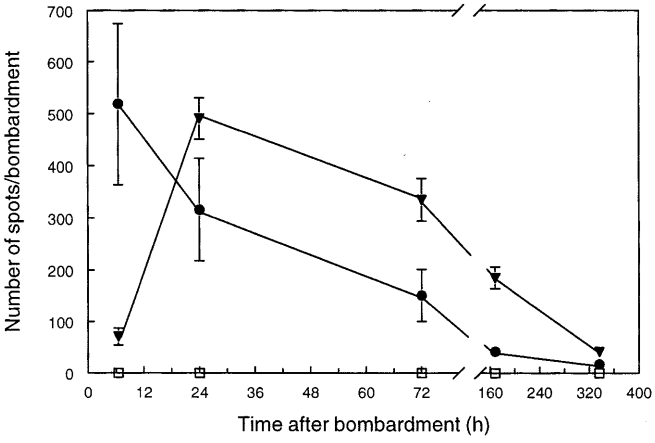

Fig. 3 Expression of *smRS-gfp* and *mgfp5-ER* over time. Embryogenic soybean tissue was bombarded with the pUC-based DNA constructions pUCGUS (□), *smRS-gfp* (●), or *mgfp5-ER* (▼) and maintained on D20 medium. Each point represents the mean ± SE for $n = 3$ bombardments with ten clumps of tissue/bombardment

et al. 1988a,b). We recommend evaluation of transient GFP expression from 6.5 to 24 h after bombardment since the 48 h time point used for determination of transient CAT and GUS activity is not optimal for GFP.

The recent availability of an ER-targeted GFP prompted us to test this construction in soybean. GFP activity was observed in soybean tissue following particle bombardment or *Agrobacterium*-mediated transformation with the binary vectors pBIN 35S-*mgfp4* and pBIN 35S-*mgfp5-ER* (data not shown). However, since GFP expression was low following particle bombardment of these binary constructions, *mgfp5-ER* was cloned into a smaller, pUC-based vector that was more appropriate for particle bombardment. The magnitude and timing of expression of the pUC-*mgfp5-ER* construction was compared with that of *smRS-gfp* in a subsequent study (Fig. 3). Although the time of peak expression was different for the two plasmids, the number of transient events was similar at their respective times of peak expression, with smRS-GFP exhibiting spots that appeared to be of greater intensity than the ER-targeted protein. Peak expression of mGFP5-ER was observed later than that of smRS-GFP. In addition, the ER-targeted GFP exhibited a delay in the loss of expression compared to smRS-GFP. The delay in expression of *mgfp5-ER* may reflect a requirement for secretion and retention of the protein in the lumen of the ER for proper folding and maturation.

If GFP is to be used as a marker to select transformed tissue, it may be necessary to expose tissue to a light source for extended periods of time. Tissues subjected to prolonged blue light exposure exhibited similar numbers of transient events as the control, which had only been exposed to the blue light source for the brief period necessary to monitor expression (Fig. 4). These results were encouraging and suggest that it may be possible to expose GFP-expressing soybean tissue to high-intensity light for fairly long periods without significant loss of activity of this scorable marker.

Stable transformation

To generate stably transformed tissue, soybean cultures were bombarded with the *smgfp* plasmid construction along with a plasmid containing a chimeric hygromycin phosphotransferase gene. Five hygromycin-resistant clones were initially tested for the presence of the *smgfp* by PCR. All of the clones tested produced the expected (~0.4-kb) band, whereas DNA from untransformed soybean cultures did not yield such a product (data not shown). Hybridization of digested soybean genomic DNA with the coding region for *smgfp* verified the presence and integration of the gene (Fig. 5). The complex hybridization patterns observed with DNA from transgenic clones is typical for stably transformed soybean tissues generated via particle bombardment (Hadi et al. 1996).

Under blue light excitation, these stably transformed cultures exhibited distinctive green fluorescence indicating expression of GFP. Since high levels of GFP expression could potentially have toxic effects on plant tissue (Haseloff et al. 1997), the growth of two stably transformed clones expressing GFP was compared with that of untransformed tissue. The GFP-expressing clones showed no reduction in growth when cultured with or without hygromycin over a 3-week period (Table 2). Apparent lack of toxicity due to GFP in stably transformed soybean tissues confirms similar effects with transiently transformed soybean cells observed earlier in this study. Although expression of

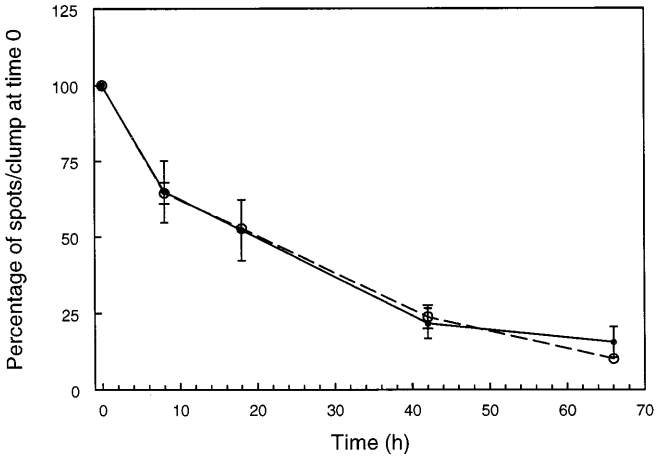

Fig. 4 Effect of prolonged exposure to blue light on GFP expression in bombarded soybean cultures. Soybean embryogenic cultures were bombarded with the *smRS-gfp* construction and incubated for 5.5 h on D20 medium. Tissue was then exposed to blue light for 1-h periods starting at time 0 (when high levels of GFP expression were visible) and repeated 9, 18, and 42 h later. The number of GFP-expressing foci was determined prior to each one hour period of exposure, and finally at 66 h after the first exposure to blue light. Control (●) tissues, which were bombarded but not subjected to prolonged blue light exposure, were monitored for GFP expression at the same times as 'exposed' (○) tissues. Data are presented as the percentage of the number of GFP-expressing areas at time 0. Each point represents the mean ± SE for $n = 5$ clumps of tissue

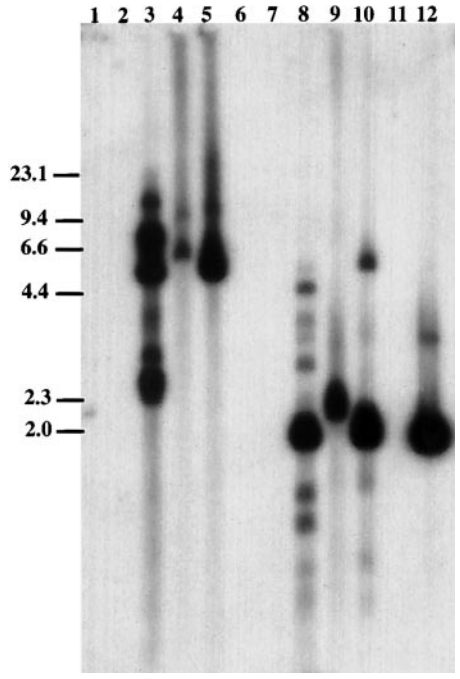

Fig. 5 Southern analysis of embryogenic suspension cultures transformed with *smgfp*. *Lanes 1* and *11* were each loaded with 1 μg of λ/*Hin*dIII DNA and *lane 6* was not loaded with DNA. DNA (10 μg) was digested with *SacI* and loaded in *lane 2* (untransformed culture), *3* (clone 2.1), *4* (clone a.1), and *5* (clone a.2). *Sac*I cleaves the plasmid once downstream of the *smgfp* coding sequence. DNA (10 μg) digested with *Hin*dIII and *Eco*RI was loaded in *lane 7* (untransformed culture), *8* (clone 2.1), *9* (clone a.1), and *10* (clone a.2). As a positive control, 100 pg of *smgfp* plasmid DNA, cleaved with *Hin*dIII and *Eco*RI, was electrophoresed in lane 12. The *Hin*dIII-*Eco*RI double digest releases the intact *smgfp* expression unit. DNA was transferred to a nylon membrane following electrophoresis through a 0.8% agarose gel and hybridized with a *Bam*H1-*Sac*I fragment consisting of the *smgfp* coding region.

GFP did not adversely affect growth of *in vitro* tissues, the effects of GFP expression on physiology and growth of plant tissues under field conditions need to be evaluated.

Following transfer of embryogenic tissue to M6 medium, smGFP expression could be visualized in developing embryos (data not shown). Stably transformed *smgfp* clones were also regenerated into plants. Not all of the pieces of tissue from a given stably transformed clone or from developing embryos exhibited GFP expression. Unfortunately, GFP expression could not be visualized clearly in tissues from plants transformed with *smgfp*. The inability to visualize *smgfp* expression in regenerated plants may be due to developmental and cell-type-specific expression of the CaMV 35 S promoter in soybean plants (Yang and Christou 1990), or because of background fluorescence that could interfere with visualization of expression with the GFP Plus detection system. Lack of clear GFP expression in plants does not appear to be unique to soybean since GFP expression was not easily visualized in developed leaves of rice (Vain et al. 1998). Similarly, background fluorescence in maize leaf and root sections precluded effective visualization of GFP in these tissues (van der Geest and Petolino 1998).

Table 2 Growth of untransformed soybean embryogenic suspension cultures, and two clones stably transformed with *smgfp*. Tissue (0.1 g/flask) was inoculated into 33 ml of medium and the fresh weight was measured 7, 14, and 21 days later. Clone a.1 was grown in FN medium either without hygromycin or with hygromycin, and the untransformed culture and clone 1.4 were grown in the absence of the antibiotic. The mean ± SE for $n \geq 3$ replicate samples was determined at each time point

	Hygromycin B (mg/l)	Fresh weight of tissue (mean ± SE) (g)		
		7 days	14 days	21 days
Untransformed	0	0.23 ± 0.02	0.42 ± 0.05	0.76 ± 0.12
Clone a.1	0	0.23 ± 0.02	0.44 ± 0.05	0.77 ± 0.09
Clone a.1	7.5	0.24 ± 0.02	0.47 ± 0.05	0.82 ± 0.06
Clone 1.4	0	0.25 ± 0.01	0.51 ± 0.03	0.94 ± 0.06

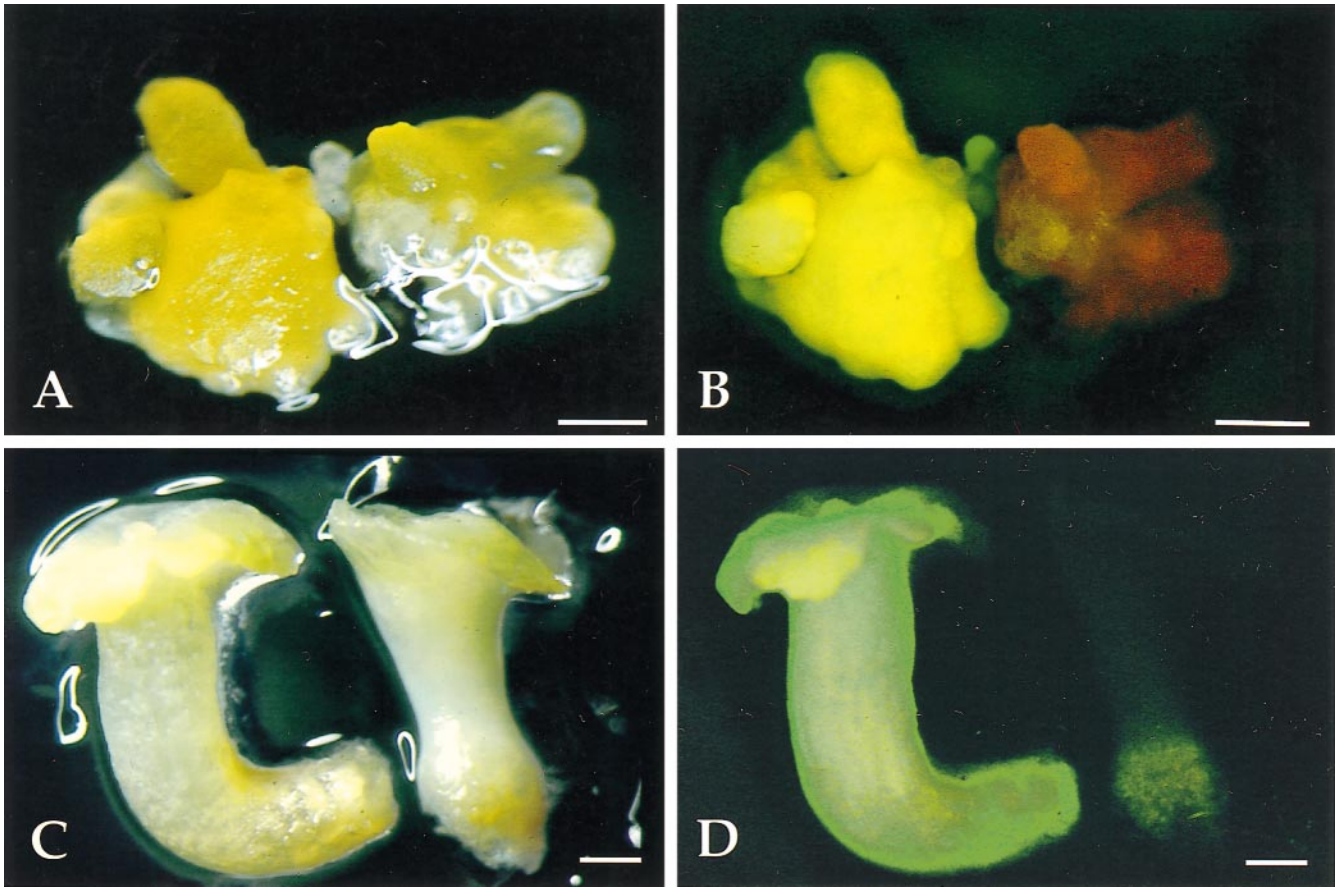

Fig. 6 GFP expression in stably transformed soybean cultures. Embryogenic suspension cultures (**A**, **B**) and developing embryos (**C**, **D**) were photographed under white (**A**, **C**) or blue light (**B**, **D**). In each panel, transformed tissues (expressing smRS-GFP) and untransformed tissues are on the left and right side, respectively (*bars* 0.5 mm)

Embryogenic cultures bombarded with pHOG were also isolated following selection on hygromycin. These clones produced more intense green fluorescence upon blue light excitation than tissues expressing smGFP, and smRS-GFP expression was clearly visible in embryogenic cultures and developing embryos (Fig. 6). Although roots of some regenerated plants exhibited distinct smRS-GFP expression, expression in the aerial portions of the plants was less pronounced. Increased fluorescence output due to the S65T mutation in smRS-GFP from *E. coli* has been reported previously (Davis and Vierstra 1998). Similarly, in maize, the use of an engineered *gfp* gene with the S65T mutation resulted in a fluorescence signal that was more than 100-fold higher than the wild-type gene (Chiu et al 1996). Experiments are underway to transform and regenerate plants from younger cultures as the older, transformation-competent cultures used in this study are only capable of producing sterile plants (Hadi et al. 1996).

GFP promises to be an exciting new tool for the optimization of transformation procedures in soybean. The ability to detect this protein in living cells non-destructively may permit the development of new transformation methodologies by early evaluation of transgene expression in putative clones obtained under various conditions. In addition, GFP could be valuable for molecular studies including promoter analysis and protein localization.

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