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Use of green fluorescent protein for detection of transformed shoots and homozygous offspring

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Abstract Using tobacco as a model system, the data obtained demonstrated that the green fluorescent protein (GFP) can be used as a visual selection marker for transformed tissues. Based on differences in the intensity of GFP fluorescence, homozygous and hemizygous states could be easily visualized in seeds and seedlings of the T1 generation. These results were confirmed by genetic analysis. Optimized conditions for GFP analysis of stable transformants are presented.

Key words Green fluorescent protein · Marker gene · *Nicotiana tabacum* · Plant transformation · Reporter gene

Introduction

Marker genes allowing the visual detection of transgene expression, such as *uidA* and *luc*, are commonly used to analyze transgenic plants, most often in combination with selectable markers which are required for the transformation process. β-glucuronidase (GUS), the *uidA* gene product (Jefferson et al. 1987), is one of the most extensively used visual markers in plant cell biology. However, the histochemical GUS assay is destructive for the tissue and therefore not suitable for direct visual selection of transformed plants. Luciferase, encoded by the *luc* gene, can be monitored in vivo (Ow et al. 1986) but requires an exogenous substrate (luciferin) and emits light only at very low intensity (Ow et al. 1986). Its use as a visual selection marker depends on the availability of sophisticated low-light-level video equipment.

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* requires no exogenous substrate for its detection. When excited by blue or UV light, GFP emits visible light of high intensity without requiring additional cofactors other than oxygen (Prasher 1995). GFP can easily be detected with standard laboratory equipment such as fluorescence microscopes (Davis and Viestra 1998; Hasseloff et al. 1997), dissecting microscopes equipped to detect fluorescence, or even under normal-room fluorescent lighting (Morise et al. 1974).

GFP has been extensively used in plants to study the expression patterns of promoters (Nagatani et al. 1997; Sheen et al. 1995) and to follow short- and long-distance movements of proteins and viruses (Itaya et al. 1997). Reports on GFP-expressing transgenic plants are still rare (Hasseloff et al. 1997; Rouwendal et al. 1997) and despite the extensive literature on GFP expression in plants, its use as a marker for the selection of transgenic shoots under direct visual control has not been described. Indeed, GFP expression has in some cases been reported to be incompatible with efficient plant regeneration (Hasseloff et al. 1997).

In a study designed to optimize conditions for the use of GFP as a visual selection marker, we have followed its expression from early transformation events of tobacco leaf discs through to the T2 generation, with particular emphasis on detection of fluorescence patterns in intact plantlets, a requirement for fast and efficient selection. No specific negative effects of GFP expression were revealed by this study. On the contrary, this marker allowed successful screening for primary transformants and visual analysis of T1 and T2 progeny at the seed, seedling, and whole-plant level. Indeed, for all the events analyzed here, the intensity of fluorescence could be used to discriminate the homozygous and hemizygous states.

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Materials and methods

Plant transformation

Agrobacterium tumefaciens strain LBA 4404 was transformed by electroporation with plasmid pHB2892 (Fig. 1A). This plasmid contains the S-GFP gene, optimized for human codon usage (Chalfie et al. 1994), under the control of the double 35S cauliflower mosaic virus promoter. Tobacco leaf discs (cv. Samsun NN) were transformed and regenerated as described by Horsch et al. (1985). All transgenic plants described here originated from independant calli. Regenerated calli and shoots, selected on kanamycin, were screened for GFP expression at the macroscopic level using an epifluorescence dissecting microscope (Leica MZ12 460–500 nm/510 nm). Pieces of transgenic leaves were also examined using an upright fluorescence microscope (Nikon E800) with long-pass (460–500 nm/505 nm/510 nm) or band-pass (460–500 nm/505 nm/510–560 nm) filters. Plants transformed with a plasmid devoid of the GFP gene (p35SGUSINT; Vancanneyt et al. 1990) and produced in the same experiment were used as negative controls. Photographs were taken on Fujichrome Sensia II 400 film with automatic exposure control.

Plant DNA extraction and analysis

Two grams of leaves from individual transgenic plants were used for DNA extraction according to the protocol of Dellaporta et al. (1983). Genomic DNA (25 μg) was digested with *Eco*RI and subjected to Southern analysis according to Sambrook et al. (1989). The blots were hybridized with the 0.5 kb *Nco*I fragment of the *npt*II gene isolated from the vector pRT99 (Töpfer et al. 1988). The probe was labelled with ^{32}P-dCTP (Amersham) using the rediprime DNA labelling system (Amersham).

Progeny screening

T1 and T2 seeds were generated by selfing. T1 seeds were allowed to germinate on medium containing micro- and macroelements, vitamins (Murashige and Skoog 1962), 1% sucrose, and 0.8% Difco-agar. In preliminary experiments, kanamycin (300 mg/l) was added to the germination medium to optimize the screening. In a second step, seeds and plantlets were only screened for GFP fluorescence using the epifluorescence dissecting microscope. Seedlings were divided into three classes (high, low, and no fluorescence) according to their level of GFP fluorescence. T1 plants of each class were transferred into the greenhouse and their segregating progeny (T2) were analyzed for GFP expression.

Results and discussion

Screening of GFP-positive calli and shoots

The evolution of GFP fluorescence was followed during all stages of the transformation and regeneration process in order to evaluate the suitability of GFP for a visual selection protocol. Although GFP is naturally absent from wild-type tobacco plants, autofluorescence with similar spectral characteristics does occur in wounded tissues. For this reason, unambiguous distinction was difficult between leaf discs treated with agrobacteria carrying pHB2892, a plasmid encoding GFP, and those treated with a control construct, immediately after co-culture. However, 2 weeks after transformation, the characteristic green fluorescence was easily

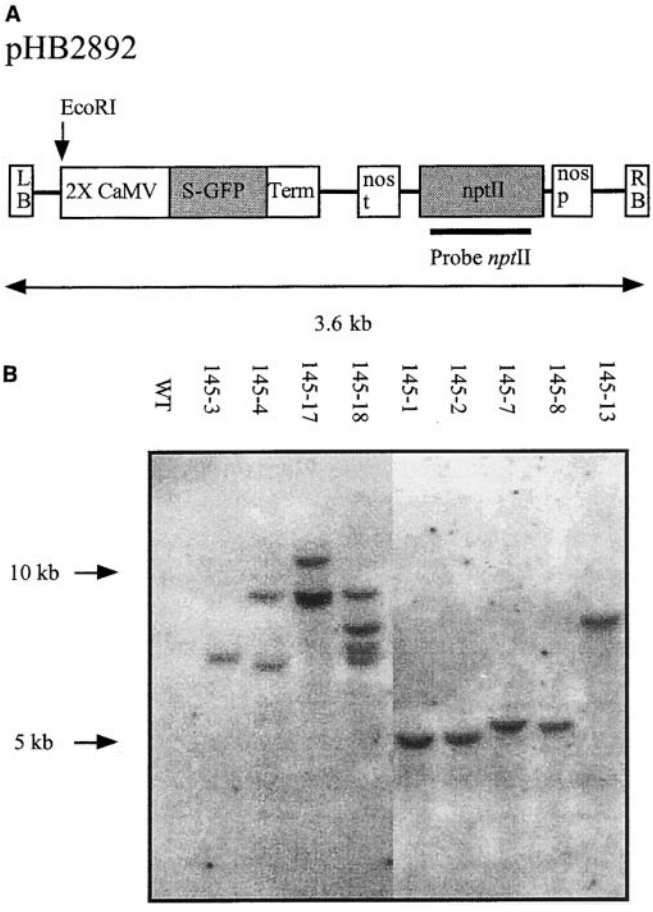

Fig. 1A, B Southern analysis of primary transformants. **A** Structure of the introduced T-DNA from plasmid pHB2892. Expression of the *npt*II gene was controlled by the *nos* promoter (*nos p*) and terminator (*nos t*). The S-GFP gene was cloned between the double 35S CaMV enhancer and the 35S CaMV terminator (*Term*). The 0.5-kb *Nco*I fragment of the *npt*II gene was used as probe. **B** Southern analysis of nine primary tobacco transformants. The wild type (*WT*) tobacco, Samsun NN, was used as negative control. Blots were hybridized with the *npt*II probe

detectable in the calli developing on transformed leaf discs (Fig. 2A). Although weak yellowish or greenish fluorescence occurred in control calli, the identification of GFP-expressing calli was unambiguous. In contrast, the red chlorophyll autofluorescence limited the detection level for GFP fluorescence in shoots regenerating from GFP-positive calli when equipment with long-pass cut-off filters was used. The use of appropriate band-pass filters alleviates this problem (Fig. 2B, C). Under all conditions, GFP-positive tissue was more readily identified in calli and the young leaf primordia of shoot tips than in older, more developed parts of regenerating shoots (Fig. 2D). The higher cytoplasmic density in young tissues may explain why GFP was detected more easily there than in older leaves where the vacuole, devoid of GFP, constitutes the largest part of the cell.

In contrast to the results of Hasseloff et al. (1997) who observed a reduced regeneration frequency from

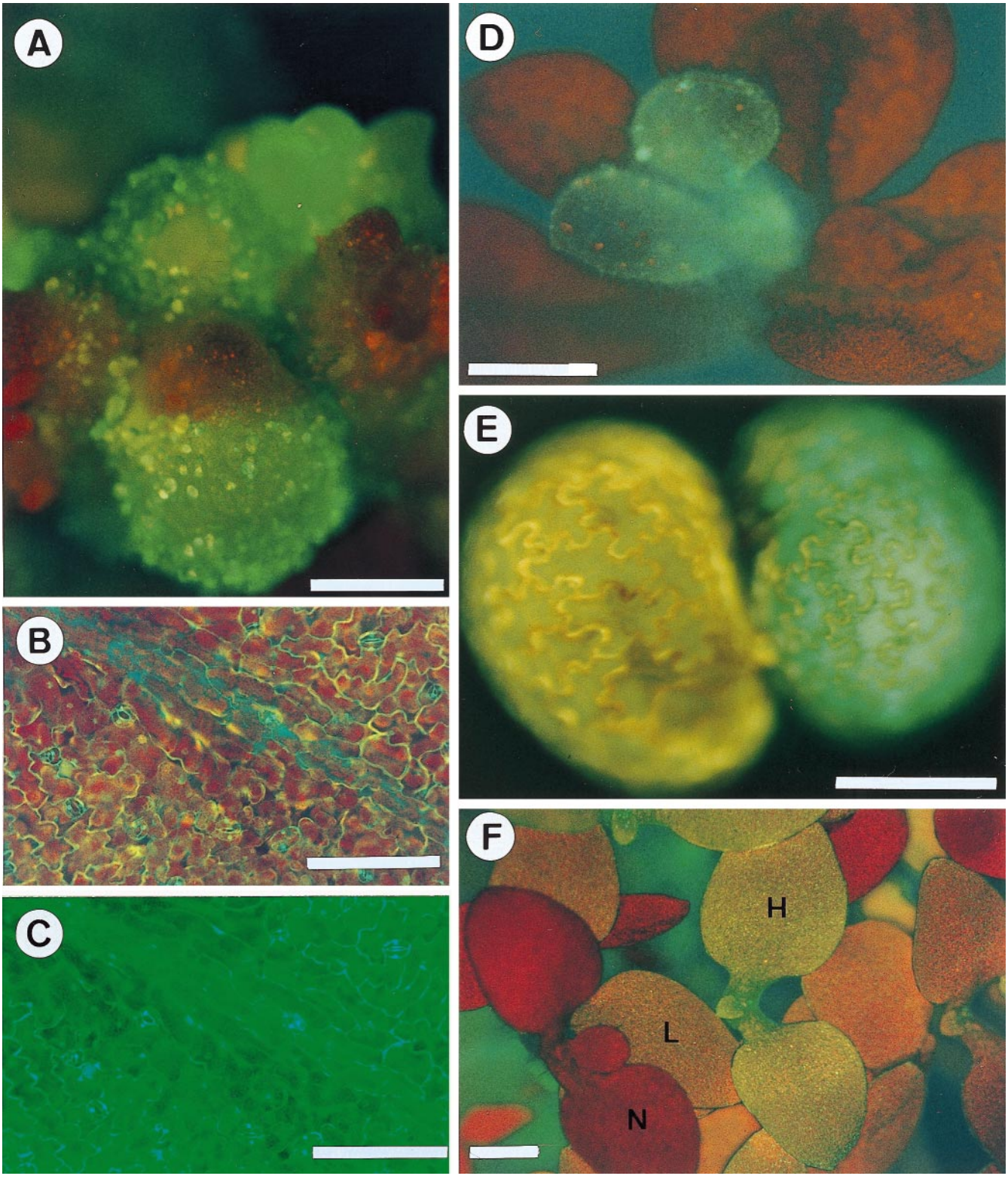

Fig. 2 **A** Calli developing on a tobacco leaf disc treated with agrobacteria containing plasmid pHB2892 observed under the epifluorescence dissecting microscope (bar 5 mm). **B** GFP-expressing leaf (2 weeks old) observed under the upright microscope using the long-pass filter combination (bar 500 μm). **C** GFP-expressing leaf (2 weeks old) observed under the upright microscope using the band-pass filter combination (bar 500 μm). **D** Six-week-old regenerant from a tobacco leaf disc treated with agrobacteria containing plasmid pHB2892 observed under the epifluorescence dissecting microscope (bar 1 cm). **E** Wild-type (*left*) and GFP-expressing (*right*) tobacco seed observed under the epifluorescence dissecting microscope (bar 1 mm). **F** Eight-day-old tobacco seedlings (T1 of plant 145–13). Note the three classes with high (*H*), low (*L*) and no (*N*) fluorescence (bar 1 mm)

the brightest GFP-expressing calli in *Arabidopsis* transformation experiments, we observed no difference in the regeneration process, in quantity, quality or duration, between tobacco leaf discs treated with agrobacteria containing plasmid pHB2892 coding for GFP and those treated with agrobacteria containing a control construct devoid of GFP (data not shown).

Molecular characterization of the transgenic GFP tobacco lines

Shoots with green GFP fluorescence visible in the shoot tips at a very early stage of their development were transferred to the greenhouse and nine putative transgenic T0 plants were randomly selected from 20 putative transgenic T0 plants. Their transgenic nature was confirmed by Southern analysis (Fig. 1B). Six plants had integrated the T-DNA in one locus (plants 145–1, 145–2, 145–3, 145–7, 145–8, 145–13), two plants in two loci (plants 145–4 and 145–17) and one plant in four loci (plant 145–18). Plants 145–1, 145–3, 145–4, 145–7, 145–13, 145–17 and 145–18 were confirmed as independant transformants. No false positives were found using this procedure. We conclude that a GFP-based selection is possible and yields reliable results. The screen is best performed on very young stages of the regenerated shoots.

Fluorescence in the T1 progeny

The progeny (T1) of five independant transformants, 145–1, 145–3, 145–7, 145–13 (one locus) and 145–4 (two loci), were analyzed for the presence of GFP fluorescence at different stages of their development, from the seed to the whole plant, and compared to wild-type plants.

Seeds of control plants (wild type for GFP) showed a strong yellow seed coat fluorescence. However, this autofluorescence was clearly different from that characteristic for GFP (Fig. 2E). After germination, only the red chlorophyll auto-fluorescence was detectable in aerial tissues. No green fluorescence was found in healthy and unwounded tissues of wild-type seedlings. In contrast, a green fluorescence visually indistinguishable from GFP fluorescence was observed in and below the crown region and throughout the root system of wild-type tobacco seedlings. This is in contrast to the roots of *Arabidopsis* where no such artifactual fluorescence was detected (Hasseloff et al. 1997). The natural presence of green-fluorescing compounds makes the detection of GFP in tobacco roots delicate, requiring carefully controlled observations.

No artifacts obstructed the detection of GFP in the aerial parts of plants 145–1, 145–3, 145–7, and 145–13, where GFP fluorescence was easily and unambiguously detected in the young leaves. As development progressed, chlorophyll autofluorescence increasingly masked the GFP fluorescence which thus became more difficult to detect in tissues comprising more differentiated cells, exactly as previously observed with the primary transformants (see above).

T1 seeds harvested from the different transgenic plants could be clearly distinguished from wild-type seeds on the basis of the green fluorescence of their embryos, visible through the seed coat (Fig. 2E). Three different classes of fluorescence were observed in each population of 8-day-old T1 seedlings derived from plants 145–1, 145–3, 145–7, and 145–13, all possessing the T-DNA in a single locus: high, low, or none (Fig. 2F). This observation is similar to that of Leffel et al. (1997) who observed classes of different fluorescence in their plants and could correlate the fluorescence level with the amount of GFP. In our experiments, high-level fluorescence was observed in 25%, low-level fluorescence in 50% of the progeny, while 25% of the seedlings showed no fluorescence (Table 1). High and low levels of GFP fluorescence were always correlated with kanamycin resistance and presence of the T-DNA, while the absence of GFP fluorescence was always correlated with kanamycin sensitivity and absence of the T-DNA (data not shown). This 1:2:1 segregation is consistent with the assumption that the progeny segregated for a single dominant locus, i.e., a single-copy transgene. Progeny of plant 145–4 which harbors the T-DNA in two loci fell into at least four different classes of fluorescence intensity. Although easily detectable in the dissecting microscope, these different levels of GFP fluorescence were difficult to group into individual classes without specialized equipment allowing quantification of the fluorescence levels. Nevertheless, the ratio of fluorescing to non-fluorescing plants (15:1) was in agreement with the hypothesis of two independent dominant loci (Table 1).

Genetic analysis of the progeny of T1 plants

Four visually selected T1 plants of each of the three previously identified fluorescence classes were individually followed and their progeny (T2) screened for

Table 1 Classes of green fluorescence intensities of the Tl progeny derived from 145–1, 145–3, 145–7, 145–13, and 145–4 plants. Chi-square values were calculated for a 1:2:1 segregation for plants 145–1, 145–3, 145–7, 145–l3, and for a 15:1 segregation for plant 145–4. For all tests, $P>0{,}05$

Plant	Number of explants in green fluorescence class			χ^2
	High	Low	None	
145–1	26	45	29	1.18
145–3	25	52	23	0.24
145–7	25	47	28	0.54
145–13	24	51	25	0.32
145-4	187		13	0.02

Table 2 Correlation between the fluorescence intensity classes of the Tl plants and the segregation of their progeny (T2). Percentages were calculated based on germinating seeds (±SE). For each of the four selected T1 plants, four T2 plants of each fluorescence class were individually followed and their progeny analyzed. About 400 seedlings were observed for each Tl plant

Fluorescence class of the T1 plants	T1 plant derived from	T2 progeny	
		GFP+ (%)	GFP− (%)
High	145–1	100	0
	145–3	100	0
	145–7	100	0
	145–13	100	0
Low	145–1	74.4±1.8	25.6±1.1
	145–3	73.8±1.1	26.2±0.8
	145–7	75.6±1.8	23.4±1.3
	145–13	74.2±2.3	25.8±2.0
None	145–1	0	100
	145–3	0	100
	145–7	0	100
	145–13	0	100

GFP fluorescence. The progeny of T1 plants belonging to the high-fluorescence class were uniform and 100% of the analyzed seedlings showed a high level of fluorescence (Table 2). Such T1 and T2 plants thus appear to be homozygous for the T-DNA. Similarly, the progeny of the T1 plants belonging to the non-fluorescing class were uniformly lacking the fluorescence characteristic of GFP (Table 2). The non-fluorescing class therefore corresponds to wild-type segregants. T1 plants from the low-fluorescence class produced offspring of heterogenous phenotypes which fell into the three classes of high, low, and no fluorescence (Table 2). This result confirms the hypothesis that low-level fluorescence is indicative of hemizygous plants.

Thus, simple observation of GFP fluorescence in an epifluorescence dissecting microscope not only permitted the identification of transformed tobacco plants but also enabled separation of homozygous from hemizygous plants. Even though a confirmation by genetic analysis remains desirable, the enrichment for either class of plants is considerable, reaching 100% in our experiments. Compared to a classification based on the genetic analysis of a large number of plants, the gain in greenhouse space and time is not negligible.

In conclusion, we have shown that GFP can indeed be used for the visual selection of transformed tissues after *Agrobacterium*-mediated gene transfer. Artifacts caused by endogenous fluorescence could be recognized in appropriate control experiments and have been no serious obstacle. For progeny of several independent events analyzed here, the quantitative nature of GFP fluorescence allowed discrimination of homozygous and hemizygous seeds and seedling using a simple visual screen.

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