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The green fluorescent protein (GFP) as a vital screenable marker in rice transformation

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Abstract An engineered green fluorescent protein (GFP) from the jellyfish *Aequora victoria* was used to develop a facile and rapid rice transformation system using particle bombardment of immature embryos. The *mgfp4* gene under the control of the 35*s* Cauliflower Mosaic Virus promoter produced bright-green fluorescence easily detectable and screenable in rice tissue 12–22 days after bombardment. Visual screening of transformed rice tissue, associated with a low level of antibiotic selection, drastically reduced the quantity of tissue to be handled and the time required for the recovery of transformed plants. GFP expression was observed in primary transformed rice plants (T_0) and their progeny (T_1). We describe various techniques to observe GFP in vitro and in vivo. The advantages of this new screenable marker in rice genetic engineering programmes are discussed.

Key words Green fluorescent protein · Visual screening · Transformation · *Oryza sativa*

Introduction

Progress in transformation technologies for monocotyledonous species has been achieved by improvements in transgene design (Callis et al. 1987), DNA delivery methods (Sanford et al. 1987; Hiei et al. 1994), and by the utilization of screenable (Jefferson et al. 1987; Wright et al. 1996) and selectable (Gordon Kamm et al. 1990, Fromm et al. 1990) markers. Vital marker genes will undoubtedly contribute further to the development of transformation technologies by serving as tools for visual monitoring of transgene expression in transformed tissues and plants.

The green fluorescent protein (GFP) from the jellyfish *Aequora victoria* has several significant advantages over alternative visual marker genes. Its expression can be detected in real time, in living cells and organisms simply by light excitation (see Cubitt et al. 1995 for a review). GFP does not require a substrate and is not toxic compared to firefly luciferase (LUC, Ow et al. 1986) and the β-glucuronidase assay (GUS, Jefferson et al. 1987) respectively. The small size of GFP (26.9 kDa) facilitates the construction of protein fusions (Wang and Hazelrigg 1994) and is advantagous for the development of virus-based vectors (Baulcombe et al. 1995). GFP allows monitoring of gene expression and protein locatization at the sub-cellular, cellular and plant level. New GFP genes with brighter fluorescence (Heim et al. 1995; Siemering et al. 1996), improved thermostability (Siemering et al. 1996), and altered excitation (Delagrave et al. 1995; Ehrig et al. 1995; Siemering et al., 1996) and emission (Heim et al. 1994) spectra have been produced by mutagenesis. These lead to the utilisation of several vital marker genes in combination (Rizzuto et al. 1996) and has promoted GFP-based applications in heterologous systems.

Early reports described wild-type GFP as a vital marker gene in bacteria, *Caenorhabditis elegans* and *Drosophila* (Chalfie et al. 1994; Inouye and Tsuji 1994; Wang and Hazelrigg 1994). GFP was subsequently engineered (Haseloff and Amos 1995; Pang et al. 1996) to maximize its transient (Hu and Cheng, 1995; Niedz et al. 1995; Sheen et al. 1995; Plautz et al., 1996; Reichel et al. 1996, Tian et al. 1997) and stable expression (Haseloff and Amos, 1995; Chiu et al. 1996; Pang et al. 1996) in various plant tissues and species. GFP has also been employed as a vital marker gene in transformed wheat and maize plants (Pang et al. 1996) but, to-date, no monocotyledonous transformation system based on

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GFP has been described for the facile identification of transformation events.

Identification and selection of transformation events generally relies on the differential growth of transformed versus non-transformed tissues or plants under drug selection. Depending on the transformation system used, selection may take several months and requires the utilisation of high levels of selective agent to eliminate escapes. This is particularly the case for monocotyledonous species. In the case of rice, the definitive identification of transformed clones or plants generally occurs 6–12 weeks after particle bombardment, and high levels of antibiotics or herbicides are utilized during the entire process (Christou et al. 1991; Cao et al. 1992). Because different rice varieties exhibit different sensitivities towards selective agents it is cumbersome to define optimum uniform selection conditions in developing genotype-independent transformation procedures. A further refinement of transformation protocols based on visual screening would certainly result in reducing labour and cost input, particularly in applied production systems involving transgenic plants. In this paper we describe the use of GFP for early selection (12–22 days following bombardment) and easy handling and monitoring of transformed rice clones and plants over several generations.

Material and methods

Rice transformation

Ten-to-fifteen-day-old immature seeds from indica cultivar TN1 were harvested from greenhouse-grown rice plants. After seed sterilization, the embryos were cultured on a CC-based medium and bombarded as previously reported (Christou et al. 1991; Vain et al. 1998). The plasmids pBIN35S-mGFP4 (NOSpro::*npt*::NOSpolyA + CaMV35Spro::*mgfp4*::NOSpolyA) obtained from Dr. Jim Haseloff (Haseloff and Amos 1995) and pJIC2001 (CaMV35Spro::*aphIV*::SpolyA + CaMV35Spro::*gusA*::NOSpolyA) were used for the stable transformation of rice in co-transformation experiments.

Two days after particle bombardment, the immature embryos were subcultured on a CC-based medium supplemented with 0, 25 or 50 mg/l of hygromycin. Twelve days after bombardment, embryogenic calli initiated from the scutellum were dissected into small segments and subsequently transferred at 10-day intervals onto fresh medium containing similar levels of hygromycin. Visual selection of transformed green-fluorescent tissue was performed as described below. Plants were regenerated by subculturing embryogenic fluorescent calli on CC medium without 2,4-D, supplemented with 40 mg/l of hygromycin. Plantlets were germinated in tubes on Murashige and Skoog (1962) medium supplemented with 40 mg/l of hygromycin, then transferred to the greenhouse as described previously (Vain et al. 1998).

Visual selection of GFP-fluorescent tissue in vitro

Observation of rice tissues cultured in vitro was carried out using a dissecting microscope placed in a laminar flow hood to ensure aseptic conditions. Visual selection of GFP-fluorescent tissue was performed as early as 12 days after bombardment and was repeated every time the tissue was transferred onto fresh medium. Visual selection was performed by dissecting and subculturing only green-fluorescent sectors onto fresh medium. Two systems were used for GFP monitoring in vitro. The first system consisted of a hand-held long-wave ultraviolet lamp (UVP B-100AP) placed in a laminar flow hood, facing the rice tissue and the air flow, thus leaving both hands free for dissection. A large screen of transparent UV-absorbent Perspex VE (ICI, UK) was placed over the dissecting microscope as a protective shield against ultraviolet radiation. The screen was made of a $80 \times 65 \times 0.3$ cm piece of Perspex VE with two openings in the centre to allow access to the lenses of the dissecting microscope. A $15 \times 12 \times 0.3$-cm metal plate coated with soft rubber on both sides and with two openings in the centre was placed between the Perspex screen and the binocular microscope to prevent friction. The screen was cleaned with 70% ethanol before use. This system can be used with any dissecting microscope and requires only a moderate investment (US$ 650). The second system consisted of a MZ12 Leica dissecting microscope with a fluorescent module (Leica #10 446093). The appropriate wavelength was adjusted using a filter block containing a 425/60 nm excitation filter, a 470 nm dichromatic beam splitter and a G6457 emission barrier filter, over a high-voltage mercury lamp. Rice tissues were observed uncovered.

Detection of GFP expression by fluorescence microscopy

GFP expression was monitored at higher magnification using a Nikon Microphot-5A fluorescent microscope equipped with a Nikon B-2A filter block containing a 450–490 excitation filter and a BA520 emission barrier filter. A SLWD $10 \times$ Nikon objective lens with a NA of 0.21 was used.

GUS detection

GUS activity was determined according to Jefferson et al. (1987).

Southern blot hybridization

Genomic DNA was isolated from young leaf tissue of rice plants using a standard CTAB extraction protocol. Five micrograms of DNA were digested overnight with 40 units of *Eco*RI and *Hin*dIII, fractionated through a 0.8% agarose gel and blotted onto a nylon Hybond-N + membrane. Membranes were hybridized with a probe generated by PCR-amplification of the *mgfp4* gene. The band of the expected size was gel-purified and oligo-labelled in the presence of ^{32}P using standard protocols. Hybridization was performed using a Dextran sulphate hybridization mix, at 65°C. The filters were washed in $0.1 \times$ SSC at 65°C.

Results and discussion

Detection of *mgfp4* expression in vitro

In this work, most of the experiments were performed using an inexpensive system for GFP observation, consisting of a hand-held long-wave UV lamp and a transparent Perspex shield. Due to its broad-wavelength range and its wide light-diffusion angle, the hand-held lamp produced only limited energy in the wavelength range useful for mGFP4 excitation (major peak at 395 nm and secondary peak at 475 nm; Haseloff and

Amos 1995). Therefore, this system only allowed identification of rice tissues expressing GFP at high levels. Using the hand-held UV lamp, fluorescent GFP sectors from rice calli could be identified and selected 22 days after bombardment. Observation of rice tissues expressing GFP at lower levels, could be performed only using focused and intense blue-light sources in the 450–490 nm range. The MZ12 Leica dissecting microscope equipped with a fluorescent module allowed observation of a wide range of GFP expression levels both in vitro and in vivo. This system allowed the screening of GFP fluorescent tissue as early as 12 days after bombardment. It was also very useful for observing GFP expression in transgenic rice plantlets, and allowed us to perform segregation studies in situ.

Visual selection of transformation events in vitro using GFP

Visual selection using GFP was performed on bombarded rice tissue cultured either in the absence of a selective agent (hygromycin) or under sub-optimal (25 mg/l)/optimal (50 mg/l) levels. Following bombardment very little fluorescence could be observed during the first 10 days of culture, other than pale-yellow autofluorescence (Fig. 1 a), orange fluorescence in necrotic tissues, or GFP transient expression. Twenty two days after bombardment, large GFP fluorescent sectors (Fig. 1 e) could be observed and dissected from rice calli grown at all levels of hygromycin tested (0, 25 and 50 mg/l) using a simple hand-held ultraviolet lamp for GFP excitation. Visually selected fluorescent rice tissue was used for subsequent clonal propagation or direct regeneration of transformed plants. Independent experiments in wheat transformation using firefly luciferase also confirmed that a 3-week culture period following particle bombardment is required for optimum identification of stably transformed tissues (Lonsdale et al. 1998).

Visual selection drastically enhanced the efficiency of rice transformation by reducing the quantity of tissue handled under our regular protocol (by a factor of 4) and the time required to recover transgenic plants (by a factor of 2) (Table 1). We did not observe a significant difference in transformation frequency between visual screening and selection involving hygromycin alone (Table 1). This was probably due to the use of the hand-held ultraviolet lamp which restricted GFP detection to highly expressing tissues. Ultraviolet radiation may also have damaged rice tissues during observation. In other experiments, the utilisation of high intensity blue-lighting sources (such as the Leica fluorescent module) allowed the identification and dissection of small green-fluorescent groups of somatic embryos as early as 12 days after bombardment, improving further the efficiency of rice transformation by identifying additional transformants more easily and rapidly.

The established transformed rice clones exhibited very bright lime-green fluorescence when observed under fluorescent microscopy (Fig. 1 b). In the absence of hygromycin the selected GFP fluorescent tissue could not be maintained or amplified easily in a clonal manner using consecutive cycles of visual selection. In contrast, both sub-optimal (25 mg/l hygromycin) and optimal (50 mg/l hygromycin) concentrations of the selective agent associated with visual selection permitted the rapid establishment of transformed GFP-expressing clones, from which transformed plants could be easily regenerated.

Expression of GFP in transformed T_0 and T_1 rice plants

Transgenic rice plants were regenerated from visually selected GFP-positive calli. *Mgfp4* expression did not appear to interfere with plant regeneration, the development or the fertility of transformed plants. Using *mgfp4* driven by the CaMV35*s* promoter, high levels of GFP fluorescence could be observed primarily in the root system (Fig. 1 d). Expression was limited in chlorophytic tissues, such as developed leaves, but could be easily detected in the leaves of seedlings grown in the dark. The limited strength of the CaMV35*s* promoter in rice might be responsible for the low apparent expression of GFP in leaf tissue. The use of highly constitutive promoters and introns lead to GFP expression in all plant tissues of transgenic wheat, maize, tobacco and *Arabidopsis* (Pang et al. 1996). The pBINmGFP4 construct used in this study was primarily designed for gene-expression studies in dicotyledonous species and it is reasonable to expect that GFP expression may be improved using stronger promoter sequences.

GFP fluorescence was observed in T_1 embryos and seedlings (Fig. 1 f). Segregation studies at the expression level, using GFP fluorescence followed by histochemical GUS staining, showed both Mendelian and non-Mendelian inheritance of GFP and GUS expression in the progeny (Fig. 1 g). Most deviations from the expected 3 : 1 ratio were due to transformed seedlings expressing GUS but not detectable levels of GFP (observed using a simple hand-held ultraviolet lamp for GFP excitation).

Southern-blot analysis confirmed the integration of *mgfp4* in rice genomic DNA of T_0 and T_1 plants.

Conclusions

There is an increasing demand for alternative selectable and screenable markers in genetic-engineering programs. Advances in our understanding for engineering complex pathways and, in the future, multigenic traits make it necessary to increase our repertoire of such

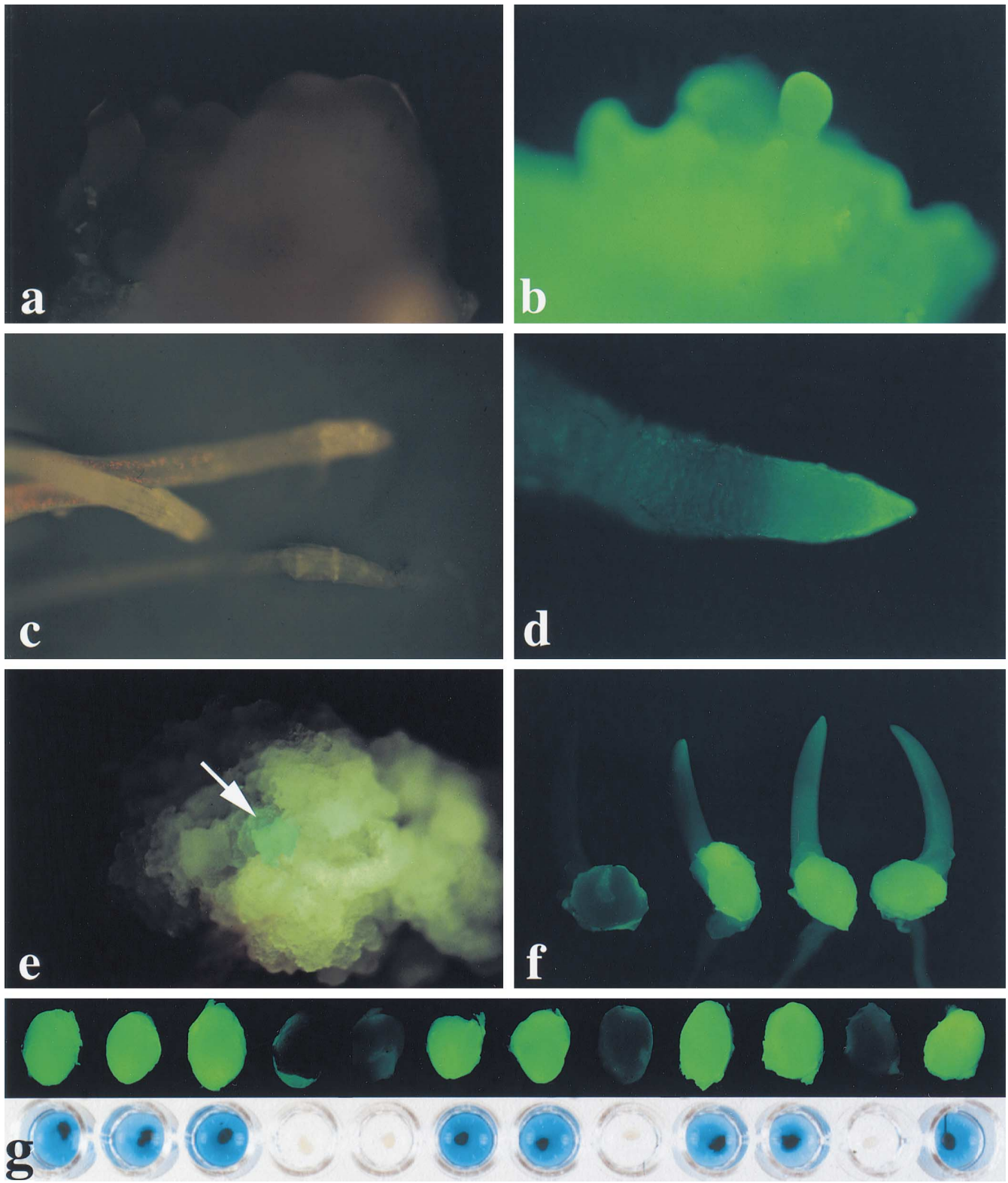

Fig. 1a–g mGFP4 as a vital screenable marker in rice transformation. Observations were performed using a Nikon Microphot fluorescent microscope (**a–d**) or a Leica MZ12 dissecting microscope with a fluorescent module (**e–g**); **a** autofluorescence of untransformed embryogenic callus 2 months after induction; **b** green fluorescence of transformed embryogenic callus 2 months after bombardment with *mgfp4*; **c** autoflurescence of untransformed roots; **d** green fluorescence of transformed root (T_0 plant regenerated from GFP-fluorescent callus); **e** GFP-fluorescent sector in rice callus 22 days after bombardment with *mgfp4*; **f** fluorescence of segregating T_1 seedlings. Left: one untransformed seedling; right: three seedlings transformed with *mgfp4;* **g** GFP and GUS expression of segregating T_1 embryos. Each embryo was first observed for GFP fluorescence (*top*) then assayed histochemically for GUS expression (*bottom*)

Table 1 Rice transformation efficiency with and without visual screening

Days after bombardment	Hygromycin selection only		Visual selection using GFP[a]	
	Number of lines[b]	Number of calli[b]	Number of lines[b]	Number of calli[b]
12	93	507	92	513
22	65	448	6	12
32	56	402	4	14
42	50	346		
52	39	279		
62	31	223		
Total	334	2205	102	539
Transgenic lines	3.6		4.2	

[a] Visual selection was performed using a simple hand-held ultraviolet lamp for GFP excitation
[b] Number of independent callus lines and calli handled for 100 rice immature embryos bombarded

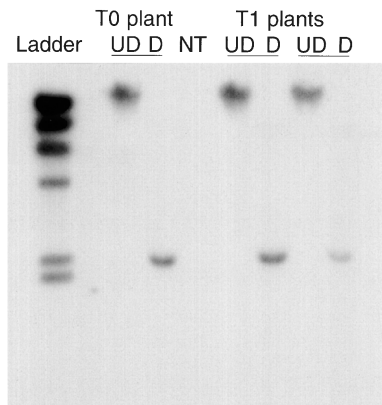

Fig. 2 Southern-blot analysis of GFP-fluorescent T_0 and T_1 rice plants. DNA was either undigested (*UD*) or digested with *Eco*RI and *Hin*dIII (*D*). The *Eco*RI and *Hin*dIII sites are flanking the 2-kb CaMV35*s*-*mgfp4*-NOSpolyA sequence in plasmid pBINmGFP4. Non-transformed rice DNA (*NT*) was used as a negative control. The membrane was probed with the *mgfp4* gene.

genes, in parallel with the development of a better understanding of factors which control the concerted expression of multiple transgenes. Markers such as GFP are particularly valuable in this regard (see Cubitt et al. 1995 for a review). Vital marker genes also provide us with a means of identifying transformation events early, and in a non-destructive manner. The utility of such markers is not limited by the gene-delivery method. GFP will certainly contribute to further improvement of meristem-based transformation systems (Bechtold et al. 1993; Lowe et al. 1995) and therefore will make an important contribution to the development of universal genotype-independent transformation technologies. We believe that marker genes such as GFP will play an essential role in removing the gene-transfer constraint from fundamental science and also in applied programmes involving genetic engineering.

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