Expression of *Aequorea* green fluorescent protein in plant cells

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Abstract The coding region of the green fluorescent protein (GFP) from *Aequorea victoria* has been fused to the cauliflower mosaic virus 35S promoter and introduced into maize leaf protoplasts. Transient expression of GFP was observed. In addition, the coding region of GFP was fused to an *Arabidopsis* heat shock promoter and co-transformed with another construct in which GFP has been replaced with chloramphenicol acetyltransferase (CAT). The heat-induced expression of GFP in maize protoplasts parallels that of CAT. While GFP was expressed in both dark-grown and green maize leaf protoplasts, no green fluorescence was observed in similarly transformed *Arabidopsis* protoplasts.

Key words: Green fluorescent protein; *Arabidopsis*; Maize; Heat shock promoter; Transient expression

1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* absorbs violet/blue light (λ_{max} = 395 nm with a minor peak at 470 nm) and fluoresces green (λ_{max} = 508 nm) [1,2]. The GFP chromophore is formed through intramolecular polypeptide cyclization and oxidation [3]. Besides light, no substrate or cofactor is required for fluorescence [4,5]. Real-time in vivo visualization makes GFP a highly desirable reporter gene. It has been expressed in *Escherichia coli* and the touch receptor neuron cells of *Caenorhabditis elegans* and the expressed proteins showed identical emission spectra to that of the natural GFP [6]. More recently, chimeric proteins of GFP and cellular proteins have been shown to fluoresce green while retaining the functions of the cellular proteins in transformed cells [7,8].

In plant research, the commonly used reporter genes include *CAT* [9], β-glucuronidase (*GUS*) [10] and firefly luciferase (*LUC*) [11]. Although these reporter genes are suitable for quantitative analysis, invasive methods are required for detecting CAT and GUS. *LUC* is the only reporter gene whose activity can be quantified using non-invasive methods and has been used successfully as a screenable marker to isolate circadian clock mutants in *Arabidopsis* [12]. However, in vivo visualization of LUC activity requires that the substrate, luciferin, be supplied to the plants. On the other hand, no additional reactants are required for the fluorescence emission when GFP is excited by violet/blue light or even ambient light [6]. Furthermore, GFP mutants with shifted wavelengths of absorption or emission have been isolated [3,13] that offer the further advantage of multiple labeling of living cells. The successful expression of GFP in *C. elegans* and the exciting recent development of the GFP systems have prompted us to investigate the possibility of using GFP as a reporter gene in plants.

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We show here that GFP is detected in maize protoplasts transformed with GFP driven by cauliflower mosaic virus 35S promoter (P35S). We then use GFP as a reporter gene to assay the heat-inducibility of an *Arabidopsis* heat shock promoter (Phs) in maize and in *Arabidopsis* protoplasts. Interestingly, while GFP was expressed in maize protoplasts isolated from either dark-grown or green seedlings, no green fluorescence is detected in *Arabidopsis* protoplasts. The lack of expression of GFP under the control of various promoters in transgenic tobacco is also discussed.

2. Materials and methods

2.1. Chemicals

Taq DNA polymerase was obtained from Promega Madison, WI). Other enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Ransom Hill Bioscience (Ramona, CA). pGFP10.1 was kindly provided by Drs. Chalfie and Prasher (Columbia University, New York, NY; and Otis Air National Guard Base, MA). Cellulase-RS and macerozyme-R10 were obtained from Karlan (Torrance, CA). Polyethylene glycol 3350 (PEG) was obtained from Sigma (St Louis, MO).

2.2. Plant growth and protoplast isolation

Maize (hybrid strain FR9Cms × FR37; Foundation Seeds, Champaign, IL) seedlings were grown as described [14]. Dark-grown leaves were cut from the first and second leaves from 10-day-old seedlings grown in the dark at 25°C. Green leaves at the same developmental stages were cut from 10-day-old seedlings grown in constant light (500 $\mu E \cdot M^{-2} \cdot S^{-1}$, obtained from cool white fluorescent lamps). *Arabidopsis thaliana*, ecotype 'Columbia', seedlings were grown in greenhouse for 3–4 weeks and rosette leaves were used. Prior to harvesting the leaves, both green maize seedlings and *Arabidopsis* plants were dark adapted for 24 h. Maize protoplasts were isolated as described [14]. *Arabidopsis* leaves were sliced finely with a razor blade and digested in enzyme solution containing: 10 mM MES, 0.4 M mannitol, 8 mM $CaCl_2$, 10 mM $MgCl_2$, 1% cellulase, 0.25% macerozyme, 10 mM β-mercaptoethanol and 0.1% BSA. After 2–3 h shaking at 40 rpm, the protoplasts were filtered through 100 μm mesh and spun at 50 × *g* for 5 min to pellet the protoplasts. Protoplasts were then washed sequentially in 133 mM $CaCl_2$ and 167 mM mannitol, 67 mM $CaCl_2$ and 333 mM mannitol.

2.3. Transformation procedures

Electroporation and culture conditions were as previously described [14]. Approximately 3×10^5 maize protoplasts in 0.3 ml solution containing 0.6 M mannitol and 25 mM KCl were used in each electroporation experiment. 20 μg of each DNA construct were added to the protoplasts. Electroporation was performed with a BioRad Gene Pulser set at 125 μF and 150 V. The samples were kept on ice for 10 min before transferring to culture medium. Maize culture medium was as described [14]. Protoplasts were incubated in the dark at 25°C.

PEG-mediated transformation of maize and *Arabidopsis* were based on published procedures [15]. Each transformation experiment contained approximately 10^6 protoplasts and 20 μg plasmid DNA of each construct. After transformation, protoplasts were incubated in the dark at 25°C.

2.4. Protoplast incubation and heat shock conditions

After transformation, protoplasts were incubated at 25°C for 12 h. Protoplasts co-transformed with P35SGFP and P35SCAT constructs, samples were taken at this time for counting the green fluorescent

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protoplasts and viable ones, and assay for CAT activities. Protoplasts co-transformed with PhsGFP and PhsCAT constructs, 0.5-h heat shock treatments were given at this time and the protoplasts were allowed to recover at 25°C for additional 2 or 4 h before samples were taken for further analyses. Temperatures for heat shock treatment were at 35°C for *Arabidopsis* and at 42°C for maize if not otherwise specified.

2.5. Detection of GFP and CAT

GFP expression in protoplasts was examined by fluorescence microscopy using an Olympus VANOX AHBT3 (Olympus, Tokyo, Japan) with filter set IB. An Optronic LX-450A Digital Colour Video Camera system was used to record microscopic images. Digital pallette CI5000S (Polaroid, Cambridge, MA) was used to convert digital images to slides.

Protoplasts were stained with 0.1% phenosafranin [16] in 0.6 M mannitol for viability assays. Green fluorescent protoplasts and viable protoplasts were counted with a hemocytometer. CAT assays were performed as described [17]. CAT activities of each sample were converted to cpm/number of viable protoplasts.

2.6. GFP constructs

Two oligonucleotide primers, 5'AGTAAAGGAGAAGAAC3' and 5'TTTATTTGTATAGTTCATC3', were made according to the published sequence of GFP [18]. These primers were used in polymerase chain reactions (PCR) to amplify from pGFP10.1 [6] a sequence starting from the second codon and ending at the fourth nucleotide 3' to the stop codon of GFP. The CAT sequence in BlueCATKS [19] was replaced by the amplified GFP sequence, giving rise to the construct P35SGFP. Fig. 1A shows this construct and Fig. 1B the junction sequence where the ATG of GFP is restored.

A 298-bp oligonucleotide 5' to the translational start site of *Arabidopsis* HSP81-1 was amplified by PCR from *Arabidopsis* genomic DNA with two primers 5'GGAGTCTCGAAACGAAAAGAAC3' and 5'CGCAACGAACTTTGATTC3' according to the published sequence [20]. This amplified product, Phs, was used to replace the P35S in the construct P35SGFP. The construct PhsGFP is shown in Fig. 1C. Fig. 1D shows the construct of PhsCAT in which CAT has replaced the GFP in PhsGFP.

3. Results

3.1. GFP expression in electroporation and PEG-transformed maize protoplasts

Dark-grown maize protoplasts were co-transformed with P35SGFP and BlueCATKS by two different methods, electroporation and PEG-mediated transformations. Fig. 2 compares the green fluorescent protoplasts and the background fluorescence of the two protoplast populations, transformed by PEG (Fig. 2A,B) and by electroporation (Fig. 2C,D). PEG-mediated transformation showed less background fluorescence and appeared to have a higher transformation efficiency. The higher efficiency of PEG method was further verified by the higher number of green fluorescent protoplasts and the higher CAT activity (Table 1).

3.2. Regulated expression of GFP

We are interested in determining the heat shock temperatures for the induction of Phs, an *Arabidopsis* heat shock promoter, in maize. When PhsGFP and PhsCAT were co-transformed (by PEG method) into protoplasts of dark-grown maize, the expression of both GFP and CAT (assayed 4 h after heat shock treatment) were at the basal levels at temperatures as high as 35°C. At 42°C, the heat shock temperature of maize plants [21], the CAT activity increased about 300-fold (Table 2A).

3.3. GFP expression detected in green maize but not in Arabidopsis protoplasts

To investigate the potential of using GFP as a reporter gene in *Arabidopsis*, PhsGFP and PhsCAT were co-transformed into *Arabidopsis* protoplasts. CAT expression was at the basal level at 25°C and increased about 6-fold at 35°C (Table 2B) which is the heat shock temperature of intact *Arabidopsis* plant [20]. However, in these protoplasts, no green fluorescent protoplasts were observed. To investigate the possibility that the lack of GFP detection may be due to the interference of chlorophylls and other pigments in the chloroplasts, similar experiments were conducted with green maize protoplasts. Green fluorescent protoplasts were clearly detected, although in fewer protoplasts than when dark grown seedlings were used (Fig. 2B,D; Table 2A). The CAT activities in the protoplasts of green maize were even higher than that of the dark-grown maize seedlings (Table 2A).

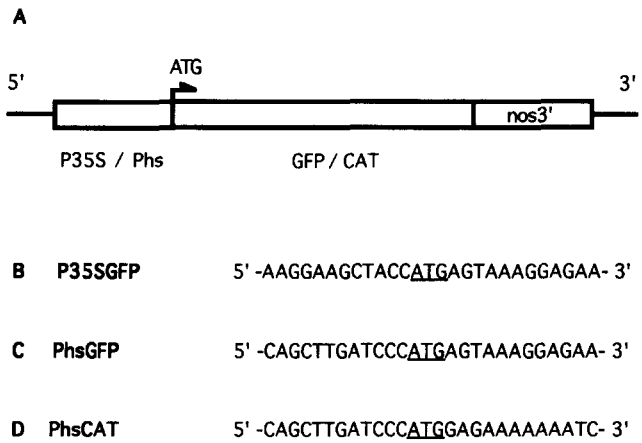

Fig. 1. Plasmid constructs. (A) Schematic drawing of the constructs used in transformation experiments: P35S or Phs promoter driving either GFP or CAT, nos 3' is the 3' flanking region of nopaline synthase gene. (B–D) The junction sequences of each of the constructs. These three constructs are derived from BlueCATKS (19) in which P35S is fused to CAT.

4. Discussion

GFP has been used successfully as a reporter gene in widely divergent organisms [6–8]. The non-invasive method of detecting the expression of GFP offers great potential for using it as a screenable marker for mutant selection. In addition, GFP has been used as a visible marker in fusion proteins to trace the temporal and spatial expressions of the proteins of interest [8]. To assess the potential of using GFP as a reporter gene in higher plants, we conducted experiments to express GFP in monocots and dicots using transient and stable transformation systems.

Two promoters, a constitutive promoter (P35S) and an inducible promoter (Phs), were used to drive the expression of GFP in transient expression systems of maize and *Arabidopsis*. Another reporter gene CAT, under the control of the same promoters, were used in co-transformation experiments to serve as internal controls. Electroporation and PEG-mediated transformation methods were compared by using P35S and Phs constructs. PEG method is more efficient and the protoplasts exhibit less background fluorescence. Because the intensity among green fluorescent protoplasts varies greatly, background fluorescence obscured the detection of those protoplasts that fluoresce less intensely. The background fluores-

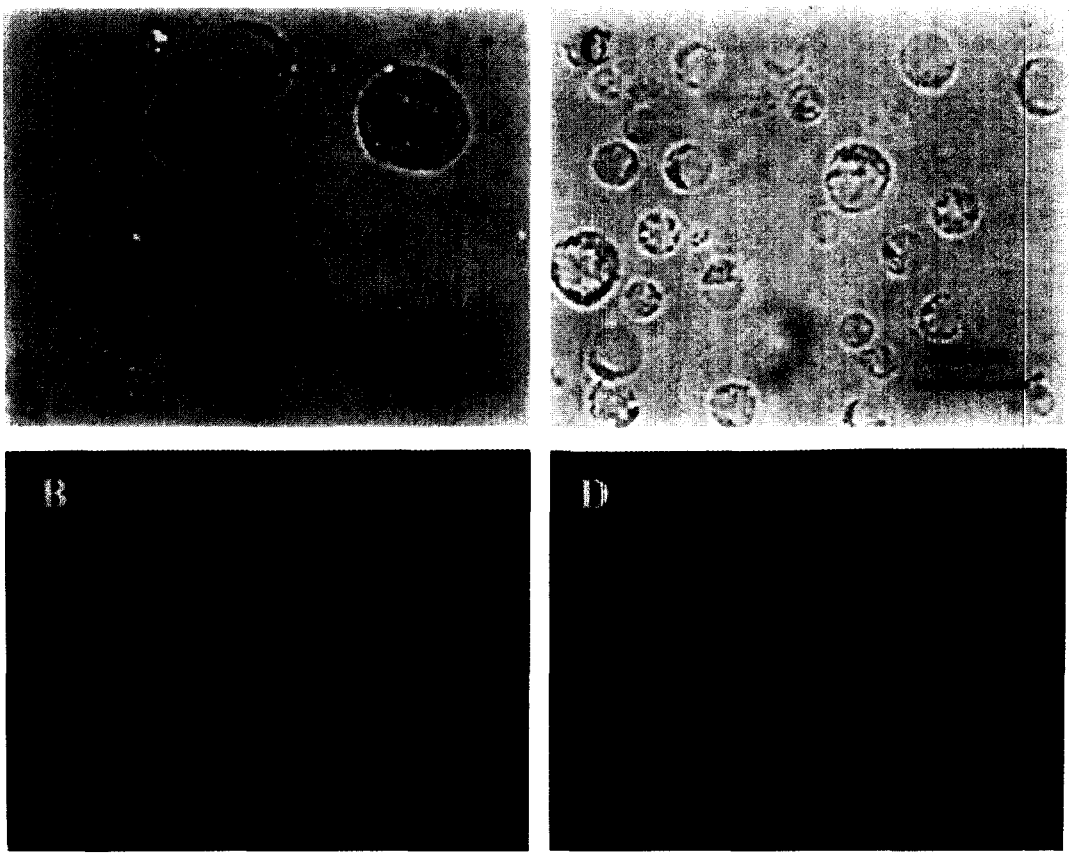

Fig. 2. GFP detection in transformed maize protoplasts. Dark grown maize protoplasts were co-transformed with P35SGFP and P35SCAT, by PEG-mediated transformation (A,B) or by electroporation (C,D). A is bright field and B is the same sample under IB excitation (490 nm spectral range), C is bright field and D is the same sample under IB excitation.

cence is probably due to severe damage (Fig. 1D) caused by electroporation. Phenolic compounds can accumulate in plant cells in response to injury [22]. Approximately 4 h recovery after heat shock treatment is required for detecting any fluorescent protoplasts (Table 2A). CAT activity can be detected after only 2 h (Table 2A). The longer lag time for detecing GFP than CAT was also observed in protoplasts co-transformed with P35SGFP and P35SCAT (data not shown). This difference may be due to slow formation of the GFP chromophore through the intrapeptide cyclization [3].

Until this study, the expression of GFP as a reporter gene has been tested only in non-photosynthetic organisms. After we had observed expression of GFP in dark grown maize protoplasts, we tested the GFP expression in protoplasts made from greenhouse-grown *Arabidopsis*. While CAT expression was observed and was heat inducible when driven by Phs, no green fluorescence was observed in protoplasts co-transformed with PhsGFP (Table 2B). These *Arabidopsis* protoplasts contain abundant chloroplasts in which pigments such as chlorophylls absorb light in the blue region of the spectra, thus, potentially filtering the light for GFP excitation. To assess the potential interference of chlorophyll on GFP detection, green maize protoplasts were co-transformed with PhsGFP and PhsCAT. Comparison of CAT activity in dark-grown and green maize protoplasts gave similar values. However, the percentage of green fluorescent protoplasts in viable protoplasts were about one-third that observed in dark grown protoplasts (Table 2A). These results suggest that chloroplasts may interfere with, but do not abolish, the detection of GFP. Although we do not know what caused the failure of detecting GFP in *Arabidopsis* protoplasts, GFP also failed to express in another dicot species, tobacco. We have created transgenic tobacco plants each carry P35SGFP, PhsGFP or TobRB7Δ0.6GFP (a tobacco root-specific promoter) [23]. No GFP was detected in multiple independent transformants (at least 15 for each construct; data not shown). The fact that green fluorescent cells were not detected in the transgenic roots carrying TobRB7Δ0.6GFP strongly supports the hypothesis that chloroplasts are not the major cause that prevents GFP detection. Experiments such as measuring

Table 1
Comparison of PEG and electroporation transformations

Cotransformed plasmids	GFP expression (GF/V) $\times 10^2$ (n)	CAT activity (cpm/10^3 V)	Relative GFP expression (GFP/CAT) $\times 10^2$
PEG			
P35SGFP/P35SCAT	6.1 (1255)	103.8	5.9
PhsGFP/PhsCAT	9.2 (1248)	295.3	3.1
No DNA	0 (1250)	0.5	0
Electroporation			
P35SGFP/P35SCAT	0.7 (762)	23.0	3.0
PhsGFP/PhsCAT	1.1 (432)	54.6	2.0
No DNA	0 (596)	0.5	0

GF, green fluorescent protoplasts; V, viable protoplasts; n, viable protoplasts examined. Conditions see Section 2.4. Duplicated countings for GF gave less than 10% error; duplicated CAT assays gave less than 10% error.

Table 2
Heat shock induced expression of GFP/CAT in maize and *Arabidopsis* protoplasts cotransformed with PhsGFP/PhsCAT

(A) Maize protoplasts

Temperature	2 h		4 h	
	GFP expression (GF/V) × 10^2 (n)	CAT activity cpm/10^3 V)	GFP expression (GF/V) × 10^2 (n)	CAT activity cpm/10^3 V)
Dark grown maize				
25°C	0 (1279)	1.0	0 (1261)	0.9
30°C	0 (1263)	0.8	0 (1242)	0.7
35°C	0 (1256)	1.0	0 (1257)	1.0
42°C	0 (1237)	285.2	9.2 (1248)	295.3
Green maize				
25°C	0 (478)	1.8	0 (510)	1.8
30°C	0 (459)	6.5	0 (481)	4.9
35°C	0 (472)	5.1	0 (445)	11.7
42°C	0 (467)	237.9	3.7 (432)	464.2

(B) *Arabidopsis* protoplasts

Temperature	4 h		6 h	
	GFP expression (GF/V) × 10^2 (n)	CAT activity cpm/10^3 V)	GFP expression (GF/V) × 10^2 (n)	CAT activity cpm/10^3 V)
25°C	0 (165)	21.6	0 (154)	32.2
35°C	0 (155)	124.7	0 (142)	92.0
42°C	0 (178)	23.5	0 (137)	14.1
No DNA	0 (149)	0.8	0 (150)	0.8

GF, green fluorescent protoplasts; V, viable protoplasts; n, viable protoplasts examined. Conditions see section 2.4. Duplicated countings for GF gave less than 10% error; duplicated CAT assays gave less than 10% error.

mRNA and protein expression of GFP will be required to understand further at what level the expression of GFP is blocked.

We are interested in studying the heat shock response of Phs in various plant species. Phs directs the expression of a HSP81-1, a heat shock protein from *Arabidopsis*. The heat shock temperature to induce the expression of HSP81-1 in *Arabidopsis* plants is around 35°C [20] which is also the heat shock temperature for inducing PhsCAT expression in transiently transformed *Arabidopsis* protoplasts (Table 2B). However, the expression of the same construct was not induced in transiently transformed maize protoplasts at 35°C, but rather was induced at 42°C, which is the heat shock temperature of maize plants [21] (Table 2A). These results showed that the heat shock temperatures for inducing Phs is determined by the species into which this promoter is introduced. The transient expression systems using protoplasts further showed that the temperature specificity is cell autonomous.

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Note added in proof

After submission of this manuscript, Niedz, R.P., Sussman, M.R. and Satterlee, J.S. reported in Plant Cell Report 14 (1995) 403–406, GFP expression in transiently transformed protoplasts made from nonphotosynthetic suspension culture of *Citrus sinensis*.