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F. Locatelli · C. Vannini · E. Magnani · I. Coraggio · M. Bracale

Efficiency of transient transformation in tobacco protoplasts is independent of plasmid amount

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Abstract We describe an optimized protocol for the transient transformation of tobacco protoplasts mediated by polyethylene–glycol (PEG). As expected, the quantitative β–glucuronidase (Gus) activity driven by pCaMV-Gus was dependent on the amount of plasmid used. Nevertheless, we demonstrate by an immunodetection method that transformation efficiency did not depend on the amount of plasmid used but on the limitation imposed by cell competence. In fact, we obtained the same percentage of transformed cells (about 60%) using a wide range of plasmid concentrations (0.1–10 μg per test). Finally, we show that, when we used two plasmid types in a mixture at a concentration ranging from 0.1 to 10 μg for each, all transformed cells expressed proteins encoded by both plasmids. Transient expression and co-transformation experiments are routinely used methods and, probably, the major results from this work were assumed by many researchers in this field, but our data experimentally support this assumption.

Keywords PEG-mediated DNA uptake · Protoplasts · Tobacco · Transformation efficiency · Transient expression

Abbreviations *AMCA:* Aminomethylcoumarin · *6–BAP:* 6-Benzylaminopurine · *CaMV35S:* Cauliflower mosaic virus 35S promoter · *Cat:* Chloramphenicol acetyltransferase · *DAPI:* 4,6-Diamidino-2-phenylindole · *FDA:* Fluorescein diacetate · *GFP:* Green fluorescent protein · *Gus:* β-Glucuronidase · *MES:* 2-[*N*-Morpholino]ethanesulfonic acid · *MUG:* 4-Methyl-umbelliferyl-glucuronide · *ORF:* Open reading frame · *PEG:* Polyethylene glycol · *PS:* Plasmolysis solution

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F. Locatelli · E. Magnani · I. Coraggio (✉)
Istituto di Biologia e Biotecnologia Agraria,
CNR, via E. Bassini 15, 20133 Milan, Italy
e-mail: immacolata.coraggio@ibba.cnr.it
Tel.: +39-02-23699427
Fax: +39-02-23699411

C. Vannini · M. Bracale
Dipartimento di Biologia Strutturale e Funzionale,
Università dell'Insubria,
via J.H. Dunant 3, 21100 Varese, Italy

Present address:
E. Magnani, Department of Plant and Microbial Biology,
University of California,
111 Koshland Hall, Berkeley, CA 94720-3102, USA

Introduction

Transient expression assays have been applied to study characteristics and functions of genes, regulatory *cis*-acting elements, subcellular localization and transactivation activity of transcription factors (Giovinazzo et al. 1992; Howard et al. 1992; Busk et al. 1997; Di Sansebastiano et al. 1998; Schwechheimer et al. 1998; Locatelli et al. 2000). Plant tissues and cells can be transiently transformed by several procedures, such as particle bombardment, DNA uptake mediated by polyethylene glycol (PEG), electroporation and microinjection (Sanford et al. 1993; Bilang et al. 1994; Holm et al. 2000). Although transient assays allow for the study of most gene functions independently of cellular type, some problems arise from variability within the system, such as the number of transformed cells and the amount of plasmid per cell.

This variability results in a more dramatic effect in co-transformation experiments in which several plasmids are used. In previous experiments, we used up to four different plasmids in the same transformation assay in order to determine whether the transcription factor Mybleu was able to modulate the Opaque2 activity on the *b32* promoter (Locatelli et al. 2000). It was therefore necessary to optimize transformation conditions in order to obtain the best and the most reproducible expression of individual constructs.

Although several papers on transient transformation methods have been published, only a few of them report the transformation efficiency as a percentage of cells

expressing the plasmid-encoded gene. An efficiency ranging from 40% to 60% was reported for both tobacco and *Arabidopsis* PEG-mediated protoplast transformation using 50 μg of plasmid DNA/test; a lower but comparable transformation efficiency was reported for soybean (25%) (Chapel and Glimelius 1990; Howard et al. 1992; Abel and Theologis 1994). Rasmussen and Rasmussen (1993) reported a dramatically low efficiency in rapeseed (2 protoplasts/million).

In a paper concerning the specific accumulation of green fluorescent protein (GFP) in a non-acidic vacuolar compartment, Di Sansebastiano et al. (1998) reported a transformation efficiency for tobacco protoplasts ranging from 60% to 80%. They used 10 μg plasmid/test (6×10^5 cells) and found that the transformation efficiency increased when protoplast concentration decreased.

We were interested in understanding whether transformation efficiency depends on plasmid amounts or on cellular competence, and whether transient protoplast transformations mediated by several plasmids are independent events.

Although the quantitative β-glucuronidase (Gus) activity was strictly correlated to the plasmid amounts used for tobacco protoplast transformation, we found that the percentage of transformed cells (about 60%) remained unchanged when a range of plasmid concentrations between 0.1 and 10 μg/test was used and that, in co-transformation experiments, all cells were co-transformed regardless of the plasmid amount.

Materials and methods

Plant material

In vitro shoot cultures of *Nicotiana tabacum* (cultivar Petit-Havana, SRI) were maintained on MS agar medium (Murashige and Skoog 1962) containing 20 g/l sucrose, at 25°C, with a light/dark cycle of 16/8 h.

Plasmid constructs

Figure 1a shows a scheme of plasmids used for transient transformation assays. pCaMVGus (5.3 kb), pCaMVCat (4.2 kb) and pCaMVMybleu (4.4 kb) expressing, respectively, Gus, chloramphenicol acetyltransferase (Cat) and Mybleu open reading frames (ORFs) under the control of the constitutive cauliflower mosaic virus promoter (CaMV35S) have been previously described (Fromm et al. 1985; Giovinazzo et al. 1992; Locatelli et al. 2000).

Plasmid DNA was purified using the "Qiagen Plasmid Kit", according to the manufacter's instructions.

Transient expression in tobacco protoplasts

All operations were performed in sterile conditions. Tobacco leaves were cut from shoot cultures grown in vitro and mesophyll protoplasts were isolated according to the protocols of Nagy and Maliga (1976) and Potrykus and Shillito (1986) with some modifications. In particular, the top sides of the leaves were brushed with carborundum powder, washed with distilled water and put, upside down, in Petri dishes containing 15 ml of plasmolysis solution [PS: 0.5× Murashige and Skoog salts; 0.5 *M* mannitol; 14 m*M* $CaCl_2$; 10 mg/l 6-benzylaminopurine (6-BAP); pH 5.8].

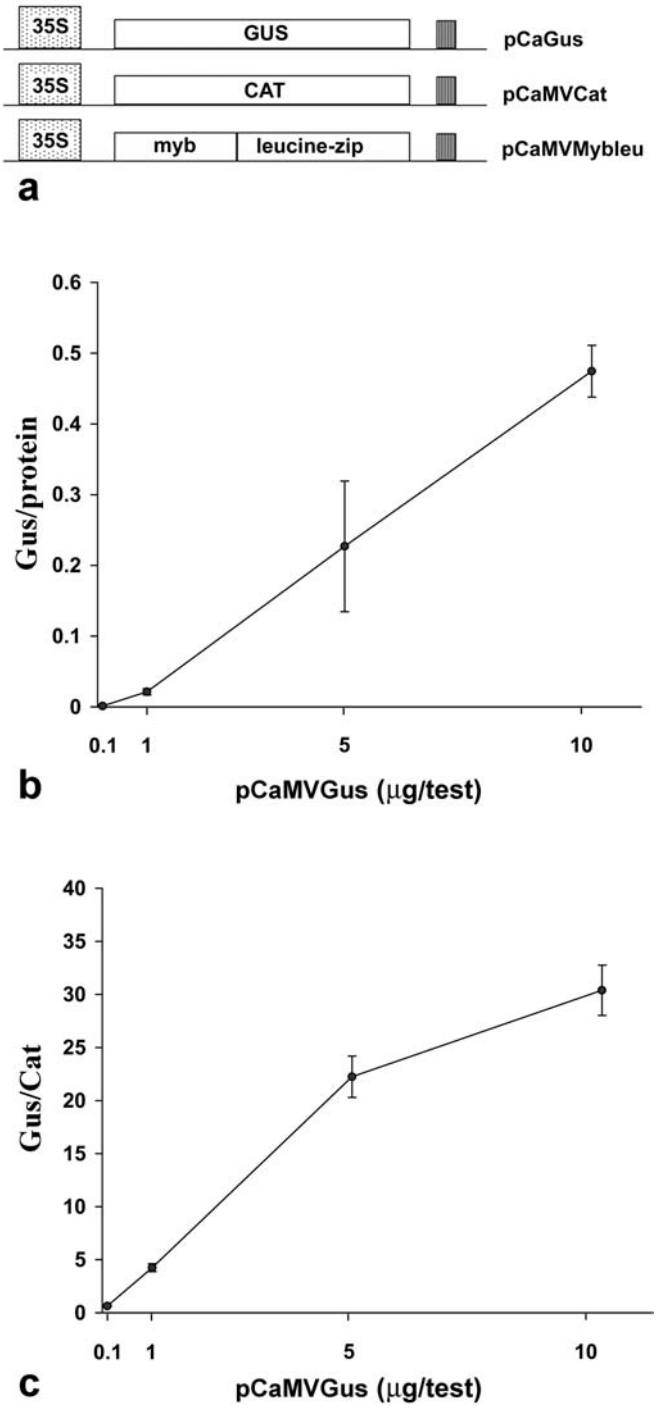


Fig. 1a–c Gus activity is proportional to the amount of the pCaMVGus plasmid used to transform tobacco protoplasts. **a** Schematic drawing of the plasmids used in tobacco protoplast transformations. The *dotted rectangles* represent CaMV35S promoters (35S). The *open rectangles* represent ORFs. The *hatched rectangles* represent *Nos* terminator. **b** Tobacco protoplasts were transformed with different amounts of pCaMVGus (indicated in μg/test). Gus/protein represents the ratio of Gus activity (picomoles MUG hydrolyzed per minute) to total protein (μg). The *bars* represent standard deviations of four independent transformation events. **c** Tobacco protoplasts were transformed with 10 μg/test of pCaMVCat and with different amounts of pCaMVGus (indicated in μg/test). Gus/Cat represents the ratio between ratio of the co-expressed Gus (picomoles MUG hydrolyzed per minute) and Cat (milliunits) activities. The *bars* represent standard deviations of four independent transformation events

After a 2-h incubation at room temperature, PS was replaced by an equal volume of enzyme mixture (10 g/l Cellulase Onozuka R10; 5 g/l Macerozyme in PS). Petri dishes were incubated overnight in the dark at 25°C. After incubation, the enzyme solution was discarded and 15 ml of K3 medium (Nagy and Maliga 1976) was gently spread on the leaf tissue to release protoplasts. After filtration through nylon filters (180 μm and 85 μm), 8 ml of the solution containing protoplasts was distributed in 10 ml polystyrene centrifuge tubes and overlaid with 1 ml of W5 buffer (154 m*M* NaCl; 5 m*M* KCl; 125 m*M* $CaCl_2$; 5 m*M* glucose; pH 5.8). After gentle centrifugation (10 min at 80 *g*) protoplasts floating at the interface were collected, washed with W5 (3/1 v/v), pelleted by centrifugation (10 min at 80 *g*) and resuspended at approximately 3×10^6 protoplasts/ml in Ma-Ca buffer (0.5 *M* mannitol, 20 m*M* $CaCl_2$, 0.1% w/v 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 5.8). The viability of protoplasts was checked by the fluorescein diacetate (FDA) method (Widholm 1972). PEG-mediated transformation was performed according to the protocol of Bilang et al. (1994), with minor modifications. Plasmid DNA and 30 μg of herring sperm carrier DNA were added to one million protoplasts in Ma-Ca buffer and mixed with an equal volume of 40% w/v PEG 4000 in CMS (100 m*M* $Ca(NO_3)_2$; 400 m*M* mannitol; pH 8.5). After incubation at room temperature for 30 min, with occasional gentle shaking, protoplasts were diluted with W5 (1:15 v/v), collected by centrifugation (10 min at 100 *g*), resuspended in 0.6 ml of K3, and incubated in the dark at 25°C for 40 h. After dilution with W5 (1:15 v/v), protoplasts were centrifuged (10 min at 100 *g*) and resuspended in W5 at a final concentration of about 300 protoplasts/μl. A total of 1,300–1,500 protoplasts were spotted onto nitrocellulose membrane (PROTRAN BA 85, 0.2 μm, Schleicher and Schuell) previously wet with W5. Finally, nitrocellulose filters, placed on 3MM Whatman paper, were air dried and kept at room temperature until used for immunodetection.

Immunodetection

Immunodetection and 4,6-diamidino-2-phenylindole (DAPI) staining were performed as previously described (Locatelli et al. 2000). Filters were treated with the primary anti Mybleu (Locatelli et al. 2000, 1:100 dilution) or anti Gus (anti GUS, 5prime-3prime, Boulder, Calif., 1:100 dilution) primary antisera, followed by the goat anti rabbit alkaline phosphatase-conjugated secondary antiserum (Sigma, 1:200 dilution). Alkaline phosphatase conjugated antibodies were then detected using 4-nitroblue tetrazolium chloride.

For Gus fluorescent detection, filters were treated with the anti Gus primary antiserum, followed by the goat anti rabbit aminomethylcoumarin (AMCA) conjugated secondary antiserum (Jackson ImmunoResearch, 1:1000 dilution).

After DAPI staining (2 mg/ml), filters were examined and photographed with a Zeiss Axioplan fluorescence microscope using the excitation filter LP400 for AMCA and DAPI. Preimmune serum was used as negative control.

Protein, Gus and Cat determination

Protein concentration from protoplast samples was determined according to Bradford (1976) by means of a Bio-Rad Protein Assay Kit.

Gus was assayed by the fluorescence method of Jefferson (1987), using 1 m*M* 4-methyl-umbelliferyl-glucuronide (MUG) as a substrate, on aliquots of the protoplast extracts. Gus activity was determined as picomoles MUG hydrolyzed per minute.

Chloramphenicol acetyltransferase activity was assayed by the diffusion of the reaction product (^{3}H-acetylated-chloramphenicol) into scintillation fluid (Sambrook et al. 1989). Fifty microliters of cell extract was mixed in a plastic scintillation vial with 200 μl of freshly prepared Cat reaction mixture, containing 25 μl 1 *M* Tris-HCl pH 7.8, 50 μl 5 m*M* chloramphenicol, 0.1 μCi ^{3}H-labelled acetyl coenzyme A (185 mCi/mmol; 50 μCi/ml; Amersham). Five milliliters of water-immiscible scintillation fluid (OCS-Amersham) was added, and the vials were placed for 5 h at 37°C and counted every hour. Cat units were calculated from DPM increases on the basis of a reference curve obtained in the same conditions using a standard Cat preparation (4,800 U/ml) from *Escherichia coli* (Sigma). In our conditions, the reaction was linear up to 0.6 mU enzyme/reaction mixture.

Gus expression was reported as both a ratio between Gus activity (picomoles MUG hydrolyzed per minute) and protein amount (μg), and a ratio between the co-expressed Gus (picomoles MUG hydrolyzed per minute) and Cat (mU) activities. The results are means of four independent transformation experiments.

Results and discussion

Gus activity driven by pCaMVGus in transformed tobacco protoplasts depends on the amount of plasmid used

To test whether the reporter gene expression was affected by the amount of encoding plasmids used, we planned to transform tobacco protoplasts with different amounts of a reporter gene-expressing plasmid.

Our unpublished results showed that, in tobacco protoplast co-transformation experiments, the addition of increasing amounts (10–60 μg/million protoplasts) of a plasmid carrying the CaMV35S promoter (pCaMVNeo, Fromm et al. 1986) did not affect the expression of 10 μg of pCaMVCat or pCaMVGus, indicating that the presence of several plasmids carrying the same strong promoter region (CaMV35S) did not influence the expression of the individual constructs. To determine the best way to express the activity of the Gus reporter gene, we performed co-transformation experiments with pCaMVGus and pCaMVCat plasmids (5 μg/million protoplasts each). Gus activity was determined fluorometrically on protoplast extracts as picomoles MUG hydrolyzed per minute. The value was referred either to total protein (μg), or to Cat activity (milliunits) determined in the same extract. The statistical evaluation of the results among five repeats of the same transient expression experiment showed that the variability of Gus expression was almost 30% when related to the total protein, and less then 10% when related to Cat activity co-expressed in the same protoplasts.

Therefore, we transformed 1 million protoplasts/test with increasing amounts (0.1, 1. 5 and 10 μg) of pCaMVGus and with 10 μg/test of pCaMVCat as internal reporter (Fig. 1a) and Gus activity was expressed with respect to total protein (Fig. 1b) and also as a ratio between the co-expressed Gus and Cat activities (Fig. 1c). As expected, when the Gus activity was referred to the protein amount, standard deviations were higher than when referred to the Cat activity. Nevertheless, in both cases the Gus activity strictly depended on the pCaMVGus amount (Figs. 1b, c).

Table 1 Percentage of transformed protoplasts. Protoplasts were transformed with different amounts of pCaMVGus and pCaMVMybleu (μg of plasmids used are indicated at the top; for co-transformed cells, μg of each plasmid are indicated). Antisera used for immunodetection are indicated on the left. Means and standard deviations are expressed as percentages. Numbers in brackets indicate means and standard deviations, expressed as percentages, referred to each transformed protoplast sample diluted with an equal amount of mock transformed protoplasts

Plasmid amount (μg per test)	0.1	1	5	10
Anti-Gus	59.17±6.35	59.48±6.96	56.82±4.09	57.00±6.32
[30.55±2.32]	[26.67±0.83]	[27.07±4.27]	[29.56±4.18]	
Anti-Mybleu	59.00±7.57	59.78±3.78	62.08±7.03	57.75±2.8
[30.11±6.42]	[29.23±5.39]	[27.47±3.32]	[29.89±4.29]	
Both anti-Gus and anti-Mybleu	59.31±5.45	60.99±4.34	59.86±7.54	56.17±2.84
[29.82±4.08]	[26.67±0.86]	[25.83±2.51]	[30.89±4.13]	

The percentage of transformed tobacco protoplasts is almost 60% and does not depend on the amount of plasmid used

The linear relationship between the Gus activity and the pCaMVGus plasmid amount might depend on the number of transformed cells or on the level of expression per cell. In other words, the limiting factor may be the number of protoplasts or the plasmid amount. In order to discriminate between these two hypotheses, we performed Gus and Mybleu immunodetections on protoplasts transformed with increasing amounts of pCaMVGus and pCaMVMybleu respectively (Fig. 2).

In pCaMVGus-transformed samples, the Gus-expressing protoplasts, evidenced by phosphatase-conjugated (Fig. 2, row anti-Gus a) or by AMCA-conjugated (Fig. 2, row anti-Gus b) secondary antisera, were counted (five fields of 180 protoplasts each). Means of expressing cells, highlighted by the AMCA-conjugated secondary antiserum, and their respective standard deviations are reported in Table 1. The results obtained with phosphatase-conjugated secondary antiserum were very similar and within the range of the standard deviation values reported for the other secondary antiserum.

As shown in Table 1, a high percentage of cells (about 60%) expressed Gus, regardless of the plasmid amount. This strongly suggests that the number of transformed cells does not depend on the tested amount of plasmid, but only on the cell competence for DNA uptake. To confirm the percentage of transformed cells, we diluted each transformed protoplast sample with an equal amount of mock transformed protoplasts. Means of Gus expressing cells in this case were about 30% (Table 1, values between brackets).

Analogous results were obtained for the Mybleu-expressing protoplasts, evidenced by phosphatase-conjugated secondary antiserum (Fig. 2, row anti-Mybleu and Table 1). These data indicate that neither the plasmid used (pCaMVGus or pCaMVMybleu) nor the cellular localization (cytoplasmic or nuclear) affected the percentage of cells expressing the reporter gene.

We used from 0.1 μg to 10 μg of DNA plasmid (1.8×10^{10}–1.8×10^{12} copies for a standard plasmid of 5,000 bp) to transform 10^6 protoplasts. Therefore, cell number would be strongly limiting even if their competence were 100%. As a consequence, in this range, the plasmid amount did not affect the percentage of expressing cells, but only the intensity of the expression.

These results agree with data of Di Sansebastiano et al. (1998) on the dependence of the efficiency of tobacco protoplast transformation on the use of a low protoplast density. Actually, one may suppose that protoplast concentration affects cell competence.

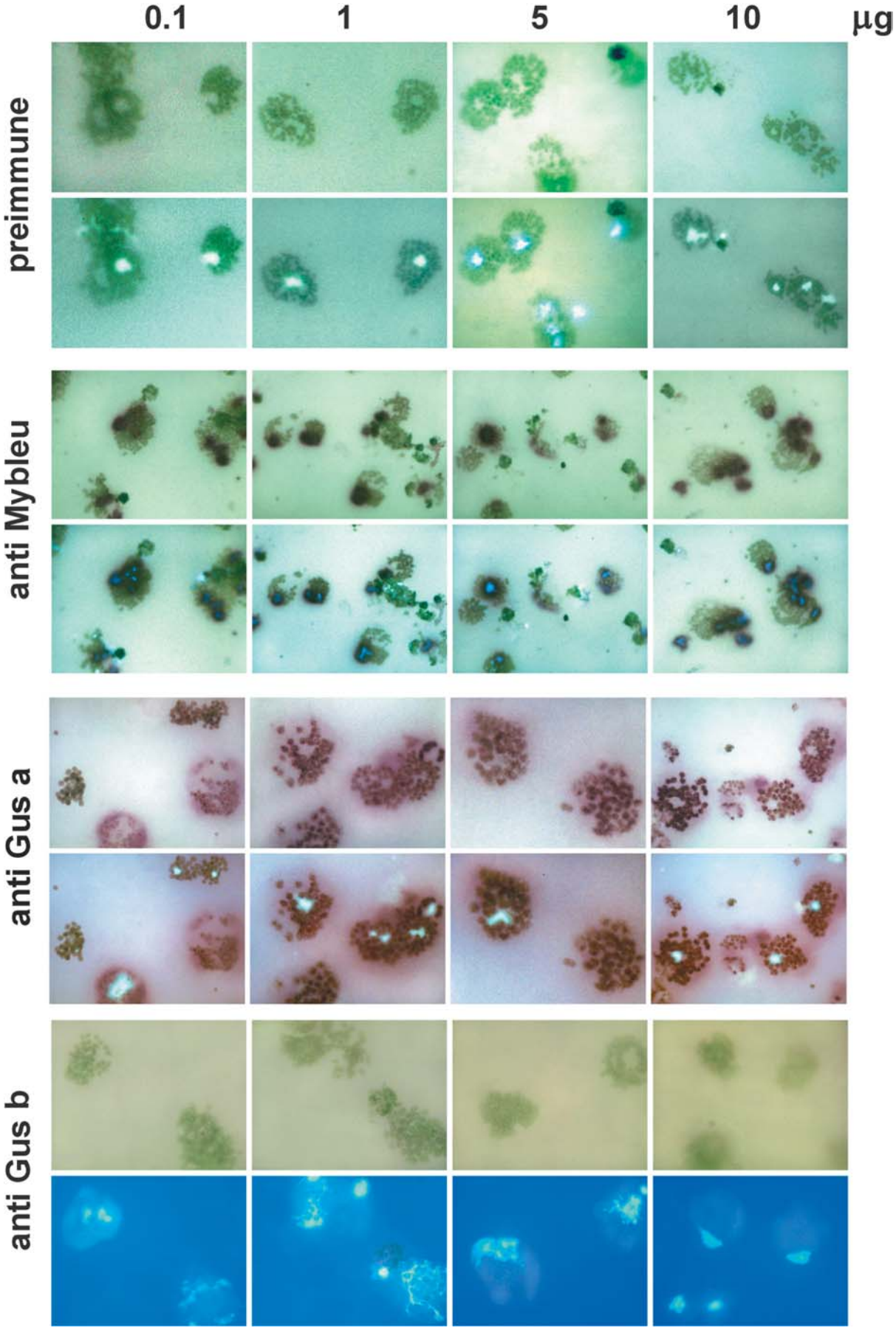
Di Sansebastiano et al. (1998) reported that the efficiency of tobacco protoplast transformation also depends on the use of CsCl-purified plasmids and that it is variable from one plasmid preparation to the next. We used plasmids purified with the Qiagen Plasmid Kit and obtained the same percentage of transformed cells, both using different preparations of the same plasmid and different plasmids. This different finding may be due to the different methods used to purify plasmids, to transform and/or to detect the reporter gene expression.

Cells transformed with a mixture of several plasmids express all the plasmid-encoded proteins

We co-transformed tobacco protoplasts with the same amount of pCaMVGus, expressing the cytoplasmic Gus protein, and pCaMVMybleu expressing the nuclear protein Mybleu (0.1–10 μg for each plasmid). Expression of both ORFs was driven by the same CaMV35S promoter sequence.

We performed immunodetection on co-transformed protoplasts using anti-Gus, anti-Mybleu and both as primary antisera. In these experiments, using a single antiserum, the immunodetection was restricted to the expected subcellular region (data not shown). Co-transformed protoplasts, assayed with both antisera, showed

Fig. 2 The percentage of transformed cells does not depend on the plasmid amount. Tobacco protoplasts, transformed with different amounts of pCaMVGus or pCaMVMybleu (indicated at the *top*), were spotted on nitrocellulose filters and treated with different antisera. Anti-Gus or anti-Mybleu were used as primary antisera. Preimmune antiserum was used as negative control. The *odd rows* show protoplasts under white light, highlighting Mybleu and Gus (anti-Gus a) phosphatase-immunostaining. The *even rows* show protoplasts under fluorescent light, highlighting both DAPI staining and Gus AMCA immunostaining (anti-Gus b)


0.1
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anti Gus a
anti Gus b

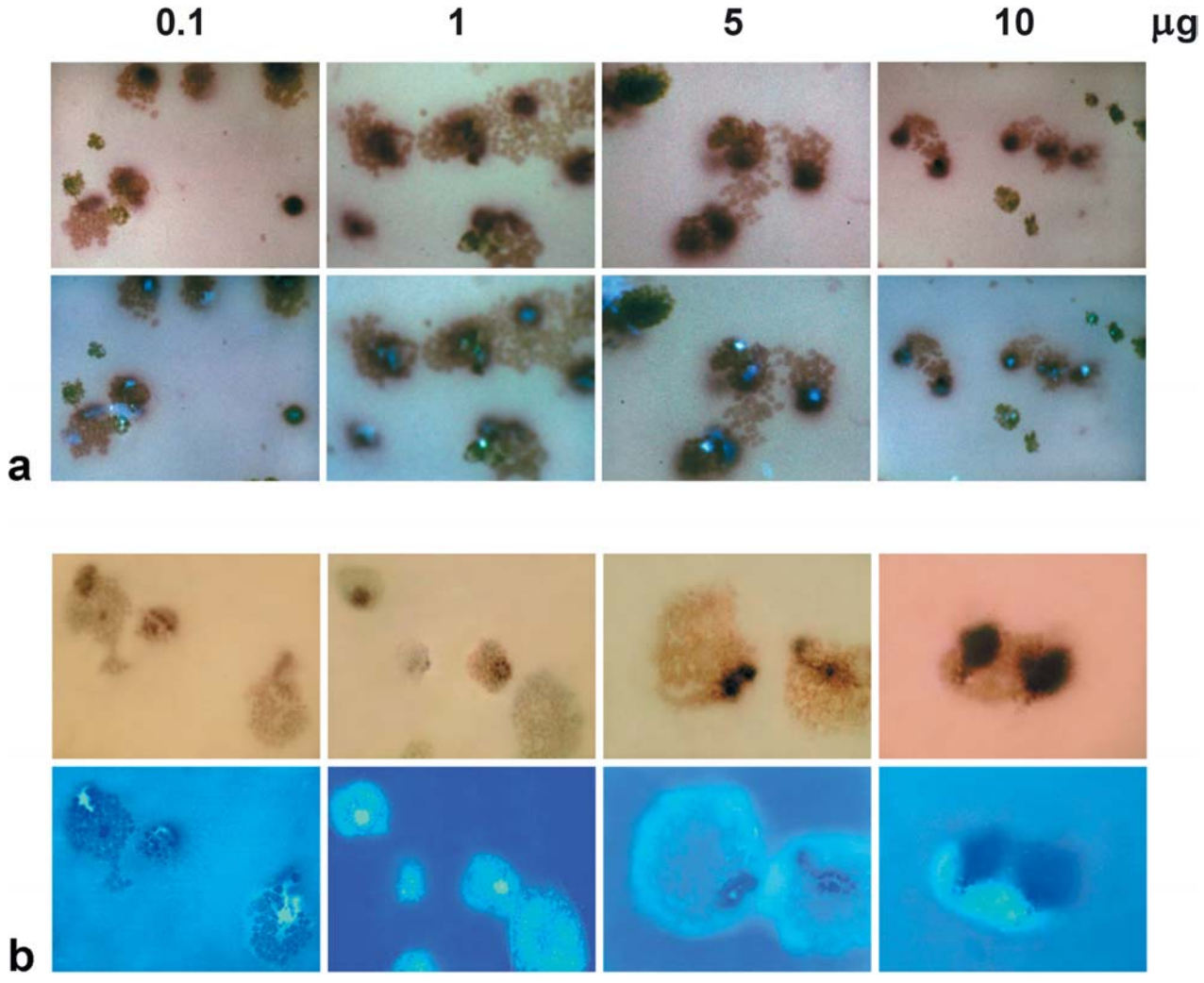

Fig. 3a, b Co-transformed cells express all the plasmid-encoded proteins. Tobacco protoplasts, co-transformed with different amounts of pCaMVGus and pCaMVMybleu (numbers at the *top* indicate μg of each plasmid), were spotted on nitrocellulose filters and treated with different antisera. Anti-Gus and anti-Mybleu were used as primary antisera. **a** The *upper row* shows protoplasts under white light, highlighting Mybleu and Gus phosphatase immunostaining. The *lower row* shows protoplasts under fluorescent light, highlighting DAPI staining. **b** The *upper row* shows protoplasts under white light, highlighting Mybleu phosphatase immunostaining. The *lower row* shows protoplasts under fluorescent light, highlighting both DAPI staining and Gus AMCA immunostaining

antigen detection both in nuclei and cytoplasm, as expected (Fig. 3). The Mybleu expression was evaluated by phosphatase-conjugated secondary antiserum, whereas Gus-expressing protoplasts were evidenced both by phosphatase-conjugated (Fig. 3a) and by AMCA-conjugated secondary antisera Fig. 3b). For each sample, protoplasts expressing only Gus, only Mybleu and both were counted (five fields of 180 protoplasts each); means and standard deviations were calculated and expressed as percentages. Since all transformed cells expressed both Mybleu and Gus, in Table 1, we reported values only for the co-expressing cells. The reported values are referred to the results highlighted by phosphatase-conjugated (Mybleu) and AMCA-conjugated (Gus) secondary antisera. The results obtained using phosphatase-conjugated secondary antiserum for both Mybleu and Gus were very similar and within the range of the standard deviation values reported for the other secondary antiserum. To confirm the percentage of transformed cells, we diluted each transformed protoplast sample with an equal amount of mock transformed protoplasts (Table 1, values in brackets).

As shown in Fig. 3 and Table 1, all transformed cells expressed both proteins even when only 0.1 μg was used for each plasmid in each test, and means of Gus and Mybleu expressing cells were about 60% and 30% for undiluted and diluted transformed cells, respectively. Data obtained for co-transformed cells did not statistically differ from those obtained for single transformants. These results indicated that (1) in co-transformation experiments, the presence of pCaMVGus plasmid did not affect the expression of pCaMVMybleu and vice versa, and (2) when a mixture of plasmids is used, proteins encoded by all of them are co-expressed in the same cells. These

findings confirmed that the limiting factor is the amount of competent cells.

Altogether, our data indicated that transgene activity is, as expected, dependent on the amount of plasmid used, whereas transformation efficiency is independent of the plasmids used (pCaMVGus or pCaMVMybleu), their concentrations (0.1–10 μg per test) and different DNA preparations. These results suggest that, at least in the range of DNA concentrations used, the limitation in cell transformation efficiency is imposed by cell competence. The finding that in co-transformation experiments all transformed cells co-expressed proteins encoded by the used plasmids further supports this hypothesis.

Plant materials and different versions of the PEG-transformation method may affect cell competence and explain the different data on transient transformation efficiency reported in literature (Chapel and Glimelius 1990; Howard et al. 1992; Rasmussen and Rasmussen 1993; Abel and Theologis 1994; Di Sansebastiano et al. 1998). Probably, those who use PEG-mediated transformation systems assume that the cell competence is the most restricting factor in transformation efficiency; nevertheless, to our knowledge, this is the first case in which this assumption has been experimentally demonstrated. We think that our results may provide useful information for those who use transient expression systems, especially in the case of co-transformation experiments.

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