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Transient gene expression in electroporated *Solanum* protoplasts

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Abstract

Electroporation was used to evaluate parameters important in transient gene expression in potato protoplasts. The protoplasts were from leaves of wild potato *Solanum brevidens*, and from leaves, tubers and suspension cells of cultivated *Solanum tuberosum* cv. Désirée. Reporter enzyme activity, chloramphenicol acetyl transferase (CAT) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, depended on the field strength and the pulse duration used for electroporation. Using field pulses of 85 ms duration, the optimum field strengths for maximum CAT activity were: *S. brevidens* mesophyll protoplasts – 250 V/cm; Désirée mesophyll protoplasts – 225 V/cm; Désirée suspension culture protoplasts – 225 V/cm; and Désirée tuber protoplasts – 150 V/cm. The optimum field strengths correlated inversely with the size of the protoplasts electroporated; this is consistent with biophysical theory. In time courses, maximum CAT activity (in Désirée mesophyll protoplasts) occurred 36–48 h after electroporation. Examination at optimised conditions of a chimaeric gene consisting of a class II patatin promoter linked to the β-glucuronidase (*gus*) gene, showed expression (at DNA concentrations between 0–10 pmol/ml) comparable to the CaMV 35S promoter in both tuber and mesophyll protoplasts. At higher DNA concentrations (20–30 pmol/ml) the patatin promoter directed 4–5 times higher levels of *gus* expression. Implications and potential contributions towards studying gene expression, in particular of homologous genes in potato, are discussed.

Introduction

Introduction of foreign genes into plant cells can be achieved by a variety of methods, including direct transfer into protoplasts using chemical [14, 21, 22] and electrical methods [6, 7, 27]. These methods can be used to study genes both transiently [6, 14] and stably integrated [7, 21, 22, 27] in plants. The former method has been used extensively in mammalian systems to study various aspects of gene regulation, such as the analysis of enhancer elements [9, 16], and in examining positive and negative regulatory control [16], in addition to being used to study the cell-specific expression of genes [16]. In contrast, transient gene expression in plants became established more recently (see [8]), and has so far been applied to study the regulation of only a few plant genes (e.g. the maize alcohol dehydrogenase 1 gene [10]).

Electroporation-mediated gene transfer appears to be one of the more general and efficient direct gene transfer techniques for obtaining transient expression and stable transformants in

plants [6–8, 27]. Here we report the development of a potato transient expression system based on electroporation of different chimaeric genes into protoplasts isolated from leaves of *Solanum brevidens* and from leaves, tubers, and suspension culture cells of cultivated *S. tuberosum* cv. Désirée. The chimaeric genes used were the reporter genes coding for chloramphenicol acetyl transferase (CAT) and β-glucuronidase (GUS), both under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the promoter of a patatin class II gene. We examined a number of parameters important in electroporation including amplitude and duration of the pulses, time lapse between electroporation and assay, and the effect of DNA concentration. The results of the transient expression of the chimaeric genes in the current study are discussed in relation to previous reports of the stable expression of the genes in transgenic plants.

Materials and methods

Plant cultures

Plant shoot and suspension cultures were maintained as previously described [4, 18, 29]. *In vitro* mini-tubers were grown from axillary node cuttings at 20° C in the dark on Murashige and Skoog medium with sucrose, 60 g l^{-1}, and benzyl amino purine, 5 mg l^{-1} [12]. Tubers were initiated 7–14 days after subculture and continued to grow for a further 2–3 months.

Protoplast isolation

Mesophyll protoplasts were isolated from *S. brevidens* and *S. tuberosum* using the methods of Nelson *et al.* [18] and Foulger and Jones [4] respectively. Protoplasts from suspension cultures were released in an enzyme mixture containing 2% Cellulase R10, 0.3% Macerozyme R10, and 0.2% Pectolyase Y23 [28]. Protoplasts capable of division and plant regeneration were isolated from *in vitro* grown mini-tubers (unpublished). Tuber discs 0.5–1.0 mm thick were placed overnight in A medium [4]. The following day they were pre-plasmolysed (20 min) and protoplasts released by incubation for 4–5 hours in an enzyme mixture containing 0.2% Pectolyase Y23, 0.5% Rhozyme HP150, 1.0% Cellulase R10, and 0.5% BSA, pH 5.6. Released protoplasts were filtered through a 100 μm aperture mesh stainless steel sieve, gently centrifuged at 40 g for 5 min, and resuspended in wash medium containing the major ions of CPW medium [5] with 10% mannitol as osmoticum, pH 5.6. Protoplasts were purified by sedimentation onto a 50% Percoll cushion (40 g for 5 min) and bands collected from the Percoll/wash medium interface and resuspended in wash medium. The protoplasts were washed (1 ×) and resuspended in electroporation medium.

Electroporation

A capacitor discharge system was used that delivered 0 to 450 V pulses at a range of capacitances from 50 to 2000 μF. Protoplasts were electroporated at a density of 0.5–1.0 × 10^{-6}/ml in a pulse medium of 20 mM KCl, with mannitol as osmoticum, pH 7.2. Pulses from the electroporator were delivered into 340 μl of protoplasts suspension in a cylindrical electrode chamber (temperature ca. 4° C, resistance 1.7 kΩ) with stainless steel electrodes 1 cm apart (DiaLog GmBH). Three replicate electroporations were carried out for each experimental parameter. The electroporated samples were pooled and incubated at room temperature for 10 min and then diluted with 10 ml VkCLG medium [4] including antibiotics (250 mg l^{-1} ampicillin; 5 mg l^{-1} gentamycin; 5 mg l^{-1} tetracycline). The protoplasts were cultured in the dark at 26° C before harvest.

Plasmids

The chimaeric plasmids used were: (1) pCaMV 35S-*cat-nos* (CaMV 35S promoter, chloramphenicol acetyl transferase (*cat*) coding region,

nopaline synthase (*nos*) terminator (Walker, unpublished); (2) pCaMV 35S-*gus*-*nos* (as construct 1, with β-glucuronidase (*gus*) coding region [13]; (3) pPOT100 (as construct 1, with a 3.8 kb class II patatin promoter [29, 30]; (4) pPOT413 (as construct 2, with a 3.8 kb class II patatin promoter). Supercoiled forms of the plasmids were used in experiments. DNA concentrations were determined by absorption measurements at 260 nm and by gel electrophoresis.

CAT and GUS assays

CAT and GUS activities were extracted by grinding thawed protoplasts (previously stored in extraction buffer at − 80° C) with sand using a glass rod. The extracts were centrifuged at high speed for 5 min and the supernatant decanted for CAT and GUS assays. CAT activity was determined as described by Gorman *et al.* [9]. Controls were run in parallel with electroporated samples in the presence of 1 unit of bacterial CAT enzyme (Sigma). GUS activity was measured according to Jefferson *et al.* [13].

Protoplast viability

After 48 h culture the number of surviving protoplasts were counted in ten random fields using a Leitz microscope. Dead protoplasts were clearly distinguishable and appeared condensed and crenated. Viability was also confirmed by staining with fluorescein diacetate [31].

Results

Characteristics of protoplasts used

The distinctive phenotypes of the different protoplast – from mesophyll tissue of *S. brevidens* and from mesophyll, tuber, and suspension culture cells of *S. tuberosum* cv. Désirée – are illustrated in Fig. 1. Tuber protoplasts contained many large

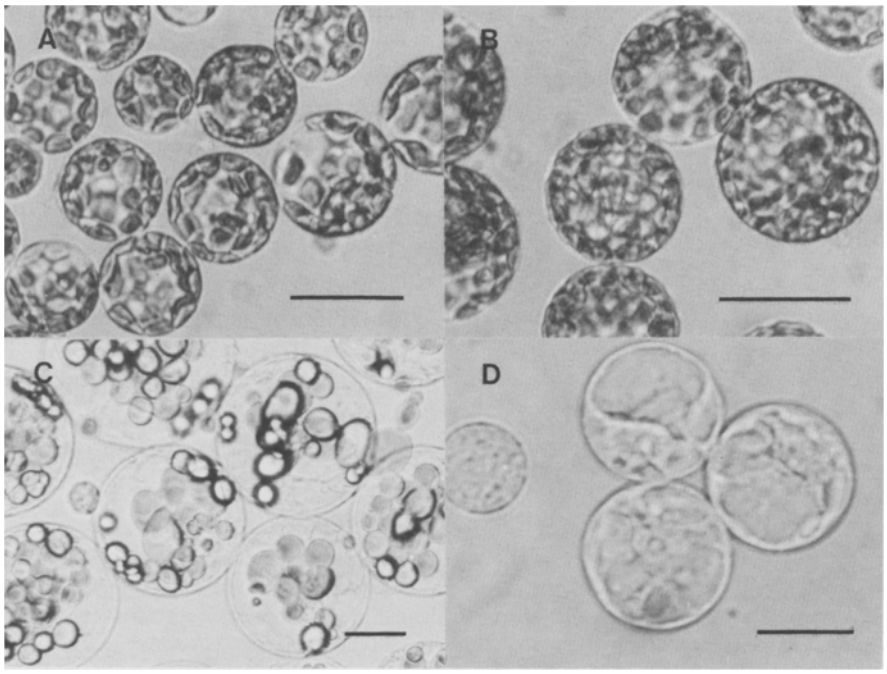

Fig. 1. Freshly isolated protoplasts from *S. brevidens* leaves (A), *S. tuberosum* cv. Désirée leaves (B), Désirée mini-tubers (C), and Désirée suspension culture cells (D). Bars represent 25 μm.

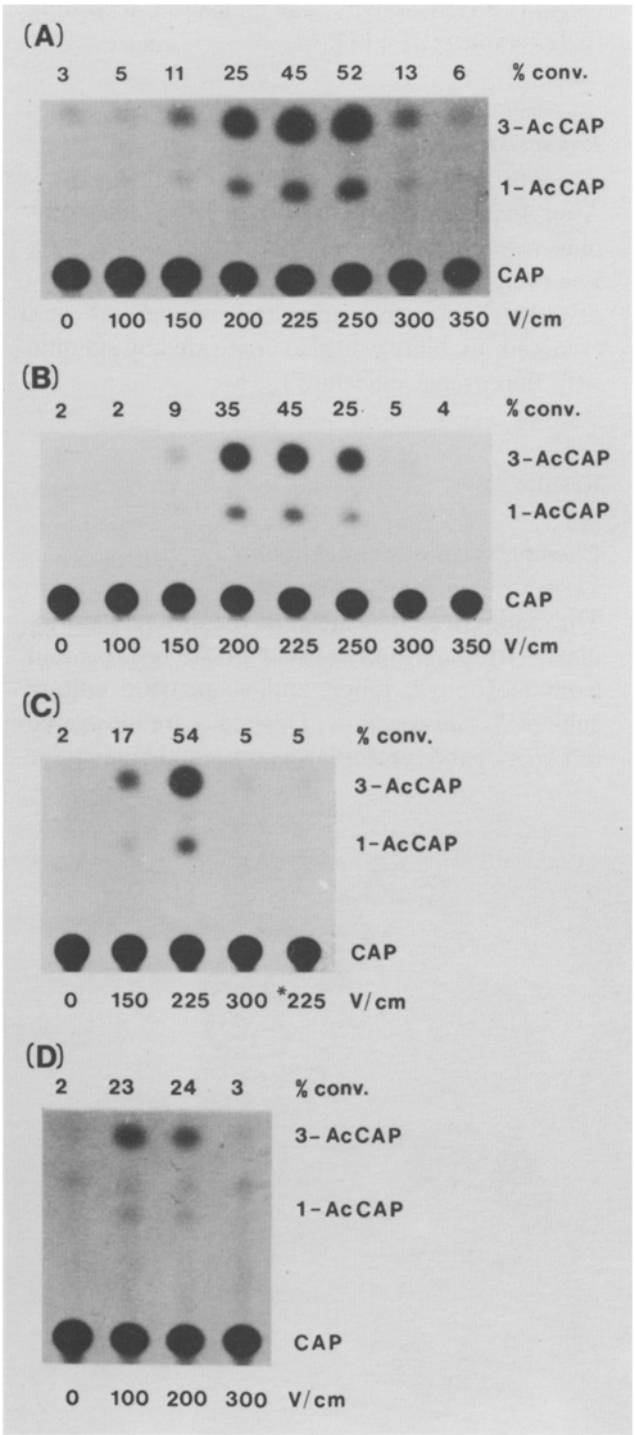

Fig. 2. Effect of field strength on extractable CAT activity from 5×10^5 protoplasts. *S. brevidens* mesophyll protoplasts (A), *S. tuberosum* cv. Désirée mesophyll protoplasts (B), Désirée suspension culture protoplasts (C), and Désirée tuber protoplasts (D). CAT activity was determined 48 h after electroporation. The pulse duration (RC) was 85 ms. The asterisk on the autoradiograph shown for suspension culture protoplasts represent an RC of 250 ms. CAP = chloramphenicol; 1-AcCAP = 1-acetyl chloramphenicol; 3-AcCAP = 3-acetyl chloramphenicol. % conv. = % of CAT converted to acetylated products estimated by scintillation counting.

starch granules. The suspension culture protoplasts were highly cytoplasmic. The average diameters (means of 100 observations) of the protoplasts were: *S. brevidens* mesophyll protoplasts, 24.4 ± 4.9 μm; Désirée mesophyll protoplasts, 27.3 ± 4.1 μm; Désirée suspension culture protoplasts, 32.5 ± 10.5 μm; and Désirée tuber protoplasts, 64.0 ± 17.7 μm.

Effect of field strength on expression of pCaMV 35S-cat-nos

For optimization of electroporation the protoplasts were subjected to three electrical pulses (RC pulse duration = 85 ms) of between 0 and 350 V/cm, with 30 s intervals between the pulses. A plasmid DNA concentration of 20 μg/ml was sufficient for routine measurement of CAT activity. The effect of field strength on CAT expression in the different protoplast are shown in Fig. 2A–D. The optimum field strengths for maximum CAT expression were: *S. brevidens* mesophyll protoplasts = 250 V/cm; Désirée mesophyll protoplasts = 225 V/cm; Désirée suspension culture protoplasts = 225 V/cm; and Désirée tuber protoplasts = 100–200 V/cm. When pulses of longer duration than 85 ms were applied, at field strengths optimum for CAT expression, extractable CAT activity decreased to low levels only marginally above control values. This is illustrated for suspension culture protoplasts in Fig. 2C, which shows the effect of increasing the pulse duration from 85 to 250 ms (obtained by increasing the capacitance from 50 to 150 μF) on CAT activity. Electroporating pCaMV 35S-*gus-nos* gave similar activity profiles in response to increasing field strength. The background GUS activity was low (data not shown).

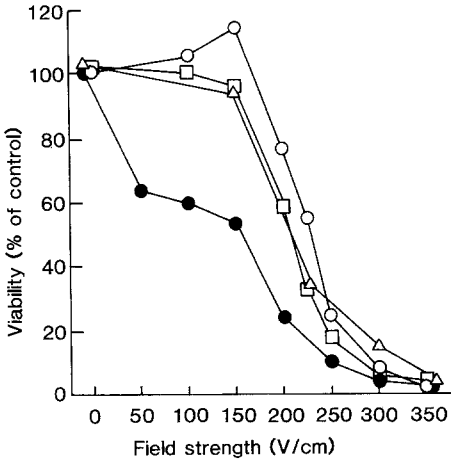

Fig. 3. Effect of field strength on protoplast viability determined 48 h after electroporation. *S. brevidens* mesophyll protoplasts (○); Désirée mesophyll protoplasts (□); Désirée suspension culture protoplasts (△); Désirée tuber protoplasts (●).

Effect of field strength on protoplast viability

The percentage of protoplasts surviving was determined 48 h after electroporation and the results are summarized in Fig. 3. The viability curves recorded were similar, except for tuber protoplasts whose viability was considerably reduced by field strengths as low as 50 V/cm. In contrast, the other protoplasts were only affected by field strengths above 150 V/cm. The data on viability correlate well with the profiles of CAT activity (Fig. 2A–D). It seems therefore that optimum CAT activity occurred when about 50% of the protoplasts were killed by the electric fields.

Time course of CAT appearance

In the previous optimization experiments the protoplasts were harvested for CAT assay 48 h after electroporation. To ascertain how early after electroporation CAT activity could be detected, pCaMV 35S-*cat-nos* was electroporated (225 V/cm) into Désirée mesophyll protoplasts and samples harvested and assayed at different times after electroporation. The results are shown in Fig. 4. CAT activity could be detected as early as 12 h after electroporation, activity thereafter steadily increased, reaching a plateau about 48–60 h after electroporation. Electroporating pCaMV 35S-*gus-nos* gave a similar time course indicating roughly the same rate of transcription,

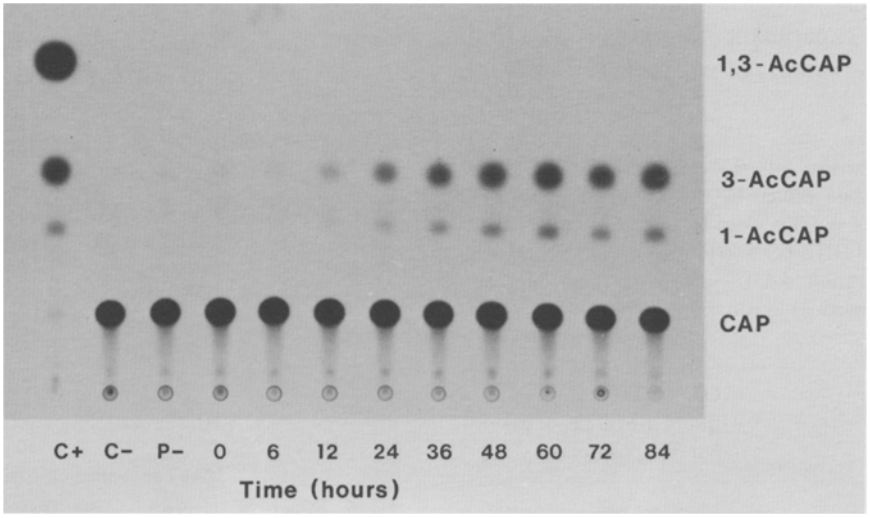

Fig. 4. Time course of CAT appearance in electroporated Désirée mesophyll protoplasts. P- represents the activity of unpulsed protoplasts after electroporation in the presence of plasmid DNA. The zero and other time points represent CAT activity after electroporation using a field strength of 225 V/cm. C+ = CAT activity of 1 unit of bacterial CATase; C− = CAT activity without protoplasts; CAP = chloramphenicol; 1-AcCAP = 1-acetyl chloramphenicol; 3-AcCAP = 3-acetyl chloramphenicol; 1,3-Ac CAP = 1,3-diacetyl chloramphenicol.

translation and protein stability over the 84 h time course (data not shown).

A comparison of the activity of pCaMV 35S-cat-nos *and pPOT100 in tuber and mesophyll protoplasts*

Plasmid DNA at a concentration of 25 μg/ml were electroporated into Désirée tuber and mesophyll protoplasts using a field strength of 150 and 225 V/cm respectively. For determining CAT activity, the protoplasts were harvested 48 h after electroporation and the results are summarized in Fig. 5. pCaMV 35S-*cat-nos* and pPOT100 were expressed at about the same level in both tuber and mesophyll protoplasts. No CAT activity could be detected with the patatin promoter in the reverse orientation. We were surprised to find that pPOT100 was directing high levels of CAT expression in mesophyll protoplasts, and this was further examined using the more sensitive GUS reporter enzyme.

Comparison of the activity of pCaMV 35S-gus-nos *and pPOT413 in Désirée mesophyll protoplasts*

The results of this comparison are summarised in Table 1. In this experiment 7.5 pmol (≃25 μg/ml of pCaMV 35S-*gus-nos*) of plasmid DNA per ml of protoplasts were electroporated and the results can therefore be directly compared in terms of the relative transcriptional activities of the two promoters. The results show that the patatin promoter is transcriptionally more active than the CaMV 35S promoter. We also found that

Table 1. A comparison of *gus* expression in Désirée mesophyll protoplasts driven by the CaMV 35S (pCaMV 35S-*gus-nos*) and a patatin class II promoter (pPOT413). 7.5 pmol of plasmid DNA per 5×10^5 protoplasts per ml was used for electroporation. 4-MU = 4-methyl umbelliferone. Results are presented as mean ± s.d. Three independent transformations.

Plasmid	GUS activity (pmol 4-MU per h per 5×10^4 protoplasts)[1]
Control	40.9 ± 1.6
pCaMV 35S-*gus-nos*	594.6 ± 39.1
pPOT413	674.4 ± 71.6

[1] One-tenth of the protoplasts originally electroporated were used in assays.

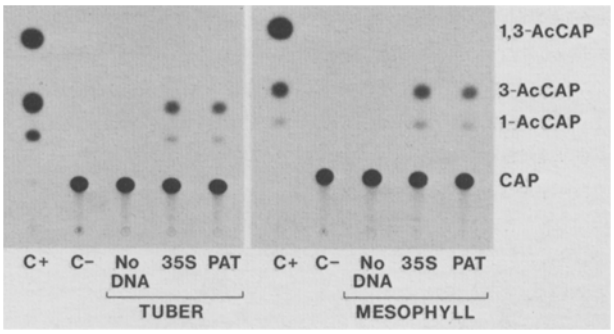

Fig. 5. A comparison of *cat* expression in tuber and mesophyll protoplasts driven by the CaMV 35S promoter (35S) and a patatin class II promoter (PAT). A plasmid DNA concentration of 25 μg per 5×10^5 protoplasts was used. The field strength for DNA delivery was 150 and 225 V/cm for tuber and mesophyll protoplasts respectively. C+ = CAT activity of 1 unit of bacterial CATase; C− = CAT activity without protoplasts; CAP = chloramphenicol; 1-AcCAP = 1-acetylated chloramphenicol; 3-AcCAP = 3-acetylated chloramphenicol; 1,3-AcCAP = 1,3-diacetylated chloramphenicol.

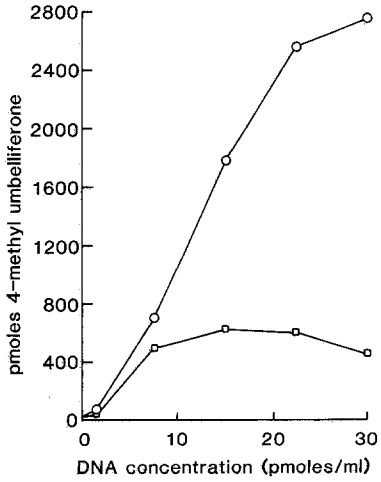

Fig. 6. Effect of electroporating increasing amounts of pPOT413 (○) and pCaMV 35S-*gus-nos* (□) plasmids into Désirée mesophyll protoplasts. GUS activity is indicated as the amount of 4-methyl umbelliferone produced per hour per 5×10^4 protoplasts. Electroporation was at the field strength optimum of 225 V/cm.

pPOT413 was more active than the CaMV 35S promoter when the protoplasts were harvested and assayed as early as 9 h after electroporation. Furthermore, there was no influence of light and dark treatment on the relative activities of the two promoters (data not shown).

On electroporating increasing amounts of plasmid DNA (up to 20–30 pmol/ml of protoplasts; 30 pmol/ml of pCaMV 35S-*gus-nos* $\simeq 100\ \mu g/ml$) the difference between the relative activities of the two promoters became more pronounced (Fig. 6). The patatin promoter continued to direct GUS synthesis up to five-fold higher levels than the CaMV 35S promoter. By comparison, GUS levels directed by the CaMV 35S promoter saturated at comparatively low concentrations of electroporated DNA (approximately 10–20 pmol/ml of protoplasts). A reduction in GUS activity was noted on electroporating very high exogenous concentrations of plasmid DNA (30 pmol/ml of protoplasts).

Discussion

We show in this paper that electroporation can be used routinely for transient expression studies with *Solanum* protoplasts. The field strength and the duration of the pulses were found to be critical for effective gene transfer. Using field pulses of 85 ms duration, the optimum field strengths for maximum reporter enzyme activity were: *S. brevidens* mesophyll protoplasts 250 V/cm; Désirée mesophyll protoplasts 225 V/cm; Désirée suspension culture protoplasts 225 V/cm; and Désirée tuber protoplasts 150 V/cm. These field strengths are lower than those previously reported in the literature [e.g. 6, 27] because of the much longer pulse duration used. The field strength optimum for maximum CAT activity correlated inversely with the size of the protoplasts electroporated, consistent with biophysical theory [19, 33]. The importance of cell size in relation to electroporation efficiency, as indicated in the present study, has also been highlighted recently in a detailed study made with different mammalian cell types [2]. However, factors other than size (e.g. membrane composition) may also be important in certain situations [2, 28].

The electroporation conditions established here can be applied to transfer genes into protoplasts of other important cultivars of *S. tuberosum*. We have found that mesophyll protoplasts of the cvs. King Edward, Maris Piper and Pirola all show a sharp optimum in CAT expression at 225 V/cm. The consistency in response is probably due to the homogeneity in size of the protoplasts; potato mesophyll protoplasts are on average about 26 μm in diameter with a small standard deviation (e.g. Désirée = $27.3 \pm 4.1\ \mu m$; n = 100).

The time course of CAT expression in the current experiments agree with those reported in carrot by Fromm *et al.* [6] and in tobacco by Okada *et al.* [20]. Generally, signals first appear after a few hours and maximum expression occurs about 24–48 hours after gene transfer. A notable exception was a recent study using tobacco mesophyll protoplasts showing maximum CAT activity occurring between 4 and 24 hours after gene transfer [23]. However, as pointed out by these authors, apparent discrepancies may be due to the different protoplast systems and DNA delivery conditions.

Optimization of electroporation enabled us to examine the feasibility of using the system to study the transient expression of homologous potato genes. For these studies we chose a member of the large patatin gene family isolated from cv. Désirée [30]. The promoter of the particular patatin gene (from clone LPOT1), was previously shown to be biologically active, in chimaeric studies using *cat* in transgenic potato, where it induced low levels of CAT expression in tubers only [29]. However, recent unpublished results (D. Twell *et al.*, manuscript in preparation) have shown that the analogous chimaeric patatin-*gus-nos* gene is expressed in leaves as well as in tubers of stably transformed potato plants, although preferential expression in tuber tissues is still maintained.

The present studies show that the patatin class II promoter from LPOT1 transcribes *cat* to comparable levels as the CaMV 35S promoter in both tuber and mesophyll protoplasts. Fusing the

patatin promoter to *gus* allowed a more accurate comparison of the relative activities of the two promoters. This showed that the patatin promoter expressed GUS to higher levels than the CaMV 35S promoter, particularly on electroporating larger amounts of plasmid DNA (20–30 pmol/ml of protoplasts). The DNA-dose response of the two promoters differed significantly, presumably reflecting varied interactions of the introduced templates with transcription factors [16, 26], although at extreme concentrations of electroporated DNA, the possibility of DNA toxicity (resulting in reduced survivability of the protoplasts [32]) may have been responsible for the eventual fall in GUS activity.

The results presented clearly show that the *trans*-acting factors that are necessary for transcription activation of patatin promoter of clone LPOT1 are present in *Solanum* protoplasts, including leaf protoplasts. Furthermore, transient gene expression studies also show that the patatin promoter is functional in leaf protoplasts of *S. brevidens*, a non-tuberising *Solanum* species (data not shown).

Several potato genes have recently been cloned including various members of the patatin multigene family [1, 17, 24, 30], wound inducible genes [15, 25] and viral genes that infect potato [11]. The system we have developed will allow experiments to be carried out, in the homologous protoplast system, to study these genes by transient assays. Recently, we have used the system developed for a functional analysis of the patatin promoter of genomic clone LPOT1. We plan to extend these studies to investigate the DNA dose response of the different mutant constructs. This will give a more complete assessment of their interaction with transcriptional factors, and thus allow more firm conclusions to be made concerning promoter strength.

Acknowledgements

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