ELSEVIER

Biochimica et Biophysica Acta 1243 (1995) 136–142

BBA Biochimica et Biophysica Acta

Lentil root protoplasts: a transient expression system suitable for coelectroporation of monoclonal antibodies and plasmid molecules

Mauro Maccarrone [a,1], Gerrit A. Veldink [a,*], Alessandro Finazzi Agrò [b], Johannes F.G. Vliegenthart [a]

[a] *Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands*

[b] *Department of Experimental Medicine and Biochemical Sciences, University of Rome Tor Vergata, Rome, Italy*

Received 30 March 1994; revised 5 August 1994

Abstract

Protoplasts were isolated from lentil (*Lens culinaris*) roots and their suitability as a transient expression system was investigated. After transfecting the protoplasts with the β-glucuronidase (GUS) gene by either electroporation or polyethylene glycol (PEG), the specific activity of the reporter enzyme and the cell viability were determined. Electroporation was more effective than PEG treatment as transfection procedure and its efficiency was affected by the plasmid length. The feasibility of electro-transferring at the same time (coelectroporation) inhibitory anti-lipoxygenase monoclonal antibodies and the GUS-carrying plasmid pBI 221 was investigated as well. The amount of transferred immunoglobulins was quantitated by ELISA and the inhibitory ability of monoclonal antibodies on the intracellular target enzyme was determined. Evidence is presented for the successful coelectroporation of immunoglobulins and plasmid DNA into lentil protoplasts, the two types of macromolecules acting independently of each other in the recipient cells.

Keywords: Protoplast; Transient expression; Coelectroporation; Monoclonal antibody; Lipoxygenase; (*L. culinaris*)

1. Introduction

Transient expression of reporter genes is a powerful tool in molecular biology, allowing the characterization of gene structure through the analysis of several regulatory elements [1]. Genes coding for key-enzymes have been recently isolated from lentil (*Lens culinaris*) seedlings. In particular, a copper amine oxidase (amine:oxygen oxidoreductase, EC 1.4.3.6) gene has been sequenced [2], the enzyme representing the first example of a mixed substrate-cofactor radical within the family of tyrosine-radical enzymes [3]. The gene coding for a lentil lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) has been isolated as well [4]. To date, for lipoxygenase very little [5] and for amine oxidase no information at all is available on the effects of the modulation of the expression in vivo by means of genetic manipulation. An homologous system for transient expression experiments, i.e. a system based on the same cell-type as the one used to isolate genes, is expected to be more effective than any other foster system. Here, the suitability of lentil root protoplasts as recipient of foreign genes was investigated, showing that the cell machinery can express exogenous DNA into active proteins.

The application of genetic engineering to two (or more) cellular functions can be limited by the competition existing between promoters of coelectroporated plasmids [6], which might compete for DNA binding transcription factors or other cellular molecules [7]. Therefore, a dramatic decrease of the cotransfection frequency of linked or unlinked genes is generally observed [8], rendering difficult the attempt of modulating the expression of two genes at the same time. On the other hand, evidence has accumulated, showing the possibility of electro-transferring monoclonal antibodies into mammalian [9] and plant [10] cells,

Abbreviations: PEG, polyethylene glycol; GUS, β-glucuronidase; GAM-AP, goat anti-mouse IgG conjugated with alkaline phosphatase; 4-MU, 4-methylumbelliferone; LP-LOX, lentil protoplast lipoxygenase; HPOD, hydroperoxyoctadecadienoic acid; BSA, bovine serum albumin

[1] Present address: Department of Experimental Medicine and Biochemical Sciences, IDI-IRCCS Unit, University of Rome Tor Vergata, Via O. Raimondo 00173, Rome, Italy.

* Corresponding author. Fax: +31 (30) 540980; Internet: veldink@accucx.cc.chem.ruu.nl.

SSDI 0304-4165(94)00124-3

where they are able to interact with the intracellular targets. Hence, it appears useful for the study of cellular metabolism.

In the present work lentil protoplasts were used to study the feasibility of coelectro-transferring antibodies and DNA molecules. Coelectroporation of plasmid DNA and monoclonal antibodies could allow the modulation of two different cellular activities, by-passing the competition problems encountered in the coelectroporation of two genes. Inhibitory anti-lipoxygenase monoclonal antibodies [10] and the GUS-carrying plasmid pBI 221 [11] were used in the coelectroporation experiments reported in this paper. Evidence is presented that the two types of macromolecules are able to act simultaneously in the recipient cells, yielding respectively inhibition of the target enzyme and transient expression of GUS.

2. Materials and methods

Chemicals were of the purest analytical grade. All media for plant culture and protoplast isolation were purchased from Sigma (St. Louis, MO, USA). Inhibitory anti-lipoxygenase monoclonal antibodies were prepared as reported [9]. Non-immune mouse serum was purchased from Nordic Immunology (Tilburg, The Netherlands), nitrocellulose filters (0.45 μm) and goat anti-mouse IgG conjugated with alkaline phosphatase (GAM-AP) were from Bio-Rad (Richmond, CA, USA).

2.1. Plasmid

pBI 221 [11], pBI 121 [12] and pAS 6, all carrying the β-glucuronidase (GUS, EC 3.2.1.31) gene under the control of the same 35S promoter, were amplified in *Escherichia coli* cells, electro-transformed according to [13]. pAS 6 consisted of a pEMBL 18 derivative in which the < it > Hin < /it > dIII/ < it > Eco < /it > RI fragment of the GUS cassette [12] was inserted. All plasmids were purified by using the Maxi-prep kit (Qiagen) according to the manufacturer's instructions.

2.2. Plant material and protoplast isolation

Lentil (*L. culinaris*) seeds were sterilized and germinated as described [10]. Protoplasts were isolated from the seedling roots after treatment with cellulase, pectinase and pectolyase as reported for lentil leaves [10].

2.3. Electroporation

Protoplasts were resuspended in 10 mM Hepes, 50 mM NaCl, 5 mM $CaCl_2 \cdot 2H_2O$ and 0.4 M mannitol, pH 7.2 (solution A), counted in a Fuchs-Rosenthal chamber and brought to a final concentration of $1.5 \cdot 10^6$ lentil protoplasts/ml. The protoplast suspension was aliquoted in 0.4 cm electroporation cuvettes (0.7 ml/cuvette), in the presence of different amounts of plasmid. Salmon sperm DNA (Sigma), sized 5–20 kb by shearing, was added as a carrier at a final concentration of 50 μg/cuvette. Samples were mixed by gentle inversion and were electroporated at a resistance of 12 Ω and different values of capacitance and field strength, using a Gene Pulser apparatus (Bio-Rad) to generate and deliver exponentially decaying pulses. The average time constant (τ) values throughout the experiments were 1.5 ± 0.2 ms (at 125 μF), 7.5 ± 1.0 ms (at 500 μF) and 13.3 ± 1.9 ms (at 960 μF). After electroporation, protoplasts were kept for 10 min on ice, washed twice in solution A by centrifugation at $70 \times g$ for 5 min and transferred to Petri dishes containing 5 ml of Kao and Michayluk culture medium [14]. After 24 h at 28°C in the dark, protoplasts were checked for viability according to Widholm [15]. Finally, they were centrifuged twice at $70 \times g$ and were resuspended in GUS extraction buffer [11]. For the coelectroporation experiments, $1.0 \cdot 10^6$ lentil root protoplasts were electroporated at 12 Ω, 960 μF and different voltage values, in the presence of immunoglobulins and/or plasmid pBI 221. After electroporation, protoplasts were washed as described above and were resuspended in 5 ml of culture medium and kept for different periods of time at 28°C in the dark. Then, protoplasts were centrifuged twice at $70 \times g$ and were resuspended in 0.1 M sodium phosphate, pH 6.8, containing 0.1% Tween 20, and disrupted by two cycles of rapid freezing (−80°C) and thawing (+25°C). Cell extracts were brought to a protein concentration of 1 mg/ml with buffer and aliquots were used for protein assay, lipoxygenase activity and immunochemical determinations.

2.4. PEG transfection

The procedure described by Saul et al. [16] was adopted, i.e. incubating root protoplasts with different amounts of plasmids in PEG solution for different periods of time and then resuspending them in culture medium [14] for 24 h at 28°C in the dark. Cell extracts were prepared for GUS assay as described above.

2.5. Enzyme assays

GUS activity was determined by the fluorimetric method of Jefferson [11] and was expressed as pmol 4-MU (4-methylumbelliferone) $\cdot$ $min^{-1} \cdot mgP^{-1}$. Lentil protoplast lipoxygenase (LP-LOX) activity was measured as reported previously [10], using linoleic acid as substrate and recording the absorbance of the product hydroperoxyoctadecadienoic acid (HPOD). LP-LOX specific activity was expressed as pmol HPOD $\cdot$ $min^{-1} \cdot mgP^{-1}$. Protein concentration of the protoplast extracts was determined according to Bradford [17], using bovine serum albumin (BSA) as standard.

2.6. Immunochemical analyses

The amount of anti-lipoxygenase monoclonal antibodies electro-transferred into lentil protoplasts was quantitated by ELISA [18]. Cell extracts (100 μg/well) were reacted with 3000-fold diluted goat anti-mouse IgGs conjugated with alkaline phosphatase (GAM-AP, Bio-Rad). Color development of the alkaline phosphatase reaction was recorded at 405 nm, using *p*-nitrophenylphosphate as substrate. The A_{405} values of the samples were within the linearity range of a calibration curve, made with pure monoclonal antibodies in the concentration range 0–300 ng/well. Controls included non-immune mouse serum as second antibody and wells coated with different amounts of BSA. Western-blotting was performed as reported [10]. The experimental points represent the mean of three independent determinations (SD < 8%). Control values from different experiments were normalized in order to reduce the variation between series [19].

3. Results

3.1. Transient expression of GUS-carrying plasmids

The effect of various experimental parameters in the transient expression experiments was investigated. Pilot electroporation experiments performed with pBI 221 showed that transient expression of the reporter gene increased with increasing plasmid concentrations, reaching a plateau at 20 μg/test. GUS specific activity was greatly affected by the capacitor size, being undetectable at 125 μF and increasing approximately 5-fold by increasing the capacitance from 500 μF to 960 μF (Fig. 1). Moreover, GUS expression increased as a function of the field strength, reached a maximum at 400 V/cm and then declined at higher voltages (Fig. 1).

The efficiency of electroporation in delivering plasmid DNA to lentil protoplasts was compared to that obtained with the PEG procedure. In the search of optimal conditions for PEG transfection, the incubation time of the cells with PEG was critical (Fig. 2). The highest transient expression values were recorded after 30 min of incubation, a condition where 70% of the protoplasts were still viable. Doubling the incubation time did not increase the GUS activity, while the cell survival was reduced to 40% of the control (Fig. 2). Moreover, the efficiency of PEG-induced transfection could not be enhanced by increasing the plasmid concentration above 10 μg/test (data not shown). Optimal PEG-mediated transfection was half the electroporation-mediated one. Thus, factors which could affect the electroporation efficiency were investigated in more detail. The possible role of the plasmid length on the reporter gene expression was examined, using three different plasmids, all carrying the same GUS cassette [12] under the control of the same 35S promoter, namely pBI 221 (5.7 kb), pAS 6 (7.0 kb) and pBI 121 (12.8 kb). Taking into account the molecular mass of the plasmids, electroporation was performed in the presence of 20 μg pBI 221 or 25 μg pAS 6 or 45 μg pBI 121 per test; thus, protoplasts were incubated with the same number of plasmid molecules in the different experiments. In Fig. 3 it is shown that larger plasmids yielded a lower specific activity of the

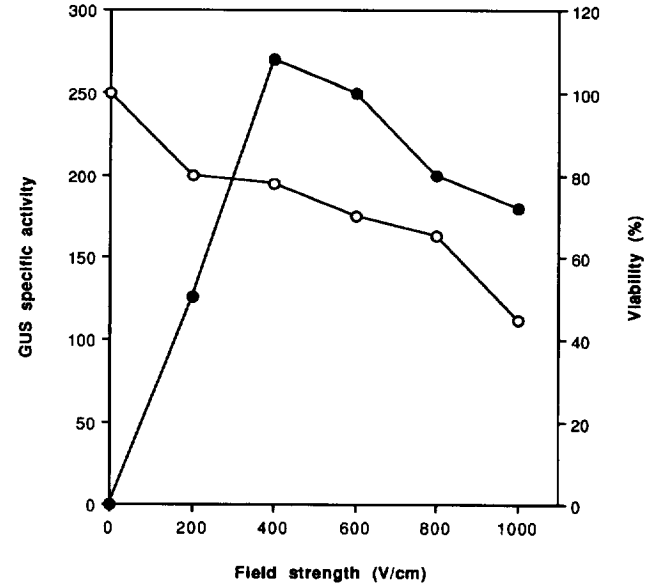

Fig. 1. Effect of field strength on electroporation efficiency. GUS specific activity (●) and viability (○) values were measured after electroporation at 960 μF and different field strengths. Experiments were performed by using 20 μg pBI 221/test. GUS specific activity was expressed as described in 'Materials and Methods'.

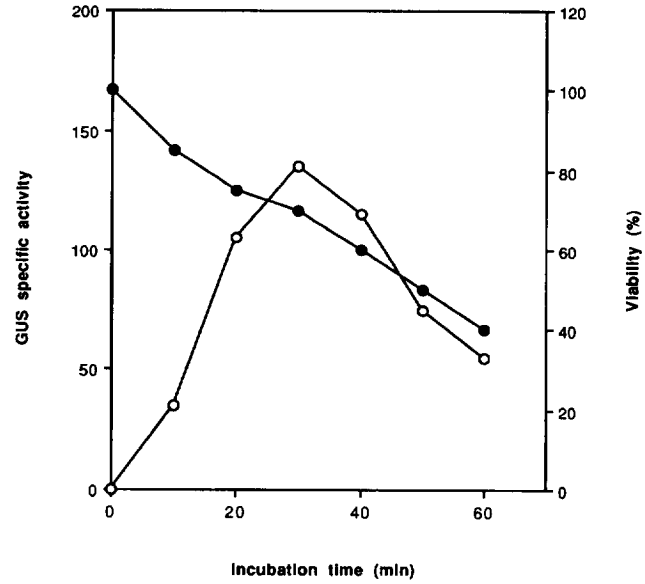

Fig. 2. Effect of incubation time on PEG transfection. Lentil root protoplasts were subjected to the PEG treatment in the presence of 10 μg pBI 221/test, then GUS specific activity (●) and viability (○) values were determined. GUS specific activity was expressed as described in 'Materials and Methods'.

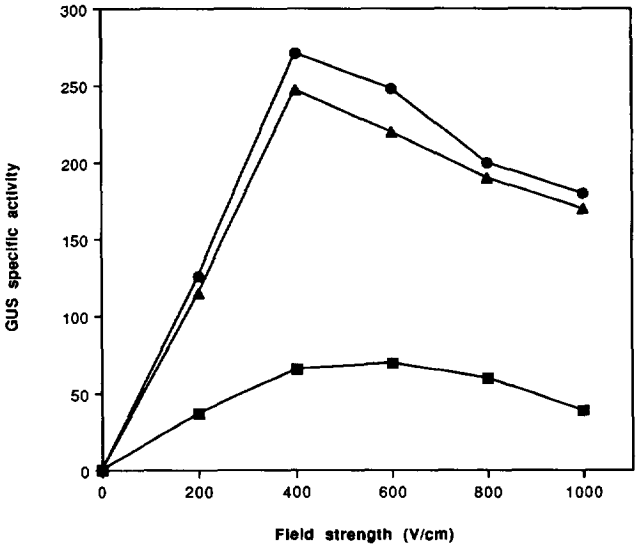

Fig. 3. Effect of plasmid length on transfection. Root protoplasts were electroporated at 960 μF in the standard electroporation mixture, containing either 20 μg pBI 221 (●) or 25 μg pAS 6 (▲) or 45 μg pBI 121 (■) per test. Viability values were not affected by the plasmid length, thus being the same as in Fig. 1. GUS specific activity was expressed as described in 'Materials and Methods'.

reporter enzyme. In particular, transfection with pBI 121 yielded a GUS expression approximately 4-fold lower than transfection with pBI 221, a plasmid approximately half the size of pBI 121. On the other hand, specific activity values slightly lower than those obtained with pBI 221 were achieved by using pAS 6, a plasmid slightly larger than pBI 221 (Fig. 3). The plasmid size did not affect cell viability, which had the same profile as shown in Fig. 1, independently of the plasmid used.

3.2. Incorporation of monoclonal antibody into lentil protoplasts

Pilot experiments were run in order to optimize the coelectroporation procedure. Immunoglobulins were detected by ELISA in extracts of lentil protoplasts which had been subjected to electroporation in the presence of 200 μg/test of monoclonal antibodies (Fig. 4). The transfer efficiency increased with increasing field strengths in the range 0–800 V/cm, and was not affected by the presence of plasmid pBI 221 (20 μg/test) in the electroporation mixture (Fig. 4). The absorbance values of the samples related to a calibration curve (Fig. 4, inset) represented the amount of incorporated antibodies. Using 200 μg immunoglobulins for the electroporation of $1.0 \cdot 10^6$ protoplasts, it was calculated that the antibody incorporation amounted to 0.20–0.74 μg, with voltages in the range of 200–800 V/cm. This gives a maximum incorporation of 0.74 pg antibody/protoplast (1.45 μg antibody/mg protein).

3.3. Interaction of monoclonal antibody and plasmid during electroporation

Lentil protoplasts were electroporated at a field strength of 800 V/cm, in the presence of 200 μg/test of monoclonal antibodies and different amounts of pBI 221, to ascertain whether increasing amounts of plasmid might interfere with immunoglobulin incorporation. It is shown in Fig. 5A that the transfer efficiency of monoclonal antibodies into the protoplasts remained the same, whatever the plasmid concentration in the mixture. When the complementary experiment was performed, by electroporating lentil cells in the presence of 20 μg pBI 221 and different amounts of antibodies, the specific activity of the reporter enzyme was found to be unaffected by the presence of immunoglobulins in the electroporation medium (Fig. 5B).

3.4. Effect of coelectroporating pBI 221 on the incorporated monoclonal antibody

LP-LOX activity was assayed in cell extracts after electroporation at 12 Ω, 960 μF and different field strengths, in the range 0–1000 V/cm, in the presence of 200 μg/test of monoclonal antibodies. The LP-LOX activity was inversely related to the amount of immunoglobulins incorporated in the same samples. In Fig. 6 it is shown that the electro-transferred antibodies were able to inhibit the intracellular target enzyme, similarly to what was observed with cell free solutions. The presence of 20 μg/test of pBI 221 did not affect the inhibition of LP-LOX by electroporated antibodies (Fig. 6).

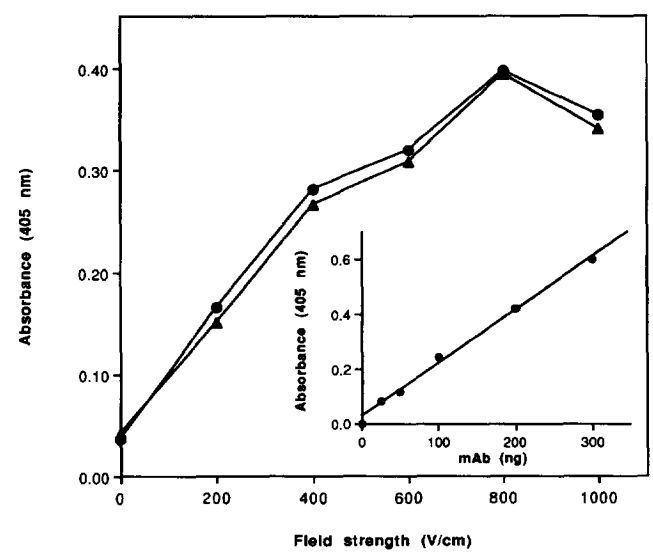

Fig. 4. Quantitation of monoclonal antibodies electro-transferred into lentil protoplasts. Monoclonal antibodies were detected by ELISA in extracts of cells cultured for 4 h after electroporation at 960 μF with (▲) or without (●) pBI 221. Inset, calibration line, drawn by using different amounts of monoclonal antibodies in the range 0–300 ng/well.

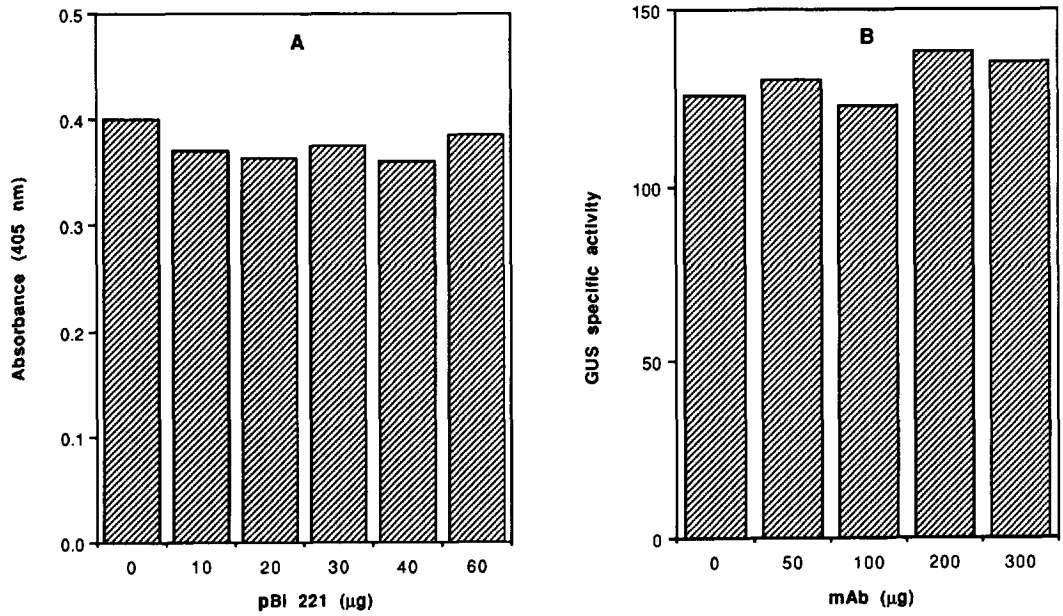

Fig. 5. Detection of monoclonal antibody and pBI 221 after coelectroporation. (A) ELISA test of extracts of lentil protoplasts subjected to electroporation in the presence of monoclonal antibodies (200 μg) and different amounts of pBI 221. (B) GUS specific activity of protoplasts electroporated in the presence of pBI 221 (20 μg) and different amounts of monoclonal antibodies. GUS specific activity was expressed as described in 'Materials and Methods'.

To investigate the possible effect of the presence of pBI 221 on the stability of the antibodies inside the cells, a time-course experiment was performed. Lentil protoplasts were electroporated at 800 V/cm in the presence of 200 μg/test of monoclonal antibodies, then they were kept in culture medium for different periods of time and cell extracts were finally analysed in the ELISA test. A linear decrease of the antibody concentration was found in protoplasts grown up to 48 h after the electric shock (Fig. 7). Moreover, immunoglobulins detected in cell extracts after 48 h of culture displayed structurally intact heavy chains,

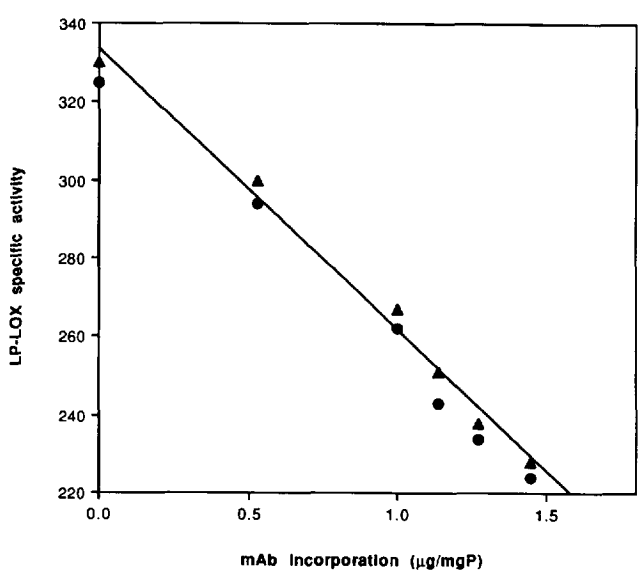

Fig. 6. Inhibition of lentil protoplast lipoxygenase by electro-transferred monoclonal antibody. Lentil protoplast lipoxygenase (LP-LOX) specific activity was assayed in cell extracts electroporated in the presence of 200 μg monoclonal antibody (●) or 200 μg monoclonal antibody + 20 μg pBI 221 (▲). Points correspond to field strengths of 0, 200, 400, 600, 1000 and 800 V/cm, respectively.

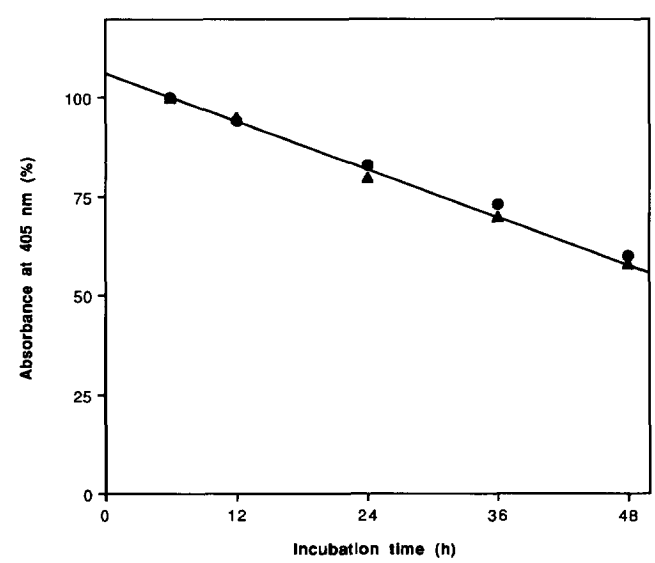

Fig. 7. Time-course of monoclonal antibody stability in lentil protoplasts. The ELISA test was performed on extracts of cells incubated for different periods of time in culture medium, after electroporation with 200 μg monoclonal antibody (full circles) or 200 μg monoclonal antibody + 20 μg pBI 221 (▲). A_{405} values, corrected for the absorbance of the non-electroporated sample (i.e., 0.040), are expressed as percentage of the maximum (i.e., 0.420).

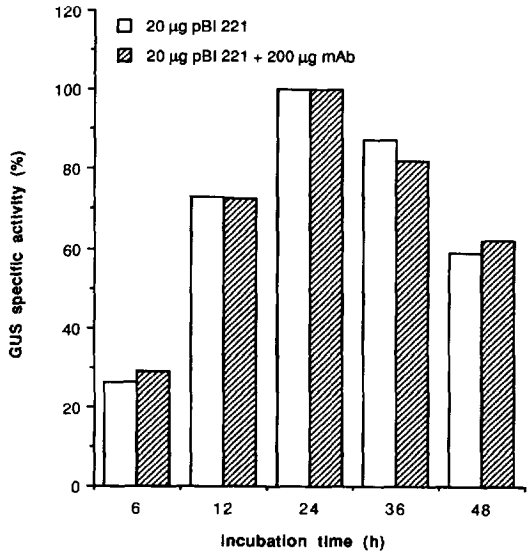

Fig. 8. Time-course of the expression of the reporter gene. GUS specific activity was assayed in extracts of protoplasts incubated for different periods of time in culture medium, after electroporation with 20 μg pBI 221 or 20 μg pBI 221 + 200 μg monoclonal antibody. GUS activity is expressed as percentage of the maximum (i.e., 140 pmol 4-MU $\cdot$ min^{-1} $\cdot$ mgP^{-1}).

as shown by Western blot analysis (data not shown). Both stability of monoclonal antibodies and integrity of their heavy chains were not affected by the presence of plasmid DNA (20 μg/test) (Fig. 7).

3.5. Time-course of GUS expression

The effect of monoclonal antibodies on the expression of the reporter gene was investigated by assaying GUS activity in lentil protoplasts electroporated at 800 V/cm in the presence of 20 μg/test of pBI 221, with or without 200 μg/test of immunoglobulins. After electroporation, protoplasts were kept for different times in culture medium, then GUS expression was determined. In Fig. 8 it is shown that the GUS gene was transiently expressed in the recipient cells, reaching a maximum after 24 h of incubation, and then decreased linearly. The profile of GUS expression did not change when monoclonal antibodies were included in the electroporation mixture (Fig. 8).

4. Discussion

Root cuttings offer several advantages over other plant organs in transfection and regeneration experiments [20]. Lentil roots were a suitable source of healthy protoplasts, yielding clean, spherical, 90% viable cells with a dense cytoplasm and an average diameter of 40 μm. Here, these protoplasts are shown to be suitable as a transient expression system, leading to measurable activities of reporter enzymes. At variance with other transient expression systems [21], electroporation is the method of choice for direct gene transfer into the root-derived cells (Figs. 1 and 2). The dependence of the transfer efficiency upon the field strength (Fig. 1) can be attributed to an increasing membrane permeability at increasing voltage values, as predicted by Laplace theory [22] and observed in other model systems [23]. Interestingly, the electroporation efficiency was dependent on the plasmid dimension as well (Fig. 3). This result, so far reported only in prokaryotic cells [24], should be taken into account when optimizing electro-transfection protocols for plant protoplasts and other eukaryotic systems, smaller vectors seeming preferable over larger ones. Such a dependency was not observed with PEG treatment and might be attributed to a slower diffusion of larger plasmids through the electropores induced in the plasma membrane during the electric pulse.

Recently, it has been reported that cellular functions can be modulated in vivo by introducing into the cells antibodies directed against specific antigens, leading to the possibility of carrying out several biochemical and physiological studies [10,25–27]. Here, it is shown that root protoplasts can be loaded with monoclonal antibodies (Fig. 4). Interestingly, no competition was observed between antibodies and plasmid molecules for the uptake through the electropores in the plasma membranes (Figs. 4 and 5). Moreover, the incorporated macromolecules did not impair each other's activity or processing in the cell (Figs. 7 and 8). Therefore, the antibody/plasmid mixtures do not show the mutual interferences observed in plasmid/plasmid [6–8] or other macromolecule/plasmid [28] mixtures. Coelectroporation of immunoglobulins and plasmids seems attractive for studying biochemical processes where the concerted action of several enzymes is involved. The combination of genetic manipulation with the use of antibodies, able to either inhibit or stimulate [29] catalysis, might allow to modulate couples of enzyme activities in the cell, overcoming the competition problems encountered in the coelectroporation of different genes. More generally, the feasibility of coelectroporating protein and DNA, reported here, might be instrumental in elucidating basic processes like trans-activation of gene expression by the coelectrotransferred protein [30]. Finally, it is noteworthy that the use of lentil protoplast technology might open the avenue to the introduction of new traits for agricultural benefit in this economically important crop, widespread all over the world but still awaiting improvement strategies [31–33].

Acknowledgements

The authors thank Dr. O. Vorst (Department of Molecular Cell Biology, Utrecht University) for the plasmid pAS 6 and Dr. G. van Duyn and Mr. R. Derksen (CIVO-TNO, Zeist, The Netherlands) for the assistance in preparing the monoclonal antibodies. They are grateful to European Community for financial support (Grant SC1-0197).

References

[1] Brough, C.L., Gardiner, W.E., Inamdar, N.M., Zhang, X.-Y., Ehrlich, M. and Bisaro, D.M. (1992) Plant Mol. Biol. 18, 703–712.
[2] Rossi, A., Petruzzelli, R. and Finazzi Agrò, A. (1992) FEBS Lett. 301, 253–257.
[3] Pedersen, J.Z., El-Sherbini, S., Finazzi Agrò, A. and Rotilio, G. (1992) Biochemistry 31, 8–12.
[4] Hilbers, M.P., Rossi, A., Finazzi Agrò, A., Veldink, G.A. and Vliegenthart, J.F.G. (1994) Biochin. Biophys. Acta, 1211, 239–242.
[5] Deng, W., Scott Grayburn, W., Hamilton-Kemp, T.R., Collins, G.B. and Hildebrand, D.F. (1992) Planta 187, 203–208.
[6] Zaghmout, O.M.-F. and Wang, W.C. (1992) Anal. Biochem. 204, 239–243.
[7] Schöler, H.R. and Gruss, P. (1984) Cell 36, 403–411.
[8] Christou, P. and Swain, W.F. (1990) Theor. Appl. Genet. 79, 337–341.
[9] Chakrabarti, R., Wylie, D.E. and Schuster, S.M. (1989) J. Biol. Chem. 264, 15494–15500.
[10] Maccarrone, M., Veldink, G.A. and Vliegenthart, J.F.G. (1992) Eur. J. Biochem. 205, 995–1001.
[11] Jefferson, R.A. (1987) Plant Mol. Biol. Rep. 5, 387–405.
[12] Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
[13] Fiedler, S. and Wirth, R. (1988) Anal. Biochem. 170, 38–44.
[14] Kao, K.N. and Michayluk, M.K. (1975) Planta 126, 105–110.
[15] Widholm, J.M. (1972) Stain Technol. 47, 189–194.
[16] Saul, M.W., Shillito, R.D. and Negrutiu, I. (1988) in Plant Molecular Biology Manual (Gelvin, S.B. and Schilperoort, R.A., eds.), A1, pp. 1–16. Kluwer Academic Publishers, Dordrecht.
[17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[18] Abdillahi, H. and Poolman, J.T. (1987) FEMS Microbiol. Lett. 48, 367–371.
[19] Zaghmout, O.M.-F. and Wang, W.C. (1992) Anal. Biochem. 204, 239–243.
[20] Lambert, C. and Tepfer, D. (1992) Theor. Appl. Genet. 85, 105–109.
[21] Dewulf, J. and Negrutiu, I. (1991) in A Laboratory Guide for Cellular and Molecular Plant Biology (Negrutiu, I. and Gharti-Chhetri, G.B., eds.), pp. 116–121. Birkhäuser Verlag, Basel.
[22] Zimmermann, U. and Vienken, J. (1982) J. Membrane Biol. 67, 165–182.
[23] Zimmermann, U. (1982) Biochim. Biophys. Acta 694, 227–277.
[24] Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988) Nucl. Acids Res. 16, 6127–6145.
[25] Uno, I., Fukami, K., Kato, H., Takenawa, T. and Ishikawa, T. (1988) Nature 333, 188–190.
[26] Chakrabarti, R., Pfeiffer, N.E., Wylie, D.E. and Schuster, S.M. (1989) J. Biol. Chem. 264, 8214–8221.
[27] Berglund, D.L. and Starkey, J.R. (1991) Cytometry 12, 64–67.
[28] Sukharev, S.I., Klenchin, V.A., Serov, S.M., Chernomordik, L.V. and Chizmadzhev, Yu.A. (1992) Biophys. J. 63, 1320–1327.
[29] Wheelock, M.J., Richards, T.J., Carroll, R.T. and Funk, M.O. (1991) Arch. Biochem. Biophys. 288, 578–583.
[30] Berkhout, B., Silverman, R.H. and Jeang, K.T. (1989) Cell 59, 273–282.
[31] Ladizinsky, G., Cohen, D. and Muehlbauer, F.J. (1985) Theor. Appl. Genet. 70, 97–1001.
[32] Saxena, P.K. and King, J. (1987) Plant Sci. 52, 223–227.
[33] Malik, K.A. and Saxena, P.K. (1992) Aust. J. Plant Physiol. 19, 731–740.