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Incorporation of fluorescein-conjugated anti-mouse immunoglobulin G into permeabilized *Nicotiana tabacum* BY-2 cells

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Abstract

A simple and efficient procedure for the transient permeabilization of cultured cells has been developed for the incorporation of a high molecular weight substance, fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (FITC-IgG, 150 kDa) into *Nicotiana tabacum* BY-2 cells grown in suspension cultures. The cells were treated with cell permeabilization solutions (CPS) comprising varying concentrations of glycerol (Gly), sucrose (Suc), ethylene glycol (EG) and dimethyl sulfoxide (DMSO). When the cells were permeabilized by treating with CPS-3 (Gly:Suc:EG:DMSO = 20:5:20:5, w/v%) containing FITC-IgG for 15 s at room temperature followed by washing with ice-cold 1 M sucrose and modified LS medium, 76% of the cells incorporated FITC-IgG and 78% of the cells remained viable according to fluorescein diacetate (FDA) staining. Most treated cells retained intact internal structure and the incorporated FITC-IgG was detected in cytoplasm and in the nuclear region. Cells of different ages were studied. Four day-old cells were found suitable for the incorporation of FITC-IgG and cell survival. Inclusion of 10 mM $CaCl_2$ in CPS-3 improved both FITC-IgG uptake and cell survival. The regrowth of treated cultures lagged for the first 3 days, then followed a growth pattern similar to the untreated controls.

Keywords: Antibody; Cell wall and plasma membrane porosity; FITC-IgG; Protein incorporation; *Nicotiana tabacum* BY-2 cell line; Permeabilization.

1. Introduction

One of the important functions of the plasma membrane and cell wall of plant cells is to work as barriers to protect the plasma from the outside environment and control the transport and entry of large molecules. Transient and reversible permeabilization of this barrier would offer several useful applications, for example, (i) bioconversions in which cofactors are required and substrates/products or both are not taken up or released by intact cells, (ii) release of intracellularly stored secondary products, (iii) in situ assay system for macromolecular synthesis (particularly proteins and nucleic acids), and (iv) blocking of a particular functional molecule or receptors by a specific antibody.

Permeabilization of plant cells has been attempted using chemical and physical treatments [1,2]. The physical treatments include the use of ultrasound [3], hypotonic shock [4], and electroporation [5,6]. Several chemicals have been shown to be effective. Soybean suspension cells have been

Abbreviations: CPS, cell permeabilization solution; DMSO, dimethyl sulfoxide; FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; LS medium, Linsmaier and Skoog's medium.

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permeabilized to calcein, FITC-bovine serum albumin (BSA) by the use of DMSO and hypotonic shock [7], dextrans and proteins of graded sizes by treating with pectinase [8], deoxynucleoside triphosphate and DNase I with L-α-lysophosphatidylcholine [9] and FITC-conjugated goat anti-rabbit IgG by the use of saponin [10]. Some organic solvents successfully permeabilized cells but cell viability tended to be rather low [2]. Methods that could permeabilize cells only transiently without hindering cell viability and growth are ideal. Presentation of quantitative information on the uptake frequency of macromolecules by permeabilized plant cells is also needed since the earlier reports lack such data.

Here we report a transient cell permeabilization procedure for high incorporation of FITC-IgG with preserved viability by the use of cocktails comprising glycerol, sucrose, ethylene glycol and DMSO. To obtain quantitative data, a homogeneous cell culture, tobacco BY-2 was employed [11].

2. Materials and methods

2.1. Cell culture

The tobacco (*Nicotiana tabacum*) BY-2 cell line was obtained from Prof. T. Nagata of Tokyo University and maintained in modified Linsmaier and Skoog's (LS) medium supplemented with 370 mg/l KH_2PO_4, 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% sucrose, in the dark at 27°C with continuous shaking [12]. In case of semi-solid medium 0.8% agar was added before autoclaving.

2.2. Cell permeabilization solutions

The cell permeabilization solutions (CPS) were composed of glycerol: sucrose: ethylene glycol: DMSO (all by w/v%) in the following ratio: CPS-1, 20:5:30:10; CPS-2, 20:5:25:5; CPS-3, 20:5:20:5; CPS-4, 20:10:10:5; CPS-5, 20:5:20:0; CPS-6, 20:10:0:10; CPS-7, 20:5:0:5; each containing 10 mM $CaCl_2$.

2.3. Permeabilization of cells

The tobacco suspension cultures were harvested on the fourth day of culture on 50 μm mesh unless otherwise noted and washed with distilled water before use. About 10 mg cells were permeabilized using 100 μl of CPS containing 1 μl of FITC-IgG (FITC anti-mouse IgG H + L(rb) (150 kDa) from Miles Scientific, USA) for 15 s at room temperature and then immediately washed twice with 1 ml of ice-cold 1 M sucrose solution containing 10 mM $CaCl_2$ and then once with 1 ml of modified LS medium. The cells were pelleted by centrifugation at $780 \times g$ for 5 min at 0°C in between the washings.

As varying factors, different permeabilization solutions, duration of permeabilization, addition of $CaCl_2$ in CPS, cell age and different diluting conditions were tested for the effects on incorporation of FITC-IgG into tobacco cells and cell viability.

The FITC-conjugated IgG mixed with CPS was passed through a Sephadex G-25 column and the eluates were monitored with a UV spectrophotometer to check for any free fluorescein originating from the FITC-IgG by the procedure described above.

2.4. Microscopy

The viability of the permeabilized cells was determined by staining the cells with fluorescein diacetate (FDA) [13]. For both viability measurements and incorporation of FITC-IgG following each treatment, 100 cells or more in each of 50 randomly selected areas (total 5000 cells) were observed for each treatment using an Olympus UV-microscope. The control cells showed only marginal autofluorescence during the 7-day culture period. The experiments were repeated three times and one data set from the replicates were shown. In the other two sets, similar results were obtained.

2.5. Regrowth of cells

For the regrowth of permeabilized cells, about 500 mg (fresh weight) of cells were permeabilized using 1 ml of CPS-3 with 10 μl FITC-IgG as described above. The cells were washed twice with 10 ml of ice-cold 1 M sucrose solution containing 10 mM $CaCl_2$. The third washing was done using modified LS medium. The cells were collected by centrifugation and blotted over two layers of filter paper. A part of the cells was used for assessment

Table 1
Incorporation of FITC-IgG into tobacco cells and survival of the cells using different cell permeabilization solutions comprising glycerol (Gly), sucrose (Suc), ethylene glycol (EG) and dimethyl sulfoxide (DMSO)[a]

Permeabilization solution	Concentrations (w/v%)				Cells showing FITC-IgG incorporation (%)	Cell survival assessed from FDA staining (%)
	Gly	Suc	EG	DMSO		
Control[b]	–	–	–	–	0	98.0 ± 4.1
CPS-1	20	5	30	10	93.9 ± 2.8	21.4 ± 1.7
CPS-2	20	5	25	5	87.4 ± 3.4	37.0 ± 1.5
CPS-3	20	5	20	5	76.4 ± 2.6	78.2 ± 2.5
CPS-4	20	10	10	5	58.3 ± 1.9	56.8 ± 3.2
CPS-5	20	5	20	0	49.6 ± 1.6	76.0 ± 2.9
CPS-6	20	10	0	10	65.7 ± 1.8	68.1 ± 4.3
CPS-7	20	5	0	5	60.0 ± 2.0	77.3 ± 1.4

[a] About 10 mg cells were permeabilized using 100 μl of CPS containing 1 μl of FITC-IgG for 15 s at room temperature and then immediately washed twice with 1 ml of ice-cold 1 M sucrose solution containing 10 mM $CaCl_2$ and then once with 1 ml of modified LS medium. The data are the mean ± SE ($n = 50$).
[b] The control cells were suspended in modified LS medium with FITC-IgG and washed with the medium.

of viability and incorporation of FITC-IgG under microscopy. The remaining cells were cultured in small clusters (each cluster weighing about 25–30 mg fresh mass) on modified LS semi-solid medium in the dark at 27°C. After 21 days, the fresh mass of the cells was measured.

The solutions were prepared using GR grade chemicals and autoclaved at 1.06 kg/cm^2 for 15 min before use. All operations were performed under aseptic conditions.

3. Results

A combination of glycerol, sucrose, ethylene glycol and DMSO at various concentrations were used for permeabilizing the tobacco BY-2 cells to FITC-IgG (Table 1). Of the various cell permeabilization solutions used, CPS-3 resulted in both higher FITC-IgG incorporation into the cells and cell survival (Table 1). Although permeabilization of cells with CPS-1 and CPS-2 resulted in high incorporation of FITC-IgG, the viability of the permeabilized cells was low. Omission of either ethylene glycol or DMSO from CPS led to reduced success.

The duration of permeabilization is critical for both higher incorporation of FITC-IgG and survival of the cells (Table 2). The optimal results were obtained using a 15 s incubation. While the uptake of FITC-IgG increased with increasing incubation period, the viability of cells decreased considerably.

The addition of $CaCl_2$ to CPS-3 resulted in improved uptake of FITC-IgG and cell survival (Table 3). The best results were obtained with 10 mM $CaCl_2$. At 100 mM $CaCl_2$ concentration, the incorporation of FITC-IgG declined.

Using tobacco cells of different ages, the highest incorporation of FITC-IgG and survival of the cells were observed with cells harvested on the fourth day of culture (Table 4). Increasing cell age (5–7 days) corresponded with decreased uptake and survival. During this period, the viability of untreated control cells also declined.

The washing of cells twice with 1 M sucrose containing 10 mM $CaCl_2$ and once with modified LS medium was better (76% cells showed FITC-IgG incoroporation and 78% cells survived) than

Table 2
Effect of the duration of permeabilization on incorporation of FITC-IgG into tobacco cells and survival of the cells[a]

Time (s)	Cells showing FITC-IgG incorporation (%)	Cell survival assessed from FDA staining (%)
10	45.2 ± 3.9	79.4 ± 3.0
15	76.7 ± 1.4	77.9 ± 2.7
20	76.6 ± 3.1	68.9 ± 1.8
25	79.2 ± 2.5	52.8 ± 3.4
30	80.4 ± 4.1	46.4 ± 2.5

[a] Experimental procedures were the same as those described in Table 1 except that cells were permeabilized with CPS-3 for different time periods.

Table 3
Effect of addition of $CaCl_2$ in the permeabilization solution on the incorporation of FITC-IgG into tobacco cells and survival of the cells[a]

$CaCl_2$ mM	Cells showing FITC-IgG incorporation (%)	Cell survival assessed from FDA staining (%)
0	54.4 ± 1.3	63.5 ± 1.7
1	61.7 ± 4.3	67.0 ± 1.4
10	73.5 ± 3.9	76.6 ± 2.7
100	66.5 ± 7.1	72.1 ± 1.2

[a] The cells were permeabilized with CPS-3 containing different concentration of $CaCl_2$ for 15 s and washed twice with 1M sucrose without $CaCl_2$ and then once with 1 ml of modified LS medium. The other experimental conditions were the same as given in Table 1.

washing twice just with 1 M sucrose plus 10 mM $CaCl_2$ (72% cells showed FITC-IgG incorporation and 71% cells survived).

Following the permeabilization with CPS-3 and washing procedures described above, the morphology of the cells remained intact and no increase in lysis of cells or destruction of subcellular organization could be observed (Fig. 1A). However, the cell morphology did not remain intact in all those CPS which resulted in sharp decline in the cell survival. The permeabilized cells showed fluorescence from FITC-IgG in the peripheral and nuclear regions as well as in cytoplasmic strands (Fig. 1B). Fig. 1C shows the plasmolyzed cells in phase view and the corresponding fluorescent image (Fig. 1D) confirmed that fluorescence was located only in the cytoplasm and not in the wall. The figure also shows that plasma membrane impermeability to osmoticum (1 M sucrose) was recovered and that the cells showed plasmolysis similar to untreated control cells. The permeabilized cells showed high viability, which was evident from the high fluorescent emission from FDA-stained cells (Fig. 2B). Separation of the mixture of FITC-IgG in CPS-3 using a Sephadex G-25 column revealed that no free fluorescein existed in the CPS-3 (data not shown).

The regrowth of the permeabilized cells was initially lower for the first 3 days (data not shown) and then followed a similar pattern as that of control (Fig. 3). A weak fluorescence from incorporated FITC-IgG was noticeable in almost all the dividing cultured cells for about 7 days which faded off later (data not shown). An increase of about 15–16-fold in fresh weight was observed for the permeabilized cells as compared to about 20-fold in the case of the untreated control cells.

Cells from suspension cultures of *Daucus carota* (var. Kuroda Gosun) were also treated with CPS using the protocol described above in order to check the applicability of the permeabilization procedure to other cell lines. The treatment with CPS-3 was found to be the best for the incorporation of FITC-IgG and FITC-dextran blue and regrowth ability which were similar to tobacco cells (data not shown).

4. Discussion

Of the permeabilization solutions used in the present study, a fine balance of glycerol, sucrose, ethylene glycol and DMSO (20:5:20:5 w/v%) present in CPS-3 was most effective in incorporating high molecular weight FITC-IgG and at the same time retaining cell viability (Table 1). As a general trend, the cell viability decreased as the concentration of the chemicals used increased (Table 1) and with longer incubation period (Table 2) while the incorporation of FITC-IgG was inversely proportional to the cell viability. Permeabilization of plant cells have been attempted using various chemicals such as DMSO, toluene, phenethyl alcohol, Triton X-100, but often resulted in the reduced viability of cells following permeabilization [2,7]. Inclusion of more than one substance in the permeabilization solution at low concentrations may have lead to higher survival. The use of multi-components at low concentrations resulted in a similar increase in the cell survival and decrease in the toxicity of the cryoprotective solutions that have similar components as CPS [14].

The permeabilization shown here may involve more than one step: modification of the cell wall and plasmalemma resulting in pore formation in the membrane, followed by the uptake of FITC-IgG through hypertonic and/or hypotonic shock. While glycerol and sucrose in CPS may provide needed osmotic stress, ethylene glycol and DMSO bring about permeation of the cell wall and plasma membranes. The organization of the pectic substances is a major controlling element in defining the sieving properties of the cell wall [8]. DMSO is often used for solubilization of polysac-

Table 4
Incorporation of FITC-IgG into tobacco cells of different ages

	Days of culture					
	2	3	4	5	6	7
% of cells showing[a] FITC-IgG incorporation	61.2 ± 4.3	70.5 ± 2.6	76.4 ± 2.6	71.3 ± 2.1	61.2 ± 3.2	54.9 ± 2.6
% cell survival[a] (FDA-stained cells)	70.4 ± 2.3	76.5 ± 2.7	78.2 ± 2.5	58.4 ± 3.9	37.8 ± 4.1	19.5 ± 1.7
% viability of untreated control cells (FDA-staining)	90.8 ± 4.9	96.8 ± 3.7	98.0 ± 4.1	89.1 ± 5.3	87.3 ± 2.1	82.9 ± 5.0

[a] The permeabilization conditions were the same as given in Table 1 except that cells of different ages were treated with CPS-3.

charides such as pectin from the cell wall and is also known to increase membrane fluidity. The presence of 10% glycerol has been reported to completely inhibit the release of cellular materials and enzymes [1] and ethylene glycol, in addition to permeabilization of cells, provides protection against dehydration injury at low concentrations.

In cells permeabilized with CPS-3, the incorporated FITC-IgG was localized both in the cytoplasm and nuclear region (Fig. 1). A similar cytoplasmic localization of incorporated calcein was observed in soybean protoplasts permeabilized with hypertonic shock (3 M) [7]. When soybean cells were permeabilized with saponin, the incorporated FITC-IgG was also visible only in the cytoplasm and not in the vacuoles [10]. Soy-

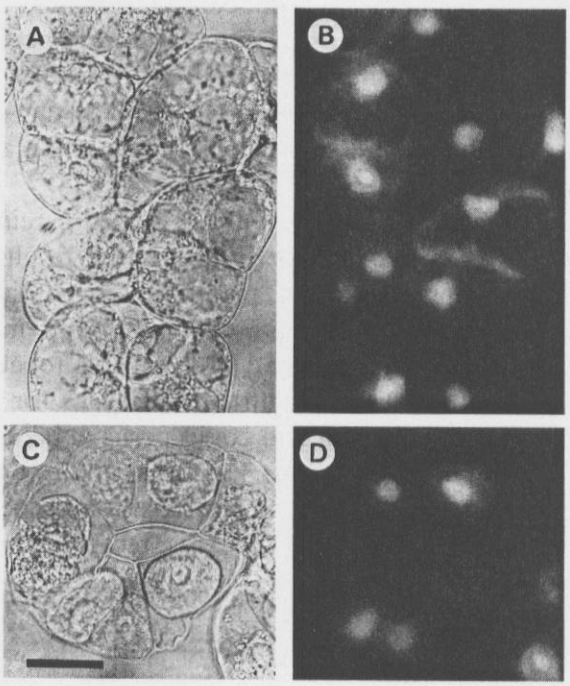

Fig. 1. Tobacco BY-2 cells permeabilized with CPS-3 in the presence of FITC-IgG. A–B, permeabilized cells after washings with 1 M sucrose followed by modified LS medium, showing intact morphology and internal organization in the phase contrast view (A) and the corresponding fluorescent image (B) shows strong fluorescence from FITC-IgG localized in the peripheral and nuclear regions as well as in the cytoplasmic strands. C–D, cells plasmolyzed with 1 M sucrose following permeabilization with CPS-3 and washing. Plasma membrane impermeability to osmoticum (1 M sucrose) was recovered (C, phase view) and fluorescence from FITC-IgG was localized in cytoplasm and not in cell walls (D, corresponding fluorescent image). The bar represents 25 μm.

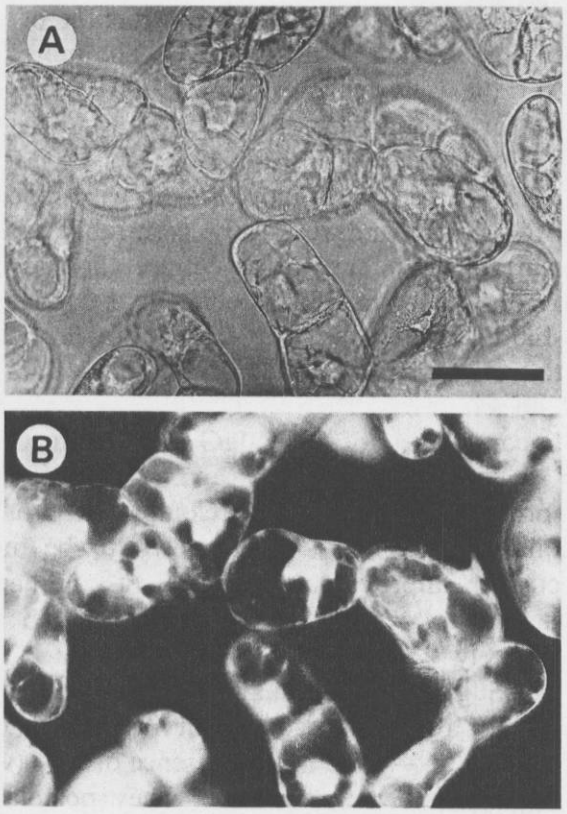

Fig. 2. A phase contrast view (A) and corresponding fluorescent image (B) of tobacco cells stained with FDA after being permeabilized with CPS-3 in the absence of FITC-IgG. The bar represents 50 μm.

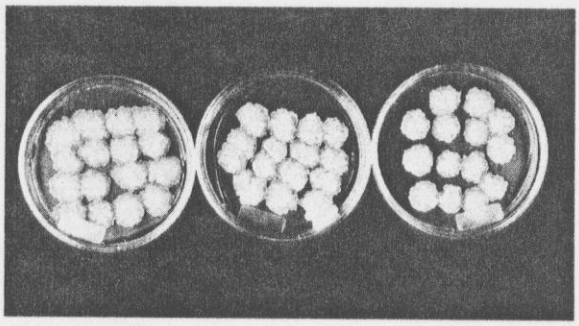

Fig. 3. Regrowth of tobacco BY-2 cells permeabilized with CPS-3 after 21 days of culture on semi-solid medium. A, untreated control cells (552 mg). B, permeabilized cells without FITC-IgG (437 mg). C, permeabilized cells with FITC-IgG (426 mg). The figures in parentheses indicate the average fresh mass of a cluster (14–16 clusters per petri dish), which initiated from 25–30 mg of cells.

bean protoplasts permeabilized with DMSO and hypotonic shock, in contrast, showed diffused fluorescence of incorporated FITC-BSA or calcein from both the cytoplasm and vacuoles [7]. Since CPS has a high osmolarity, FITC-IgG may be taken up by tobacco cells during hypertonic shock generated by CPS-3. CPS-3 permeabilized tobacco cells without lysis of cells or destruction of the inner organization (Figs. 1 and 2). The morphology of the cells remained intact and cells were viable for a long term (Fig. 3). Permeabilization with CPS-3 may be transient and reversible. Meiners et al. [10] reported that treatment with saponin can transiently and reversibly permeabilize soybean root cells in suspension culture allowing the incorporation of macromolecules in the range of 20–140 kDa, however, they did not present quantitative cell permeabilization and viability data.

The inclusion of $CaCl_2$ in CPS-3 improved both the incorporation of FITC-IgG and cell survival. Ca^{2+} is known to stabilize plasma membranes during protoplast isolation [15]. Compared to other cations, the Ca ion is also known to be most effective in improving the survival of cold-hardy plant cells following a freeze–thaw cycle to approximately $-10°C$ without cryoprotectants [16,17]. Sugawara and Sakai [18] showed that addition of $CaCl_2$ increased the survival of tobacco cells frozen to $-22°C$ in the presence of 10% (v/v) DMSO and 5% (w/v) glucose. They postulated that Ca^{2+} may be involved in repairing some deleterious changes produced in the plasma membranes during freeze–thawing since the Ca^{2+} effect was achieved even when Ca^{2+} was added to the cells immediately after thawing [18]. Ca^{2+} enhances the cell recovery from osmotic shock-induced protein release and membrane transport disturbance [19] and helps restore the normal membrane permeability following γ-irradiation [20]. More recently, extracellular $CaCl_2$ has been shown to inhibit apoptosis-like nuclear degradation and DNA fragmentation [21]. The Ca ion is also involved in the signal transduction controlling stress tolerance induction [22]. Protection mechanisms similar to some of these may be involved in the action of $CaCl_2$ in improving the cell viability and FITC-IgG incorporation.

The high incorporation of FITC-IgG and cell survival could be partly attributed to the unique characteristics of tobacco BY-2 cell line. These cells are reported to have exceptionally high growth rates and high homogeneity and the cell wall is marginally lignified as it lacks autofluorescence throughout the subculture period [11]. FITC-IgG uptake and cell survival during permeabilization and osmotic shock were dependent on the age of the BY-2 cells (Table 4). Four day-old cells had the highest FITC-IgG incorporation and cell survival.

In conclusion, the procedure described herein is a reversible type of cell permeabilization resulting in high incorporation of macromolecules where cell morphology and regrowth ability are marginally affected. The method was useful in tobacco BY-2 and carrot suspension cultured cells for incorporating antibodies and could perhaps be used for even larger molecules. Various applications may be expected such as in situ assays and locating macromolecules and blocking receptors with specific antibodies.

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