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Permeabilization of the plasmalemma and wall of soybean root cells to macromolecules

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Abstract. A technique has been developed that results in the reversible permeabilization of the cell wall and plasmalemma of soybean (*Glycine max* (L.) Merr.) root cells grown in suspension and callus culture. Cells in culture are treated with saponin (0.1 mg/ml) for 15 min at room temperature. They are then coincubated in separate experiments with fluorescent-derivatized dextrans (20–70 kDa) or fluorescein-conjugated goat anti-rabbit immunoglobulin G to ascertain the exclusion size of macromolecules capable of diffusing across the cell wall and plasmalemma into the cytoplasm. Following an incubation period of 30 min, it was observed by conventional and confocal fluorescence microscopy that all derivatized macromolecules tested (20–140 kDa) could be incorporated into the cytoplasm, but not into the vacuole. This procedure did not appear to affect cell viability adversely. A normal doubling time was observed for these cells following the permeabilization procedure.

Key words: Cell wall – Cell culture (permabilization) – *Glycine* (plasmalemma permeabilization) – Plasmalemma permeabilization – Saponin

Introduction

A variety of techniques presently exist to introduce impermeant molecules into the cytoplasm of living cells (McNeil 1989). These methods have, for the most part, been developed for use with either animal cells in tissue culture or with plant protoplasts. The procedures, therefore, are principally designed to permeabilize, in a temporary and reversible manner, the plasmalemma. Plant cells, on the other hand, are a more difficult biological material for cytoplasmic incorporation of macromolecules. The impermeant lipid bilayer of the plasmalemma, in conjunction with the molecular-sieving properties of the cell wall, have resisted most permeabilization procedures and have required more exotic approaches, such as coating micrometer-sized projectiles with the molecule to be included and then shooting these coated projectiles under high pressure into the cells. This technique has been most successfully employed for incorporation of DNA (Klein et al. 1988; McCabe et al. 1988). In a previous effort to examine the wall porosity in soybean cells (Baron-Epel et al. 1988a), we found that we could substantially enhance the trans-wall diffusion of macromolecules by mild treatment of cells grown in suspension culture with pectinase. Following such a treatment, molecules as large as soybean agglutinin were found to enter the space between cell wall and plasmalemma. More importantly, cells treated in this manner maintained their viability, as judged by their ability to undergo repeated plasmolysis (Baron-Epel et al. 1988a). These observations provided evidence that the molecular-sieving properties of the wall could be substantially modified without seriously affecting cell viability. In our initial efforts to incorporate macromolecules into plant cells, this pectinase treatment was utilized in conjunction with saponin, a plant glycoside that perturbs membranes. Such a binary treatment resulted in the incorporation of high-molecular-weight dextrans and immunoglobulins into the cell cytoplasm. Subsequent work has demonstrated that the pectinase treatment is unnecessary and that saponin (0.1 mg · ml^{-1}) by itself is capable of transiently and reversibly permeabilizing whole soybean root cells in suspension culture while maintaining cell viability.

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Abbreviations: FDA = fluorescein diacetate; FITC-20 kDa, FITC-40 kDa, FITC-70 kDa dextrans = fluorescein-derivatized 20-kDa, 40-kDa, and 70-kDa dextrans; IgG = immunoglobulin G; kDa = kilodalton

Material and methods

Cell culture, permeabilization, and analysis. Cells (SB-1 cell line) derived from soybean (*Glycine max* (L.) Merr. cv. Mandarin) roots were grown in 1B5C medium in darkness (Ho et al. 1986). Five milliliters of 4-d-old cells were pelleted by centrifugation in a tabletop centrifuge for 5 min at 460 · *g*. The supernatant was dis-

carded, and the cells were resuspended in 5 ml of fresh 1B5C medium containing 0.1 mg · ml^{-1} saponin (Sigma Chemical Co., St. Louis, Mo., USA). The cells were incubated in the saponin solution for 15 min at room temperature, washed three times by pelleting and resuspension in 1B5C medium, and incubated in a variety of fluorescein-derivatized macromolecules or fluorescein diacetate (FDA). The procedure for loading calli grown on agar was identical except for the elimination of the centrifugation step. The incubation periods and fluorescent probes employed were as follows: 10 min for FDA (Molecular Probes, Junction City, Ore., USA); 30 min for FITC-conjugated dextrans (20 kDa, 40 kDa, and 70 kDa) (Sigma); and 30–60 min for FITC-conjugated goat anti-rabbit immunoglobulin G (IgG; Bio-Rad, Richmond, Cal., USA). The FITC-conjugated goat anti-rabbit IgG was purified by chromatography on a Sephadex G–25 column prior to use, to remove possible contamination with free fluorescein. All fluorescent macromolecules were used at a concentration of 1 mg · ml^{-1}, while FDA was used at 10 μg · ml^{-1}. Following incubation, the cells from suspension culture were again washed in 1B5C medium and mounted on slides in 70% (v/v) glycerol in 1B5C medium containing 5% (w/v) of the anti-bleaching agent *n*-propyl gallate (Sigma). The slides were viewed with a Leitz Orthoplan epifluorescent microscope (Leitz, Wetzlar, FRG) using a 40× or 25× objective lens. Labeled calli were placed on coverslips and optical sectioning was performed with a 100× objective (oil immersion) utilizing the ACAS 570 Confocal Interactive Laser Cytometer (Meridian Instruments, Okemos, Mich., USA).

Cell-proliferation assay. Twenty milliliters of a 4-d-old culture of soybean root cells (SB–1) were pelleted by centrifugation at 460 · *g* for 5 min. The cell pellet was then resuspended in fresh 1B5C medium containing 0.1 mg · ml^{-1} saponin (Sigma) and incubated in this solution for 15 min at room temperature. The cells were then washed five times by pelleting and resuspension in 1B5C medium. After the final wash, the cells were resuspended in 50 ml of 1B5C medium and grown for 80 h in darkness. Cell proliferation was measured as previously described (Schindler et al. 1989). An aliquot (500 μl) of suspended cells was added to a 1.5-ml Eppendorf tube that was pre-weighed. Equal aliquots were also dehydrated and the dry weight was determined for comparison. The wet- and dry-weight measurements for each time point were performed in triplicate. Variability for both wet and dry weight between samples was less than 15%.

Results and disussion

Incorporation of FL-dextrans into the cell cytoplasm and preservation of cell viability. Soybean root cells (SB–1 cell

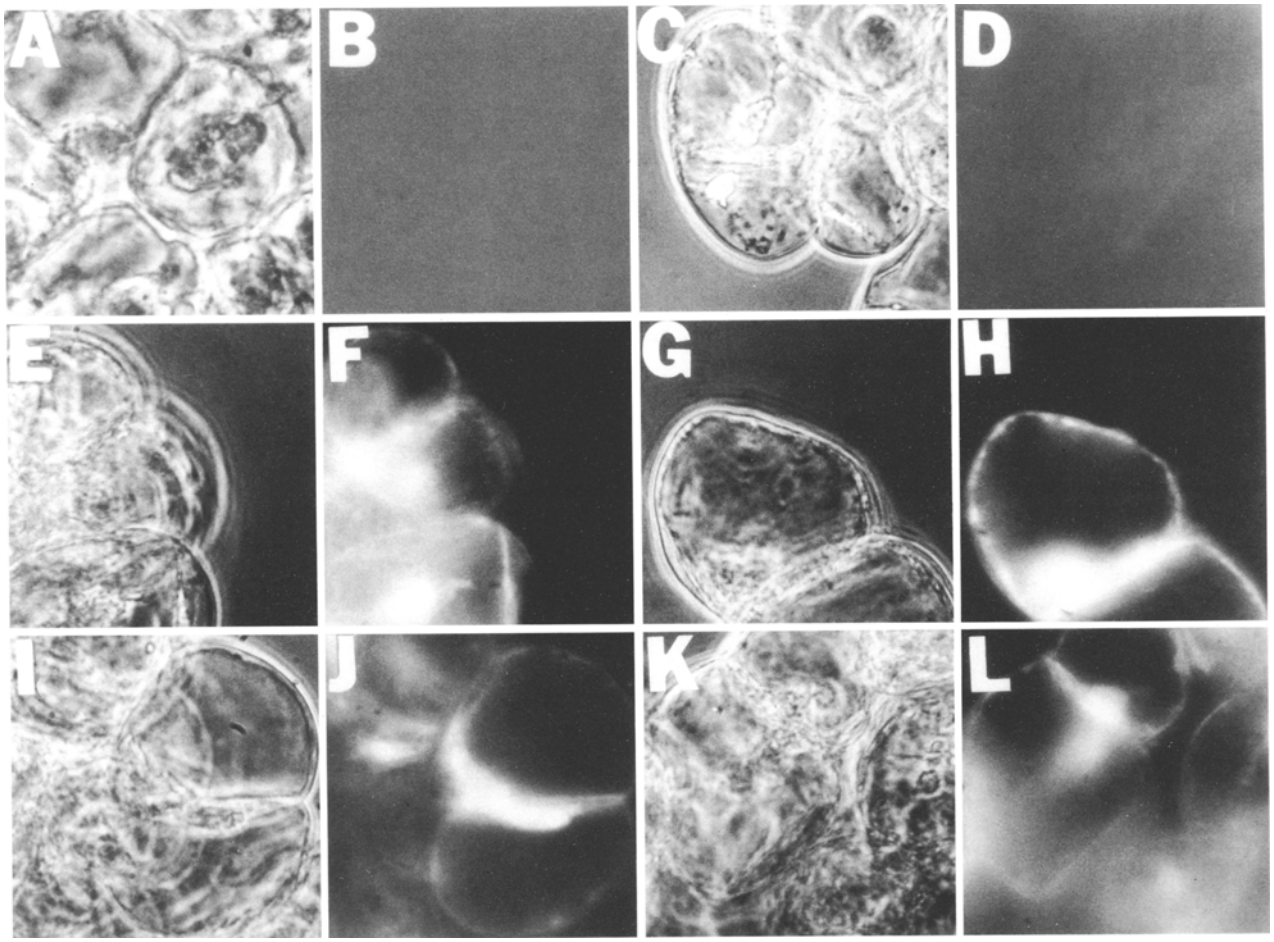

Fig. 1A–L. Fluorescent labeling of soybean cells grown in suspension culture following saponin treatment × 400. In **A, B,** cells are either treated with saponin but not incubated with fluorescently-derivatized dextrans, or **C, D,** not treated with saponin but incubated with FITC–40 kDa dextran for 30 min. In **E, F,** cells are treated with saponin and then incubated for 30 min with FITC–20 kDa dextran, while in **G, H,** cells are treated with FITC–40 kDa dextran, and in **I, J,** cells are treated with FITC–70 kDa dextran. In **K, L,** cells are incubated for 10 min with FDA. **A, C, E, G, I,** and **K** are phase views; **B, D, F, H, J,** and **L** are the corresponding fluorescent images

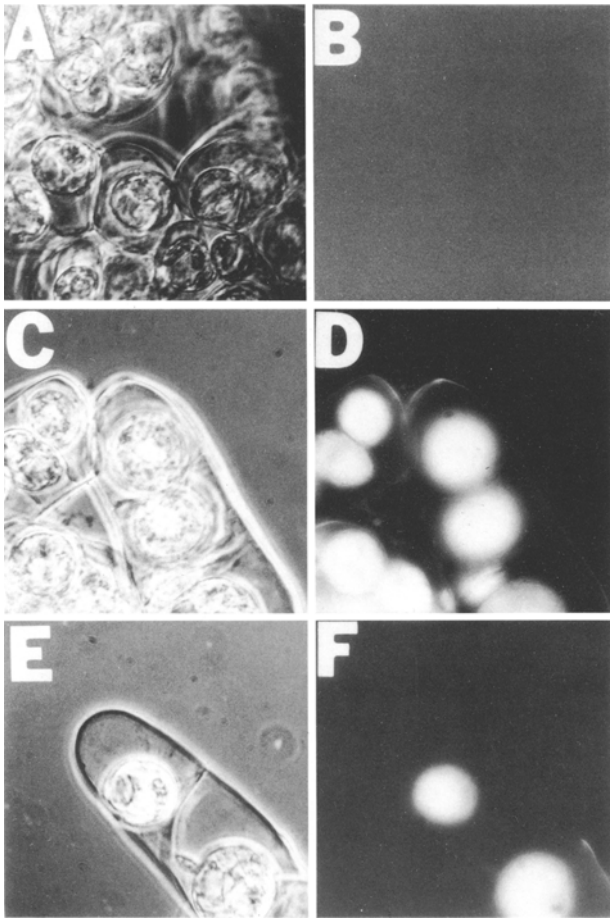

Fig. 2A–F. Cytoplasmic labeling of plasmolyzed soybean cells × 250. **A, B** show cells not treated with saponin and incubated with FITC–40 kDa dextran for 30 min and plasmolyzed as described in *Material and methods*. **C, D** show cells treated with saponin and incubated with FITC–40 kDa dextran for 30 min, followed by plasmolysis. **E, F** show cells treated with saponin and incubated with FITC–70 kDa dextran for 30 min, followed by plasmolysis. **A, C,** and **E** are phase views; **B, D,** and **F** are the corresponding fluorescent images

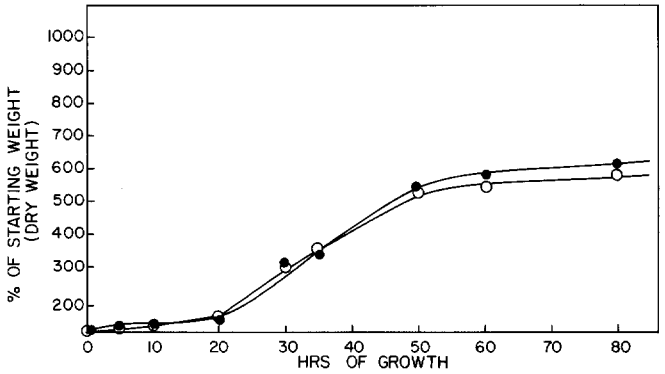

Fig. 3. Proliferation of soybean cells grown in suspension culture. The cells were cultured in 1B5C medium as described in *Material and methods* and treated with (●) or without (○) saponin prior to growth analysis. Dry weight was utilized to evaluate growth. Each point consists of the average of three samples. Variability between samples did not exceed 15%

line) were grown in suspension culture and then incubated with saponin (0.1 mg · ml^{-1}) and fluorescent probes as described in *Material and methods*. Fluorescein-derivatized dextrans of approx. 20 kDa and 40 kDa were found to incorporate into the cell cytoplasm but not into the vacuole, as shown in Fig. 1. Control cells that were saponin-treated without addition of fluorescent macromolecules were observed to be morphologically intact by phase microscopy (Fig. 1A) and to exhibit no intracellular fluorescence (Fig. 1B). Cells not treated with saponin but incubated with FITC–40 kDa dextran and then washed as described in *Material and methods* also appeared normal by phase microscopy (Fig. 1C). In addition, these cells did not exhibit intracellular accumulation of fluorescence (Fig. 1D). Saponin treatment of cells in the presence of FITC–20 kDa dextran (Fig. 1E, F) and FITC–40 kDa dextran (Fig. 1G, H) for 30 min now demonstrated the inclusion of both dextran sizes. Note the labeling of the peripheral and nuclear regions (Fig. 1F, H) and the exclusion of fluorescence from the vacuolar space (Fig. 1F, H). Experiments utilizing FITC–70 kDa dextran (Stokes radius of approx. 5.5 nm; Baron-Epel et al. 1988a) also demonstrated intracellular accumulation (Fig. 1I, J).

In an effort to demonstrate that the labeling occurs predominantly in the cytoplasmic compartment, two approaches were chosen, as illustrated in Fig. 1K, L and Fig. 2. Fluorescein diacetate [FA] has previously been shown to accumulate in the cytoplasm of soybean cells (Baron-Epel et al. 1988b). As can be seen in Fig. 1L, the intracellular localization of FDA matches that observed for the fluorescent-derivatized dextrans. In Fig. 2, cells were plasmolyzed (Baron-Epel et al. 1988a) following the permeabilization procedure to provide additional evidence that the incorporated macromolecules are localized to the cytoplasm. As shown in Fig. 2C, D for the FITC–40 kDa dextran and in Fig. 2E, F for the FITC–70 kDa dextran, fluorescence is observed in the resulting protoplasts but not the intracellular space between the plant cell wall and plasmalemma (Fig. 2D, F). A particularly important feature of this permeabilization technique is that it apparently has no serious effect on the viability of the treated cells and their ability to proliferate as shown by dry-weight (Fig. 3) and wet-weight (data not shown) determinations as an indicator of cell division (Schindler et al. 1989).

Incorporation of antibodies into the cytoplasm of living cells. Although the dextran-incorporation data provide evidence that macromolecules as large as 5.5 nm in Stokes radius can gain access into the cell cytoplasm, it was important to examine the possibility that the technique could also be utilized to incorporate antibodies into living cells. An important advantage for the study of structure-function relationships of proteins in animal cells has been the ability to microinject antibodies that could block the biological activity of proteins in living cells, potentially leading to altered cellular activity (Warner et al. 1984; Fraser et al. 1987). Such a capability would also be most useful in plant cell research, and evidence is presented in Fig. 4 demonstrating the feasibil-

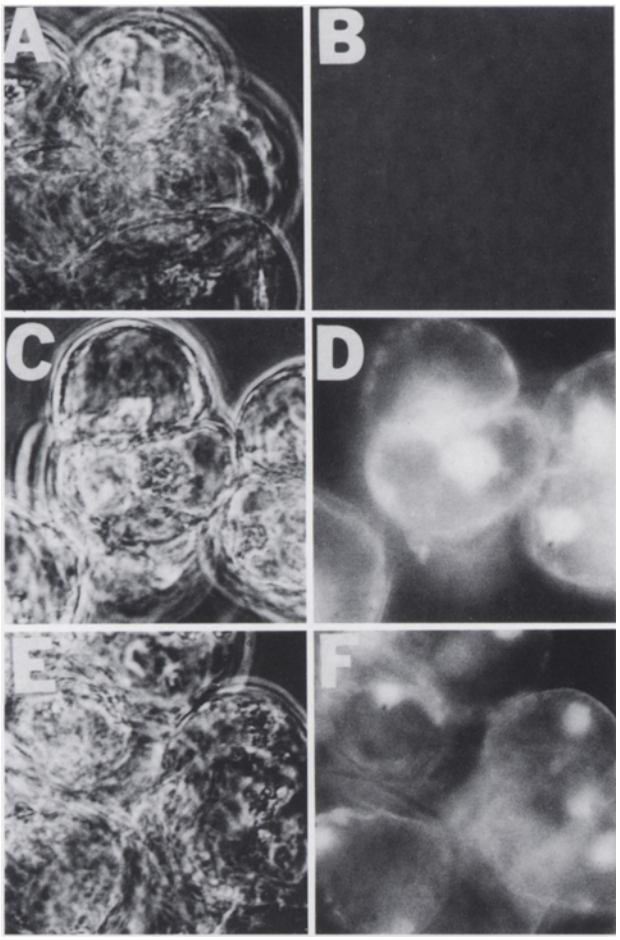

Fig. 4A–F. Fluorescent labeling of the cytoplasmic compartment of soybean cells with FITC-goat anti-rabbit IgG (× 400). Control cells were incubated with FITC-goat anti-rabbit IgG for 60 min (**A, B**) without prior treatment with saponin; or with saponin and incubated for 30 min with FITC-goat anti-rabbit IgG (**C, D**); or treated with saponin and incubated for 60 min with FITC-goat anti-rabbit IgG (**E, F**). **A, C,** and **E** are phase views; **B, D,** and **F** are the corresponding fluorescent images

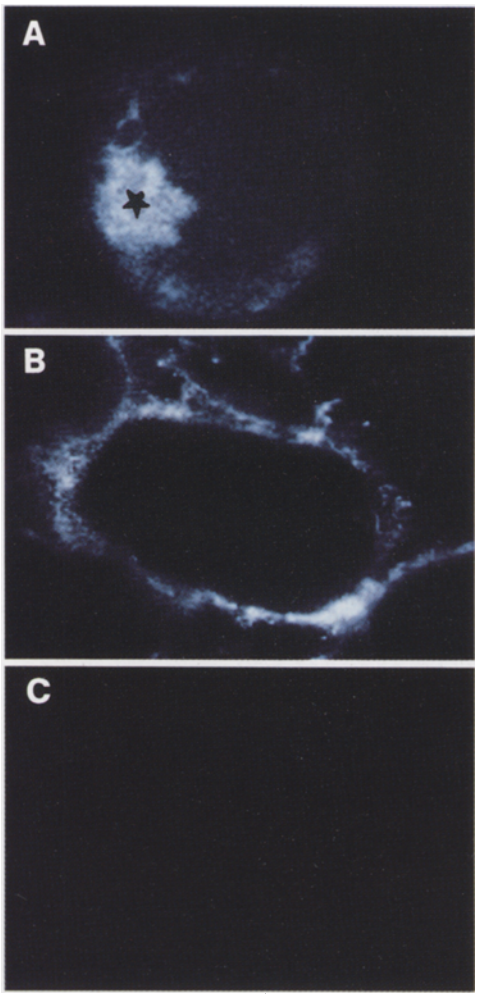

Fig. 5A–C. Confocal fluorescence section of soybean cells in callus culture. In separate experiments, soybean callus was permeabilized and then incubated with FDA (**A**) and FITC-IgG (**B**). In **C,** callus was incubated with FITC-IgG, but not permeabilized. Optical sections are approx. 1 μm thick. *Star* indicates the cell nucleus

ity of such an approach. Figure 4A, B shows control cells incubated for 60 min in FITC-conjugated goat anti-rabbit IgG without the presence of saponin. No included fluorescence is observed in Fig. 4B. In contrast, incorporation of antibody is observed following saponin treatment and incubation with the FITC-conjugated antibody for 30 min (Fig. 4C, D) and 60 min (Fig. 4E, F).

To provide additional evidence that antibody incorporation has occurred, and that the observed fluorescence was not a result of free fluorescein, confocal fluorescence microscopy was performed on soybean calli that were permeabilized utilizing the saponin procedure. Laser-scanning confocal microscopy can substantially enhance images of fluorescence localization in cells and tissues by eliminating the out-of-focus fluorescence from above or below the plane of focus. This has important advantages for providing non-destructive high-resolution images within living tissues and also for greatly minimizing the effect of tissue autofluorescence. Optical slices of approx. 1 μm are presented in Fig. 5. As shown in Fig. 5A, incorporated fluorescein can partition into the cytoplasm (labeled periphery) and the nucleus (star), but not the vacuole. Incorporated FITC-conjugated goat anti-rabbit IgG, in contrast, only labels the cytoplasm but not the nucleus or vacuole (Fig. 5B). This is consistent with the known exclusion limit for diffusion-mediated transport of macromolecules into cell nuclei (Jiang and Schindler 1988). A comparison of these two images (Fig. 5A, B) also provides evidence that free fluorescein is not a contaminating factor in the analysis of IgG localization. Figure 5C shows a portion of callus that was incubated with FITC-IgG but without the saponin treatment.

In conclusion, a procedure has been developed that results in the reversible permeabilization of the plant cell wall and plasmalemma of soybean root cells under conditions that maintain cell viability and proliferative ability. Macromolecules ranging in molecular mass from 20 kDa to 140 kDa may be specifically incorporated into the cytoplasm of living soybean cells.

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