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Introduction of Calmodulin into Suspension-Cultured Cells and Protoplasts of Soybean (*Glycine max* L.)

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대두(*Glycine max* L.) 현탁배양 세포와 원형질체 내로의 외부 Calmodulin의 도입

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In an effort to investigate the role of calmodulin (CaM) as a modulating molecule in the signal transduction system in plant cells, we established methods for introduction of purified CaM into cultured soybean cells. CaM was purified from bovine testis, and was labelled with fluorescein isothiocyanate (FITC). Suspension-cultured cells were treated with saponin (0.1 mg/mL) to permeabilize the plasma membrane and coincubated with FITC-CaM complex. Saponin pretreatment was found to increase the fluorescence in the suspension cultured cells, indicating that the FITC-CaM complex could be incorporated into the cytoplasm. Optimal conditions for introducing FITC-CaM complex into protoplasts by electroporation were established with various electric pulses. With increasing field strength, the fluorescence in the protoplasts was increased, while the viability of the protoplasts decreased. FITC-CaM complex was successfully introduced into the protoplasts by electroporation and the amount of FITC-CaM complex in the protoplasts was estimated.

Key words: electroporation, saponin-permeabilization, soybean cells

A number of methods have been developed to introduce foreign macromolecules into the living cells. These methods include microinjection, detergent-induced permeabilization, liposome fusion, electroporation and microprojective technique. Detergent-induced permeabilization is widly used for its low cost. This method is based on the selective removal of membrane cholesterol by detergents such as digitonin and saponin (Knight et al., 1986). Antibodies were successfully transferred into hydra (Fraster et al., 1987) or soybean suspension-cultured cells (Meiners et al., 1991). In the latter case, saponin used as a detergent maintains the viability of the cell, providing a valuable tool for studying the physiological responses of the living cells. Recently electroporation has been extensively used to transfer proteins as well as genetic material in mammalian cells (Chu et al., 1987: Ou-Lee et al., 1986: Potter et al., 1987). A basic concept underlying this technique is that components of the surface membrane are transiently reoriented by a brief electric pulse of high field strength, forming nanomolar-sized pores in the cell membrane. Electroporation technique can also be applicable to plant protoplasts. Compared with the microinjection and microprojective methods, electroporation is suitable for biochemical or physiological studies which require a large population of cells. Cellular processes intervening between the perception of an external signal and the manifestation of its effector system are poorly understood in plants compared with the well-established signal transduction mechanisms known for animal systems. One of the few common elements for both animal and plant signal transduction systems is calmodulin (CaM), a small, acidic protein that binds calcium ions with a high affinity. This Ca^{2+}-modulated protein is ubiquitously distributed in every eukaryotes examined (Burgess et al., 1985). It is a pleiotropic modulator of physiological function in animal cells, and a

growing body of evidence suggests that CaM plays a similar role in plants (Ling and Zielinski, 1989). Plant CaMs have the ability to activate bovine heart phosphodiesterase albeit at a reduced level to that of animal CaM (Schleicher et al., 1983). Moreover, although plant CaMs differ in amino acid composition from their animal counterparts, the sequence of amino acid is relatively well-conserved (Thompson et al., 1989). As a first step to elucidate mechanisms by which auxin signal is transduced through possible mediation of CaM in plant cells, we established methods in which purified CaM from bovine testis was introduced to soybean cells by electroporation and detergent-permeabilizing techniques. Using fluorescent-labelled calmodulin, we were able to quantitate the amount of introduced protein, allowing general and quantitative use of these methods.

MATERIALS AND METHODS

Soybean Suspension Cultures

A cell line has been established from callus induced from roots of soybean seedlings (*Glycine max* L.). The standard medium used for cell growth was B5 liquid medium supplemented with 2,4-D (1 mg/mL) and kinetin (0,1 mg/mL) (Gamborg et al., 1968) The cells were cultured in the dark at 27°C on a rotary shaker (140 rpm) and subcultured every 7 days.

Isolation and Purification of Protoplasts

The suspension cells (3 g) were treated with CPW13M (pH 5.8) solution containing 2% cellulase (Onozuka RS), 0.5% hemicellulase (Sigma), and 0.5% Macerozyme (Onozuka R-10) at 27 ± 1°C in darkness for 12 h. After incubation, the crude protoplast suspension was filtered through a 300 μm stainless mesh to discard the cell debris. Protoplasts were washed twice with CPW13M (pH 5.8) solution and purified further by sucrose density gradient with CPW21S (pH 5.8) solution. The number of purified protoplasts was counted with a hemocytometer.

Purification of Bovine Testis Calmodulin and Labelling with FITC

Bovine testis CaM was purified according to the method described by Gopalakrishnna and Anderson (1982) employing isoeletric precipitation and phenylsepharose column chromatography. Isolated CaM was dissolved in 1 mM EGTA, and separated by electrophoresis on 12.5% SDS-polyacrylamide gel. For fluorescence labelling, CaM (2 mg/mL of 100 mM $NaHCO_3$) was reacted with FITC (fluorescein isothiocyanate) (5 mg/mL of DMSO) at room temperature for 1 h, and passed through a Sephadex G-25 column. This labelling procedure was carried out in total darkness. The fluorescent values of the fractions were measured at 488 nm (excitation wavelength) and 525 nm (emission wavelength) with a fluorescence spectrophotometer (F-2000, Hitachi Ltd.).

Permeabilization with Saponin

The suspension-cultured cells were treated with 0.1 mg/mL saponin according to the method described by Meiners et al (1991). After treatment with saponin for 15 min, the cells were incubated in the absence or presence of FITC-CaM (1 mg/mL) for 30 min, and then washed three times by centrifugation.

Electroporation

Isolated protoplasts were washed and resuspended (1×10^6 cells/mL) with electroporation buffer (10 mM HEPES pH 7.2, 150 mM NaCl, 5 mM $CaCl^2$, 0.2M mannitol) with or without 70 μg/mL FITC-CaM and placed in an 0.8 mL electroporation cuvette. Electric pulses with various capacitance and field strength were given to the protoplasts with an electroporator (PG200 ProgenetorII, Hoefer Scientific Inc.). After electroporation, the cells were washed twice and stabilized at 4 for 1 h before counting with a hemocytometer or measuring fluorescence with a spectro-fluorometer.

RESULTS AND DISCUSSION

Calmodulin (CaM) is one of the acidic and heat stable Ca^{2+}-binding proteins which can be easily purified. We have purified 50 mg of CaM from 300 g of bovine testis. In order to check the purity of CaM, an isolated CaM fraction after phenylsepharose chromatography was electrophoresed in the presence of 1 mM EDTA on 12.5% SDS-polyacrylamide gel and visualized with Coomassie blue staining. The result presented in Figure 1 shows a single band of CaM with a molecular weight of 16.7kDa.

To evaluate the amount of CaM retained in the cells,

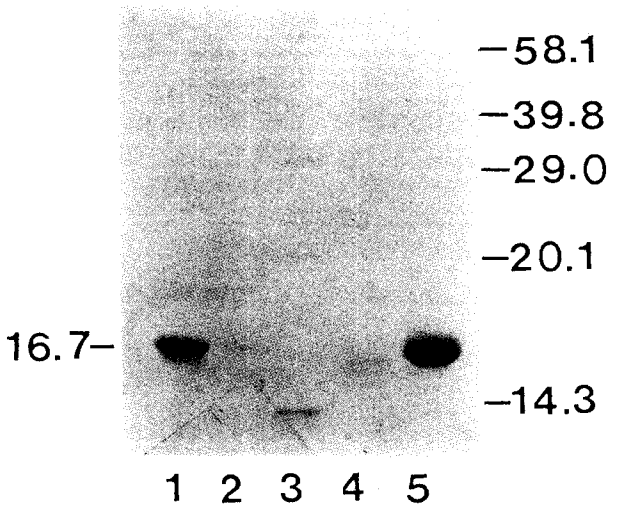

Figure 1. SDS-polyacrylamide gel electrophoretic analysis of urified bovine testis calmodulin (CaM) preparations stained with Coomassie brilliant blue. Lane 1:7 μg CaM, lane 2:2 μg CaM, lane 3: Molecular weight standards, lane 4:4 μg FITC-CaM complex, lane 5:15 μg FITC-CaM complex.

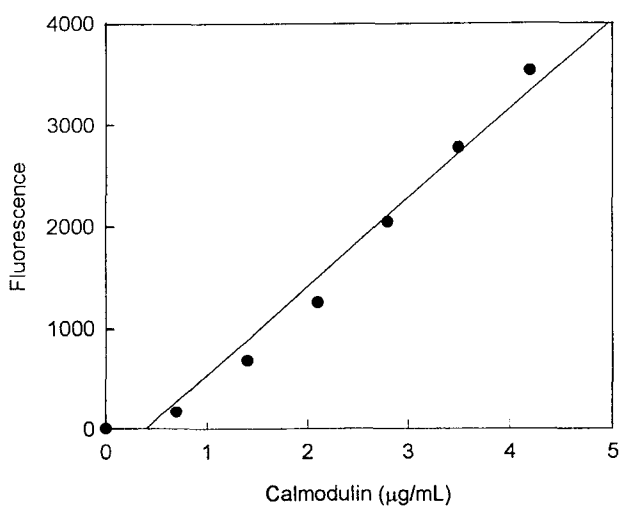

Figure 2. Relationship between fluorescence emitted by FITC-CaM complex and CaM concentrations.

purified CaM was labelled with fluorescent dye, fluorescein isothiocyanate (FITC) which can be covalently linked to a polypeptide. This compound rapidly looses its fluorescence by bleaching, and thus all the labelling process should be carried out in complete darkness. CaM-FITC complex was separated from free FITC by Sephadex G-25 column chromatography. The band of FITC-CaM complex is also shown in Figure 1 (lane 4, 5). The fluorescent intensity of the labelled complex was proportional to the concentration of CaM above 0.7 μg/mL (Figure 2). This indicates that we could quantitate the amount of CaM by measuring the fluorescent intensity of FITC-CaM complex.

Table 1. Intracellular CaM contents and protoplast viability after electroporation.

Treatment		Number of viable cells (10^6 cells/mL)	CaM introduced (μg/10^6 cells)
FITC-CaM (g/mL)	Field strength[a] (V/cm)		
0	500	0.3925	ND[b]
70	500	0.3125	6.6922
70	250	0.6650	2.0812

[a]Capacitance 1080 μF.
[b]Not determined.

Two different methods, namely saponin-permeabilization and electroporation, were established for introduction of FITC-CaM complex into the soybean cells. Soybean suspension-cultured cells were incubated with saponin and FITC-CaM complex and observed under the fluorescence microscope as described in "materials and methods". At low magnificance (100x), we could observe many cells with high flourescence (Figure 3C). The fluorescence was confined to the cytoplasm when a single cell was observed (Figure 3D). The control cells which were saponin-treated only were observed to be morphologically intact without any intracellular fluorescence (Figure 3A, B). In addition, the growth curve of soybean suspension cells was not changed by saponin treatment (Meiners et al., 1991). These results clearly indicate that this protein can be taken up by the cell upon short pulse of saponin treatment with their physiological status intact.

Naturally the cells undergo irreversible damage upon receiving strong electric pulses, and therefore one has to set compromising conditions for two opposing needs, i.e. maximal uptake of macromolecules and minimal rate of cellular death. Data in Figure 4 illustrates survival curves of the cells following electroporation with various electric pulses. The viability of protoplasts after the electroporation was determined by Evans blue staining (Smith et al., 1982). With increasing field strength, the viability of protoplasts was rapidly decreased, but changes in capacitance have no significant effect on the viability. However, at a high field strengh, increased capacitance resulted in the loss of viability. Based on these data, efforts were made to introduce FITC-CaM complex into the protoplasts by electroporation, and the results are presented in Table 1. The purified protoplasts were electroporated with FITC-CaM at different field strength and

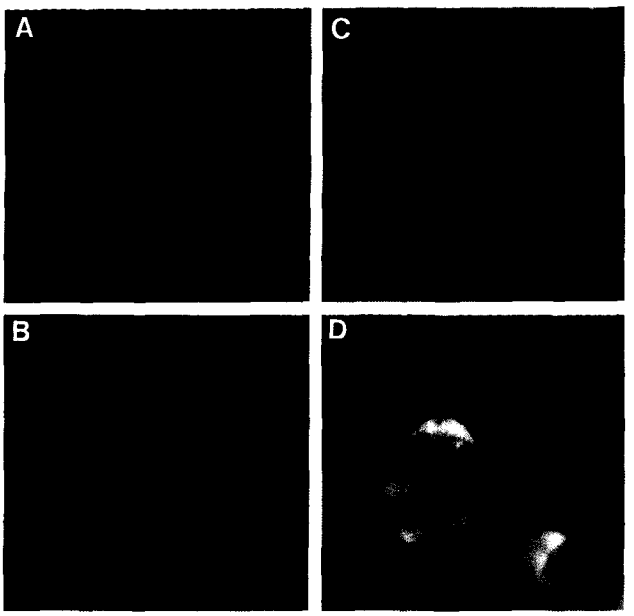

Figure 3. Fluorescent labelling of the cytoplasmic compartment of soybean cells grown on suspension culture following saponin treatment. Cells were incubated for 30 min in the abscence (A, B) or presence (C, D) of FITC-CaM after prior treatment with saponin for 15 min. Magnification: A and C, 100x: B and D, 400x.

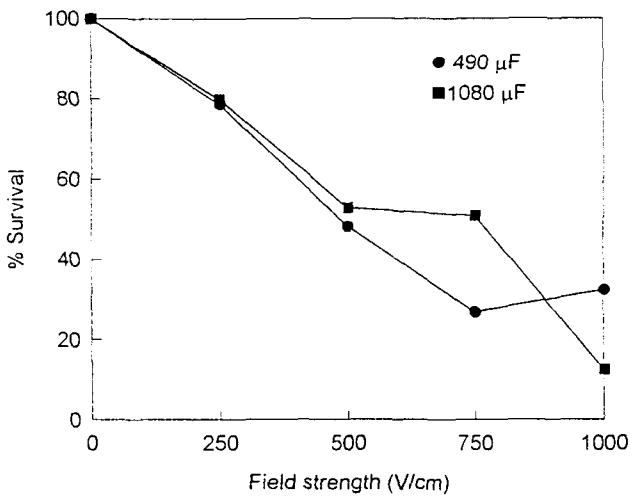

Figure 4. *Immediate survival of soybean protoplasts after* electroporation. The protoplasts (0.6 ml of 1 × 10^6 cells/mL) were electroporated at various voltages and capacitances as indicated.

the amount of incorporated CaM could be calculated from the fluorescent intensity. Table 1 indicates that the amount of incorporated CaM is propotional to the field strength. This is probably due to the increased membrane permeability at a higher strength of electric pulses. Fluorescent-microscopy of electropermeabilized protoplasts is shown in Figure 5.

In our present study, it is established that exogenous CaM could be introduced into soybean cells, and the amount of CaM introduced can also be quantified. This technique may provide a valuable tool for investigation of the role of CaM in the signal transduction pathway in plant cells.

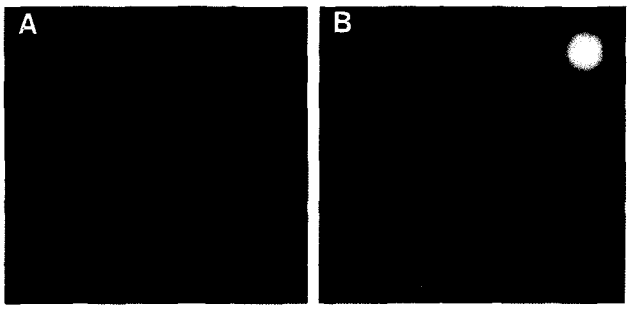

Figure 5. Fluorescent labelling of soybean protoplasts after electroporation with FITC-CaM. Electroporation was performed at 250 V/cm, 1080 μF (A: control, B: electroporated).

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적 요

식물 세포의 신호전달 기작에 있어 calmodulin (CaM)의 역할을 조사하기 위하여 외부기원의 CaM을 식물 세포 내로 주입하였다. 이를 위하여 bovine testis로부터 CaM을 분리, 정제하여 SDS-PAGE상에서 순수함을 확인하였고, 이를 세포 내로 주입한 후 실제 주입 여부를 확인하기위해 정제된 CaM을 미리 fluorescein isothiocyanate (FITC)로 표식하여 사용하였다. 또한, FITC-CaM complex의 농도에 따른 fluorescent intensity를 측정하여 주입된 CaM의 농도를 결정하였다. 대두 현탁 배양 세포를 saponin (0.1 mg/mL)이 포함된 배지에서 15분간 배양하여 permeabilization시키고, FITC-CaM (1 mg/mL)을 30분간 처리한 후 형광현미경으로 조사했을 때 세포질에서 강한 형광이 나타났으며, 이 결과로 외부의 calmodulin이 세포 내로 주입되었음을 확인할 수 있었다. 대두 현탁 배양 세포에 3가지의 세포벽 분해 효소를 처리하여 원형질체를 분리하였고, 이 원형질체를 FITC-CaM이 들어있는 완충용액에 넣고 electroporation 하였을때 capacitance와 field strength가 증가할수록 원형질체의 생존률은 감소하였으나, 세포 내의 fluorescent intensity는 증가하였다. 이것은 외부의 calmodulin이 electroporation에 의해 대두의 원형질체 내로 도입되었음을 의미하는 것이다.

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