Effects of Electro- and Chemical Fusion Treatment on Cell Wall Formation and Cell Division of Plant Protoplasts

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Abstract : The effects of electro- and chemical fusion procedures on cell wall formation and cell division of lettuce and soybean protoplasts were studied. Judging from Calcofluor White staining, cell wall formation of both lettuce and soybean protoplasts was stimulated by electro-fusion procedure 3 h after application. On the other hand, the cell wall formation of lettuce protoplasts was not affected by the chemical fusion treatment using polyethylene glycol (PEG). However, cell wall formation was inhibited with a very dilute concentration of PEG when lettuce protoplasts were mixed with PEG for 3 h followed by Calcofluor White staining. The enhancement of cell division of soybean protoplasts was observed by the electro-fusion treatment 3 days of culture. By transmission electron microscopy, it was also confirmed that the cell wall was detected on the surface of the electrofusion-treated protoplasts 1 day after culture. A cell wall was not observed on the surface of nontreated protoplasts. Moreover, the endoplasmic reticulum in the treated soybean protoplasts increased compared with nontreated ones.

Key words : Cell division, Cell wall, Fusion, Protoplast

プロトプラストの細胞壁形成及び細胞分裂に対する細胞融合処理の影響 : Salak PHANSIRI・三宅 博・前田英三・谷口 武 (名古屋大学農学部)

要 旨 : レタス及びダイズプロトプラストを電気的または化学的方法で細胞融合処理をしたとき、細胞壁形成ならびに細胞分裂に対するそれらの影響を調べた。Calcofluor White 染色法によると、細胞壁形成はレタス、ダイズ両プロトプラスト共に電気的融合処理により促進された。一方、polyethylene glycol (PEG) を用いた化学的融合法では、レタスプロトプラストの細胞壁形成に影響は無かった。レタスプロトプラストを PEG と混合して 3 時間後に Calcofluor White で染色したところ、細胞壁形成は非常に薄い濃度の PEG で阻害されることを確かめた。ダイズプロトプラストを電気的処理をすると、培養 3 日後に細胞分裂の増加が観察された。透過型電子顕微鏡観察によると、電気的融合処理をしたプロトプラストでは、1 日後に既に細胞壁が認められたが、未処理細胞では形成されていなかった。さらに、電気的処理をしたプロトプラストでは無処理に比べ小胞体が増加した。

キーワード : 細胞分裂, 細胞壁, 細胞融合, プロトプラスト。

For inducing plant protoplast fusion, some chemicals, mainly PEG, have been used for a long time. However, in recent years, electric fields have been used for protoplast fusion or electroporation in a wide variety of plant species to obtain somatic hybridization or to introduce gene or DNA into plant protoplasts.

In the case of electrofusion, although knowledge of the possible effects of electrical treatment on cell growth and development is limited, electric pulses have been shown to stimulate DNA synthesis in both cultured mammalian cells^{2,15)} and cultured plant protoplasts^{6,14)}, wall regeneration and cell division^{5,13)}, shoot formation and plant regeneration^{1,3)}. This technology has also been employed to stimulate uptake of macromolecules (i.e. ¹⁴C-inulin and calcein) to

soybean protoplasts⁴⁾.

On the other hand, chemical fusion methods, mainly using PEG, are very useful and more popular for fusion. One problem is that PEG has been known to be toxic to plant protoplasts because of its high concentration⁹⁾. However, there has been no exact study of the effect of PEG on cell activity.

In this paper, the effects of electrical and chemical fusion procedures on cell wall formation and cell division on soybean and lettuce protoplasts are examined.

Materials and Methods

1. Protoplast isolation and culture

(a) Soybean. The methods of isolation and culture of the soybean protoplasts were as previously described^{10,12)}. The protoplasts were collected from 3–5 day-old suspension culture cells derived from hypocotyl calli and

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incubated in an enzyme solution containing 2% Cellulase YC and 0.2% Pectolyase Y-23 in modified IS-1. After protoplasts were collected, they were cultured in a liquid medium (K8P)⁸⁾ at a density of 1×10^6 protoplasts/ml and cultured in dark conditions at 25°C for one week.

(b) Lettuce. Lettuce (5–7 day-old) cotyledons were cut into small pieces and protoplasts were isolated using an enzyme solution containing 2% Cellulase YC (Seishin Pharmaceutical Co., Tokyo, Japan), 0.5% Macerozyme R-10 (Kinki Yakuruto, Nishinomiya, Japan), 2.5 mM calcium chloride and 0.5 M mannitol, pH 5.6. Three hours later, protoplasts were isolated as described earlier^{10,12)}.

2. Electrofusion

Protoplast fusion was induced by Shimadzu Somatic Hybridizer SSH-1 (Kyoto, Japan) as previously described^{16,17)}.

Before fusion, protoplasts were diluted at 4×10^5 /ml with washing solution and aligned in an alternating electric (AC) field at a frequency of 1 MHz and a field strength of 200 V cm⁻¹ for 30 s. Afterwards, fusion was induced by direct current (DC) pulses of 1600 V cm⁻¹, 50 μ s duration. The distance between electrodes was 0.2 cm.

3. Chemical fusion

Protoplast fusion was induced by the method of Kao⁷⁾ with some modifications. The original PEG solution consisted of 33% PEG (MW 6000), 3.5 mM CaCl₂, and 0.7 mM KH₂PO₄, adjusted at pH 5.5. Washing solution consisted of 0.5 M mannitol and 2.5 mM CaCl₂. PEG solution (2 ml) was slowly added to the protoplast suspension (0.2 ml) in a test tube and mixed slowly. After a given time, the mixture was centrifuged at 100 $\times g$ for 3 min. The precipitated protoplasts were resuspended in washing solution followed by centrifugation. The washing procedure was repeated 3 times.

4. Electron microscopy

Untreated and electrofusion-treated soybean protoplasts were cultured for 1/2 d to 7 d. They were then collected and fixed as previously described¹¹⁾. Samples were fixed in a mixture of 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and 0.25 M mannitol with 1.25 mM CaCl₂ (pH 5.6) for 5 h at room temperature after being washed with cacodylate buffer. Then they were postfixed in

2% osmium tetroxide for 3 h, immobilized in alginate beads, dehydrated in a graded alcohol series and embedded in Epon 812 resin.

For electron microscopic staining, uranyl acetate and lead citrate were used as described elsewhere¹¹⁾.

Results

Effect of electrofusion treatment on cell wall formation of protoplasts

At the light microscopic level, the cell wall formation of untreated and electrofusion-treated protoplasts were assessed by Calcofluor White (CW) staining 3 h after isolation (control) and 3 h after electrofusion, respectively.

The control protoplasts of lettuce and soybean showed weak fluorescence of CW on their surface (Figs. 1 and 4). The fluorescence on the treated protoplasts of both plants was significantly stronger than that of the controls (Fig. 1 compared with Fig. 3 and Fig. 4 with Fig. 6).

At the electron microscopic level, after 1 d of culture the electrofusion-treated soybean protoplasts already formed cell wall layers and untreated protoplasts, however, did not have a cell wall layer (Figs. 14 and 15 compared with Fig. 13).

Effect of electrofusion treatment on promotion of cell division of soybean protoplasts

At the light microscopic level, the effect of electrofusion-treatment on the enhancement of cell division of soybean protoplasts was shown in Table 1 and Figs. 7–12. After 1 d of culture in both the treated and untreated protoplasts, only a few cells showed the first cell division. However, after 3 d of culture the electrofusion-treatment markedly enhanced cell division compared with untreated protoplasts.

At the electron microscopic level, after 1 d of culture electrofusion-treated protoplasts showed the increase in endoplasmic reticulum compared with untreated protoplasts (Fig. 15). After a long time culture, electrofusion-treated protoplasts also sustained rapid growth to form microcalli and calli.

Effect of PEG treatment on the cell wall formation of lettuce protoplasts

It is known that PEG is toxic to protoplasts at higher concentration. In this experiment,

Explanation of Figures

Figs. 1-6. Effect of electrofusion or polyethylene glycol (PEG) treatment on cell wall formation of lettuce protoplasts (Figs. 1-3) and soybean protoplasts (Figs. 4-6). Cell wall material was examined by staining with Calcofluor White 3 h after treatment.

Figs. 1 and 4 : Nontreatment. Figs. 2 and 5 : Incubation in 0.03% PEG for 3 h. Figs. 3 and 6 : Electrofusion treatment. (Bar : 100 μ m)

Figs. 7-12. Effect of electrofusion treatment on cell division of soybean protoplasts. Figs. 7-9 : Nontreatment and Figs. 10-12 : Electrofusion treatment. Figs. 7 and 10 : 1 d culture, Figs. 8 and 11 : 3 d culture, and Figs. 9 and 12 : 5 d culture, respectively. (Bar : 100 μ m)

Figs. 13–15. Electron micrographs of soybean protoplasts one day after electrofusion treatment. Fig. 13: A nontreated protoplast without cell wall (bar: 1 μ m), Fig. 14: an electrotreated protoplast with cell wall (arrow) (bar: 5 μ m), and Fig. 15: endoplasmic reticulum and cell wall (arrow) in an electrotreated protoplast. (Bar: 1 μ m)

Table 1. Electrofusion treatment on cell division of soybean protoplasts.

Days after culture		1st division	2nd division	3rd division
1d	untreated	+	—	—
	treated	+/-	—	—
3d	untreated	+	+/-	—
	treated	++	++	—
5d	untreated	++	++	—
	treated	+++	+++	++
7d	untreated	++	++	+/-
	treated	+++	+++	++
+++ : many		+/- : very few		
++ : moderate		— : none		
+ : few				

Table 2. Effect of PEG concentration on the cell wall formation of lettuce protoplasts.

PEG concentration (%)	30*	3.0	0.3	0.03	0.003	0
Calcofluor fluorescence	—	—	—	—	+/-	+

Three hours after mixing with PEG, protoplasts were washed with washing solution and stained with Calcofluor White.

(+ : clear, +/- : weak, — : none)

* After the dilution ($\times 5$) with washing solution, the protoplasts were washed.

Table 3. Effect of PEG-treated time for chemical fusion on the cell wall formation of lettuce protoplasts.

30% PEG treatment (minutes)	Cont.	10	20	60
Calcofluor fluorescence	+	+	+	+/-

Three hours after mixing with PEG and washed with washing solution followed by staining.

(+ : clear, +/- : weak, — : none)

the effect of PEG concentration on cell wall formation was examined. Fresh protoplasts were mixed with different concentration of PEG and incubated for 3 h. Table 2 indicates PEG clearly inhibits the cell wall formation of lettuce protoplasts at a very dilute solution of PEG (0.03%). Figs. 2 and 5 show no fluorescence on lettuce and soybean protoplasts.

In the next experiment, lettuce protoplasts

were treated with 30% PEG for 10, 20 or 60 min to induce fusion. The PEG-treated protoplasts were diluted with washing solution as described in the Materials and Methods section. After washing, the treated protoplasts were stained with CW to examine their cell wall formation. The results are shown in Table 3. There was no significant difference in fluorescence intensity between control and treated protoplasts treated for 10 or 20 min. However, by 60-min treatment, many protoplasts did not show fluorescence. The nontreated protoplasts indicated some fluorescence on the surface of the protoplasts. Figs. 2 and 5 indicate no fluorescence on the PEG-treated protoplasts.

Discussion

When plant protoplasts are isolated and cultured, earlier stages of protoplast development are cell wall formation and then cell division. Cell activity for these earlier two processes is very important because they may relate to the metabolic activity of the cell and to the following processes of plant regeneration. Immediately after enzymes for protoplast isolation are removed, protoplasts start to form a cell wall in order to return to the original state from an abnormal one¹⁰.

Experimentally, the detection of cell wall formation is very easy by using fluorescence microscopy. This study was performed to get more information on the physiological state of fusion-treated protoplasts by analyzing the activity of cell wall formation, the first step for

plant regeneration.

In the case of electrofusion, protoplasts make contact with each other by application of an AC electric field. In the preliminary experiments, when the AC field was applied to the protoplasts only under the conditions used here, there was no difference in cell wall formation between nontreated and AC-treated protoplasts. Therefore, AC field did not affect cell wall formation. The fusion apparatus used here applies DC pulses only after the application of an AC electric field. Therefore, it was concluded that the effect of this electrofusion procedure on cell wall formation may be induced by DC pulse application.

The mechanism of stimulation by electrofusion treatment on cell wall formation and cell division of plant protoplasts is unclear at present. In this study, the treatment markedly promoted cell wall formation and cell division of soybean protoplasts, supporting recent results that electroporation promoted protoplast division and colony formation in both monocotyledonous plant protoplasts⁵⁾ and dicotyledonous plant protoplasts^{1,3,13)}. It is suggested that high voltage electric pulses, through the mechanism of inducing pore-like openings in the plasma membrane, may facilitate uptake of ions and nucleic acids from the surrounding culture medium.

The other possible explanation for electric pulses enhancing cell division and plant regeneration in protoplasts is that the faster division rate may reflect underlying stimulation of DNA synthesis. Rech et al.¹⁴⁾ indicated that DNA synthesis, as measured by the incorporation of ³H thymidine, was indeed enhanced by electroporation. The increase of endoplasmic reticulum in the present study may be related to the stimulation of DNA or protein synthesis that is a prerequisite for cell division.

In the case of chemical fusion, it is believed that a high concentration of PEG is toxic to protoplasts. However, there were no exact explanation on toxicity to plant protoplasts. The present experiment could indicate interference for the cell wall formation of PEG at a very low concentration. However, if PEG was removed by washing within 20 min, the protoplasts did not suffer a harmful effect. For chemical fusion, protoplasts are usually treated with a high concentration of PEG for 10 min. The present study can conclude the

usefulness of PEG for protoplast fusion effectively as electrofusion. Although the chemical fusion procedure is more time consuming than electrofusion, it draws a certain result at a small expense.

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