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Efficient Transfection of Cauliflower Mosaic Virus DNA into Turnip Protoplasts by Microinjection

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Cauliflower mosaic virus (CaMV-S) DNA was introduced efficiently into turnip protoplasts by the pricking or injection method. The expression of DNA was detected by staining the treated protoplasts with CaMV-S specific fluorescent antibody 48 h after pricking or injection. The concentration of introduced DNA lower than 125 μg/ml caused no apparent damages within 48 h after the treatment of protoplasts, giving fluorescing specks in 20-35% of the survived protoplasts. The injection of DNA as low as 10 μg/ml gave fluorescing specks to the treated protoplasts. The microinjection was technically more difficult than the pricking method.

CaMV has attracted attention of many researchers as a favourable candidate of plant-DNA vector, since it has a double-stranded circular DNA in contrast with the majority of other plant viruses having RNA and, moreover, its genome DNA is relatively small in the molecular size. Thus the introduction of this DNA into protoplasts has been tried in several plants. By applying this method with a complex of a polycation and DNA,[1] we succeeded in introducing CaMV DNA into plant protoplast at a high frequency ($1\text{-}2 \times 10^{-1}$).

Thus, a simpler and more reliable method is further needed to employ a less amount of DNA and to detect an expression of DNA information within a shorter time. Such a technique will be helpful to elucidate the mechanism and capability of replication of modified DNA's and a possibility of detectable expression of information carried by these DNA's.

Recently, Yamamoto and colleagues[2] described an injection method to introduce exogenous genes into nuclei of animal cells. Their method is advantageous for studying a mechanism of expression and physiological action of the injected genes action in individual cells, if the location of injected cells is marked and recorded at the time of injection. In the present paper, it is described that by use of a slight modification of this method, we succeeded in introducing CaMV-S DNA efficiently into turnip protoplasts and detected the expression of capsid protein gene of the virus.

Materials and Methods

Viral DNA. Cauliflower mosaic virus (CaMV-S) was given by Dr. H. Tochihara (National Institute of Agrobiological Resources, Ibaragi, Japan). Virus multiplication and purification were carried out by the method described by Yamaoka et al.[3] For the purification of CaMV-S DNA, Proteinase K-SDS method was used.[4] Two ml of virus solution (1 mg/ml) was mixed with equal volume of 10 mM Tris-HCl buffer (pH 7.2) containing 4 mg Proteinase K (Merck Co. Ltd.) and 4 mg SDS. After incubation at 37°C for 2 h, SDS was added to the solution to make final concen-

tration of 1%, and then the mixture was incubated at 37℃ overnight. The solution was mixed with equal volume of phenol saturated with the same buffer and shaken vigorously at room temperature for 15 min. After centrifugation, the aqueous solution was treated with ether to remove phenol and then 2.5 volumes of cold ethanol was added to the solution to precipitate DNA, followed by overnight storage at −20℃. DNA was collected by centrifugation and resolved in a small volume of sterilized distilled water and stored at −80℃ until use.

Protoplasts. Leaves of turnip (Brassica rapa cv. Marubakomatsuna) were used for the source of protoplasts 5 weeks after sowing. Protoplasts were isolated by the procedure of Furusawa and Okuno[5]. Protoplast suspension obtained by enzyme treatment was filtrated through 4 layers of gauze and protoplasts were collected by centrifugation at $100 \times g$ for 2 min, washed twice with sterilized 0.5 M mannitol containing 0.1 mM $CaCl_2$ and resuspended in culture medium.

Pricking method. Injectoscope (Olympus, IMT-YE, Type 1, IF, Co., Osaka, Japan) was used to prick protoplasts. Microneedles were made from glass capillaries with a Narishige microelectrode puller (PN-3) under the following conditions: heater 8; magnet 9; main magnet 9. The diameter of a microneedle tip was about 0.5 μm. The microneedle was fixed to the holder, which is located in the center hole through the optical axis of the condenser, and the cells were pricked by the up-and-down movement of the condenser lens. All of the protoplasts in the 10 μl protoplast suspension in a plastic dish (Falcon 3801) were pricked once until the tip of the microneedle came in touch with the substratum. Routinely, 5-10 cells can be pricked per minute.

Injection method. By applying a method similar to the above pricking method, CaMV-S DNA solution was injected to protoplasts. Ten μg/ml CaMV-S DNA solution was sucked from the tip of microneedle, and then injected into each protoplast through the microneedle under pressure during pricking.

Determination of coat protein synthesis. After 48 h incubation, protoplasts were stained with a CaMV-S specific fluorescent antibody to detect coat protein synthesized.[1]

Results

A preliminary test showed that pricking of protoplasts or free cells was difficult when they were not fixed on the bottom of their container such as a Petri dish, because they moved around when pricking was attempted. Even if they were successfully pricked in this condition, a relatively long time is required to pull up a glass needle from the protoplasts because of their condition. The plastic Petri dish (Falcon 3801) used in this experiment, which had been developed for the purpose to attach the object materials to the bottom, led us to succeed in pricking without any troubles mentioned above. By the present trials, it was found that the density of protoplasts in their suspension was substantially significant to attach them to the bottom of the Petri dish: the density should be lower than 1×10^4 protoplasts per ml.

According to these preliminary experiments, suspensions of protoplasts were all adjusted to the density of $1\text{-}5 \times 10^3$/ml. By the pricking or injection method, it is conceivable that protoplasts objected are injured during the process (**Fig. 1a**). As indicated in **Tables 1** and **2**, however, the incidence of dead protoplasts was not significantly different between pricked protoplast groups and not pricked ones. Although this result allowed us to assume that pricking or injection might give pricked protoplasts no significant damage, the incidence of dead protoplasts was drastically elevated when the concentration of DNA in the suspension was more than 250 μg/ml. Pricked protoplasts on a Petri dish were collected to a glass slide after incubation, and fixed with acetone and stained with CaMV-S specific fluorescent antibody. In order to know whether protoplasts had been lost or not during fixation and staining, the number of protoplasts was counted after staining, and compared with that before staining. As indicated in **Table 1**, 35-60% of pricked protoplasts were successfully collected and in 20-35% of them fluorescing specks (**Fig. 1b**) were observed when the DNA concentrations of 25-125 μg/ml were applied. It was confirmed that the above pricking procedure

Table 1. Introduction of CaMV-S DNA into turnip protoplasts by the pricking method.

CaMV-S DNA conc. (μg/ml)	Number of pricked PPs[a]	Dead PPs[a] /Observed PPs[a] (%)	Fluorescent PPs[a,b] /Observed PPs[a] (%)
0	0	47/127 (37)	0 (0)
0	70	20/ 42(48)	0 (0)
25	43	11/ 26 (42)	3/15 (20)
50	120	29/108 (27)	20/79 (25)
100	130	28/106 (26)	27/78 (35)
125	36	5/ 27 (19)	5/22 (23)

[a] Protoplasts.
[b] Stained with CaMV-S specific fluorescent antibody 48h after the onset of incubation at 26° C.

Table 2. Introduction of CaMV-S DNA into turnip protoplasts by the microinjection method.

CaMV-S DNA conc. (μg/ml)	Number of injected PPs[a]	Rate of dead PPs[a] (%)	Rate of fluorescent PPs[a,b] (%)
0	58	57	0
10	100	57	22

[a] Protoplasts.
[a,b] Stained with CaMV-S specific fluorescent antibody 48h after the onset of incubation at 26℃.

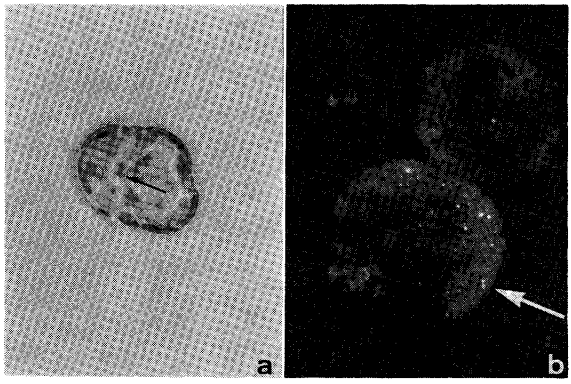

Fig. 1a. An apex of microneedle (arrow) which has been inserted into a turnip free cell. The free cell was used to illustrate clearly the location of the microneedle apex instead of protoplasts.

Fig. 1b. A turnip protoplast having fluorescent specks (arrow) incubated 48 h after pricking at the concentration of 25 μg/ml CaMV-S DNA. Protoplasts were stained with CaMV-S specific fluorescent antibody.

did not induce any non-specific fluorescent speck in controls (without the addition of DNA).

In the case of injection, fluorescing specks in protoplasts were observed even at lower concentration of DNA (10 μg/ml), while at this concentration, protoplasts with fluorescing specks were rarely obtained by pricking.

Discussion

The present results evidently indicate that CaMV-S DNA can be introduced into turnip protoplasts by physical means and the expression of its information and its replication can be detected within 48 hr after pricking or injection. During the present study it was found that the pressure in a cylinge was very important for injection in contrast with pricking, because protoplasts burst immediatly after exposed to high pressure. Thus, to avoid such a damage of protoplasts, injection should be carried out under the condition of cylinge force which causes diffusible flow-out of the DNA through the needle tip.

In a previous experiment, an attempt was made to introduce TMV RNA into tobacco protoplasts by pricking. However, a further experiment revealed that the number of cells having fluorescing specks was significantly greater than thant of actually pricked cells, suggesting that without pricking treatment TMV RNA could be incorporated into tobacco protoplasts resulting in its replication and its subsequent expression. By contrast, fluorescing specks were successfully observed only in turnip protoplasts pricked, indicating that the spontaneous introduction did not occur in the case of CaMV-S DNA. It has been accepted that the amount of liquid which can be introduced into animal cells by one trial of pricking is usually about 1.4×10^{-15} l [6]. Assuming that this was also the case of turnip cells, approximately 20 molecules of DNA could be introduced into the cell by one pricking at the concentration of 100 μg/ml of CaMV-S DNA.

A further experiment was carried out to confirm that replication, transcription and translation of CaMV-S DNA introduced might actually occur in pricked turnip cells: 400 turnip protoplasts were pricked at the concentration of 100 μg/ml CaMV-S DNA, followed by isolation of the virus particles at 0 and 48 hr after pricking, subsequent extraction of DNA, and autoradiography by using ^{32}P-CaMV-S DNA as a probe. The results indicated that CaMV-S DNA was originally introduced and replicated in the turnip protoplasts (data not shown). These results will be published elsewhere.

As described above, the present pricking method was successful in giving a high frequency of introduction of DNA (25–30%). This frequency was much higher than that as low as 10^{-5}–10^{-6} or 2%, respectively obtained by the methods by using polyethlenglycol or liposomes,[7] and by electroporation[8]. Moreover, by the present method 300 cells can be readily pricked per hour without any difficulty. This technique is much easier to master than microinjection[9,10] which requires more skill. In addition to these advantages over other techniques, it is likely that the present method might be suitable to elucidate replication function of modified DNA within a short time.

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マイクロインジェクション法によるカリフラワーモザイクウイルスのコマツナプロトプラストへの導入

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マイクロインジェクトスコープを用いて，外来 DNA を植物プロトプラストに導入できるかどうかについて検討した．カリフラワーモザイクウイルス（CaMV）DNA を所定の濃度に調製し，コマツナのプロトプラストと混合し，プリッキングを行った．CaMV DNA の導入ならびにその発現を CaMV 外被蛋白特異螢光抗体により確認した．導入 48 時間後において，コマツナのプロトプラストに螢光塊を示すものが存在した．この時間内ではプリッキングの影響はほとんど認められず，CaMV DNA 125 μg/ml の濃度で 20〜35% のプロトプラストに螢光塊の存在が確認された．なお，CaMV DNA 10 μg/ml の濃度でインジェクションを行い，同様に螢光塊を認めたが，プリッキングよりも時間を要し，多種類の組換 DNA 等の発現の有無を調べるのにはプリッキングの方が適しているように思われる．