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Uptake of Liposome-Encapsulating Plasmid DNA by Plant Protoplasts and Molecular Fate of Foreign DNA

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Summary

Conditions faborable for the uptake of artificial lipid vesicles (liposomes) encapsulating plasmid DNA by *Daucus carota* protoplasts were investigated. Incubation period necessary for the maximum uptake of liposome-DNA was in the neighborhood of 10 minutes under the circumstances where liposomes (approximately 1.28 μmoles lecithin/ml) were mixed with 5×10^6 protoplasts/ml. Results obtained by Southern hybridization indicated that the recombinant DNA vector, pBR325 was found in protoplasts after 20 hours incubation in the open circular, linear and some complexed forms. There were some variations in DNA-uptake among protoplasts from different plant species. The gradual disappearance of plasmid molecules was confirmed after 1 week culture, suggesting instability of pBR325 in prolonged cell culture.

Keywords: Liposome-DNA; Protoplasts.

1. Introduction

Artificial lipid vesicles (liposomes) have been used as vehicles to transfer several materials into plant protoplasts. Special attention has been paid to the insertion of foreign DNA into plant protoplasts with the aid of liposomes such as multilamellar vesicles (LURQUIN 1979, 1981, ROLLO *et al.* 1981), and large unilamellar lipid vesicles (LURQUIN 1981, MATTHEWS and CRESS 1981). Recently we demonstrated that a measurable amount of foreign DNA can be incorporated into protoplasts via liposomes (UCHIMIYA and HARADA 1982). Furthermore, we tracked the fate of plasmid DNA in plant protoplasts in a relatively longer period of incubation (20 hours).

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This paper reports the kinetic analysis of liposome-mediated transfer of plasmid DNA into plant protoplasts. Furthermore, molecular fate of plasmid DNA in protoplasts will be discussed.

2. Materials and Methods

The (^{32}P)-pBR322 or -pBR325 were obtained by nick-translation (RIGBY *et al.* 1977).

Liposomes encapsulating plasmid DNA (liposome-DNA) were prepared according to a method reported previously with slight modification (UCHIMIYA and HARADA 1981).

Protoplasts were prepared from suspension cultured cells of *Daucus carota*, *Vinca rosea* and *Nicotiana glutinosa* according to a method reported elsewhere (UCHIMIYA and HARADA 1981). Protoplasts (10^6–10^7/ml) were incubated with liposome (1–2 μmole lipid/ml)-DNA in 100 mM phosphate buffer (pH 7)-0.5 M mannitol solution in given periods. For long term culture, protoplasts were washed twice with a medium containing MURASHIGE and SKOOG (1962) salts, and the following (mg/l): glucose (72,000), 2,4-D(1), kinetin (0.5), thiamine · HCl (0.5), nicotinic acid (5), pyridoxin · HCl (0.5), biotin (2), folic acid (0.2), *myo*-inositol (100) and glycine (2). The pH of the medium was adjusted to 5.7.

Protoplasts were thus cultured in the same medium. After incubation, liposome-protoplasts were subjected to 0.5 M mannitol (top)-0.5 M sucrose (bottom) density gradient centrifugation ($2,200 \times g$) for 5 minutes. Pelleted protoplasts devoid of liposome-DNA were suspended in 0.5 M sucrose solution, and recentrifuged at $2,200 \times g$ for 5 minutes. In some experiments, Triton X100 (0.7%) treated nuclei were prepared from protoplasts according to a method reported elsewhere (UCHIMIYA and MURASHIGE 1977).

Protoplasts or isolated nuclei were lysed in the solution A containing 62.5 mM Tris · HCl (pH 6.8), 25 mM EDTA, 1% SDS, and 10% glycerol. Either total or TCA-insoluble count of radioactivity associated with lysates of protoplasts or of isolated nuclei was measured (UCHIMIYA and MURASHIGE 1977).

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In order to monitor the fate of plasmid DNA, protoplasts were treated with liposome-DNA (cold pBR325). Following the culture of protoplasts for given periods, cells or isolated nuclei were lysed in the solution A, and were then frozen at least 2 hours. After thawing 30 μl cell lysate was applied to a 0.7% agarose gel containing 36 mM Tris · HCl (pH 6.8), 30 mM NaH_2PO_4 and 1 mM EDTA, pH 5.8. Following electrophoresis at 25 V for 16 hours, a gel slab was stained with ethidium bromide and photographed. The same gel was then alkaline-treated, and denatured DNA was transferred to a nitrocellulose filter (SOUTHERN 1975). Using a method reported elsewhere (WAHL *et al.* 1979), a baked filter was hybridized with (^{32}P)-pBR325 probe prepared by nick-translation, and an autoradiographic image of probe hybridization was obtained.

3. Results and Discussion

In the present investigation, we tested the efficient encapsulation condition of foreign DNA by liposomes. The following procedure was found to be appropriate.

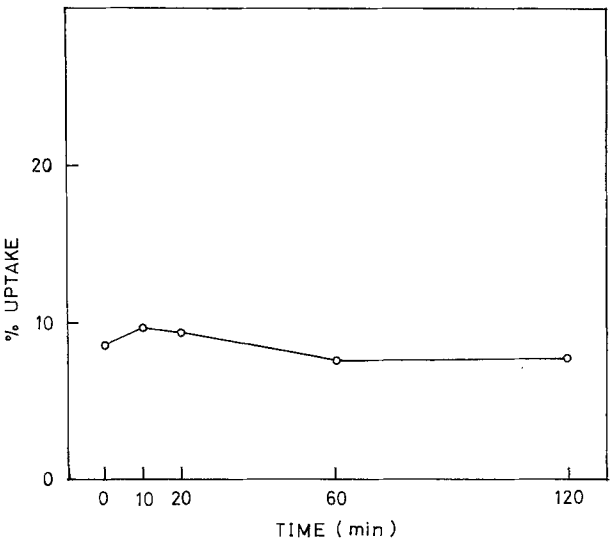

Fig. 1. Time cource of liposome-mediated uptake of (^{32}P)-pBR322 by *D. carota* protoplasts. Uptake was measured by counting acid insoluble radioactivities of protoplast lysates

One hundred μl Tris-buffer (20 mM Tris · HCl, pH 7.5) containing (^{3}H)-pBR322 (0.1-1 μg) or pBR325 (4-5 μg) were added to 300 μl diethyl ether dissolving 1 mg hydrogenated phosphatidylcholine and 0.1 mg stearylamine, which were contained in a 10 ml round-bottom flask. Following 1 minute sonication, the flask containing liposome-DNA was placed in ice for 30 minutes, then the remaining ethyl ether was evaporated under N_2 gas with a rotation of a flask at 180 rpm. Liposomes encapsulating plasmid DNA were suspended in 100 mM PB (pH 7.0)-0.5 M mannitol and centrifuged at 5,000 × g for 5 minutes. This washing procedure was repeated twice.

Time course of the liposome-mediated DNA uptake by *D. carota* protoplasts is seen in Fig. 1. Uptake occurred immediately after exposure of protoplasts to liposome-DNA and the maximum uptake was seen in 10 minute incubation. This period is relatively shorter than that of other reported instances. LURQUIN (1979) used 45 minutes incubation of multilamellar lipid vesicles containing pBR322 with *Vigna sinensis* protoplasts, and MATTHEWS and CRESS (MATTHEWS and CRESS 1981) used 1.5-2 hours for incubation of large unilamellar lipid vesicles containing plasmid DNA with *D. carota* protoplasts.

Influence of liposome concentration on the DNA uptake by *D. carota* protoplasts was tested. Efficiency of DNA uptake was saturable in the neighborhood of

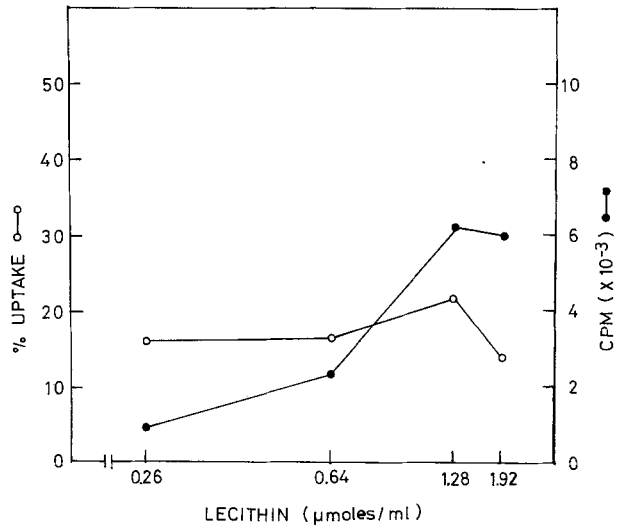

Fig. 2. Influence of liposome concentrations on the DNA "(^{32}P)-pBR322" uptake by *D. carota* protoplasts. Liposome concentrations were expressed by lipid contents of vesicles. Acid insoluble radioactivities in protoplast lysates were recorded

1.28 μmoles lecithin/ml (Fig. 2). Inasmuch as the average diameter of this type liposome is 2×10^{-1} μm, and that of *D. carota* is 26.8 μm, 7×10^4 lipid vesicles would be required to cover entire surface of a protoplast. There was a saturation of DNA uptake at 2.6×10^{-6} μmole lipid ($= 7.6 \times 10^5$ vesicles)/protoplast. Therefore, approximately 11 times the number of liposomes which presumably bind to the entire protoplast surface would be required to achieve maximum DNA uptake. Regarding the mechanism of the uptake of liposome DNA by protoplasts, one would

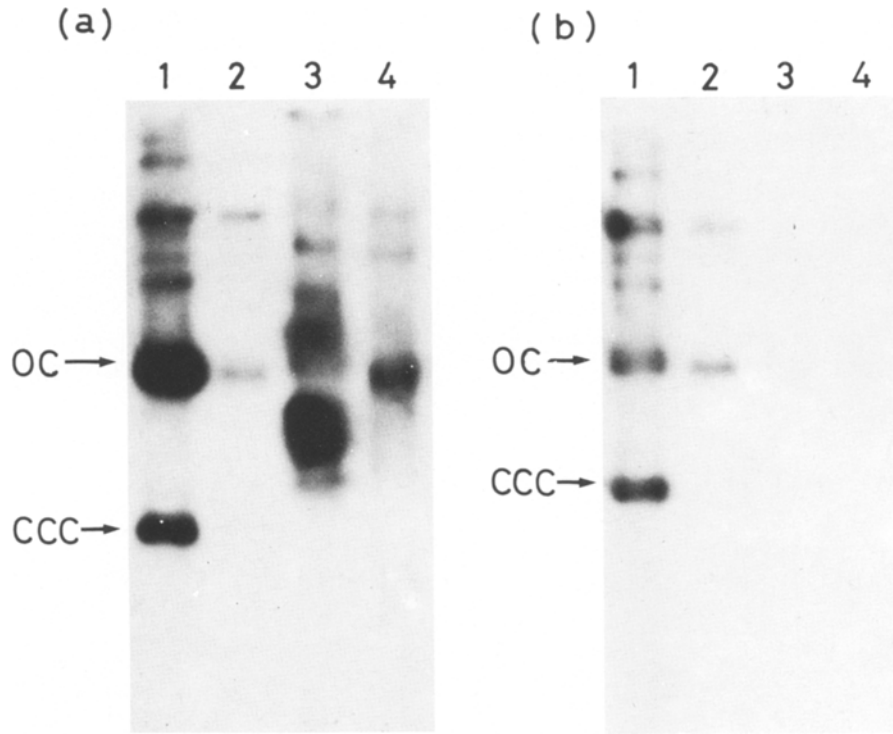

Fig. 3. Southern hybridization of DNA in the nuclear fraction of protoplasts (cells) treated with liposome-DNA for 15 minutes, followed by (*a*) 20 hours and (*b*) 1 week culture in a synthetic medium. Track *1*: pBR 325 (0.5 μg). Track *2*: *D. carota* protoplasts treated with liposome-DNA. Track *3*: *V. rosea* protoplasts treated with liposome-DNA. Track *4*: *N. glutinosa* treated with liposome-DNA

expect the adsorption of liposome-DNA on the surface of protoplasts. Since we employed positively charged liposomes, there would be some electrostatic binding of lipid vesicles to protoplast membrane. Nevertheless, our previous observation using liposomes encapsulating a fluorescent marker did not support significant adsorption of lipid vesicles or protoplasts (UCHIMIYA 1981).

Protoplasts of several plant species were treated with liposome-DNA for 30 minutes, followed by the culture of 20 hours and 1 week. As seen in Fig. 3, open circular and complexed forms of pBR325 were seen in nuclear fraction from three different plant species in 20 hour culture. Covalently closed circular form was not detected in any protoplasts tested. Since DNA encapsulated by liposomes with or without DNase treatment still contained CCC form of plasmid DNA (unpublished data), this conformational change might take place after the association of DNA molecules with protoplasts.

Our preliminary results obtained by subcellular analysis of ^{32}P-labelled DNA suggest approximately 50% of DNA taken up by protoplasts was associated with nuclear fraction after 15 minute incubation.

After 1 week culture, a trace amount of plasmid DNA was detected only in *D. carota* protoplasts. It is unlikely that bacterial plasmid ever replicate in plant cells in a long term culture. In fact, present work indicated decrease and/or disappearance of plasmid DNA after 1 week culture. Nevertheless, it is hoped that the application of recombinant technology will provide in future such vectors as capable of replicating in plant cells.

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