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Liposome-mediated delivery of tobacco mosaic virus RNA into tobacco protoplasts: A sensitive assay for monitoring liposome–protoplast interactions

(phospholipid vesicles/liposomes/intracellular delivery/nucleic acids/plant cells)

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ABSTRACT Tobacco mosaic virus (TMV) RNA was encapsulated in large, unilamellar phospholipid vesicles (liposomes), and the encapsulated TMV RNA was shown to be infectious when incubated with tobacco protoplasts under appropriate conditions. Maximal virus production in protoplasts was observed after their incubation with TMV RNA entrapped in phosphatidylserine/cholesterol liposomes. Infection was dependent on the presence of polyalcohols in the incubation mixture. Other parameters, such as the extent of vesicle binding, the cell-induced leakage of vesicle contents, and the degree of liposome toxicity were shown to be important in determining the efficiency of infectivity. Liposome-mediated delivery offers an efficient and reproducible method for introducing RNA into plant protoplasts.

Because of their ability to fuse directly with the plasma membrane or to be taken up by cells by an endocytosis-like process, liposomes have been used to introduce a variety of molecules, including drugs and enzymes into cultured mammalian cells (1, 2). Recent improvements in liposome technology (3, 4) now permit the encapsulation of large macromolecules such as nucleic acids in liposomes, and it has recently been shown that liposome-encapsulated RNA and DNA molecules can be introduced and expressed in a variety of mammalian cell lines (for review, see 5).

Liposomes offer several advantages as carriers for delivering nucleic acids into cultured cells, including (*i*) protection of entrapped RNA and DNA molecules from degradation by nucleases, (*ii*) low toxicity towards cells, (*iii*) effective utilization with a variety of cell types, and (*iv*) enhanced efficiency of expression of the encapsulated nucleic acid. These advantages have been recognized by several laboratories (6–12) which have attempted to use liposomes to introduce nucleic acids into plant protoplasts. However, to date there have been no reports describing the expression of liposome-encapsulated nucleic acids by plant cells.

We have investigated liposome–protoplast interactions in order to optimize the delivery of liposomal contents to protoplasts and to determine the potential of this delivery system for introducing nucleic acids into plant cells. This study reports on optimal conditions for delivering liposome-encapsulated tobacco mosaic virus (TMV) RNA in a biologically-active form to tobacco protoplasts as a convenient assay for monitoring the delivery of nucleic acids into plant cells.

MATERIALS AND METHODS

TMV RNA Isolation and Encapsulation in Liposomes. RNA was extracted from virus (TMV strain U_1) by the method of Diaz-Ruiz and Kaper (13) and stored in ethanol at −80°C until use. Liposomes were prepared by the method of Szoka and Papahadjopoulos (14) as modified by Fraley *et al.* (15) for the encapsulation of nucleic acids. Briefly, 500 μg of TMV RNA and 0.1 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]poly(A) in 0.33 ml of sterile buffer (5 mM Tris/50 mM NaCl/0.4 M mannitol/0.1 mM EDTA, pH 7.0) was added to 10 μmol of phospholipid dissolved in diethyl ether (1.0 ml). The sources, purity, and storage of lipids used in this study have been reported (16). The two-phase solution was briefly sonicated (≈5 sec) in a bath-type sonicator and transferred to a rotary evaporator, and the ether was removed under reduced pressure as described (15). Liposomes used for binding studies were prepared in the same manner except that either [^{3}H]dipalmitoyl phosphatidylcholine (PtdCho) (50 μCi) or [^{3}H]inulin (50 μCi) was included in the preparation as radioactive labels for the vesicle lipid or aqueous interior, respectively.

Lipid concentrations were measured by phosphate analysis (17), and RNA encapsulation was determined by monitoring an aliquot (25–50 μl) for radioactivity. Typically, between 30–40% of the TMV RNA was encapsulated by this procedure. The integrity of the encapsulated RNA was determined by extracting the RNA from liposomes by the method of Bligh and Dyer (except that isopropanol was substituted for methanol; ref. 18) and analyzing it by gel electrophoresis (19).

Protoplast Isolation, Incubation, and Culture. Tobacco protoplasts were isolated from suspension cultures of *Nicotiana tabacum* "Xanthi" (n.c.) as described by Uchimiya and Murashige (20) and were resuspended at a final cell density of 2×10^6 cells per ml in Tris-buffered mannitol solution (TBM solution; 5 mM Tris/0.5 M mannitol/0.5 mM $CaCl_2$, pH 7.0). Liposomes (10–500 nmol of phospholipid) were added to 0.5 ml of protoplasts (in a 15.0-ml plastic centrifuge tube), mixed, and allowed to incubate for 5.0 min at 25°C. TBM (4.5 ml) containing 5% (vol/vol) glycerol, 5% (vol/vol) dimethyl-sulfoxide, 5% (vol/vol) ethylene glycol, 10% (wt/vol) polyvinyl alcohol (PVA) (Polysciences, no. 2975), or 10% (wt/vol) polyethylene glycol (PEG) (M_r = 6000; British Drug House, Poole, England) was added to the protoplast suspension and allowed to incubate for 20 min. The viscous solutions then were diluted by the addition of 10 ml of TBM solution, and the protoplasts were pelleted by centrifugation (100 × *g* for 10 min). The protoplasts were washed twice with TBM solution, once with MS medium [ref. 21; B_5 vitamin (22)/0.5 M mannitol/1.5% sucrose/2 μg of (2,4-

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Abbreviations: TMV, tobacco mosaic virus; TBM solution, Tris-buffered mannitol solution; PtdCho, phosphatidyl-choline from hen egg yolks; PtdSer, phosphatidylserine from bovine brain; SteN, stearylamine; PVA, polyvinyl alcohol; PEG, polyethylene glycol; Chol, cholesterol; LD_{25}, lipid concentration that reduces cell viability by 25%.

dichlorophenoxy)acetic acid per ml] and were resuspended at a final cell density of 10^5 cells per ml in medium. The protoplast suspension (2.0 ml) was dispensed into tissue culture flasks (Falcon T3025) and cultured in the dark for 24 hr before transfer to a light intensity of approximately 2000 lx. Aliquots (100 μl in duplicate) were removed at 48 hr and stored (−20°C) for determination of virus levels.

The determination of cell-associated vesicle lipid and vesicle contents was as described except that after liposome/cell incubation, the protoplasts were washed twice by centrifugation in TBM solution containing 2% Ficoll (the liposomes cannot enter solutions of this density, whereas the protoplasts pellet during centrifugation). The cells were resuspended in 1.0 ml of TBM solution and transferred directly to scintillation vials for determination of cell-associated radioactivity.

Radioimmunoassay for Monitoring TMV Production in Protoplasts. Rabbit anti-TMV antiserum was prepared as described (23), and iodinations were performed with Enzymo Beads (Bio-Rad) according to the manufacturer's directions. Microtiter plates (Dynatech Laboratories, Alexandria, VA) were precoated with unlabeled anti-TMV antibody (10 μg/ml in 50 mM phosphate buffer, pH 9.0) and washed three times with phosphate-buffered saline (containing 1% bovine serum albumin) before use. Aliquots of protoplasts (subjected twice to freeze/thaw cycle) or dilutions of TMV were added to wells and incubated for 12–18 hr at 25°C; the wells were washed three times with phosphate-buffered saline (containing 1% bovine serum albumin), and 125 μl of the I^{125}-labeled antibody (≈200,000 cpm) was added and allowed to incubate for 3.0 hr. The wells were washed five times with phosphate-buffered saline, separated, and transferred to scintillation vials for the determination of radioactivity. The assay detects 1 ng of virus (at twice background levels); at saturating amounts of virus (>1 μg), ≈3–5% of the labeled antibody was bound. The reproducibility of the results was quite good; all experimental points were determined in triplicate, and a variation of <14% was observed between separate samples. The values from independent experiments agreed within a factor of 2.

RESULTS

A variety of different liposome preparations, including neutral (6, 10), negative (8–11), and positively charged (7, 10, 12) vesicles, have been reported to have been successfully used to introduce encapsulated materials into plant protoplasts. TMV RNA was encapsulated in each of these different vesicle types to determine which liposome composition was optimal for delivery of TMV RNA into protoplasts (Table 1). However, preliminary experiments in which tobacco protoplasts were simply incubated with these various liposome preparations resulted in no detectable virus production. The lack of infectivity of the liposome-encapsulated TMV RNA was not attributable to degradation because analysis of the encapsulated RNA on agarose gels revealed no breakdown had occurred during encapsulation (data not shown), and infection with the free RNA could be obtained by using the poly-L-ornithine method. Instead, a number of factors, including (*i*) inefficient binding of liposomes to protoplasts, (*ii*) leakage of encapsulated RNA from liposomes during incubation with cells, (*iii*) failure of liposomes to introduce their contents intracellularly, and (*iv*) possible toxic effects of liposomes on cells could account for the lack of infectivity of the encapsulated TMV RNA. These factors were examined to determine their possible influence on liposome–protoplast interactions.

Association of Vesicle Lipid and Internal Contents with Protoplasts. Liposomes containing [^{3}H]dipalmitoyl phosphatidylcholine as a trace radioactive phospholipid label were incubated with 2 × 10^5 protoplasts, and the amounts of cell-associated vesicle lipid remaining after extensive washing of the protoplasts was determined (Fig. 1A). It should be emphasized that this experimental approach does not distinguish between liposomes adsorbed to the cell surface and those taken up by protoplasts by fusion, endocytosis, or some other process; therefore, these vesicles are referred to as cell-associated. Positively charged liposomes [PtdCho/stearyl amine (SteN) liposomes] were found to associate with protoplasts to a much greater degree than either neutral PtdCho or negatively charged [phosphatidylserine (PtdSer)] liposomes (Fig. 1A). The level of binding of the various liposome preparations (Fig. 1A) was ≈10- to 100-fold greater than had been observed previously with mammalian cells (15, 24, 25), suggesting that inefficient binding is not a likely cause for the lack of infectivity of encapsulated TMV RNA.

Table 1. Infectivity of TMV RNA encapsulated in various liposome preparations

Vesicle lipid composition	Encapsulation efficiency*	Infectivity†	Infectivity‡
PtdSer	15.2	<1	85
PtdCho	17.7	<1	<3
PtdCho/SteN	18.3	<1	<1
PtdSer/Chol	16.4	<1	503

TMV RNA (500 μg) was encapsulated in liposomes (10 μmol of lipid) prepared by reverse-phase evaporation. A small amount of radioactive [^{3}H]poly(A) is included to permit precise calculations of encapsulation efficiency and TMV RNA concentrations. Unencapsulated TMV RNA was separated from liposomes by centrifugation (flotation) on discontinuous Ficoll gradients (15). Ficoll solutions were prepared in 5 mM TRIS/0.5 M mannitol/0.1 mM EDTA, pH 7.0. Rapidly growing suspension cultures were centrifuged (200 × g), resuspended at twice the original volume in 2% Cellulysin (Calbiochem)/1% Driselase (Kyowa Hakko Kogyo, Tokyo, Japan)/0.5% Macerase (Calbiochem)/0.5 M mannitol (pH 5.7), and incubated for 5–6 hr. The resulting protoplasts were separated from debris and undigested cells by successive passage through 100, 150, and 200 mesh filters (Small Parts, Miami, FL) and were washed twice by centrifugation (100 × g for 5 min) with the above buffer. Protoplasts (10^6) were incubated with 400–500 nmol of lipid (5 μg of RNA) in the presence and absence of 10% polyvinyl alcohol and were assayed 48 hr later for virus production.

* The amount of encapsulated RNA is expressed as μg/μmol of phospholipid.

† The infectivity of liposome-encapsulated TMV RNA in the absence of PVA treatment (ng of virus per 10^5 protoplasts).

‡ The infectivity of liposome-encapsulated TMV RNA after treatment with 10% PVA (ng of virus per 10^5 protoplasts).

Because liposomes are known to exhibit increased permeability during their incubation with both mammalian cells (15) and plant protoplasts (6), it is important to measure the amount of vesicle contents that become cell-associated to determine if cell-induced leakage can account for the lack of infectivity. For this purpose, liposomes containing [^{3}H]inulin (Fig. 1B) were used to determine the association of vesicle contents with protoplasts under conditions identical to those used in Fig. 1A to determine vesicle lipid association with cells. The use of inulin as a marker for vesicle internal contents is preferable to using radioactively-labeled RNA because it eliminates possible artifacts associated with the binding or uptake of free RNA (or nucleotides) by protoplasts, which may have leaked from the liposomes during the incubation. The amount of [^{3}H]inulin (expressed as vesicle lipid equivalents) associated with protoplasts was highest for the PtdCho/SteN liposomes (Fig. 1A). By comparing the amounts of cell-associated vesicle lipid and contents, it could be determined that PtdCho/SteN liposomes retained 48% of their contents after incubation with protoplasts. PtdSer and PtdCho liposomes retained 10.4% and 30% of their

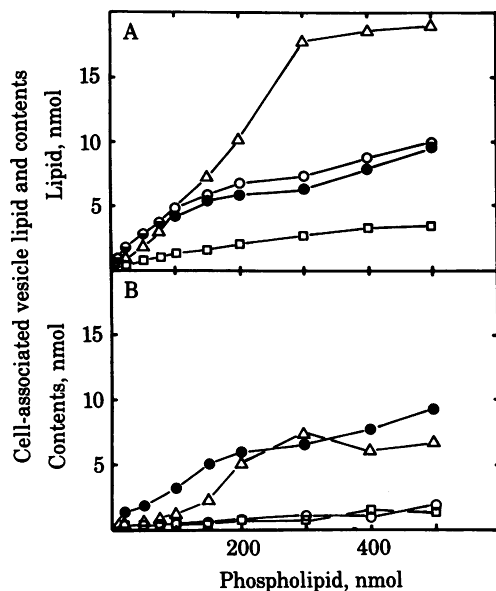

FIG. 1. Association of vesicle lipid and internal contents with protoplasts. Liposomes, containing either [^{3}H]dipalmitoyl PtdCho (5 μCi/μmol of lipid) or [^{3}H]inulin (0.15 μCi/μl) to label vesicle lipid or internal contents, respectively, were prepared. Liposomes (10–500 nmol of lipid) were incubated with protoplasts (2 × 10^5 cells) for 20 min. The amount of cell-associated radioactivity was determined after centrifugation of protoplasts through 2% (wt/vol) Ficoll buffer. (*A*) Protoplast-associated vesicle lipid (nmol) after incubation of protoplasts with increasing amounts of radioactively labeled PtdSer (○), PtdCho (□), PtdCho/SteN (△), and PtdSer/Chol (●) liposomes. (*B*) Protoplast-associated vesicle contents (expressed as vesicle lipid equivalents) after incubation of protoplasts with increasing amounts of radioactively-labeled liposomes.

contents, respectively. These results confirm and extend those of Lurquin (6), which demonstrated that a large fraction of liposome contents (≈70%) are released during a short incubation with cowpea protoplasts, and they indicate that vesicle leakage is an important parameter that could influence the efficiency of liposomal delivery. However, the problems of leakage could be minimized by the addition of Chol during liposome preparation.

Effects of Different Liposome Preparations on Protoplast Viability. Several investigators (7, 10) have reported significant loss of protoplast viability after their incubation with liposomes, and this could dramatically influence the apparent efficiency of delivery as monitored by TMV RNA infectivity. The effects of increasing liposome concentrations on protoplast viability were determined by using the fluorescein diacetate technique (26) to monitor viable cells 48 hr after incubation with liposomes (Fig. 2). Positively charged liposomes were found to be more toxic (LD_{25} = 135 nmol of lipid) than either neutral (LD_{25} = 645 nmol of lipid) or negatively charged liposomes (LD_{25} = 330 nmol of lipid); however, at lower lipid concentrations (≈100–200 nmol) these cytotoxic effects were minimal and would not be expected to interfere with virus replication.

Effect of Lipid Composition and Incubation Conditions on Liposome-Mediated Delivery of TMV RNA to Protoplasts. These results show that a significant amount of liposome contents became cell-associated under conditions in which protoplast viability was maintained, yet the lack of detectable virus production indicates that significant intracellular delivery of the encapsulated TMV RNA did not occur. The efficiency of liposome-mediated delivery to various mammalian cell lines has been shown to be increased greatly by exposing the cells to agents such as glycerol, PEG, dimethyl sulfoxide, and ethylene

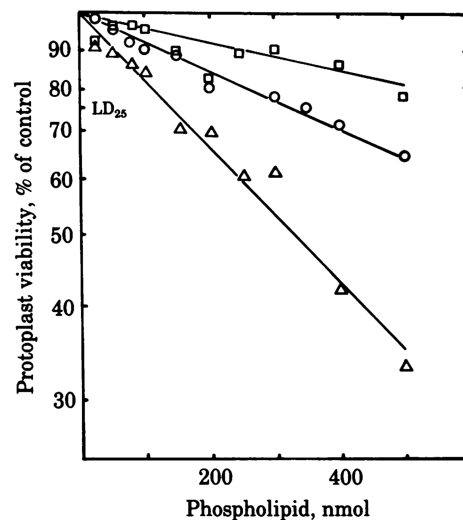

FIG. 2. Effect of different liposome preparations on protoplast viability. Empty liposomes were incubated with protoplasts (1 × 10^6 cells) as described in the legend to Table 1. Cells were then washed and resuspended in medium (2 × 10^5 cells per ml). Forty-eight hours after the incubation with liposomes, the percentage of viable protoplasts (expressed as % of control viability) was determined by using the fluorescein diacetate technique (26). The average viability of nontreated control cells was ≈80%. ○, PtdSer; □, PtdCho; △, PtdCho/SteN. LD_{25}, the lipid concentration which reduces cell viability by 25%.

glycol after their incubation with liposomes (ref. 15, unpublished data). Therefore, a variety of postincubation treatments were examined for their ability to stimulate liposome delivery and to increase the infectivity of encapsulated TMV RNA. Of the various treatments examined, exposure of tobacco protoplasts to dilute PVA solutions proved most effective in stimulating TMV RNA infectivity and was studied in detail (Table 2). The PVA treatment was specific for PtdSer liposomes (Table 1), and the relative infectivities of the various liposome preparations at different liposome concentrations is shown in Fig. 3. The infectivity of PtdSer liposomes could be enhanced substantially (5- to 6-fold) by including an equimolar concentration of cholesterol (Chol) in the preparation (Table 1 and Fig. 3). Chol had no effect on vesicle binding to protoplasts (Fig. 1*A*) but significantly increased the amount of vesicle contents that became cell-associated (Fig. 1*B*) from 10.4% to 90%. Adding Chol to either neutral or positively charged liposome preparations had no effect on delivery (unpublished observations).

Table 2. Effect of various treatments on the infectivity of liposome-encapsulated TMV RNA

Treatment	Infectivity*	Reference†
Glycerol	<1	15
PEG	205	6, 15
Dimethyl sulfoxide	<1	15
Ethylene glycol	<1	Unpublished data
PVA	503	27

PtdSer/Chol liposomes containing 16.9 μg of RNA per μmol of lipid were prepared. Vesicle lipid (400 nmol; 5 μg RNA) was incubated with protoplasts (2 × 10^6) for 5.0 min in 0.5 ml of TBM. TBM (4.5 ml) containing glycerol, PEG, dimethyl sulfoxide, ethylene glycol, or PVA was added to the cells and allowed to incubate for an additional 20 min. Protoplasts were washed, resuspended in media, and assayed 48 hr later for virus production.

* Expressed as ng of virus per 10^5 protoplasts. Conditions were described in Table 1 and in *Materials and Methods*.

† Previous reference to the use of these treatments either with mammalian cells (ref. 15; unpublished data) or plant protoplasts (6, 27).

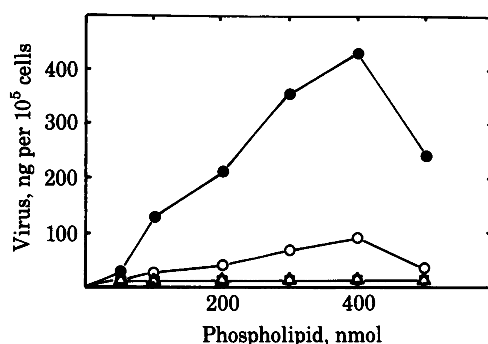

FIG. 3. Infectivity of TMV RNA encapsulated in various liposome preparations. Liposomes containing TMV RNA were prepared as described in the legend to Table 1. Vesicle lipid (10–500 nmol) was incubated with protoplasts (1×10^6 cells) for 5.0 min prior to the addition of 4.5 ml of a PVA solution (10% wt/vol). The cells were washed, resuspended in media, and assayed 48 hr for virus production. ○, PtdSer; □, PtdCho; △, PtdCho/SteN; ●, PtdSer/Chol liposomes.

It should also be noted that the PVA treatment enhanced the infectivity of TMV but not that of TMV RNA (Table 3). That liposomes are directly involved in the delivery process (i.e., fusion or endocytosis) is suggested by the observations (Table 3) that empty liposomes did not promote the infectivity of free TMV RNA and that encapsulated TMV RNA was introduced into protoplasts by a mechanism that protects the RNA from digestion by ribonuclease added to the medium.

DISCUSSION

In this paper we demonstrated the expression of liposome-encapsulated nucleic acids in plant protoplasts. Several studies have shown that radioactively labeled nucleic acids entrapped in liposomes can become cell-associated after their incubation with protoplasts (6–11), and cell fractionation and autoradiography have been used in attempts to localize the labeled nucleic acids within the cell. Although the results of these earlier studies were encouraging, they also were ambiguous because the assays used could not distinguish between adsorption of liposomes to the cell surface and intracellular delivery (1, 2). In addition, it has been demonstrated that damaged protoplasts (29) and isolated nuclei (30) may bind substantial amounts of nucleic acids. As a result, inference that the intracellular delivery of radioactive DNA sequestered in liposomes has occurred based strictly on the localization of radioactivity in nuclei could give misleading results. The use of a biological assay for monitoring liposome-mediated delivery overcomes these uncertainties and allows for the unambiguous determination of conditions that favor intracellular delivery.

Table 3. Infectivity of TMV and TMV RNA under various incubation conditions

RNA preparation	Incubation*	RNA, μg	RNase†	Infectivity‡
TMV (whole virus)	—	0.25	–	<1
	PLO	0.25	–	6000
	PVA	0.25	–	90
TMV RNA	—	5.0	–	<1
	PLO	5.0	–	135
	PVA	5.0	–	<1
	PLO	5.0	+	5
TMV RNA plus empty PtdSer LUV	PLO	5.0	–	<1
TMV RNA in PtdSer/Chol LUV	—	5.0	–	<1
	PVA	5.0	–	480
	PVA	5.0	+	450

Protoplast incubations and virus assay were carried out as described in the text. Liposomes containing TMV RNA were prepared from PtdSer and Chol and contained 16.9 μg of TMV RNA per μmol of lipid. Empty liposomes, prepared identically, were added at a concentration of 450 nmol per incubation. LUV, large unilamellar vesicles; PLO, poly(L-ornithine).

* Treatment with PVA was described in the text; incubations in the presence of PLO were as in ref. 28.

† RNA preparations were preincubated with ribonuclease (10 μg) for 15 min prior to their addition to protoplasts.

‡ Expressed as ng of virus per 10^5 protoplasts.

Our results indicate that negatively charged (PtdSer/Chol) liposomes are the most efficient carrier for introducing TMV RNA into tobacco protoplasts. This observation is somewhat surprising because protoplasts are highly negatively charged (31), but the superiority of PtdSer/Chol liposomes can at least be partially understood in terms of a combination of their high affinity for cells (Fig. 1*A*), resistance to protoplast-induced leakage of vesicle contents (Fig. 1*B*), and their relatively low toxicity (compared with positively charged liposomes) to protoplasts (Fig. 2). These observations are not limited to tobacco cells because similar results have been obtained for the delivery of liposome-encapsulated cowpea mosaic virus RNA to cowpea protoplasts (unpublished data).

The role of Chol in enhancing the infectivity of liposome-encapsulated TMV RNA correlates with its ability to reduce the extent of vesicle leakiness during incubation with cells. Therefore, the net result is an increased amount of encapsulated TMV RNA that remains available for intracellular delivery. Similar effects of Chol in enhancing simian virus 40 DNA delivery to mammalian cells have been observed (15). These results are in conflict with those of Ostro *et al.* (8), which indicate that the inclusion of Chol in negatively-charged liposomes actually decreases the extent of liposomal delivery. It should be emphasized, though, that the latter study utilized an assay based on the uptake of radioactively labeled DNA.

The absolute efficiency of virus production after liposome-mediated delivery of TMV RNA to protoplasts (≈400–500 ng of virus per 10^5 protoplasts; Table 3) is comparable or greater than values reported for TMV RNA infection mediated by polycations (Table 2; ref. 28) or alkaline pH (32); however, absolute comparisons are difficult to make because of the different cell preparations used in these studies (suspension cells vs. leaf tissue). Under optimal conditions, ≈20% of the tobacco protoplasts become stained with fluorescein-labeled anti-TMV antibody (33) 48 hr after incubation with liposome-encapsulated TMV RNA. Other important advantages of liposome-mediated delivery are its high degree of reproducibility and the fact that once the RNA is encapsulated in liposomes, it remains stable for several weeks and may be used for many experiments. Finally, although minor changes in incubation conditions are required for maximal liposome delivery in other protoplast systems (cowpea, petunia, tobacco mesophyll, etc.), this method appears to be generally applicable to use with all plant species.

The mechanism by which the PVA and PEG treatments stimulate intracellular delivery of liposomal contents is not yet clear. These agents are known to induce protoplast-protoplast fusion (27, 34) and also may be able to facilitate liposome-protoplast fusion as well. Alternatively, PEG and PVA may stimulate liposome uptake by an endocytosis-like process, as has been suggested for the action of glycerol in enhancing liposome delivery with mammalian cells (15, 35). Although such processes have not been clearly elucidated in plant cells, there have been several reports describing the uptake of large particles by protoplasts (34, 36), particularly after treatment with PEG. Our ef-

forts are directed towards understanding these aspects of liposome–protoplast interaction and increasing further the efficiency of liposome-mediated delivery to plant cells.

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