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Corn Root Protoplasts

ISOLATION AND GENERAL CHARACTERIZATION OF ION TRANSPORT[1, 2]

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ABSTRACT

A method was developed for the large scale and rapid isolation of intact viable corn root protoplasts. Pure and metabolically active protoplasts were collected using a flotation technique. Vital staining tests, light and electron microscopy, and measurements of basic metabolic processes indicated that the isolated protoplasts were metabolically active, and that the plasmalemma and other organelles were well preserved. The isolated protoplasts performed normal, active ion transport functions. Time course of K^+ and inorganic phosphate ($H_2PO_4^-$) influx and the effects of external pH, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, fusicoccin, and diethylstilbestrol on K^+ and inorganic phosphate influx and net H^+ efflux in isolated protoplasts correlated well with data obtained on root segments. Data presented indicated that isolated protoplasts from roots can be used to gain additional insights into the mechanism of ion transport in plant cells.

In recent years, plant protoplasts have become increasingly important in plant biology research (see reviews 5, 23, 25). For example, protoplasts have been used in the studies of intracellular localization of metabolic processes (2, 3, 18), cell wall biosynthesis (21), organelle isolation and characterization (2, 3, 13, 18, 19), and protoplast fusion in genetic research (4). Because of the lack of methodology in the large scale isolation of viable root protoplasts, no studies have yet been conducted on ion uptake in isolated protoplasts from root cells. Recent reports by Mettler and Leonard (15, 16) indicated that isolated protoplasts from cultured tobacco suspension cells showed normal ion transport properties. These authors suggested that the use of isolated protoplasts for studies on ion transport is feasible. We now describe methods in this study for large scale and rapid isolation of protoplasts from corn root tissue. Data presented indicate that these protoplasts carry out normal ion transport.

MATERIALS AND METHODS

Protoplast Isolation. Two-cm (0.5–2.5 cm from the tip) root segments from 3-day-old etiolated corn (*Zea mays* L. B73 × Missouri 17) seedlings (12) were split down the middle with a razor blade and washed once with 0.1 mM $CaCl_2$ solution. Surface sterilization with ethanol was avoided, since preliminary studies showed that this treatment inhibited cell wall digestion by cellulase during the isolation procedure. As shown diagrammatically in Figure 1, the segments were then incubated in a cell wall digestion enzyme solution (3 g tissue/20 ml enzyme solution) consisting of 2% Cellulysin (Calbiochem), 1% hemicellulase, and 0.5% pectinase in a solution of 0.2 mM $CaCl_2$ and 0.6 M mannitol at pH 5.5 for 3.5 to 4.5 h with constant shaking (50 cycles/min) at 30 C. After the cuticle and some undigested materials were removed with a pair of forceps or a spatula, the remaining crude protoplast mixture was layered onto 20 ml of a solution containing 5% Ficoll (type 400), 25 mM Mes (pH 5.5), 0.2 mM $CaCl_2$, and 0.5 mM DTT[3] in 0.7 M mannitol. The mixture was then centrifuged at 400*g* for 10 min to pellet the starch grains and other large debris. The isolation procedure was continued by transferring the upper layer, which still contained most of the intact protoplasts, to another centrifuge tube where they were diluted with 15 to 20 ml of 0.7 M mannitol containing 25 mM Mes (pH 6.0), 0.2 mM $CaCl_2$, and 0.5 mM DTT (protoplast suspension solution). Protoplasts were immediately spun down at 2,000*g* for 10 min and then washed once with an additional 20 ml of suspension solution.

Final protoplast pellets were resuspended in 7 ml of the protoplast suspension solution to which 10% Ficoll had been added, and 7 ml each of 8, 5, and 0% Ficoll-containing protoplast suspension solutions were then layered on top in sequence. The Ficoll gradients were then centrifuged at 300*g* for 20 min. Based on light microscopic analysis, intact viable protoplasts were found at the interface of the 0 and 5% Ficoll gradients. These were removed with a pipet, washed once with 20 ml protoplast suspension solution, followed by a 10-min, 2,000*g*-pelleting centrifugation. Morphologically, intact protoplasts could also be found in other interfaces of the Ficoll gradient. However, vital staining tests showed that most of these protoplasts were not viable. With the exception of incubation, all procedures were performed at room temperature. All solutions used were sterilized by passing them through a 0.45-μm Millipore filter.

Electron Microscopy. Final protoplast pellets were resuspended and fixed overnight in 5 ml cold 5% glutaraldehyde in Milloning's phosphate buffer (17) at pH 7.0. Fixed protoplasts were spun down at 1,000*g* for 3 min and washed five times with cold phosphate buffer. Postfixation was done with 1% cold OsO_4 in phosphate buffer (pH 7.2) for 1 h. After gradual dehydration with ethanol, the protoplast pellet was infiltrated with Spur (Polysciences, Inc.) and polymerized overnight at 16 C. Protoplast sections were double-stained with uranyl acetate, followed by lead citrate, and examined with a Zeiss model 9 electron microscope.

Respiration and Proton Transport Measurements. Protoplast respiration was measured polarographically at 30 C with a ther-

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[2] Preliminary results had been reported at the "Plant Membrane Transport Workshop," Toronto, Canada, July, 1979.

[3] Abbreviations: DTT: dithiothreitol; FCCP: carbonyl cyanide *p*-trifluoromethyoxyphenyl-hydrazone; FC: fusicoccin; PCMBS: *p*-chloromercuribenzenesulfonic acid; DES: diethylstilbestrol.

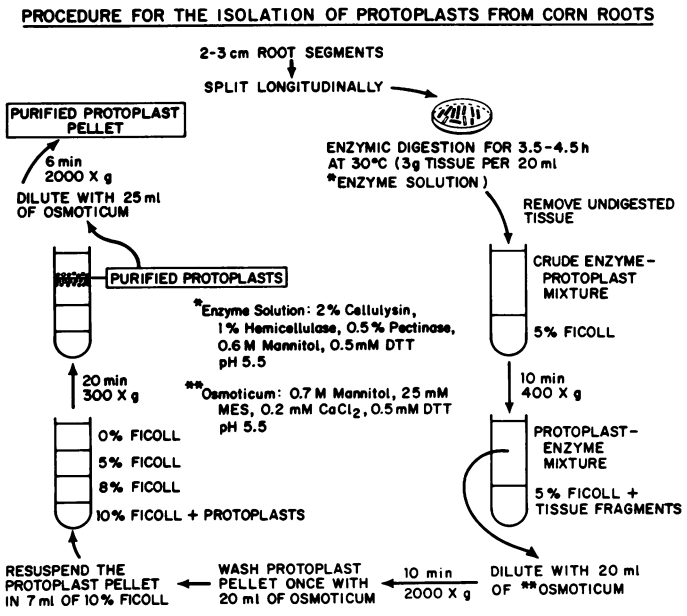

FIG. 1. Procedure for the isolation of protoplasts from corn roots.

mostatic reaction vial containing 2 ml of 0.7 M mannitol, 1 mM KCl, 0.2 mM $CaCl_2$, and 25 mM Mes buffer (pH 6.0). To prevent breakage of protoplasts, the magnetic stirring speed was set at a minimum while still maintaining a stable response of the O_2 electrode. A similar setup with a pH electrode was used for the external pH change measurements, but the Mes concentration was reduced to 1 mM (pH 6.0). Net (apparent) H^+ efflux rates were calculated as previously reported (12). About one million protoplasts in 50 μl of reaction medium were used in each assay. Less than 5% of the protoplasts were broken at the end of each assay.

Ion Uptake Measurements. A supporting and separating medium consisted of 50 μl of silicone oil with a density of 1.045 g/ml at the bottom, followed first by 50 μl of 0.7 M mannitol, 0.2 mM $CaCl_2$, and 25 mM Mes buffer (pH 6.0), and then 50 μl of 1.044 g/ml silicone oil was prepared in a 400-μl microfuge tube. Two hundred μl of absorption solution, containing about 0.5 μCi radioisotope of ^{86}Rb or ^{32}P in a solution of 0.65 M mannitol, 0.2 mM $CaCl_2$, 25 mM Mes buffer (pH 6.0) (in pH experiments, Tris and HCl were used to adjust pH), and either 1 mM KCl for K^+ uptake or 1 mM KH_2PO_4 (1 mM K_2HPO_4 was used to adjust pH) for Pi uptake, was then layered on the top of the supporting and separating medium. Uptake by protoplast suspension (about 0.5 million protoplasts) to each absorption solution was followed by incubation at 30 C. Uptake was terminated by spinning the protoplasts through the supporting and separating medium with a Beckman Microfuge B for 30 s; protoplasts then being packed at the bottom of the microfuge tubes which contained protoplasts were cut, and radioactivities for each tip were counted. With the exception of time course study, uptake was terminated 15 min after the addition of protoplast suspension. Amount of ion accumulated in protoplasts was calculated against the radioactivity of 10 μl standard absorption solution with a correction factor (see discussion below). Comparisons were made between the method described here and that of Mettler and Leonard (15); no significant difference was found between fluxes and the inhibitory effect of FCCP. All experiments were confirmed by one or more repetitions.

RESULTS AND DISCUSSION

Protoplast Isolation. Until the present time no method has existed for the large scale preparation of viable root protoplasts. Therefore, it has not been possible to study ion uptake in root protoplasts. Application of fungal enzyme mixture for the digestion of cell walls has been widely used to isolate protoplasts from plant tissue (4, 6, 25). Unlike the case in most tissues (3, 4, 6), our primary studies found that Cellulysin, used as the only enzyme source, did not digest root tissue well within 10 h. The addition of hemicellulase and pectinase greatly increased the cell wall digestion rate, and large amounts of protoplasts were released within 5 h. Removal of the epidermis was found to be necessary for the fast digestion of the cell walls inside the tissue. However, removal of epidermis was a time-consuming process. I found that simply cutting the root tissue in half was sufficient to allow penetration of the enzyme mixture.

Essentially no microorganisms were found in the protoplast suspension after the protoplasts were purified through the Ficoll flotation steps. Therefore, surface sterilization with ethanol or the addition of antibiotics, which are methods commonly used during protoplast isolation, were not needed. As pointed out by Vasil (23), the addition of antibiotics could cause undesirable side effects. Preliminary results also showed that surface sterilization with ethanol caused strong inhibition of cell wall digestion and stopped the release of protoplasts.

The purity of the protoplast preparation and the viability of the protoplasts were examined under the microscope and with vital staining tests. Little or no cell debris or other free organelles were evident in the protoplast preparation (Fig. 2). High cytoplasmic streaming activity was observed in isolated protoplasts. Protoplasts accumulated neutral red inside the vacuole and excluded Evan's blue, indicating their viability (6, 23). Also, the plasmalemma and other organelles were well preserved (Fig. 2).

For every g fresh weight of root tissue, more than one million

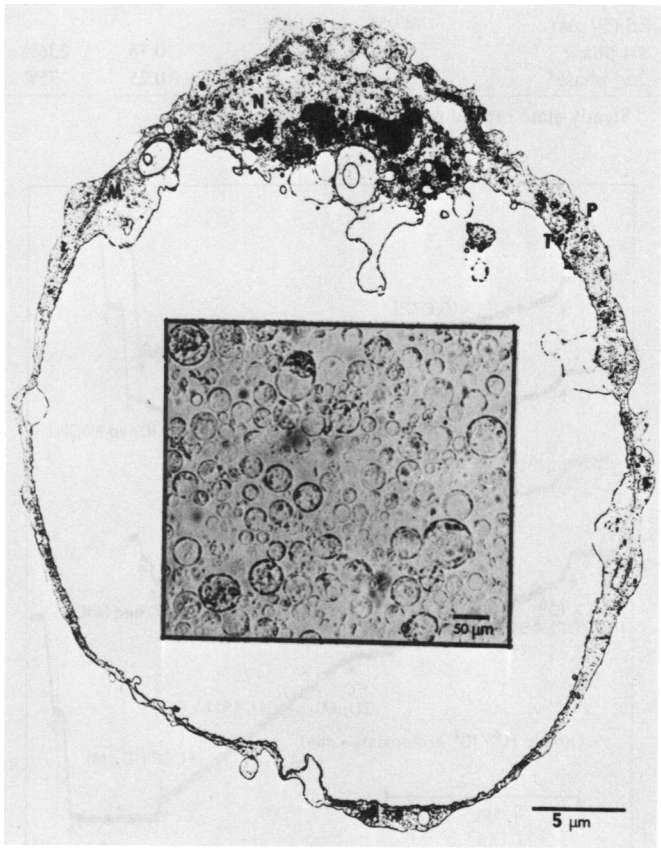

FIG. 2. Electron micrograph and light micrograph (insert) of corn root protoplast. ER, endoplasmic reticulum; M, mitochondria; N, nucleus; P, plasmalemma; T, tonoplast.

protoplasts were isolated with this procedure. By comparing the number of cells inside the tissue with electron microscopic sections of root segments, I estimated that about 10% of the cells were released as intact and viable protoplasts.

Respiration. In addition to the vital staining tests, respiratory activities were used to examine protoplast viability. All reagents tested showed a similar effect on the respiration of protoplasts and root segments (Table I). FC and PCMBS had no effect on the respiration of both root segments and protoplasts for at least the first 20 min. FCCP uncoupled the root segments and protoplasts, and respiratory activity increased. However, in the protoplast suspension, 10 μM FCCP doubled the respiratory rate initially, and this was followed by a strong inhibition reflecting a typical response to a high uncoupler concentration (8). A similar uncoupling effect was found with 50 μM DES. Data showed that protoplasts were more sensitive to uncouplers than that of root segments. Table I also showed that only 1%, but not 10%, of the respiratory rate was recovered in the protoplasts, suggesting that some kind of substrate for respiration might be required in the protoplast suspension for a maximal activity, or that cell membrane had been slightly altered as suggested by the increasing of sensitivity toward the uncouplers.

Table I. *Corn Root Respiration*

	Root Segments		Protoplasts	
	μmol O_2/g fresh wt·h		*μmol O_2/10^6 protoplasts·h*	
Control	31.91		0.33	
FC (10 μM)	31.58	99%	0.31	95%
PCMBS (0.2 mM)	32.12	101%	0.33	100%
FCCP (5 μM)	48.52	152%		
1st phase			0.58	177%
2nd phase[a]			0.19	58%
DES (50 μM)	47.55	149%		
1st phase			0.78	236%
2nd phase[a]			0.25	75%

[a] Steady-state rate 10 min after addition of reagents.

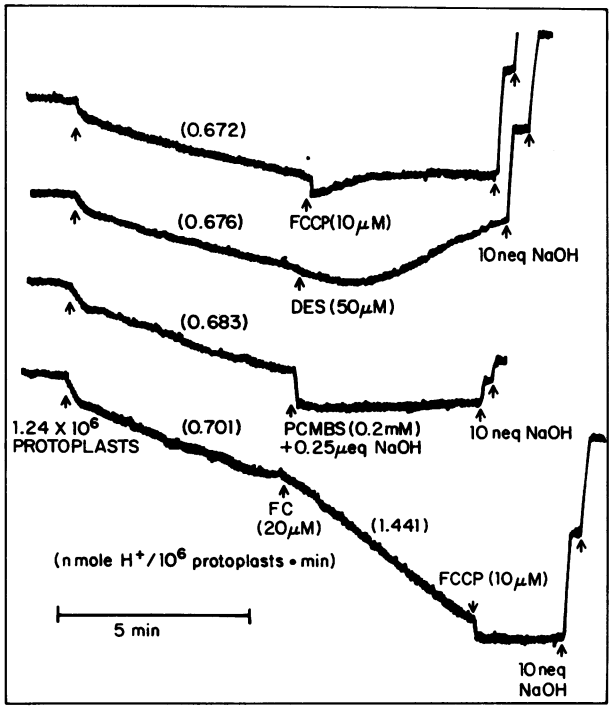

FIG. 3. Recorder traces of net (apparent) proton efflux in corn root protoplast suspension.

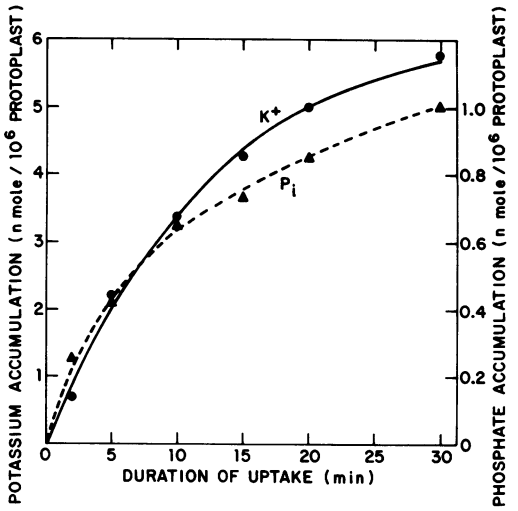

FIG. 4. Time course of K^+ and Pi accumulation in corn root protoplasts.

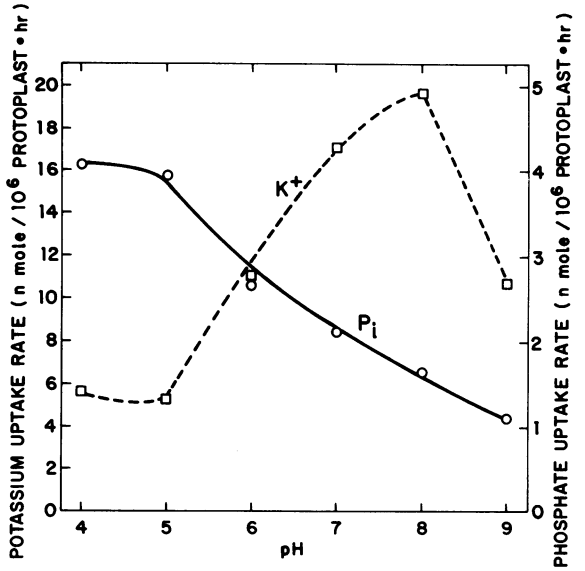

FIG. 5. Effect of pH on K^+ and Pi uptake in corn root protoplasts.

Proton Transport. An electrogenic proton pump located in the plasmalemma is believed to be directly involved in the ion transport process in plant cells (9–11, 14, 20, 22). The removal of cell wall would not be expected to have any effect on the proton pump of the cell. Acidification of the medium, which reflected the net proton efflux, was observed in the protoplast suspension (Fig. 3). This net proton efflux was sensitive to several chemical probes for the plasmalemma ATPase. The uncoupler, FCCP, stopped the H^+ efflux and equilibrated the H^+ concentration across the membrane. DES is suggested to inhibit H^+/K^+ exchanging ATPase (1, 12) but not OH^-/Pi antiporter activity (12). Therefore, DES should have a different effect on the H^+ pump than that of the uncoupler. DES altered the net H^+ flux direction (from efflux to influx) after a short (about 1 min) lag (Fig. 3). The nonpermeant—SH reagent, PCMBS, stopped the net H^+ flux as a result of the inhibition of both H^+/K^+ and OH^-/Pi exchangers (12). The addition of PCMBS also caused a great increase in buffering capacity of the medium, the small change in the external pH (if there was any) may not be detectable. A stimulator of the electrogenic proton pump, FC (14), doubled the apparent H^+ efflux (Fig.

Table II. *Potassium and Phosphate Uptake in Corn Root Protoplasts*

		K^+ (^{86}Rb) (nmol/10^6 protoplast·h)				Pi ($H_2{}^{32}PO_4^-$) (nmol/10^6 protoplast·h)			
		Observed	%	Calculated[a]	%	Observed	%	Calculated[b]	%
Control		12.23	100	11.67	100	2.40	100	1.98	100
+FC	(20 μM)	48.26	395	47.70	409	2.62	109	2.20	111
+FCCP	(5μM)	3.60	29	3.04	26	1.18	49	0.76	38
	(10 μM)	1.11	9	0.55	5	0.98	39	0.51	26
+DES	(50 μM)	4.85	40	4.29	37	1.73	72	1.31	66

[a] K^+ calculated rate was obtained by subtracting 0.56 (see text for detail) from each observed rate.
[b] Pi calculated rate was obtained by subtracting 0.42 (see text for detail) from each observed rate.

3, bottom curve). A similar effect of FC on H^+ was found in root segments (12). These data strongly suggest that, inasmuch as isolated protoplasts display an active electrogenic proton pump, other active ion uptake function can be predicted.

K^+ and Pi Uptake. The active accumulation of ions is the most direct evidence for the intactness and viability of protoplasts. Since the isolated protoplasts performed normal electrogenic proton pump activity (Fig. 3), protoplasts would also be expected to accumulate ions. Unlike that of root segments (12), the amount of K^+ and Pi accumulated in protoplasts leveled off after 30 min (Fig. 4). A similar fast leveling-off phenomenon has been reported in sugar and amino acid uptake in isolated pea leaf protoplasts (7).

Preliminary results showed that protoplasts traveling through the labeled absorption solution would occlude some nonabsorbed isotopes and misrepresent the accumulation rate. Accordingly, protoplasts were quickly spun through cold absorption medium containing 10 μM FCCP to measure the occlusion rate in each experiment. An average of 0.28×10^{-15} mol/protoplast and 0.21×10^{-15} mol/protoplast was calculated to be the amount of K^+ and Pi, respectively, which was occluded in protoplast pellets. These figures were used to correct the amount of ion accumulated in the protoplasts. Due to the fragility of the protoplast and the tight packing of protoplasts on the microtube tips, rewashing to reduce the contaminated isotope (nonabsorbed isotope) was not possible. When nonpermeant [^{3}H]inulin was used as a marker, the protoplast pellets from the FCCP-treated protoplasts had the same amount of ^{3}H activity as that of normal protoplast pellets.

Figure 5 shows that, between pH 4 and 8, protoplast K^+ uptake increased with increasing pH, whereas Pi uptake decreased. Similar observations have been reported in corn root segments (12). A sudden drop of K^+ uptake at pH higher than 8 reflects the damage of protoplasts, since pH 8 was commonly used in vacuole isolation (2, 13, 24).

The response of K^+ and Pi uptake to different chemical probes in protoplasts corresponded very well with that obtained for root segments. FCCP, FC, and DES were found to affect K^+ and Pi uptake differentially in root segments (12). In protoplasts (Table II), FCCP at 10 μM inhibited K^+ uptake by 90 to 95% and Pi uptake by 60 to 70%. FC quadrupled the K^+ uptake, whereas Pi uptake was only slightly increased; DES inhibited K^+ uptake by 60%, but with 30% inhibition of Pi uptake. Similar degrees of inhibition and stimulation and differential effects by FCCP, DES, and FC on K^+ and Pi uptake in corn root segments were reported before (12). The data strongly indicated that isolated protoplasts display similar ion transport properties to that of the root segments.

CONCLUSION

The method described proved to be feasible for a large scale and rapid isolation of intact viable root protoplasts. The total isolation could be done within 6 h. The protoplasts isolated were metabolically active. The plasmalemma and other organelles were well preserved. The isolated protoplasts performed almost identical active ion transport characteristics as that of the tissue from which they were derived. Data presented indicate that isolated protoplasts can be used to gain additional insights into the mechanism of ion transport in plant cells.

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