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H^+-pumping activities of plasma membrane and tonoplast from *Quercus rubra* roots

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Introduction

Climatic conditions in France are favorable for red oak *(Quercus rubra)*, which is being used increasingly in afforestation. But edaphic restriction, calcifuge property nitrogen nutrition (low level of NO_3^- accumulation) and sensitivity to high pH, suggested a contribution of membranes (plasmalemma and tonoplast) to these physiological properties. Herein we present the first results concerning *in vitro* identification of these membranes.

Two membrane fractions were collected from $d = 1.165$ g·cm^{-3} and $d = 1.108$ g·cm^{-3} steps of sucrose density gradients from 12 000–130 000 x *g* pellets. An aqueous 2 polymer phase system was used to obtain a better purification. The plasma membranes were identified in the high density fraction by the APTc (phosphotungstic acid–CrO_3) stain associated with vanadate-sensitive Mg^{2+} ATPase (Mg^{2+} adenosine triphosphatase) (pH 6.5). Tonoplast was characterized in the low density fraction by its negative reaction to APTc, the nitrate-sensitive Mg^{2+} ATPase (pH 8) and the presence of a PPase (pyrophosphatase).

ATP induces quenching of ACMA (9-amino-6-chloro-3-methoxy-acridine) fluorescence in both fractions and requires Mg^{2+}; the quenching is collapsed by NH_4^+ and nigericin. The initial rates of quenching (600–700% quenching·mg^{-1} prot·min^{-1} for plasmalemma and 500% for tonoplast) indicate very good coupling between hydrolytic and pumping activities, but the K_m were different.

Materials and Methods

Plant material

Roots were excised from young plants (3 wk) and chilled in cold aerated grinding medium.

Membrane isolation

50 g fresh weight of roots were homogenized in 150 ml of a grinding medium containing 0.25 M sucrose; 25 mM MES-Tris-carbonate (2-(*N*-morpholinoethanesulfonic acid-tris-(hydroxymethyl)aminoethane) (pH 7.2); 3 mM EGTA (ethyleneglycol (amino-2-ethyl) tetraacetic acid); 1 mM DTT (dithiothreitol); 0.5% BSA (bovine serum albumin); 5 mg·l^{-1} trypsin inhibitor, 5% PVP (polyvinyl pyrrolidone). After filtration (4 layers of gauze), the homogenate was centri-

fuged at 12 000 x *g* for 15 min and the supernatant at 130 000 x *g* for 30 min to prepare the microsome pellet.

For plasma membranes, the pellet was suspended in medium (0.25 M sucrose, 5.6% PEG (polyethylene glycol) 4000, 5.6% dextran, 10 mM KH_2PO_4, 30 mM NaCl, pH 7.8) of an aqueous 2-polypmer phase system. The membranes of the upper phase were centrifuged on a 16/22/30/38 (%, w/w) discontinuous sucrose density gradient at 80 000 x *g* for 60 min. The membranes were collected from the 30/38 interface.

For tonoplasts, the procedure was in inverse order. The microsomes were centrifuged on the sucrose density gradient and the 16/22 interface membranes were washed in the aqueous 2-polymer phase system. In this case, the lower phase was collected.

ATPase and PPase assays

ATPase activity was measured in a standard reaction mixture containing 40 mM Tris-MES (pH 6.5 or 8), 50 mM KCL, 3 mM $MgSO_4$, 200 μM Na_2MoO_4, 3 mM ATP-Tris. The reaction was carried out at 23°C for 10 min with 10–40 μg of membrane protein in a final volume of 600 μl.

PPase activity was measured in a standard reaction mixture containing 40 mM BTP-MES (pH 8), 50 mM KCl, 3 mM $MgSO_4$, 1 mM BTP-PP_i. The reaction was carried out at 23°C for 30 min with 30–50 μg of membrane protein in a final volume of 500 μl. After incubation, P_i release was determined according to Ames' method (1966).

Fluorescence assay

The decrease in internal pH of vesicles was assayed by the quenching of ACMA. Membranes (10–20 μg) were added to a fluorescence assay solution: 10 mM Tris-MES or BTP-MES, pH 6.5 or 8, 100 mM KCl, 3 mM $MgSO_4$, 2 μM ACMA (final volume: 4 ml). After addition of Mg^{2+}–ATP (or BTP-PP_i) the decrease in fluorescence at 500 nm was monitored with a Jobin Yvon JY 3D spectrophotometer at an excitation wavelength of 430 nm.

Protein estimation

Proteins were measured using the dye-binding method of Bradford (1976), with BSA as the protein standard.

Results

The 2 fractions were separated from ER (endoplasmic reticulum) (antymicin A-insensitive NADH cytochrome *c* reductase), mitochondria (cytochrome *c* oxidase), golgi (latent IDPase). The estimated contamination was around 15% (data not shown).

High density membranes (plasma membranes)

The fraction consisted of vesicles and shreds stained by phosphotungstic acid–CrO_3 (specific stain) with a β-GSII (β-glucan synthetase II) activity (data not shown). The ATPase activity (Table I) was stimulated by K^+ (50 mM), inhibited by vanadate, insensitive to NO_3^- with good specificity for ATP. Maximum activity was obtained at pH 6.5 (K_m value 0.7 mM). 30% of the ATPase was apparently latent and stimulated by TX–100 (0.02%). This part of the plasma membrane vesicles appeared to be sealed in a right-side out orientation. The capacity of ATP-driven H^+-transport across membranes (quenching of ACMA, Table I) reflected the formation of an interior acidification of the membrane vesicles (inside-out vesicles); quenching was blocked by nigericin and the sensitivities of the H^+-transport toward the inhibitors, vanadate (Fig. 1) and DES (data not shown), were quite similar to those of ATPase activity, but the K_m were different.

Low density membranes (tonoplast)

The fraction consisted of vesicles and most of them were not stained by phosphotungstic-acid–CrO_3. The ATPase activity (Table II) was stimulated by Cl^-, inhibit-

Table I. Properties of ATPase in high density (1.18) membranes (plasma membranes).

	ATPase activity		*Initial rate of quenching*
	μmol P_i·mg^{-1} protein·h^{-1}	*%*	*% quenching·min^{-1}·mg^{-1} protein*
control (ATP, 3 mM)	14.5	100	600–700
– KCl	9.9	68	
+ Na_3VO_4 (100 μM)	4.3	30	
I_{50}: 10 μM			I_{50} 3 μM
+ KNO_3^- (50 mM)	13.9	95	
+ gramicidin D (10 μM)	14.9	106	
+ nigericin (0.2 μg·ml^{-1})			0
+ Tx–100 (0.02%)	19.4	134	
+ oligomycin (10 μg·ml^{-1})	12.4	86	
+ NaN_3 (0.2 mM)	12.3	85	
ITP (3 mM)	7.2	50	
UTP (3 mM)	5.4	37	
GTP (3 mM)	5.3	36	
AMP (3 mM)	0.0	0	
PP_i (3 mM)	0.1	0.6	
V_{max} app.: 15 μmol P_i·mg^{-1} protein·h^{-1}			700
K_m app.: 0.7 mM			0.2 mM
pH optimum: 6.5			

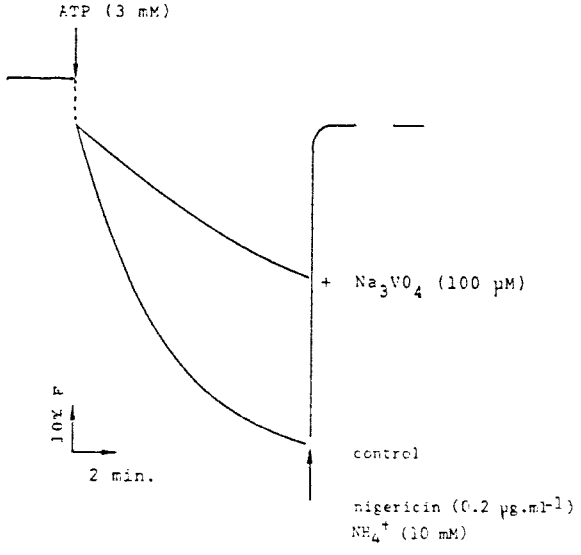

Fig. 1. High density membranes. ATP-dependent quenching of ACMA fluorescence (see Materials and Methods for assay medium).

Table II. Properties of ATPase and inorganic pyrophosphatase in low density (1.08) membranes (tonoplast vesicles).

	Activity		*Initial rate of quenching*
	μmol P_i·mg^{-1} protein·h^{-1}	*%*	*% quenching·min^{-1}·mg^{-1} protein*
ATPase			
control (ATP, 3 mM)	27.6	100	450–500
+ KCl (+K.MES 50 mM)	19.7	71	300–350
+ KNO_3^- (50 mM)	11.7	42	250–300
+ Na_3VO_4 (100 μM)	18.6	68	
+ gramicidin D (10 μM)	30.1	110	
+ nigericin (0.2 μg·ml^{-1})			0
+ oligomycin (10 μg·ml^{-1})	23.9	87	
+ Tx–100 (0.02%)	32.8	120	
ITP (3 mM)	7.3	26	
UTP (3 mM)	6.4	23	
GTP (3 mM)	8.5	30	
ADP (3 mM)	3.7	13	
AMP (3 mM)	2.8	10	
V_{max} app.: 29.2 μmol P_i·mg^{-1} protein·h^{-1}			450–500
K_m app.: 0.32 mM			0.67 mM
pH optimum: 6.5–8.5			
PPase			
control	5	100	100–200
– KCl (NaCl 50 mM)	0.2	4	nd
+ FK (10 mM)	0.1	2	nd
V_{max} app.: 5 μmol P_i·mg^{-1} protein·h^{-1}			100–200
K_m app.: 0.25 mM			

ed by NO_3^- (60%) and slightly inhibited by vanadate (plasma membrane contamination). The ATPase had high specificities for ATP, with a K_m value of 0.32 mM. Quenching of ACMA (initial rate: 500%) was collapsed by nigericin and inhibited by NO_3^-. K_m value of pumping: 0.67 mM (Fig. 2).

An inorganic pyrophosphatase activity stimulated by K^+ was detected. F^- (10 mM) induced total inhibition. This hydrolysis mediated a distinct proton translocation (initial rate: 100–200%) (Fig. 3).

Conclusion

The present work shows that 3 distinct types of electrogenic proton pumping, 2 ATPases and 1 PPase, are present in the

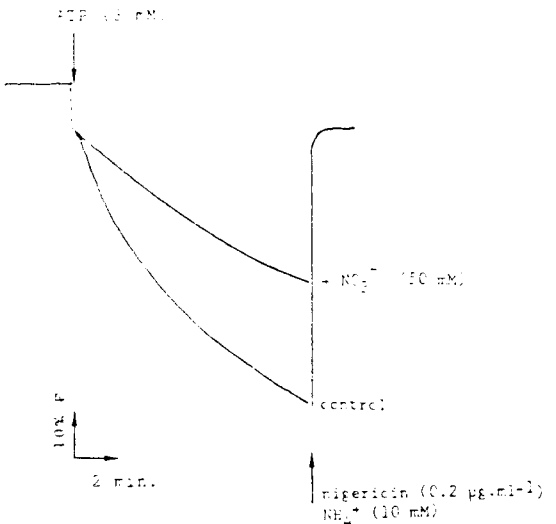

Fig. 2. Low density membranes ATP-dependent quenching of ACMA fluorescence (see Materials and Methods for assay medium).

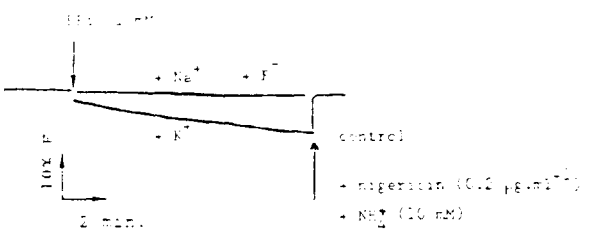

Fig. 3. Low density membranes. Pyrophosphatase-dependent quenching of ACMA fluorescence (see Materials and Methods for assay medium).

microsomal fraction of *Q. rubra* roots, as have been found in other plant materials (Sze, 1984). The H^+-translocating ATPases differ from each other in the following respects: the one in the low density membrane fraction is stimulated by Cl^-, inhibited by NO_3^- and relatively insensitive to cations and vanadate; whereas the other in the high density membrane fraction is stimulated by K^+, relatively insensitive to anions and inhibited by vanadate (Tables I and II). Based on these properties, the low density membrane ATPase may be of tonoplast origin and the other of plasma membrane origin. The PPase activity (stimulated by K^+ and inhib-

ited by F^-) confirms the tonoplast identification (Wang *et al.*, 1986).

About 30% of the ATPase activity in the tonoplast fraction was sensitive to vanadate (Table II). The data suggest a partial contamination by plasma membranes.

Vanadate and nitrate have similar effects on ATPase and H^+-pumping (Figs. 1 and 2). Thus the H^+-pumping observed is driven by ATP hydrolysis. The difference between hydrolysis and pumping K_m is not clear, the heterogeneity in the accessibility of the substrate to the catalytic site (shreds, inside-out and right-side-out) could explain these results.

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