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EFFECT OF THE PHOTOPERIOD ON THE PLASMA MEMBRANE ATP-DEPENDENT H^+-PUMPING ACTIVITY OF SPINACH PETIOLES

BY

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ABSTRACT

Effect of the photoperiod on the plasma membrane ATP-dependent H^+-pumping activity of spinach petioles. - The plasma membrane from spinach (*Spinacia oleracea*, cv.Nobel) petioles was purified by phase partitioning and the use of enzyme markers showed that this fraction was highly enriched in plasma membrane vesicles. This fraction was almost devoid of phosphohydrolase activities originating from endomembranes (tonoplastes, mitochondria, non specific phosphatases and golgi apparatus). The ATP-dependent H^+ pumping activity in this fraction was Mg^{2+} and K^+ dependent and was not stimulated by valinomycin. It was unsensitive to nitrate (tonoplaste ATPase inhibitor) but sensitive to vanadate (plasma membrane ATPase inhibitor). This activity was specific for ATP and had a pH optimum around 6.5. The kinetic constants (Km_{app} and V_{max} for ATP) of the H^+ pumping activity of the purified plasma membrane were determined from plants grown in short days (vegetatives) and from similar plants induced by 24 h of continuous light. Whereas Km_{app} have not been changed after light induction, values of V_{max} and the activity in the presence of 0.5 mB ATP have been increased suggesting a regulation of the enzyme after light induction.

Key-words: H^+-ATPase, Plasma membrane, *Spinacia oleracea*.

Abbreviations: ADP, Adenosine diphosphate; ATP, Adenosine triphosphate; BSA, bovine serum albumin; BTP, bis-tris-propane (1,3-bis (tris (hydroxymtehyl) methylamino) propane); DTT, dithiothreitol; GTP, Guanosine triphosphate; IDP, inosine diphosphate; Mes, 4-morpholinoethane sulfonic acid; PNPP, p-nitrophenylphosphate; PMSF, phenylmethylsulfonylfluoride; PP_i, Pyrophosphate; Tris, Tris (hydroxy methyl)-aminomethane; UDPG, uridine diphosphoglucose.

INTRODUCTION

The plasma membrane of cells plays a crucial role in the perception, transduction and transport of environmental signals within the cell leading to a particular response. One of the important enzymes which seems to control growth, development, nutrient transport and stomata mouvements is the H^+-pump of the plasma membrane (SERRANO, 1989). Reflecting the physiological importance of this activity, it is to be expected that the enzyme is subject to strict regulation *in vivo* by plant growth factors such as plant hormones, growth-modifying pathogens and light (ASSMANN *et al.*, 1985; BIDWAI *et al.*,

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1987; CLELAND, 1987). This enzyme belongs to the P-type family of cation-translocating enzymes characterized by formation of phosphorylated intermediate, inhibition by vanadate and structural similarity in several conserved domains (SERRANO, 1989). It generates an electric potential and pH gradient (H^+ motive force) that drive solute mouvements cross the plasma membrane. In plants (e.g. tobacco, tomato, *Arabidopsis thaliana*) the plasma membrane H^+-ATPase is encoded by a multigene family, members of which are differentially regulated from one tissu to another within the plant and in a same tissu in response to different developmental or environmental conditions (PARDO & SERRANO, 1989; HARPER *et al.*, 1990; ROLDAN *et al.*, 1991; HASE, 1993; EWING & BENNETT, 1994).

Different studies underwent on spinach demonstrated that the plants could be induced to flowering by different chemical and physical ways (AUDERSET & GREPPIN, 1976). In this paper, we report the purification of the plasma membrane from spinach petioles (*Spinacia oleracea*, cv.Nobel). The highly purified plasma membrane fraction suitable for biochemical studies was characterized by enzyme markers and the activity of the H^+-ATPase was first characterized and tested after flowering induction of the plants.

MATERIAL AND METHODS

Plant Material

Spinach (*Spinacia oleracea*, cv.Nobel) plants at the vegetative stage were grown in a phytotron for 4 weeks under non inductive short days (SD) of 8 h (8:00 am to 4:00 pm; 400 $\mu mol.m^{-2}.s^{-1}$) and 16 h dark (4:00 pm to 8:00 am). The light was provided by white fluorescent tubes (40 W, 244332 Sylvania USA). The temperature was set at 20 ± .1°C and the relative humidity at 80 ± 5% during the light period and 60 ± 5% during the darkness respectively. Floral induction was initiated by continuous light treatment (24 h) of plants grown in SD for 4 weeks (AUDERSET & GREPPIN, 1976).

Preparation of microsomal vesicles

Crude microsomal vesicles were prepared as described by DE MICHAELIS & SPANSWICK (1986). Petioles (12 g) were harvested and homogenized at 4°C in a medium (4 ml per g fresh weight) containing 250 mM sucrose, 2 mM EGTA, 2 mM $MgSO_4$, 2mM Na_2 ATP, 10% (v/v) glycerol, 1 mM PMSF, 2mM DTT, 0.5% (w/v) BSA, 25 mM BTP-MES adjusted to pH 7.6. The homogenate was filtered and centrifuged at 6,000 x g (7,000 rpm, Sorvall, SS-34 rotor) for 10 min at 4°C. The resulting supernatant was filtered through one layer of Miracloth (475855 Calbiochem) and centrifuged at 30,000 x g for 30 min at 4°C. The pellet (crude microsomes) was resuspended in phosphate buffer (KH_2PO_4: Na_2HPO_4 5mM pH 7.8) and subjected to plasmalemma purification by partitioning in an aqueous polymer two-phase system (12 g system) as described by KJELLBOM & LARSSON (1984). The upper phase was washed one time in a fresh lower phase and the resultant upper phase U_2 was diluted three times in a resuspension medium containing 250 mM sucrose, 10% glycerol, 1 mM DDT, 0.2% BsA, 2.5 mM

BTP-MES pH 7. The plasma membrane vesicles were recovered by centrifugation at 30,000 x g for 30 min at 4°C and resuspended in the same medium. The plasma membrane vesicles were stored at - 80°C until used.

Proton pumping activity

The initial rate of acridine orange absorbance quenching was utilized to measure the plasmalemma proton translocation activity as described by BENNETT & SPANSWICK (1983). This activity was expressed in arbitrary unit (AU) min^{-1} mg^{-1} protein. Absorbance was measured with a Perkin-Elmer 320 spectrophotometer at the wavelength of 495 nm. Thawed membrane vesicles (about 20 µg proteins) were incubated at room temperature in 1 ml of 25 nM BTP-Mes pH 6.5, 20 µM acridine orange, 0.25 µM valinomycin, 50 mM KBr and 10 mM $MgSO_4$. The reaction was started by addition of 1.5 mM Na2 ATP.

ATPase activity

The plasmalemma ATPase activity was measured at 37°C during 30 min in the presence of 50 mM KCl, 5mM $MgSO_4$, BTP-Mes 25 mM pH 6.5. The reaction was started by addition of 1.5 mM Na_2 ATP. The membrane vesicles were pelleted at 12,000 rpm (microfuge Sorvall) for 10 min at 4°C and the inorganic phosphate released by ATP hydrolysis was determined by adding 0.2 ml of the resultant supernatant to 0.5 ml of 0.24% (w/v) ammoniummolybdate (in 0.5 M H_2SO_4), 0.96% (w/v) ascorbic acid and 0.8% (w/v) trichloroacetic acid. After incubation at 36°C for 10 min the absorbance at 660 nm was measured.

Other assays

Glucan synthase II was determined after KAUSS & JEBLICH (1985). UDPG sterol-glycosyl transferase was determined after CHANSON *et al.* (1984). NADH dependent cytochrome C reductase was determined after PALMGREN *et al.* (1990). Cytochrome C oxidase was determined after HODGES & LEONARD (1972).

Protein determination

Membrane aliquot (30 µl) was diluted in 1 ml of cold water and centrifuged at 96,100 x g (Beckman, T-50 rotor) for 30 min at 5°C. The pellet was resuspended in cold water (160 µl), and protein was determined using Bio-Rad solution. 50 µl of protein suspension were diluted in 750 µl of water and 200 µl of Bio-Rad solution were added. The resulting solution was mixed and incubated at room temperature for at least 5 min. The OD at 595 nm was measured and proteins were quantified using BSA as standard.

Reproducibility of the data

Each measurement was repeated three times and standard deviation (SD) calculated. For determination of kinetic constants (Km_{app} and V_{max}) and the activity of the H^+-pumping in the presence of 0.5 mM ATP, extraction and purification of the plasma membrane vesicles was repeated two or three times (see captions of figures).

RESULTS AND DISCUSSION

Plasma membrane vesicles prepared by aquous phase partitioning were characterized by plasma membrane markers (glucan Synthase II, UDPG sterolglycosyltransferase, proton pumping ATP-dependent) and a possible contamination by endomembranes determined by other markers (NADH-cytochrome C reductase for endoplasmic reticulum, NADH-cytochrome C oxidase for mitochondria). As shown in table 1, the plasma membrane markers were more present in the U_2 fraction than in the endomembranes fraction (lower phase L). However, the NADH-cytochrome C reductase activity was more present in the L fraction than in the U_2 fraction, and the cytochrome C oxidase activity was absent in the U_2 fraction. The IDPase and the pyrophosphatase activities were also absent in this fraction (Tab. 2, Tab. 4), showing that the U_2 fraction is highly plasma membrane enriched.

The H^+-pumping activity of the purified plasma membrane showed a pH optimum around 6.5 (data not shown), similar to that described for other plant species (DE MICHELIS & SPANSWICK, 1986; BELLAMINE *et al.*, 1994; OLIVARI *et al.*, 1993), and requires ATP as source of energy than other phosphorylated substrats tested. No activity was measured in the presence of GTP, ADPO, PNPP, IDP or PPi (Tab. 2). This activity was Mg^{2+} and K^+ dependent, and was not stimulated by valinomycin (Tab. 3). It was insensitive to 50 mM KNO_3 (vacuolar ATPase inhibitor) and about 50% inhibited by only 20 µm vanadate (plasma membrane ATPase inhibitor) (Tab. 3). The ATP phosphohydrolase Mg-dependent activity of the purified plasma membrane was almost unsensitive to nitrate, Na-molybdate (non specific phosphatase inhibitor) and azide (mitochondrial ATPase inhibitor), and showed that the purified plasma membrane fraction was almost devoid of phosphohydrolase activities originating from endomembranes as assessed by the effects of nitrate (tonoplast), azide (mitochondria), sodium molybdate and p-nitrophenyl-phosphate (non-specific phosphatases) or IDP (golgi apparatus) (Tab. 4). These results showed that the ATP hydrolysis and the proton transport into the purified vesicles were mostly due to the plasma membrane ATPase activity.

Kinetic constants (Km_{app} and V_{max} for ATP) of the H^+ pumping activity were determined from plants grown in short days and from similar plants induced by 24 h of continuous light (Tab. 5). Whereas Km_{app} have not been changed after light induction, V_{max} and the activity in the presence of 0.5 mM ATP have been increased (Fig. 1) suggesting a regulation of the enzyme after light induction of spinach petioles. An enhanced synthesis of the polypeptide or of more active isozyme during plant development may result in the increase of the ATPase activity (ROLDAN *et al.*, 1991; HASE, 1993), but other mechanisms of regulation (e.g. phosphorylation, release of C-terminal domain) may be taken into account (PALMGREN, 1991).

RÉSUMÉ

La membrane plasmique des petioles d'épinard (*Spinacia oleracea*, cv.Nobel) a été purifiée par partition de phase, et l'utilisation des enzymes marqueurs a montré que cette fraction est hautement enrichie de vésicules de la membrane plasmique. Cette

fraction est presque dépourvue des activités phosphohydrolases originaires des endomembranes (tonoplastes, mitochondries, phosphatases non spécifiques et appareil de golgi). L'activité H^+ ATPase dans cette fraction est Mg^{2+} et K^+ dépendante, et n'est pas stimulée par la valinomycine. Elle est insensible au nitrate (inhibiteur de l'ATPase du tonoplaste) mais sensible au vanadate (inhibiteur de l'ATPase du plasmalemme). Cette activité est spécifique pour l'ATP et possède un pH optimum de 6.5 environ. Les constantes cinétiques (Km_{app} et V_{max} pour l'ATP) de l'activité enzymatique ont été déterminées chez des plantes cultivées en jours courts (végétatives) et des plantes induites par 24 h de lumière continue. Alors que Km_{app} n'a pas changé après induction, les valeurs de V_{max} et de l'activité en présence de 0.5 mM ATP ont augmenté, ce qui suggère une régulation de l'enzyme après induction lumineuse.

TABLE 1

Characterization of plasma membrane purified by two phase partitioning. Enzyme markers of membranes present in upper phase (U2) and lower phase (L) after two-phase partitioning of a crude microsomes preparation from spinach petioles. U2 phase represents the purified plasma membrane fraction. L phase represents the endomembranes fraction. The values are the specific activities ± SD. The values betwen parenthesis represent the enrichment relative to the specific activities of the crude microsomal fraction.

Specific activity of enzyme markers	Crude microsomes	U_2	L
Glucane synthase II (Dpm min^{-1} μg^{-1} prot.)	53.34 ± 3.04 (1)	83.42 ± 3.85 (1.56)	59.98 ± 4.23 (1.12)
UDPG-sterol transferase (Dpm min^{-1} μg^{-1} prot.)	3.03 ± 0.13 (1)	7.74 ± 0.55 (2.55)	1.61 (0.53)
NADH dep-cyt.C reductase (nmol min^{-1} mg^{-1} prot.)	152.3 ± 0.004 (1)	82.7 ± 0.01 (0.54)	135.2 ± 0.004 (0.89)
Cyt.C oxidase (nmol min^{-1} mg^{-1} prot.)	83.3 ± 0.01 (1)	0.00 ± 0.00 (0)	136.5 ± 0.003 (1.64)

TABLE 2

Effect of some phosphorylated substrats on H^+-pumping activity of the purified plasma membrane. The reaction was started by addition of 1.5 mM of the substrat, and the activities were expressed in AU min^{-1} mg^{-1} protein ± SD. All the substrats were sodium salt and the reaction medium contained 10 mM $MgSO_4$.

Substrate	Initial rate of substrat-dependent quenching of acridine orange absorbance (AU min^{-1} mg^{-1} protein)
ATP	22.8 ± 1.54
GTP	00
ADP	00
PNPP	00
IDP	00
PPi	00

TABLE 3

H^+-pumping activity of the purified plasma membrane from spinach petioles. The activity was tested for the effect of magnesium, valinomycin, potassium, nitrate (in this case the KCl was omitted) and vanadate. The results were expressed in specific activities (AU min^{-1} mg^{-1} protein) ± SD and values between brackets represent the percentage of the activities to control. The reaction was started by addition of 1.5 mM ATP sodium salt.

Incubation medium	Specific activity of H^+ pumping (AU min^{-1} mg^{-1} protein)
Control	41.96 ± 2.70 (100)
Incubation medium without:	
- Mg^{2+}	00
- Valinomycin	41.56 ± 4.02 (99)
- KBr	00
Incubation medium with:	
KNO_3 (50 mM)	42.11 ± 2.97 (100)
Na_3VO_4	
20 µM	21.69 ± 1.38 (52)
100 µM	00
200 µM	00

TABLE 4

ATP hydrolysis activity of the purified plasma membrane from spinach petioles. The activity was assayed in the presence of 50 mM KCl and 5 mM $MgSO_4$ (control) or with the modifications indicated in the table. When the effect of KNO_3 was tested, the KCl was omitted. The reaction was started by addition of 1.5 mM ATP, PNPP or IDP. All the substrats were sodium salt. The results were expressed in specific activity (µmol P_i min^{-1} mg^{-1} protein) ± SD. Values between brackets represent the percent of the specific activity to control.

Treatment	ATP hydrolase activity ± SD (µmol P_i min^{-1} mg^{-1} protein)
Control	0.448 ± 0.009 (100)
- Mg^{2+}	0.046 ± 0.004 (10)
+ KNO_3 (50 mM)	0.423 ± 0.011 (94)
+ Sodium molybdate (1 mM)	0.550 ± 0.01 (105)
+ NaN_3 (5 mM)	0.477 ± 0.008 (106)
PNPP (1.5 mM)	0.045 ± 0.003 (9)
IDP (1.5 mM)	0.070 ± 0.014 (16)

TABLE 5

Kinetic constants of the H^+-pumping activity of the purified plasma membrane from spinach petioles. Values of apparent Michaelis-Menten constant (Km_{app}), maximal activity (V_{max}) of the plasma membrane ATP dependent H^+-pumping activity from vegetative plants and plants induced by 24 h of continuous light. Km_{app} is expressed in mM ATP and V_{max} was expressed in AU min^{-1} mg^{-1} protein.

Treatment	Vegetative	Induced
Exp. N° 1		
Km_{app}	0.531	0.660
V_{max}	41.62	69.91
Exp. N° 2		
Km_{app}	0.329	0.350
V_{max}	33.80	69.82

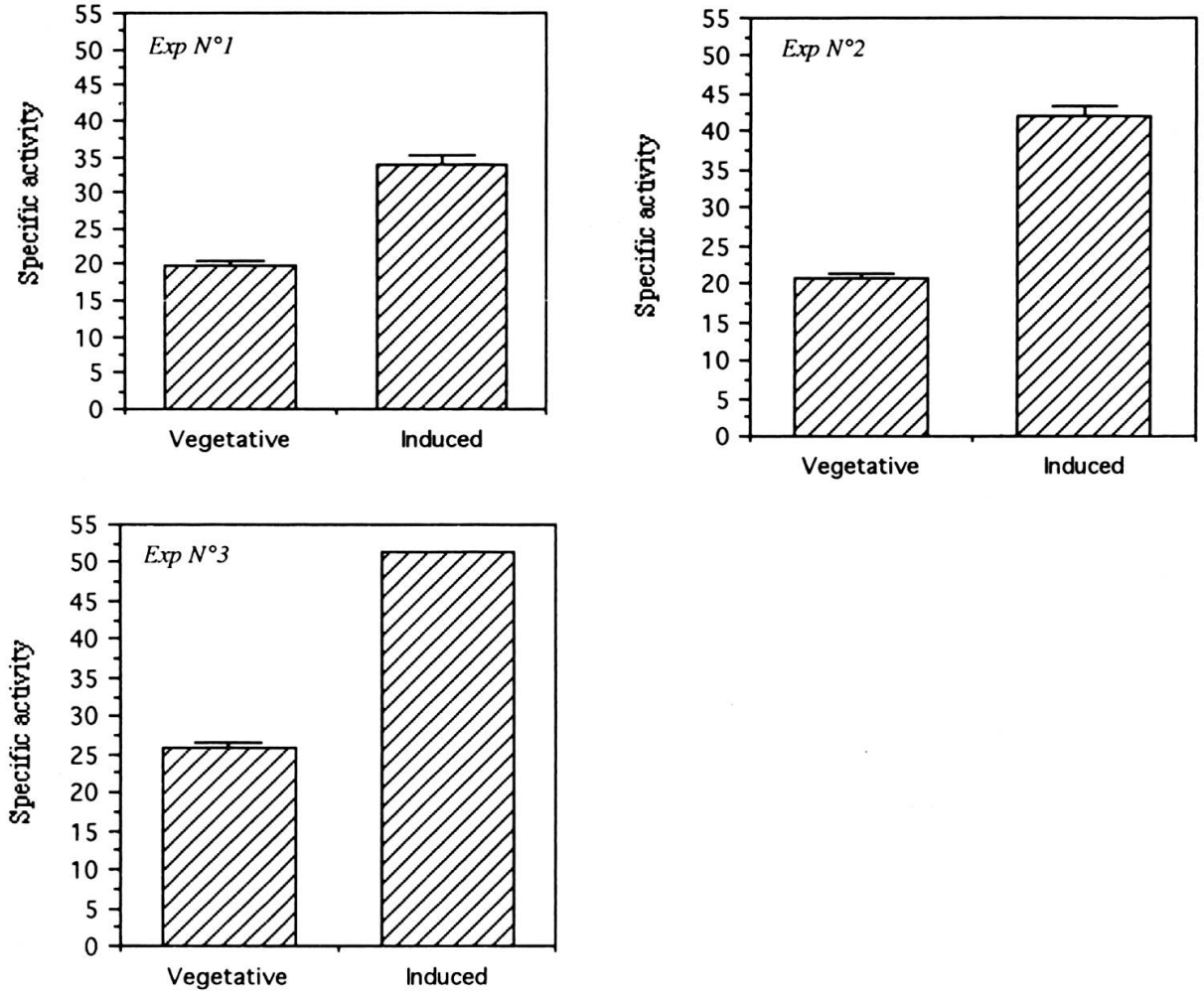

FIG. 1.

Activities in the presence of 0.5 mM ATP of the plasma membrane ATP dependent H^+-pumping from vegetative plants and plants induced by 24 h of continuous light. They were expressed in AU min^{-1} mg^{-1} protein ± SD. The experiments were repeated three times (Exp N° 1, N° 2, N° 3) with a novel preparation of the plasma membrane.

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