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Phosphatase and ATPase activities in isonuclear lines of cytoplasmic male-sterile and male-fertile petunia*

M. Perl, D. Swartzberg, and S. Izhar

Department of Plant Genetics, ARO, The Volcani Center, Bet-Dagan 50 250, Israel

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Summary. Soluble and membrane-bound fractions of plant leaves, cell suspension cultures and seedlings of petunia were examined for phosphohydrolase activity on *p*-nitrophenyl phosphate (pNPPase) and adenosine triphosphate (ATPase). One cytoplasmic male-sterile (CMS) and one fertile (F) line was examined for each tissue. Both pNPPase and ATPase exhibited a broad optimal activity between pH 5.5–7.0 for the membrane-bound fraction and between 4.5–7.0 for the soluble fractions. The activity of both were inhibited by divalent ions including Mg^{2+}. At pH 7.2, the activities on various triphosphonucleotides were similar and they were hydrolyzed by a rate of 20–50% of that of ATP. Significant differences between CMS and F extracts were: (a) higher activities in CMS membranes; (b) lower Ea (energy of activation) values for activities in CMS membrane functions; (c) seedling and cell-culture CMS extracts exhibited a higher sensitivity to high temperature denaturation; (d) the hydrolase activity on mono- and triphospho-cytosine compounds was significantly higher in CMS than in F membranes.

Key words: ATPase – Cytoplasmic male sterility – Petunia – Phosphatase

Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait, leads to the abortion of the pollen mother cells during gametogenesis and microsporogenesis. The biochemical activities of anthers that characterize CMS plants may result from impaired membrane structure and function (Bino 1985; Evenor 1988), followed by inefficient transport abilities (Perl et al. 1992).

Plant cell-wall-associated enzymes, which are grouped under the name of acid phosphatases (EC 3.1. 3.3), may be involved in nutritional uptake. They are widely distributed in various plant tissues and cell types (Duff et al. 1991), are inducible by water stress and Pi availability (Goldstein et al. 1988) and may be excreted into the environmental medium (Goldstein et al. 1988; Ueki 1978). Phosphatases may also play a role in the mobilization of nutrient reserves (Flinn and Smith 1967) and in the active transport of sugars and other materials across the membranes (Figier 1968).

Relatively higher acid phosphatase activities have been demonstrated in the shriveled seeds of an intergenic hybrid of triticale than in plump normal seeds (Ching et al. 1984, 1987). High phosphatase activity may deplete the energy supply for biosynthesis and degrade the substrates for the membrane components required for tissue integrity (Ching et al. 1987). The study presented here characterizes *p*-nitrophenyl phosphatase (pNPPase) and adenosine triphosphatase (ATPase) activities in cell cultures, seedlings and plant leaves of petunia, revealing some differences between CMS and fertile (F) lines that may explain the possible role of these activities in the process of sterilization of the plant.

Materials and methods

Cell suspension cultures of petunia (*Petunia parodii Steere*) lines 3699 (fertile, F) and 4544 (cytoplasmic male sterile, CMS) were generated by growing surface-sterilized stem segments in liquid UMIA medium as described by Connett and Hanson (1990).

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Correspondence to: M. Perl

The cells were harvested by centrifugation or by a glass-fiber filter under vacuum suction, homogenized in 50 m*M* ice-cold TRIS-HCl buffer, pH 7.2 (extraction buffer) and centrifuged at 18 000 *g* for 15 min at 4 °C. The supernatant (soluble fraction) was mixed with an equal volume of glycerol and kept at −18 °C for enzyme examination. The pellet was resuspended in the extraction buffer plus 4% Triton × 100 (v/v) and incubated on ice for 20 min. After centrifugation (18 000 *g*, 15 min), the supernatant was discarded and the pellet washed twice with 20 vol of the extraction buffer. The final pellet was resuspended in 50% glycerol in the extration buffer and kept at −18 °C. Earlier experiments had shown that glycerol does not interfere with the examined enzyme activities and that the prescribed method is suitable for preserving the activities intact for at least several weeks.

For sterile seedlings, seeds were surface sterilized and sown aseptically on Murashige and Skoog medium supplemented with sucrose and agar in test tubes as described in Moreno et al. (1985). Seedlings approximately 8 weeks old were washed, homogenized and fractionated as described above.

Plants were obtained by planting petunia seedlings on 50% peat and 50% vermiculite in greenhouses. Leaves were taken from 6- to 7-week-old growing plants and homogenized; fractions were prepared as described for the cell cultures.

pNPPase was investigated by two methods: (a) Routinely, 0.1 ml of tissue extract was added to a preheated (37 °C) 0.9-ml aliquot of 10 m*M* *p*-nitrophenol (pNPP) in 0.3 *M* TRIS-HCl buffer, pH 7.2, and the change in absorbance at 400 nm was recorded automatically in a double-beam, temperature-controlled (37 °C) spectrophotometer. For activity calculations, the extension coefficient of pNPP under the experimental pH conditions was quantitated. (b) To preincubated 10 m*M* pNPP in buffer, 0.1 ml tissue extract was added. After a 2-min incubation at 37 °C 1 ml of 0.5 *M* Na_2CO_3 was added and the absorbence at 400 nm was measured (Ec = 18/m*M*).

ATPase activity was measured, if not otherwise stated, by adding 50 µl tissue extract to 0.2 ml 10 m*M* adenosine triphosphate (ATP) in 0.3 *M* TRIS-HCl buffer, pH 7.2. After a 15-min incubation at 37 °C, 25 µl of 50% trichloroacetic acid was added; the mixture was then centrifuged (18 000 *g*, 10 min), and 50-µl and 100-µl samples of the supernatant were examined for Pi (inorganic phosphate) content by the Baginski method as described by Esmann (1988). Each experiment included a tube incubated in the absence of enzyme and another incubated in the absence of ATP. Two standard concentrations (50 and 100 nmol) were also run concomitantly.

Protein concentrations were examined in the pellets of 5% trichloroacetic acid obtained from the soluble fraction or in aliquots of the membrane fractions by the method of Bradford (1976). For the membrane fractions the absorbance of the particles per se were taken into account in the calculation of the activities.

Results

Profiles of the effect of pH on pNPPase and ATPase activities are depicted in Fig. 1. Although the data are for seedling extracts, similar profiles have been obtained for cultured cell and plant extracts. In cell extracts the maximal activity is slightly shifted toward the alkaline range (6.0–7.5) for both activities. It is clearly demonstrated that ATPase activity decreases under low pH conditions, while the pNPPase is highly

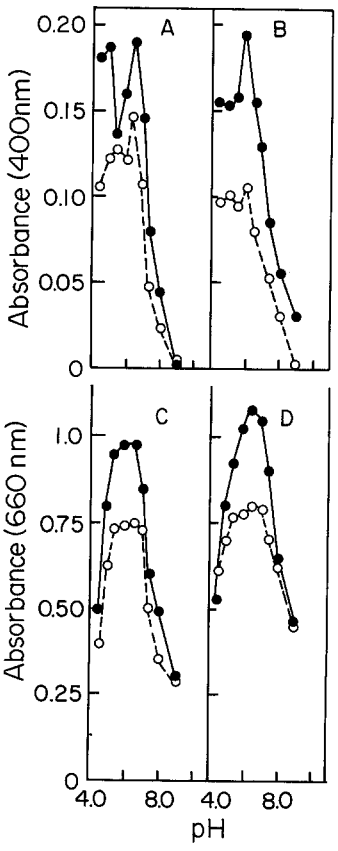

Fig. 1A–D. The effect of pH on pNPPase and ATPase activities in seedlings. **A, B** pNPPas; **C, D** ATPase; **A, C** supernatant; **B, D** membrane fractions. ● CMS, ○ F. For details see Materials and mehods

active between pH 4.0 and 7.0, indicating a multiple activity on pNPP.

Some characteristics of pNPPase and ATPase are presented in Table 1. The apparent Km (Michaelis constant) values for both substrates do not differ significantly between the soluble and the membrane activities, or between CMS and F enzyme sources. Likewise, there are no differences between CMS and F activities in the apparent Ki (Inhibitor characteristic constant) values for some inhibitory examined ions, though there are some differences between plant and cell extracts.

A noticeable difference between F and CMS membrane-ATPase is depicted in Fig. 3. The low concentration effect and the competitive inhibition profile of molybdate is similar in both extracts, but the activity of the F membrane-ATPase reveals the presence of an isoform with a non-competitive inhibition pattern at relatively high concentrations of molybdate. Other significant differences shown in Table 1 are between the Ea (energy of activation) values for CMS and F membrane fractions, indicating a higher temperature sensitivity for the F membrane-

Table 1. Some characteristics of ATPase and pNPPase activities in plant and cultured cell extracts

	Supernatant				Membrane			
	Plant		Cell		Plant		Cell	
	CMS	F	CMS	F	CMS	F	CMS	F
Apparent Km values (mM)[a]								
For ATP	2.2(0.9)[d]	2.3(10)	2.5(0.6)	3.3(0.6)	3.5	2.9	1.5	2.1
For pNPP	2.8(0.5)	2.2(1.1)	3.1(0.7)	2.6(1.0)	2.5	2.5	2.2	1.3
Ea values[b]								
For ATPase	5300(950)	6600(1100)	7000(1000)	6500(800)	5300(400)	7200(800)	7800(1100)	10000(1200)
For pNPPase	ND	ND	ND	ND	6100	7900	8200	11000
Apparent Ki values (mM)[c]								
$ZnCl_2$ (ATPase)	1.2	1.2	2.5	1.5	0.5	1.2	2.5	2.2
NH_4VO_3 (ATPase)	0.1	0.2	1.0	0.5	0.1	0.1	1.0	0.5
$MgSO_4$ (ATPase)	2.0	1.0	0.5	0.5	1.5	1.3	2.0	1.5
$CaCl_2$ (ATPase)	2.5	2.5	1.5	1.5	2.5	2.5	2.5	2.5
K_2HSO_4 (pNPPase)	2.0	2.2	3.8	3.2	2.0	3.0	1.8	0.9

[a] Data from three experiments for supernatants and the average of two experiments for membranes
[b] Data from three to four experiments for ATPase and data from one experiment for pNPPase. Ea Energy of activation calculated from Arrhenius plots
[c] All data are the average of two experiments
[d] SD indicated in brackets

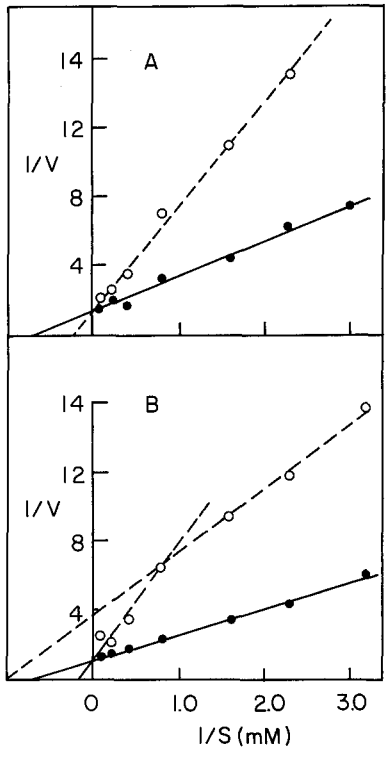

Fig. 2A–B. The effect of ammonium molibdate on ATPase activity on Triton-treated membranes from CMS (**A**) and F (**B**) cultured cells at various concentrations of ATP. The data of one experiment are presented by a Lineweaver Burke plot. ● No additions, ○ + ammonium molibdate (5 μ*M*). For details see Materials and methods

ATPase as compared with the CMS membrane-ATPase. Figure 3 shows that the CMS ATPase activities in cells and seedlings are less stable at high temperature incubation (55 °C) as compared with F activities. In plant extracts the CMS seems to be more stable, though both the membrane and the supernatant activities are more susceptible to high temperature as compared with seedlings and cell extracts.

Substrate specificity was examined for a variety of tri- and mono-phosphonucleotides (Table 2). It is

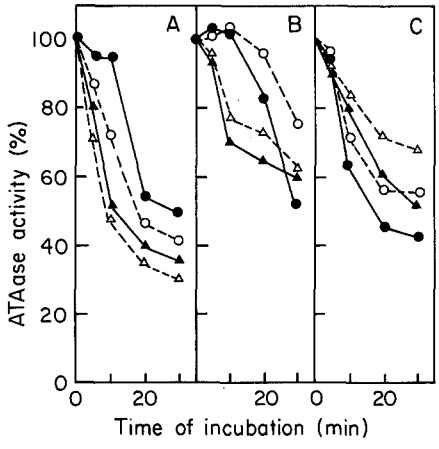

Fig. 3A–C. The effect of preincubation of tissue extracts at 55 °C on ATP activities. **A** Plant leaves, **B** cultured cells, **C** seedlings. *Closed symbols* CMS, *open symbols* F, *circle* membranes, *triangles* supernatant. For details see Materials and mehtods

Table 2. Relative phosphohydrolase activities (percentage of ATPase) on various substrates in the membrane fractions of CMS and F petunia plant leaves, seedlings and cultured cells. (All at 10 m*M* substrate)

Substrates	Plant leaves		Seedlings		Cultured cells	
	CMS	F	CMS	F	CMS	F
ATP	100	100	100	100	100	100
GTP	97	93	83	103	100	79
CTP	135(14)[c]	110(12)	100(3)	94(14)	88(16)	100(8)
UTP	94	100	102	97	93	98
AMP	25	54	33	29	36	36
GMP	45	38	43	43	65	55
CMP	57(10)	40(12)	32(6)	9(8)	50(7)	39(3)
UMP	24	32	31	35	35	38
TMP	26	32	19	32	63	43
XMP	78	57	35	65	57	45
Deoxy CMP	52(11)	29(8)	26(4)	13(5)	45(6)	35(3)
3′ AMP	38	32	9	0	55	56
PEP[a]	83	113	19	21	51	61
pNPP[b]	200	150	125	143	158	157
Glucose-1-ph	22	31	18	16	22	27

[a] Phosphoenolpyruvate
[b] *p*-Nitrophenylphosphate
[c] Data from three to four experiments (in brackets: SD); all other data are averages of two experiments

Table 3. The distribution of protein concentrations, ATPase and pNPPase in CMS and F seedlings (A) and in plant leaves and cultured cells (B) of petunia

A. Seedlings	Supernatant		Pellet[a]		Triton-treated membranes[b]	
	mg/g FW	% of total	mg/G FW	% of total	mg/g FW	% of total
Protein						
CMS	11.5	84.5	2.2	15.5	0.79	5.5
F	10.4	81.0	2.3	19.0	0.64	5.0
	µmol/h per gFW		µmol/h per gFW		µmol/h per gFW	
ATPase activity						
CMS	287	85	52	15.0	38	11
F	234	84	46	16.0	21	7.5
pNPPase						
CMS	344	83	72	17.0	49	12
F	306	85	53	15.0	30	8.4

B. Plant leaves and cultured cells	Percentage of total Protein		ATPase		pNPPase	
	CMS	F	CMS	F	CMS	F
Plant leaves						
Supernatant	66	77	50	70	53	66
Triton-treated membranes	16	19	38	21	37	22
Cultured cells						
Supernatant	56	63	31	29	30	26
Triton-treated membranes	21	23	49	41	46	39

[a] Washed pellet
[b] Extracted pellet with 4% Triton × 100

worthwhile to notice: (a) the relatively high and similar phosphohydrolase activities on all four triphosphonucleotides in the membrane fractions of plants, seedlings and cells; (b) the significantly higher phosphohydrolase activities on cytosine monophosphonucleotides in CMS membranes than in F membranes. This was not found in the supernatant fractions (not shown).

The distribution of pNPPase and ATPase activities among the soluble and membrane-bound fractions is presented in Table 3. This table shows the full data for the seedling fractions and the percentages for plants and cells that have been calculated similarly to those for seedlings. The data indicate that in all of tissue sources examined, CMS membrane fractions contain a higher percentage of the total ATPase and pNPPase activity examined under our conditions. Since the percentage of protein in plants and cells are lower in the CMS membrane fractions, the high specific activities (per protein) in those fractions are even more emphatic.

Discussion

The data presented show that CMS tissues, have higher membrane- (or cell-wall)-bound phosphohydrolase activity than F tissues. Although acid phosphatases are defined by their high activity under low pH conditions, it has been shown that the optimal pH may be shifted by the presence of various compounds, including a purified different isoform of phosphatase (Paul et al. 1987). Thus, the activities demonstrated here may represent other phosphohydrolases rather than acid phosphatase. The examined hydrolase activities seem to be triphosphonucleotide specific, although they exhibit a higher activity on the artificial substrate pNPP, which is commonly used for acid phophatase determination.

Ching and her coworkers (1987) have shown that shrunken endosperm of mutants of *Triticale* seeds, which are a result of mitotic errors in the endosperm during early coenocytic period (Thomas et al. 1980), exhibit significantly higher pNPPase and ATPase activities than plump endosperms. They have shown that this increased phosphohydrolase activity may be a result of membrane degradation in the shriveled tissue, which leads to a reduction in energy supply, to nutrient deficiency and finally to shriveled seeds. The higher membrane-bound ATPase activity in CMS tissues seems to play a similar role in depleting nutritional substrates (Perl et al. 1992), leading to anther degradation and male sterility. On the other hand, some isoforms of phosphohydrolases are regulatory and inducible by water, Pi and probably by other substrate deficiencies. They are important for Pi production and transport (Barrett-Lenard et al. 1982; Goldstein et al. 1988). Thus, the possibility that the higher CMS phosphohydrolase activity described in this work is a result of a prestarving status of the CMS tissues rather than the cause for its increase may not be excluded.

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