BIOLOGIA PLANTARUM 41 (3): 321-330, 1998

Isolation and preliminary characterization of ATPase from olive calli grown at different auxin/cytokinin ratio

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Abstract

ATPase activity was studied in calli from olive (*Olea europaea* L.) petioles cultured in media modified in their auxin/cytokinin ratio in order to induce different morphogenetic responses. Addition of 0.54 μM α-naphthaleneacetic acid (NAA) or 14 μM zeatin (ZEA) did not induce any morphogenesis in calli and proton pump activity *in vivo* was very low, while calli produced roots at 27 or 11 μM NAA + 0.28 μM ZEA and possessed clearly detectable proton pump activity. ATPase activity associated with microsomes isolated by differential centrifugation from different callus cultures had the same pH optimum and similar sensitivity toward nitrate and azide. However, microsomes isolated from non-morphogenetic calli had higher specific ATPase activity which was very poorly (6 %) inhibited by vanadate. Also, the fractionation of these microsomes on a continuous sucrose gradient showed two peaks of ATPase activity, the more pronounced one co-purifying with the Golgi marker enzyme, Triton-stimulated UDPase activity, suggesting thus the presence of very high ATPase activity in Golgi secretory vesicles. On the contrary, ATPase activity of microsomes from calli producing roots was more sensitive to vanadate (30 - 40 % inhibition). Furthermore, the component of ATPase activity attributable to Golgi secretory vesicles was less abundant.

Additional key words: Golgi apparatus, morphogenesis, α-naphthaleneacetic acid, plasma membrane, zeatin.

Received 19 November 1997, *accepted* 20 April 1998.

Abbreviations: BSA - bovine serum albumine; BTP - bis-tris-propane (1,3-bis(tris(hydroxy-methyl)-methylamino)-propane); DTT - DL-dithiothreitol; EGTA - ethylene glycol bis(β-aminoethyl ether)-N,N'-tetracetic acid; MES - (2(N-morpholino) ethanesulphonic acid); NAA - α-naphthaleneacetic acid; pm - plasma membrane; PMSF - phenylmethylsulphonyl fluoride; PVPP - polyvinyl-polypyrrolidone; ZEA - zeatin.

Acknowledgement: The authors acknowledge financial support of the C.N.R. and M.I.R.A.A.F.

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Introduction

The proliferation and regeneration of plant tissues can be controlled by exogenous auxins and cytokinins. The manipulation of the auxin/cytokinin ratio is a well established technique in callus cultures to induce shoot and root organogenesis in several species, included woody species (*e.g.* Krikorian 1995). Callus cultures exposed to relatively high auxin and low cytokinin concentrations are induced to produce roots, whereas in the presence of low auxin concentrations and high cytokinin concentrations shoots are formed. At intermediate auxin/cytokinin ratio only proliferation occurs. However, very little information is available on physiology and biochemistry of calli under different hormonal treatments. Furthermore great variability exists among different plant species in their response to the hormonal treatment (Hammerschlag and Litz 1990) and until now the use of hormones for inducing organogenesis is considered "an art". Previous studies on olive showed that when zeatin prevailed over α-naphthaleneacetic acid (NAA) in growth medium, petioles produced only callus, whereas in the presence of relatively high concentrations of NAA, together with low concentrations of zeatin, root formation occurred (Rugini and Fedeli 1990).

In the present paper, we describe a preliminary characterization of ATPase activities associated with microsomes isolated from olive calli cultured at different auxin/cytokinin ratios. It is well known that plasma membrane H^+-ATPase plays a central role in plant growth and development and evidence exists of a relationship between plant development and ATPase activity in membranes (Santon *et al.* 1991); in addition it has been shown that this enzyme is activated after hormone treatments and it is supposed to be a part of the signal trasduction pathway (Palmgren 1991).

The aim of the paper mass to find whether the induction of a different morphogenetic response in olive calli is paralleled by changes in proton extrusion activity measured *in vivo* and in the level of ATPase activities associated with isolated microsomes.

Materials and methods

Plants: The calli were obtained from petioles of olive (*Olea europaea* L.) cv. Frantoio shoots grown *in vitro* on *Olive Medium* (Rugini 1984). Seven petiole explants were placed in Petri dishes (100 mm diameter) containing 20 cm^3 MS medium (Murashige and Skoog 1962) supplemented with 4 % sucrose and the following concentrations of hormones:
medium A - NAA 0.54 μM, zeatin 14 μM;
medium B - NAA 27 μM, zeatin 0.28 μM;
medium C - NAA 11 μM, zeatin 0.28 μM.
The above hormone concentrations were used on the basis of their morphogenetic response. The pH of all media was adjusted to 5.8 with NaOH before adding 0.6 % agar and the media were autoclaved for 20 min at 121 °C. The cultures were placed

into a growth chamber at 28 °C in the dark for 6 weeks before harvesting and were transferred after 4 weeks on a fresh medium.

"*In vivo*" measurement of proton efflux: The assay was performed using 2-week-old calli which did not show any visible organogenesis. Three g of calli were washed in distilled water for 30 min and then transferred to 10 cm^3 of distilled water adjusted to pH 6.3 with BTP. Proton extrusion from calli was measured by recording the pH change of deionized water. Samples were kept under constant stirring and the pH variation was detected by a *Pharmacia* recorder. When the steady state of the reaction was reached, KCl, which is known to enhance H^+ extrusion (Lado *et al.* 1976), was added to a final concentration of 20 mM.

Isolation of microsomal vesicles: Microsomes were isolated from olive calli according to Varanini *et al.* (1995). Briefly, 10 g of olive calli, deprived of all roots, were washed twice in 0.1 mM $CaSO_4$ and homogenized. The homogenate was subjected to differential centrifugation steps: twice 1 000 *g* for 5 min (supernatant recovered), 13 000 *g* for 20 min (supernatant recovered) and 82 500 *g* for 30 min (pellet recovered). The pellet (microsomes) was gently resuspended in 0.8 cm^3 of a medium containing 0.25 M sucrose, 10 % (v/v) glycerol, 1 mM DTT and 2 mM BTP titrated to pH 7.0 with MES, quickly frozen in liquid N_2 and stored at -80 °C until used. All the preparation steps were performed at 4 °C.

Sucrose density gradient: Continuous sucrose gradients (20 - 50 %, m/m) were made up in 17 cm^3 tubes with ice cold 20 and 50 % (m/m) sucrose solution in 5 mM BTP-MES buffer (pH 7.4) containing all the protectants present in the homogenizing medium. Gradients were carefully loaded with 1 cm^3 of microsomes (1.3 mg of protein) and then centrifuged at 4 °C for 21 h at 95 000 *g*. The long centrifugation times ensure that the membranes have reached their isopycnic densities. After centrifugation, the gradients were fractionated into 1 cm^3 fractions which were quickly frozen in liquid N_2 and stored at -80 °C until used.

Enzyme assays: ATP-hydrolyzing activity was measured by determining the release of inorganic phosphate according to Forbusch (1983). The reaction mixture contained 50 mM MES-BTP (pH 6.5), 5 mM $MgSO_4$, 5 mM ATP-BTP (pH 6.5), 0.6 mM Na_2MoO_4 and, when present, 100 mM KCl in a final volume of 0.6 cm^3. The assay was started with adding the reaction mixture to the enzyme and run for 30 min at 38 °C. ATPase activity of membrane vesicles preparations was tested in the presence of diagnostic inhibitors: 100 µM vanadate (plasma membrane ATPase inhibitor), 1 mM sodium azide (mitochondrial ATPase inhibitor) or 100 mM KNO_3 (tonoplast ATPase inhibitor). Triton-stimulated UDPase activity, marker enzyme activity for Golgi membranes, was assayed in a reaction mixture containing 30 mM MES-BTP (pH 6.5), 3 mM UDP, 3 mM $MnSO_4$ and 0.03 % Triton X-100, when present. The assay was run for 30 min at 38 °C. PPase activity was determined by assaying for Pi released in 5 mM $MgSO_4$, 100 µM $Na_4P_2O_7$, 0.6 mM Na_2MoO_4, 100 mM KCl,

when present, and 50 mM MES-BTP at pH 8.0. The reaction was run for 30 min at 38 °C.

Protein assay: Protein content was determined by the Coomassie staining method described by Bradford (1976) using BSA as a standard.

Results

Morphological observations: At the harvest, the calli, yellowish in colour, appeared as quite friable masses having almost the same fresh mass regardless the composition of the growth media. However, different media strongly influenced morphogenesis (Table 1). In medium A, calli did not show any differentiated tissues, while in media B and C they differentiated roots (70 % and 80 %, respectively). Calli from media B and C showed an average of 4 roots per callus with a length of about 2 - 3 cm.

Table 1. Effect of auxin/cytokinin ratios in the growth medium on growth and organogenesis in olive callus cultures.

NAA [μM]	ZEA [μM]	Calli harvested [number]	Fresh mass [g callus^{-1}]	Calli with roots [number]	Root formations [%]
0.54	14.00	414	1.43	0	0
27.00	0.28	403	1.40	282	70
11.00	0.28	391	1.40	313	80

***In vivo* proton efflux:** Calli grown on different media possessed different capability in acidifying external solutions (Fig. 1). In the absence of 20 mM KCl (basal proton extrusion activity) the calli grown on medium A (low auxin/cytokinin ratio) did not show any detectable proton efflux. When KCl was added the pH of the solution started to decrease with a drop of 0.3 units after 30 min, when the steady-state level of the reaction was reached. On the contrary, the calli grown in the media with high auxin/cytokinin ratio (media B and C) showed a remarkable basal proton efflux activity which determined the decrease of the pH value of deionized water of about 1.20 and 0.70 units, respectively. The addition of KCl at the steady-state of the reaction (95 and 47 min, respectively) produced a further acidification of the solution by 0.14 units, in the case of calli from medium B, and 0.19 units, in the case of calli from medium C.

ATP-hydrolyzing activity: Microsomes greatly differed in the specific ATPase activity. The enzyme activity was higher in microsomes from calli grown on medium A [5.28 nmol(Pi) mg^{-1}(protein) s^{-1}] than in microsomes from media B and C [1.47 and 0.66 nmol(Pi) mg^{-1}(protein) s^{-1}, respectively]. When the assay was run in the presence of the plasma membrane ATPase inhibitor vanadate (100 μM), ATPase activity was decreased in microsomes from media A only by 6 %. On the contrary, in

Table 2. Effect of inhibitors on ATPase activity [nmol(Pi) mg^{-1}(protein) s^{-1}) of microsomes isolated from olive calli. A, B and C refer to microsomes isolated from olive calli exposed to low (medium A) or high (media B and C) auxin/cytokinin ratio. Values in brackets represent percent ATPase activity. Data are means from an experiment run in triplicate representative of 4 independent preparations of microsomal vesicles. SD did not exceed 5 % of the means.

Treatment	A	B	C
Control (pH 6.5)	5.28 (100)	1.47 (100)	0.66 (100)
V_2O_5 100 μM	4.96 (94)	1.04 (71)	0.39 (60)
Control (pH 8.0)	2.51 (100)	0.79 (100)	0.46 (100)
KNO_3 100 mM	2.28 (91)	0.68 (86)	0.39 (86)
NaN_3 1 mM	2.31 (92)	0.66 (83)	0.40 (87)

the presence of the same inhibitor, ATPase activity of microsomes isolated from calli grown in medium B was reduced by 30 % and that of microsomes from medium C by 40 %. The effect of nitrate, an inhibitor of tonoplast ATPase, was similar in all the microsomes with inhibition ranging from 10 to 14 % (Table 2). These figures were not modified by changing the assay pH from 8.0 to 6.5 (not shown). The inhibition caused by sodium azide, a compound able to inhibit mitochondrial ATPase, did not exceed 17 % (microsomes isolated from calli grown on medium B) and inhibited ATPase activity of microsomes cultured on media A and C by 8 and 13 %, respectively.

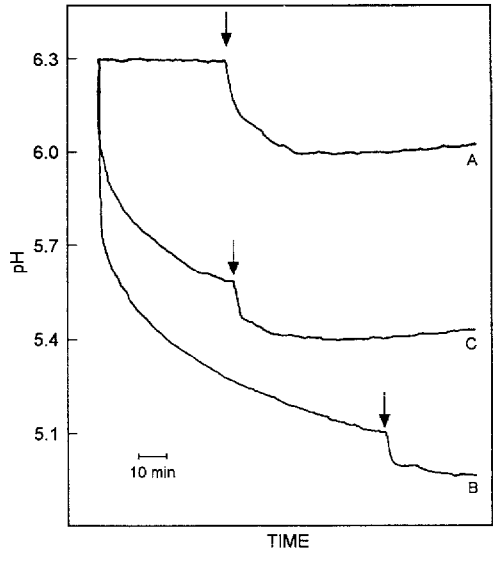

Fig. 1. pH record of the external solution upon submerging olive calli in deionized water. Calli (3 g), grown on media A, B and C (A, B and C traces, respectively), were washed for 30 min in deionized water and transferred into 10 cm^3 of deionized water adjusted to pH 6.3 with BTP. Samples were kept under constant stirring and when the steady state of the reaction was reached, KCl at final concentration of 20 mM was added (*arrow*).

Effect of vanadate: The effect of vanadate on ATPase activity of microsomes isolated from olive calli was also evaluated at concentrations of the inhibitor ranging from 25 and 250 μM (Fig. 2*a*). The increase of vanadate concentration in the assay from 25 to 60 μM increased the inhibition of ATPase activity reaching the values of 6, 30 and 40 % for microsomes from calli grown on media A, B and C, respectively. Concentrations of vanadate as high as 250 μM did not cause any further increase in the inhibition of ATPase activity in any of the membrane preparations.

Effect of pH: ATPase activity was pH dependent in the pH range between 5.0 and 8.5 for all membrane preparations with an optimum pH of 6.5 (Fig. 2*b*).

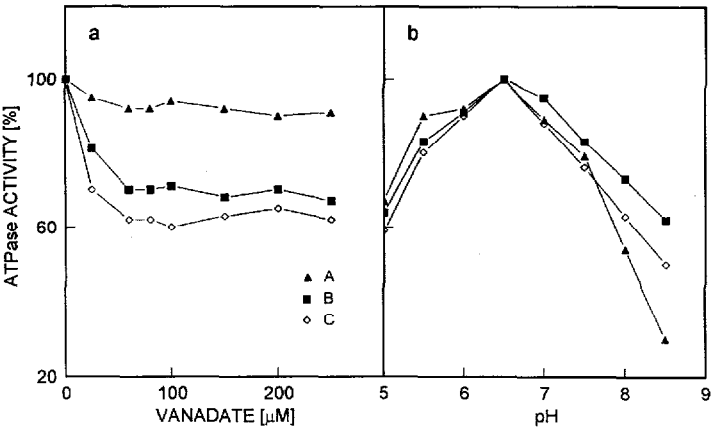

Fig. 2. Effect of increasing concentration of vanadate (25 - 250 μM) (*a*) and effect of pH (*b*) on ATPase activity of microsomes isolated from olive calli from different media. A, B and C refer to microsomes isolated from olive calli exposed to low (medium A) or high (media B and C) auxin/cytokinin ratio. The pH of the assay was adjusted by varying the ratio of BTP to MES (50 mM final concentration). Data are means from an experiment run in triplicate representative of 4 independent preparations of microsomal vesicles. SD did not exceed 5 % of the means.

Continuous sucrose density gradient: Linear sucrose gradient of microsomes (Fig. 3*a,b*) from medium A showed a major peak of ATPase activity [12.05 nmol(Pi) cm^{-3} s^{-1}] in the light end of the gradient (108 - 110 g cm^{-3}). Another peak of ATPase activity [1.33 nmol(Pi) cm^{-3} s^{-1}] in the denser region of the gradient (1.17 g cm^{-3}) was observed. The distribution of the vanadate-sensitive ATPase activity showed only one peak occurring at the density of 1.17 g cm^{-3}; azide and nitrate sensitive ATPase activities were broadly distributed along the gradient and showed both a very low level of activity. PPase activity (tonoplast marker), and Triton-stimulated UDPase activity (a marker for Golgi secretory vesicles) peaked at the density of 1.08 g cm^{-3} and 1.10 g cm^{-3}, respectively. Continuous sucrose gradient of microsomes from medium B produced a distribution of ATPase activities and marker enzymes similar to that revealed in microsomes from medium A. However, the major peak of bothATPase and Triton-stimulated UDPase activities were significantly lower (4 - 5 times) than those obtained from the gradient of microsomes from medium A.

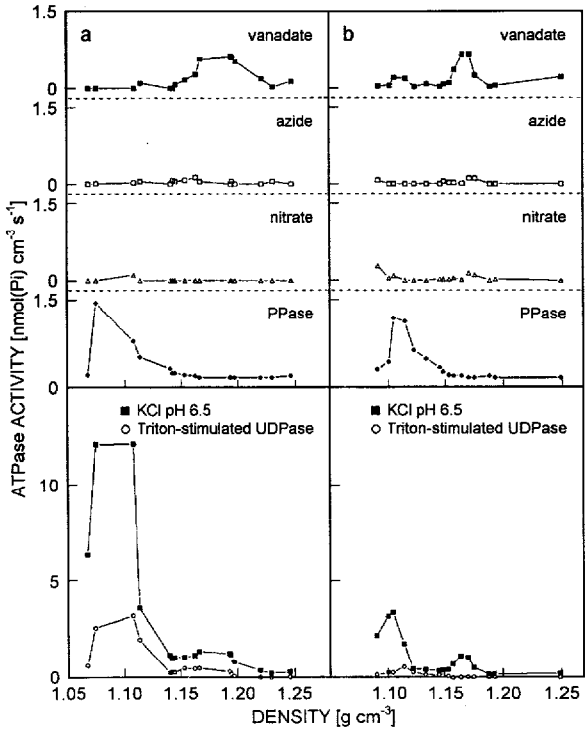

Fig. 3. Distribution of ATPase activities, PPase and Triton-stimulated UDPase activities on linear 20 to 50 % (m/m) sucrose gradients (*a* and *b* refer to olive calli grown on media A and B, respectively). Upper panels, vanadate, azide and nitrate were calculated as the difference in ATPase activity assayed in the absence or presence of 100 μM vanadate, 1 mM sodium azide and 100 mM KNO_3. Middle panel, PPase activity was calculated as the difference in PPase activity assayed in the absence or presence of 100 mM KCl. Lower panel, ATPase activity at pH 6.5 and Triton-stimulated UDPase activity. UDPase activity was evaluated as the difference in activity measured in the presence or absence of the detergent Triton X-100 at the concentration of 0.03 %. Data are means from an experiment run in triplicate representative of 4 independent preparations of microsomal vesicles. SD did not exceed 5 % of the means.

Discussion

In the first set of experiments we studied H^+ extrusion, an activity which is considered to be dependent on the operation of plasma membrane H^+-ATPase (Kotyk *et al.* 1991) in olive calli grown on media only altered in their hormonal composition. The results indicate that calli grown on a medium characterized by a low auxin/cytokinin ratio (medium A) did not show any detectable basal H^+ extrusion activity whereas those grown on media having high auxin/cytokinin ratio were able to significantly decrease the pH of the solution bathing the tissue. Since the composition of the growth media only differed in the concentration of hormones it is

reasonable to conclude that the exposure to low auxin/cytokinin ratio can lead to development of cells in which the activity of proton pump is very low or absent.

Results obtained *in vitro* with microsomes isolated from calli grown on different media indicate that the presence of different hormonal ratios influenced the characteristics of membrane associated ATPases. Microsomes isolated from calli cultured on medium A showed a high specific ATPase activity with only a slight sensitivity to inhibitors vanadate, nitrate and azide. In particular the component of ATPase activity inhibited by vanadate, a specific inhibitor of plasma-membrane H^+-ATPase (Gallagher and Leonard 1982), was only 6 % and this value remained constant even at a vanadate concentration as high as 250 μM. These results seem to be independent of olive cultivar, since in preliminary experiments carried out on calli from several olive cultivars (Dolce Agogia, Moraiolo and Leccino) we obtained a similar behaviour. In the same way, as tested in preliminary experiments, modification of the composition of grinding media did not lead to an increase of the vanadate-sensitive component of the ATPase (not shown). Furthermore, ATPase activity was not affected by sodium molybdate in microsomes from the different callus cultures (data not shown) thus indicating the absence of acid phosphatase in isolated membranes (Gallagher and Leonard 1982).

However, after fractionation of microsomes from calli grown on A medium on a continuous sucrose gradient a considerable peak of Triton-stimulated UDPase activity [3.19 nmol(Pi) cm^{-3} s^{-1}), a marker of Golgi secretory vesicles, was observed thus indicating that membrane vesicles originating from Golgi apparatus are present. The presence of vesicles from this organelle could at least in part explain the high level of ATPase activity not affected by inhibitors of tonoplast, mitochondrial and plasma membrane ATPases. In fact, it has been shown that ATPase from Golgi secretory vesicles was insensitive to azide, nitrate and vanadate (Ali and Akazawa 1986, Chanson *et al.* 1984). Furthermore, the peak of latent UDPase activity almost overlapped that of inhibitor-insensitive ATPase activity and was clearly separated from that of vanadate sensitive-ATPase. Therefore, treatment with low auxin/cytokinin ratio seems to produce calli with an unusual high ratio of ATPase associated with Golgi apparatus and with plasma-membrane. In this condition tissues seem not to be able to acidify external solution. In microsomes from media B and C characterized by specific ATPase activity 3 to 8 times lower than that from medium A the component inhibited by vanadate was 30 and 40 % at pH 6.5, respectively, indicating a relatively higher ATPase activity attributable to plasma-membrane ATPase. Interestingly, after the fractionation on sucrose gradient of microsomes obtained from olive calli cultured on medium B a peak of Triton-stimulated UDPase was detected with an activity 4 times lower than that measured in the gradient of membrane vesicles from calli grown on medium A. Furthermore, the distribution of other markers and activitiy was very similar in both gradients.

In conclusion, our data quite clearly demonstrate that the exposure of olive calli to substrate with low auxin/cytokinin ratio, a condition which does not induce morphogenesis, can give rise to development of cells possessing very scarce proton pump activity *in vivo*, a relative low plasma-membrane ATPase and considerable amount of ATPase attributable to Golgi secretory vesicles. On the contrary, calli

cultured in the presence of high auxin/cytokinin ratio, a treatment inducing root formation, possess clearly detectable proton pump activity *in vivo* and a relative higher plasma-membranes ATPase activity. Results do not allow to clarify the mechanisms leading to this event, however, it is interesting to note that numerous reports (Napier and Venis 1995) suggest a role of auxin in the control of secretion. Furthermore, Hager *et al.* (1991) demonstrated that auxin induces a large increase in secretion of pm H^+-ATPase. Results obtained by continuous sucrose gradient indicates that the major peaks are due to membranes of Golgi and plasma membranes. Furthermore, the relative enrichment of ones compared to the others might represent the alteration of the membrane flow in both directions. The elucidation of the role of different membranes in relation to morphogenetic characteristics in olive calli is an interesting area for future research and could contribute to the explanation of the mechanisms involved in hormone induced differentiation.

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