Cytochemical localization of ATPase plasma membrane and acid phosphatase by cerium-based method in a salt-adapted cell line of *Pisum sativum*

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Abstract

In order to study a possible application of cerium-based techniques in plant cells, ATPase and acid phosphatase activities have been compared in two cell lines of *Pisum sativum* calli, one sensitive to NaCl and the other selected to be grown under salinity (85 mM NaCl). ATPase activity was unchanged and localized in the plasma membrane of both cell lines. Acid phosphatase activity was significantly increased in the salt-selected line and localized in the cell walls, Golgi complex, multivesicular bodies and vacuoles. These results indicated a possible involvement of both activities in the maintenance of cell growth in the selected line under saline conditions.

Key words: Acid phosphatase, *Pisum sativum*, plasma membrane ATPase, salt stress, ultrastructure.

Introduction

The cellular adaptation to salinity is a serious problem that affects the physiology and biochemistry of the cells at different levels. Acid phosphatases and ATPases have been widely studied in plant and cell cultures under salt stress (Venekamp, 1989; Reuveni *et al.*, 1993), although the role of these enzymes is not completely understood.

Acid phosphatases are, in general, non-specific enzymes, which can use different substrates in their reactions, while ATPases are more selective. The mechanism which regulates acid phosphatase distribution and activity is unclear, although their abundance may be regulated by the level of phosphate in the environment and their activity is clearly influenced by local polyelectrolytes (Tu *et al.*, 1990). Although the role of vacuolar acid phosphatase is unclear, the secreted enzyme may be used to scavenge phosphate from organic sources under phosphate-limiting conditions (Duff *et al.*, 1994).

The most commonly used cytochemical method for localizing of ATPase and acid phosphatase enzymes is ion capture. Lead has been widely used to trap the phosphate released at the enzymic site as an insoluble phosphate precipitate. However, these techniques using lead have been criticized by several authors (Hoefsmit *et al.*, 1986; Kantz *et al.*, 1988; Chauhan *et al.*, 1991) because they may produce false positives and some enzymes are sensitive to lead. Nevertheless, these techniques have been widely used in plant and animal cytochemistry (Veenhuis *et al.*, 1980; Hall *et al.*, 1992). An azo dye method has also been developed for the localization of acid phosphatases. This method makes use of the very broad substrate specificity of phosphatase, which include α- and β-naphthyl phosphates and has been adapted for electron microscopy (using a mercury-substituted diazoate) and applied to the study of acid phosphatases in plants (Sexton and Hall, 1991). Recently, cerium-based methods have been extensively used in the ultrastructural localization of several phosphatases and ATPases in animal tissues (Hulstaers *et al.*, 1983). However, few studies have applied this technique to the localization of acid phosphatases and ATPases in plants (Record and Griffing, 1988; Hall *et al.*, 1992). Cerium penetrates tissues slowly and reacts directly with phosphate to form insoluble, finely divided deposits of cerium phosphate which are easily detectable by electron microscopy.

The aim of this work is to provide new evidence concerning the localization of acid phosphatase and...
ATPase activities using a cerium-based method instead of lead, diazonium salt or indigo dye methods. The activities of these enzymes were also tested in order to compare the results with those of previous biochemical studies using pea cells (Olmos et al., 1994; Olmos and Hellin, 1996b). It is considered that cytochemical techniques may help clarify the function of these enzymes in cell adaptation to salinity.

Materials and methods

Cell cultures

The pea callus cultures used in this work (Pisum sativum L. cv. Challis) were from two cell lines; salt-sensitive calli and calli from a line adapted to 85.5 mM NaCl. The culture medium and growth conditions were as described in Olmos et al. (1994). Both types of calli were cultured on solid medium. Samples were taken 21–25 d after subculture and were used for cytochemistry and electron microscopy studies.

ATPase activity

Samples of the two cell lines for H⁺-ATPase were fractionated according to Hall and Nelson (1990). Calli were homogenized with a cold pestle and mortar in 50 mM HEPES-TRIS buffer (pH 7) containing 300 mM mannitol using a tissue/volume ratio of 2 g ml⁻¹. The homogenate was filtered through two layers of muslin and centrifuged at 1000 g for 15 min at 4°C. The pellet was resuspended in the same medium with 500 mM NaCl and centrifuged as before. The process was repeated six more times using a homogenization medium without NaCl. Finally, the resultant pellet was resuspended in the reaction medium. H⁺-ATPase activity was measured as the release of Pi from ATP (LeBel et al., 1978). Samples were incubated with 100 μM vanadate as the control.

Acid phosphatase activity

The acid phosphatase was extracted in a 25 mM TRIS-MES buffer (pH 7.2) containing 5 mM EDTA and 250 mM sucrose. Acid phosphatase activity was measured as the release of pNPP from pNPP (LeBel et al., 1978).

Phosphorous determination

Phosphorous concentrations of both types of callus were assessed by spectrophotometry of the ammonium phosphomolybdenum complex (Kitson and Mellon, 1944) of cell extracts according to Piqueras et al. (1996).

Cytochemistry

Cerium-based methods were used for ATPase localization (Chauhan et al., 1991). Small cell clusters from the control and sensitive calli of Pisum sativum were collected and fixed in a mixture of 2% (v/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, at 0°C for 1 h. The material was then washed for 2 h in an ice bath with 100 mM cacodylate buffer, pH 7.2 (three changes). Sections were preincubated in 1 mM CeCl₃, 50 mM KNO₃, and 50 mM TRIS-malate buffer, pH 7.0. Control sections were preincubated in 1 mM CeCl₃ in 50 mM TRIS-malate buffer containing 0.2 mM Na₃VO₄. Na₃VO₄ (0.2 mM) had also been added to the incubation reaction mixture. Incubation without ATP was also used as control. After incubation, the samples were washed in 50 mM TRIS-malate buffer (one change) for 15 min and then in 100 mM cacodylate buffer (three changes) for 1 h, at 0°C before being left overnight in a fridge. Sections were postfixed in a mixture containing 1% OsO₄ in 100 mM cacodylate buffer, pH 7.2, for 2.5 h at 4°C.

Acid phosphatase was located using the cerium-based procedure of Record and Griffing (1988) as described by Hall et al. (1992). Thin sections were fixed in 2.5% (v/v) glutaraldehyde in 100 mM cacodylate buffer, pH 7.2, at 0°C for 2 h. The sections were then washed for 1 h in an ice bath with 100 mM cacodylate buffer (two changes). The material was preincubated in 100 mM acetate buffer, 2 mM CeCl₃, pH 5.0 for 1 h in a water bath at 37°C. This was followed by a 30 min incubation at 37°C in a water bath using a reaction mixture containing: 1 mM β-glycerophosphate, 2 mM CeCl₃ and 100 mM acetate buffer, pH 5.0. Control treatments without sodium β-glycerophosphate and CeCl₃ were made. The first rinse after incubation was in the preincubation mixture (15 min), followed by three more washes with acetate buffer only. Tissue was post-fixed with 1% OsO₄ for 2.5 h. For conventional electron microscopy, samples were fixed for 2.5 h at 4°C in a 0.1 M Na-cacodylate buffer (pH = 7.2) mixture of 2.5% glutaraldehyde and 4% paraformaldehyde. Tissue was post-fixed with 1% OsO₄ for 2.5 h.

Samples from all methods were dehydrated in a graded alcohol series and embedded in Spurr’s (1969) resin. Blocks were sectioned on a Reichert ultramicrotome. Thin sections for transmission electron microscopy were collected on copper grids and stained with uranyl acetate followed by lead citrate (Reynolds, 1963). For cytochemistry, the thin sections were not counterstained with lead or uranyl acetate. The cell ultrastructure was observed with a Zeiss EM10 and Zeiss EM109.

Quantitative method

Electron micrographs with a final magnification of ×17 000 singularity were brought to an automatic image analyser (Leica Q500MC), which, by segmenting the levels of grey in the micrograph, sharply discriminated the areas of positive cytochemical reaction. A quantitative approach for the estimation of enzyme activity used morphometric sampling methods, in which the area of the reaction deposit relative to a constant arbitrary profile area of plasma membrane (ATPase) or cell wall (APase) was determined (Sánchez-Aguayo et al., 1991). Measurements were taken from 10 micrographs of each sensitive and adapted cell.

Results

ATPase localization

Figure 1A shows cells from a sensitive callus with cerium precipitates on the plasma membrane, whereas the tono-
plast did not present any reaction. There was a little non-specific staining on cell walls. Plasmodesmata were stained by cerium precipitates (Fig. 1B), although no such reaction was observed in nuclei, mitochondria, or plastids.

The salt-adapted line showed a similar reaction to the sensitive line on plasma membrane (Fig. 1C). However, neither tonoplast nor cell walls showed any reaction. Sometimes small vesicles showed precipitates on the membrane (Fig. 1D). Plasmodesmata were reactive as in the sensitive cell line. Controls from neither cell line showed precipitate on either plasma membranes or other areas (Fig. 1E).

Acid phosphatase localization

In the sensitive cell line the acid phosphatase reaction was mainly located in the cell wall, although intercellular spaces were also stained (Fig. 2A). Sometimes Golgi complex, RER and small vesicles were stained (Fig. 2B).

The salt-adapted line showed a strong reaction on the cell walls (Fig. 2C). The intercellular spaces was less stained than in the sensitive cell line. Some multivesicular bodies and vesicles showed cerium precipitates (Fig. 2D). Other organelles presented similar patterns to the sensitive line. Controls from neither cell line showed reaction in the tissue (Fig. 2E).

Table 1 shows the morphometric quantification of ATPase activity associated with the plasma membrane in both sensitive and adapted cells. No significant differences were found between the two cell lines. Biochemical analysis carried out on plasma membrane ATPase activity confirmed the above results. Table 1 also shows the morphometric data of phosphatase activity associated with cells walls. The mean values observed of adapted cells were significantly higher than those of sensitive cells. In addition, biochemical data have demonstrated that acid phosphatases are induced in adapted-cells exposed to salt with a 2-fold increase over that observed in sensitive cells. It was also observed that the phosphorous levels of adapted-cells decreased significantly after 21 d of subculture.

Discussion

In recent work, the use of cell cultures for the study of different stresses has been widely used (Collin and Dix, 1990). Similarly, a cell line of Pisum sativum adapted to 85 mM of NaCl (Olmos et al., 1994; Olmos and Hellin, 1996a, b) was characterized. Based on the results reported here, using image analysis, it is thought that these enzymes could be localized and quantified by means of cerium precipitates. Morphometric data (Table 1) show good correlation with the biochemical results obtained. The cerium concentration used (2 mM) did not apparently affect the activities of the either enzyme, whereas previous work using lead as the precipitation substrate led to a substantial inhibition of these activities (Kantz et al., 1988). Cerium-based techniques present less of a problem with non-specific reactions in the medium without substrate (Figs 1E, 2E) and, unlike the lead method, did not show nuclear staining.

ATPase localization

In all cells of both, sensitive and salt-adapted cell lines, ATPase was mostly located in the plasma membrane. In a recent work, Sanchez-Aguayo et al. (1991) working with tomato roots under salt stress observed a differential staining pattern, and so reactivity depended on the localization of the cell in the root. In salt-adapted pea cells, the stain pattern of the ATPase of the plasma membrane was similar to that observed in sensitive cells. In this paper, using partial purification of plasma membranes, it is shown that the specific activity of the plasma membrane ATPase was maintained in adapted cells, which confirms that such activity was unchanged in the salt-adapted line of Pisum sativum. In Lycopersicon esculentum, ATPase activity in plasma membranes was greatly decreased by salt treatment in the different root zones compared with control plants (Sanchez-Aguayo et al., 1991). Maintenance of plasma membrane ATPase activity has been reported to be important in the transport mechanisms which permit salt adapted cells to assimilate Na⁺ and Cl⁻. Reuveni et al. (1993) observed a change in the kinetic properties of ATPase in tobacco cells adapted to salt as a possible adaptation mechanism to saline environments.

Acid phosphatase localization

Acid phosphatases are known to act under salt stress by maintaining a certain level of Pi (Szabo-Nagy et al., 1992), which can be co-transported with H⁺ along a gradient of proton motive force (Sakano et al., 1992). The exact role of these phosphatases, however, remains unclear. It follows that alternative functions for external phosphatases must exist, perhaps as part of a salvage mechanism, which permit salt adapted cells to reabsorb. Extracellular phosphatases may also have arisen from the abundance of insoluble mineral Pi in soils.
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since it is not normally available for absorption by plants (Lefebvre et al., 1990).

Extracellular acid phosphatases appears to be the most important isozymes of acid phosphatases in the cells of in vitro cultures (Halperin, 1969; Lefebvre et al., 1990). In this respect, the cytochemical results agree with biochemical analysis, indicating an increase in acid phosphatase in adapted calli. The acid phosphatase activity that we measured in vitro is probably the main source of the acid phosphatase activity observed cytochemically. This increase in extracellular acid phosphatase activity may be considered as a cellular response to the Pi deficiency observed in adapted cells. An increased level of phosphatase activity accompanied by a decrease in phosphorous under salt-stress has been reported in some varieties of wheat calli (Szabo-Nagy et al., 1992) and other whole plants (Trivedi et al., 1991; Duff et al., 1994). It is considered that the phosphorous deficiency observed in our selected pea calli (nearly 25% of the observed in sensitive cells) can serve as a signal for the induction of acid phosphatase, particularly extracellular acid phosphatase (Lefebvre et al., 1990). The acid phosphatase reaction products of the golgi complex were found on both cis and trans cisternae and in associated vesicles. The secretion molecules destined for the extracellular matrix or plasma membrane began, as for the lysosomal pathway, in the ER and the Golgi complex. Similarly, Record and Griffing (1988) reported the involvement of acid phosphatases in the endocytic pathways in soybean protoplasts.

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References


