Plasmalemma ATPase activity modifications induced by traumatisms in *Bidens pilosa*

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Abstract

It has previously been shown that cotyledonary pricks induced modifications of ion levels (H⁺ and K⁺) in hypocotyl cells of *Bidens pilosa*. These modifications differed according to the light quality: H⁺ levels increased and K⁺ levels decreased in white light (WL), whereas H⁺ levels decreased and K⁺ levels increased in blue light (BL). In this study, in order to determine the mechanism responsible for these ionic modifications, plasma membrane vesicles have been isolated and characterized from hypocotyl cells. The effects of light quality and cotyledonary pricks on plasmalemma ATPase activity (EC 3.6.1.3) were studied.

Cotyledonary pricks induced, in WL, rapid (5 min) and transient (restoration in 60 min) inhibition of plasmalemma ATPase activity. Conversely, in BL, a rapid and transient stimulation was observed. These results suggest that, in *Bidens pilosa*, plasmalemma ATPase is involved in 'short-term' ionic level modifications induced by traumatisms.

Key words: ATPase activity, short-term ionic regulation, growth inhibition.

Introduction

One of the common responses of plants to mechanical stimuli is the reduction of their growth (Jaffe, 1988). In previous work (Pichon et al., 1993), it was shown that cotyledonary pricks induced hypocotyl growth inhibition under white light (WL) or blue light (BL) conditions in young plantlets of *Bidens pilosa* L. This growth inhibition is associated with modifications of ionic levels (H⁺, K⁺) in hypocotyl cells, and with rapid modifications of hypocotyl cellular development (elongation, division). Nevertheless, these modifications differ according to ionic culture medium composition and to light quality. When plants are cultivated on rich ionic medium (Cera III), no modification of hypocotyl growth or of ionic levels is observed after cotyledonary pricks. However, on poor ionic medium (deionized water), modifications of hypocotyl growth and of ionic levels are observed after pricks. Moreover, in WL, opposite ionic movements were observed after pricks: an intracellular H⁺ increase and K⁺ decrease correlated with a rapid inhibition of cell elongation and mitotic activity. In contrast, in BL, a decrease of H⁺ and increase of K⁺ intracellular levels correlated with a rapid stimulation of radial-cell growth and cell division is observed (Pichon et al., 1993).

In plants, there are two main sources of pH modification: 'biochemical' (production or consumption of protons) and 'biophysical' (net fluxes of H⁺ or OH⁻ across lipid membranes) mechanisms (Kurkdjian and Guern, 1989). One of the most efficient biophysical systems of pH regulation is the plasmalemma H⁺-ATPase, which is able to pump out H⁺ ions (Sze, 1984; Serrano, 1984). Moreover, plasmalemma ATPase activity is able to modify cellular levels of other ions such as K⁺ (Serrano, 1988) or Ca²⁺ (Briskin, 1990). Several authors have found the plasmalemma H⁺-ATPase to be involved in the long-term regulation of cytoplasmic pH (Felle, 1989; Guern et al., 1990), but the short-term regulation of pH₁ has most commonly been found to be the result of...
other mechanisms, such as membrane transporters (Felle, 1989), redox systems (Pétel and Gendraud, 1987; Barr, 1988) or biochemical mechanisms (Davies, 1986). Moreover, ATPase activity is strongly pH dependent (Szé, 1984; Marré and Balarin-Denti, 1985).

In this work, in order to study the involvement of the plasmalemma ATPase in the cellular ionic-level modifications observed after various treatments (culture medium composition, light quality and mechanical stimulus), plasma membrane vesicles from hypocotyl cells were isolated and characterized and the variations in the ATPase activity (ATP hydrolysis) were studied in these various conditions.

Materials and methods

Plant material

Plants of Bidens pilosa L. were cultivated according to the method and the conditions (WL or BL) previously described (Desbiez et al., 1987; Pichon et al., 1993). Five days after achenes imbibition (D5), half of the plants were transferred to a low ionic medium: deionized water (experimental condition necessary to obtain significant hypocotyl growth inhibition after cotyledonary pricks). The other plants were always cultivated on rich ionic medium: Cera III de Homes (experimental condition in which only a little hypocotyl growth inhibition was observed after cotyledonary pricks) (Desbiez et al., 1983). At D6, 50% of plants have been pricked (4 pricks on each cotyledon), the others were used as control samples.

Membrane preparation

At D6, hypocotyl fragments (first cm below the cotyledonary node) isolated from 1000 plants (7 g) were harvested 0, 5 and 60 min after the cotyledonary pricks and ground in 5 cm$^3$ of 100 mol m$^{-3}$ TRIS-HCl buffer (pH 8.0), 500 mol m$^{-3}$ mannitol, 10 mol m$^{-3}$ β-mercaptoethanol, 2 mol m$^{-3}$ EDTA, and 0.5% (w/v) bovine serum albumin and then filtered on four layers of cheesecloth and centrifuged for 10 min at 10 000 g (Sorval, SS34 rotor). The supernatant was centrifuged for 1 h at 100 000 g (Beckman, T80, 14610). The pellet was resuspended in 1 cm$^3$ of 100 mol m$^{-3}$ TRIS-HCl (pH 8.0), 5 mol m$^{-3}$ β-mercaptoethanol. This fraction, corresponding to the microsomal fraction, was purified by a two-phase partition system (Widdel, 1987) to obtain a plasmalemma-enriched membrane fraction (final fraction obtained by two-phase partitioning) was that of UDPG-glucose-sterol-D-glucosyltransferase (EC 2.4.1.35) (Quantin et al., 1980) and 1,3-β-glucan synthase (EC 2.4.1.34) (Fink et al., 1987), by a marker of the endoplasmic reticulum: NADH-cytochrome c reductase (EC 1.6.99.3) (Askerlund et al., 1988) and of the Golgi system: latent IDPase (EC 3.6.1.1) (Hodges and Leonard, 1974) and (ii) by inhibitors of ATPase activities (EC 3.6.1.3): vanadate (Dupont et al., 1981), nitrilotriacetic acid (O’Neill and Spanswick, 1984) and sodium azide (Lamant et al., 1989); ATPase inhibitors of, respectively, plasmalemma, tonoplast and mitochondria. (iii) The percentage of ‘inside out’ and ‘right side out’ vesicles present in the membrane fraction was estimated by measurement of ATPase activity before and after four freezing and thawing cycles (Palmgren et al., 1990; Sandstrom et al., 1987). (iv) The $V_m$ and $V_m$ values of ATPase were determined using increasing concentrations of ATP (from 0.05 to 0.8 mol m$^{-3}$ under WL and from 0.2 to 2 mol m$^{-3}$ under BL). ATP hydrolysis was determined after 1 min reaction, as described below. $K_m$ and $V_m$ values were determined graphically from the Lineweaver-Burk data plot. These measures were performed on control plants cultivated on Cera III under WL or BL conditions.

Measurements of ATPase activity were made in a final volume of 0.5 cm$^3$ of 20 mol m$^{-3}$ MES-TRIS (pH 6.5), 5 mol m$^{-3}$ MgSO$_4$ and 12 mmol m$^{-3}$ ATP to which 5 μg of membrane proteins were added to start the reaction. After 5 min, the remaining ATP was measured by bioluminescence (Pradet, 1967). Bioluminescence measurements were monitored on a Lumat LB 9501 (Berthold Inc.) using the Boehringer Manheim ATP bioluminescence CLS kit. The difference between this value and the total initial ATP gave the quantity of ATP hydrolysed during 5 min. The measures were performed 0, 5 and 60 min after cotyledonary pricks for plants cultivated on Cera III and on deionized water and under WL or BL conditions. ATPase activity was expressed as nmoles hydrolysed ATP mg$^{-1}$ protein min$^{-1}$.

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical treatment

Each experiment has been reproduced 5-fold with 1000 plants. Statistical calculations (mean value and standard error) were performed according to Lamotte (1967).

Results

Characterization of isolated membranes

In order to characterize the membranes isolated from hypocotyl cells, enzymatic markers of cellular membranes were used (Table 1). The only activities detected in the plasma membrane fraction (final fraction obtained by two-phase partitioning) was that of UDPG-glucose-sterol-β-glucosyltransferase and 1,3-β-glucan synthase (enzymatic markers of plasmalemma). No activity of NADH-cytochrome c reductase (enzymatic marker of endoplasmic reticulum) and of latent IDPase (enzymatic marker of Golgi asparatus) was detected within the technical limits of sensitivity. However, specific inhibitors of ATPase activity were also used (Table 2). With 0.3 mol m$^{-3}$ vanadate, an inhibitor of plasmalemma ATPase activity, a drastic inhibition of activity was observed compared with the controls. In contrast, nitrate or azide (ATPase inhibitors, respectively, of tonoplast and mitochondrial internal membrane) had no significant action against ATPase activity. These results were obtained on
Mechanical stimuli and plasmalemma ATPase activity

Table 1. Enzymatic markers of membranes

Measurements were performed on final membrane fraction (20 μg membrane proteins for UDP-glucose sterol transferase and 50 μg for the other enzymatic activities). Activities were expressed as nmol 14C-glucose incorporated mg−1 protein min−1 (for UDPG-sterol transferase and β-1,3-glucane synthetase) and as nmol reduced cytochrome c mg−1 protein min−1 (for NADH-cytochrome c reductase) and as nmol inorganic phosphate released mg−1 protein min−1 (for latent IDPase).

Values were obtained on control plants cultivated on deionized water, in white light. Values are the mean of 5 measurements ± standard error.

<table>
<thead>
<tr>
<th>Enzymatic activity (nmol mg−1 protein min−1)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,3-glucane synthase (plasmalemma)</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>UDP-glucose transferase (plasmalemma)</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase (endoplasmic reticulum)</td>
<td>0*</td>
</tr>
<tr>
<td>IDPase latent (Golgi apparatus)</td>
<td>0*</td>
</tr>
</tbody>
</table>

* Within the limits of sensitivity of the technique.

Table 2. Effects of ATPases specific inhibitors

Measurements were performed on final membrane fraction (without freezing and thawing cycles). ATPase specific activity was expressed as nmol of hydrolysed ATP mg−1 protein min−1. Values were obtained on control plants cultivated on deionized water in white light. Values are the mean of 5 measurements ± standard error.

<table>
<thead>
<tr>
<th>ATPase activity (nmol mg−1 protein min−1)</th>
<th>Inhibition percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.0 ± 3</td>
</tr>
<tr>
<td>Vanadate (0.3 mol m−3) (plasmalemma)</td>
<td>51.0 ± 0.4</td>
</tr>
<tr>
<td>Nitrate (50 mol m−3) (tonoplast)</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>Azide (0.5 mol m−3) (mitochondrial membrane)</td>
<td>10.7 ± 0.3</td>
</tr>
</tbody>
</table>

Table 3. Effect of freezing and thawing cycles on ATPase activity

Measurements were performed on the final membrane fraction before and after freezing and thawing cycles (FTC). ATPase activity was expressed as nmol mg−1 protein min−1. Percentage of 'inside out' (ISO) vesicles = [activity before FTC/activity after FTC] × 100, percentage of 'right side out' (RSO) vesicles = 100 − percentage of 'inside out' vesicles. Values were obtained on control plants cultivated on deionized water in white and in blue light. Values are the mean of 5 measurements ± standard error.

<table>
<thead>
<tr>
<th>ATPase activity (nmol mg−1 protein min−1)</th>
<th>White light</th>
<th>Blue light</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase activity before freezing</td>
<td>11 ± 3</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>ATPase activity after freezing and thawing cycles</td>
<td>51 ± 4</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>'ISO' percentage</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>'RSO' percentage</td>
<td>79</td>
<td>77</td>
</tr>
</tbody>
</table>
Effect of cotyledonary pricks and light quality on ATPase activity

The ATPase activity of control plants cultivated under WL was higher than the ATPase activity of control plants cultivated under BL. This result was observed whatever the ionic composition of the culture medium: Cera III (Fig. 2) or deionized water (Fig. 3).

After the cotyledonary pricks, no modification of ATPase activity was observed, whatever the light quality, when plants were cultivated on Cera III (Fig. 2). In contrast, when plants were cultivated on deionized water (Fig. 3), modifications of ATPase activity were observed after cotyledonary pricks. When plants were cultivated under WL, the cotyledonary pricks induced a rapid (5 min) and transient (restored in 60 min) decrease of the ATPase activity. In contrast, when plants were cultivated under BL, pricks induced a rapid and transient increase of ATPase activity.

Discussion

To test if the plasmalemma H\(^+\)-ATPase was involved in the modifications of cellular ionic levels (H\(^+\) and K\(^+\)), observed in Bidens pilosa (Pichon et al., 1993), a two-phase partitioning method has been used to isolate plasma membrane. Using enzymatic markers of membranes and specific inhibitors of ATPase activity, two-phase partitioning is an efficient method to obtain highly purified plasmalemma from Bidens hypocotyl cells with a low level of contamination by tonoplast (5%) and internal membrane of mitochondria (2%).

In control plants, plasmalemma ATPase activity of hypocotyl cells differed according to the light quality, being higher in WL than in BL. These results were obtained after four freezing-thawing cycles, a treatment which disrupted membrane vesicles (equivalent to 100% of ‘inside out’ vesicles). This difference was, therefore, not due to a light quality effect on vesicle conformation (percentage of ‘inside out’ vesicles). Moreover, similar values of ‘ISO’ percentage were observed on native membrane fractions, whatever the light quality. The higher ATPase activity observed in WL than in BL could be due to a higher affinity of the enzyme for its substrate (ATP) and to its higher \(V_m\) in WL condition.

The difference in ATPase activity correlates with difference in pH\(_c\) and K\(^+\) level detected in intact cells by Pichon et al. (1993): values of pH\(_c\) and cellular K\(^+\) concentration are higher in WL than in BL (Pichon et al., 1993). The ATPase activity would be responsible for these ionic modifications in hypocotyl cells, induced by light quality. This represents a mechanism involving the long-term regulation of pH\(_c\) by ATPases (Felle, 1989; Guern et al., 1990; Petel et al., 1992).

In pricked plants cultivated on deionized water, rapid (5 min after cotyledonary traumatisms) and transient (restoration 60 min after traumatisms) modifications of hypocotyl cells plasmalemma ATPase activity were observed. These modifications were opposite according to the light quality. A decrease of pump activity was observed in WL, and an increase in BL.

Cotyledonary pricks in intact cells are proposed to elicit similar changes in ATPase activity and ionic variation (Bonnin et al., 1989; Pichon et al., 1993) to those found in our in vitro system. This proposal is supported by two findings. Firstly, in both systems the reduction of ATPase activity in WL is associated with a decrease of K\(^+\) levels and an increase in H\(^+\) concentration; whilst increased enzymatic activity in BL is correlated to an
increase of K\(^+\) and a reduction of the H\(^+\) concentration. Secondly, the modifications to hypocotyl cell ion concentrations (both in vivo and in vitro) and to ATPase activity (in vitro) both take place after a 5 min time lag.

In Bidens pilosa, the plasmalemma ATPase seemed to be responsible for ‘short-term’ intracellular ionic modifications. This hypothesis was supported by the fact that these ATPase activity and ionic (H\(^+\), K\(^+\)) modifications were observed only when plants were cultivated on deionized water, culture conditions corresponding to low ionic levels in tissues (Desbiez et al., 1989; Bonnin et al., 1989).

In contrast, on Cera III (high tissue ionic levels), neither ATPase activity modifications nor variations of ionic levels were observed. The variations of H\(^+\) and K\(^+\) levels, induced by ATPase activity, would induce modifications of cellular development (mitotic activity and cell elongation) and would be linked to hypocotyl growth inhibition (Pichon et al., 1993). Proton extrusion into the cell wall by ATPase leads to wall loosening and so contributes to expansion growth of plant cells. ATPase activity reduction by a mechanical stimulus causes a decrease in proton pumping into the wall space, reducing cell wall loosening and, therefore, cellular growth.

Lastly, BL, without cotyledonary prickings, induces hypocotyl elongation (Gaba and Black, 1979) and cellular expansion (Cosgrove, 1988) inhibition, related to intracellular pH and K\(^+\) level modifications (Pichon et al., 1993). The present data show that cotyledonary prickling leads to an additional modification of both pH\(_{c}\) and K\(^+\) level, which appears to be a specific response to mechanical stimuli.

The present study indicates that the plasmalemma ATPase was involved in the long-term regulation of pH\(_{c}\) and K\(^+\) concentration and, in an original way, in Bidens pilosa, it is possible to induce rapid modifications of intracellular H\(^+\) and K\(^+\) levels, in response to pricks.

**Acknowledgements**

P Bonnin, O Pichon and G Péetel would like to dedicate this work to the memory of Dr MO Desbiez, deceased in January 1995.

**References**


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