FEBS Letters 456 (1999) 22–26 FEBS 22372

Role of the 14-3-3 proteins in the regulation of H^+-ATPase activity in the plasma membrane of suspension-cultured sugar beet cells under cold stress

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Received 23 April 1999; received in revised form 7 June 1999

Abstract All higher plants possess highly specific binding sites for fusicoccin, a metabolite of the fungus *Fusicoccum amygdali* Del. These sites are harboured in the plasma membranes and formed by a 14-3-3 protein dimer associated with the C-terminal autoinhibitory domain of H^+-ATPase. We considered the fusicoccin binding to plasma membranes to be an indicator of complexation between the 14-3-3 dimer and H^+-ATPase, we assessed the effect of cold stress on the interaction of these proteins in suspension-cultured sugar beet cells and protoplasts derived from these cells. In both objects, upon lowering the temperature to 0–4°C, a portion of the cytoplasmic 14-3-3 proteins became associated with the plasma membrane, which showed an increasing amount of ATPase/14-3-3 complexes and enhanced ATPase activity. Association between ATPase and 14-3-3 resulted in a several-fold rise in the H^+ efflux from protoplasts and intact cells. We suppose that regulation of the H^+ pumping under changing external conditions may be based on the interaction between H^+-ATPase and the 14-3-3 proteins.

Key words: Cold stress; Plasma membrane; H^+-ATPase regulation; 14-3-3 Protein

1. Introduction

Plants develop under varying environmental conditions and have a special system of protection against external influences, which enables them to react adequately to the changes in temperature, humidity, illumination etc. A special role in this protective system is played by the plasma membrane H^+-ATPase [1]. Hydrolysing ATP, this enzyme extrudes protons to create a transmembrane electrochemical potential of hydrogen ions, which is then used by the plant cells to provide energy for ion and metabolite transport.

The activity of the proton pump in the plant plasma membrane is not constant, being modulated by all the important factors controlling the plant physiology [2].

In the plasma membrane, H^+-ATPase is part of a large complex of 500–800 kDa of a yet uncertain composition [3,4]. It is, however, known that the ATPase C-terminal domain, which shows autoinhibitory action [5], interacts in the membrane with a dimer of 14-3-3 proteins [6–8]. These proteins are hydrophilic, conserved and widely represented in animal and plant cells [9]. They themselves do not show enzymic activity and are believed to act as regulators or adapters [10]. Suggestions have been made about the regulatory role of the 14-3-3 proteins in H^+-ATPase functioning [4,6,11,12], but the regulatory mechanism remained obscure.

In the plant plasma membrane, the protein complex including H^+-ATPase exhibits a unique property: highly specific binding of fusicoccin (FC), a metabolite of the fungus *Fusicoccum amygdali* Del [13]. The FC binding site (FCBS) is in the 14-3-3 dimer [14]. Interestingly, the dimer as such does not bind FC [4,15] and acquires this ability only on interaction with the ATPase C-terminal domain. The affinity and the number of FCBSs are not constant, but change as the cells are subjected to various stimuli [16]. The lability of FCBS is especially vivid on protoplasts. Acidification of the cytoplasm causes a rise in affinity [17], whereas a decrease of the osmotic pressure in the protoplast incubation medium elicits a significant number of new FCBSs [18]. As these stimuli mimic abiotic stress, we supposed that 14-3-3 proteins may take part in regulating the H^+-ATPase under stress conditions and tried to check the influence of another external factor, temperature, on the interaction between these proteins and H^+-ATPase, using the binding of FC to the plasma membrane as an index of complexation between them. Indeed, a lowered temperature proved to cause an increase in the amount of 14-3-3 dimer/H^+-ATPase complexes in the plasma membrane of cultured sugar beet cells or protoplasts, with a concomitant rise in H^+-ATPase activity and redistribution of 14-3-3 proteins between the cytoplasm and the plasma membrane.

2. Materials and methods

2.1. Protoplast preparation

Sugar beet (*Beta vulgaris* strain 2n) root cells were grown as a suspension culture in a Shenk-Hildebrandt medium in the dark at 25°C with constant shaking at 100 rpm in round-bottom flasks, with 21-d passaging. Cells were harvested by centrifugation and incubated under the above-specified conditions for 75 min in 0.4 M sorbitol, 1 mM $CaSO_4$, 10 mM potassium ascorbate (pH 5.5) containing (all w/v) 0.4% driselase, 0.1% cellulysin, 0.2% cellulase R-10 and 0.1% macerozyme. The resulting protoplasts were washed twice by 5 min centrifugations at $100\times g$ and suspended in 0.4 M sorbitol, 1 mM $CaSO_4$, 2.5 mM K_2SO_4 and 20 mM MES-BTP (buffer A, pH 6.3). The protoplasts retained viable for at least 4 h and up to 90%, therefore, remained intact throughout the experiment, as judged by fluorescein staining and exclusion of Evans blue. These protoplasts responded to FC by acidification of the medium and alkalisation of the cytosol, which was blocked by erythrosine B.

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PII: S0014-5793(99)00923-0

2.2. Cold treatment

2.2.1. Cells. The cell suspension was diluted with an equal volume of the Shenk-Hildebrandt medium cooled to 0°C and kept on ice for 4 h with constant shaking at 100 rpm. Then, microsomes and plasma membranes were isolated. Control cells were diluted with the medium at 26°C and kept for 4 h under normal culturing conditions.

2.2.2. Protoplasts. Freshly prepared protoplasts were diluted with an equal volume of buffer A (final density 1–1.5·10^6 cells/ml), cooled to 0°C and kept on ice. Plasma membranes were obtained from protoplasts subjected to cold stress for 1 h. Protoplasts chilled for 1 h were transferred to room temperature, after which aliquots were taken at indicated moments to measure the binding of [^{3}H]dihydroFC (dhFC).

2.3. Membrane isolation

2.3.1. Cells. Suspension-cultured cells were washed with 5 mM EDTA, suspended 1:10 (w/v) in 20% (v/v) ethylene glycol, 10 mM EDTA, 5 mM EGTA, 10 mM DTT, 400 mM sucrose, 10 mM NaF, 30 mM β-glycerophosphate, 1 mM PMSF, 50 mM MES-Tris (pH 7.2) (buffer B) with polyvinyl polypyrrolidone to 10% of the cell mass and homogenised in an IKA Ultraturax device. The microsomal fraction was isolated by differential centrifugation 10/80 and suspended at 2–3 mg protein/ml in 5 mM KH_2PO_4 (pH 7.8), 0.33 M sucrose, 3 mM KCl. The plasma membrane was purified by aqueous two-phase partitioning in Dextran T500-PEG 3350 6.2%, according to [19]. Isolated plasma membrane was suspended in 10% sucrose (w/w), 5 mM EDTA, 20 mM DTT, 20 mM Tris-MES, pH 7.2, and stored at −70°C. Membrane proteins were measured essentially after Bradford [20] with trypsin as the standard.

2.3.2. Protoplasts. Protoplasts (1.5·10^7) were washed twice (100×*g*, 5 min), transferred into 5 ml of buffer B plus 5 μg/ml of the protease inhibitor mixture (leupeptin, aprotinin, antipain) and sonicated for 10 s at 0°C in an MSE unit with immersed probe at a medium setting. The homogenate was centrifuged for 10 min at 10 000×*g* and the supernatant was then centrifuged at 120 000×*g* for 30 min. The plasma membrane was purified as described above.

2.4. [^{3}H]dhFC binding

2.4.1. Protoplasts. dhFC binding was conducted in 100 μl of protoplast incubation medium containing 10^5 protoplasts at 25°C for 20 min. Non-bound label was removed by vacuum filtration [17]. Binding parameters were evaluated with the Enzfitter 1.05 program (R.J. Leatherbarrow, Elsevier-Biosoft, Cambridge, UK).

2.4.2. Membranes. The binding assays were conducted in 100 μl 50 mM Tris/MES (pH 6.0), 5 mM $MgSO_4$, containing 30 μg of microsomal or 10 μg of plasmalemmal protein, for 30 min at room temperature. All the subsequent procedures were carried out as described for protoplasts. The results were averaged over several independent triplicates.

2.5. Extracellular acidification and H^+-ATPase activity assays

Protoplasts (2·10^6) were placed in 2 ml 0.4 M sorbitol, 1 mM $CaSO_4$, 2.5 mM K_2SO_4, 2 mM MES-BTP (pH 6.3) containing 1 μM of a non-permeating fluorescent pH indicator, 5(6)-carboxyfluorescein, and fluorescence was measured at 530 nm upon excitation at 490 and 440 nm. The F_{490}/F_{440} ratio was calibrated in nmol H^+. Cells (0.5 g fresh weight) were placed in 7 ml of 1 mM $CaSO_4$, 2.5 mM K_2SO_4, 2 mM MES-BTP (pH 6.3) and the pH of the medium was recorded continuously.

The ATP hydrolytic activity was assayed by incubating 20 μg of plasmalemmal protein at 30°C in 1 ml of 40 mM Tris/MES, 5 mM $MgSO_4$, 50 mM KNO_3, 1 mM sodium molybdate plus 2 μg oligomycin. The reaction was started with the addition of 3 mM ATP/Tris to the assay solution and stopped after 30 min with 400 μl of 25 mM ammonium molybdate in 2% H_2SO_4. Then, 40 μl of 10 mM 1-amino-2-naphthol-4-sulfonic acid (Fiske and Subbarow reducer) were added to the reaction mixture and free P_i was measured at 815 nm after the colour developed at 22°C for 30 min. The assays were carried out in the pH range 6.5–7.2.

2.6. SDS-PAGE and Western blotting

For 14-3-3-assays, 5 μg of plasma membrane, 10 μg of microsomes and the corresponding amount of supernatant were diluted with sample buffer and heated to 95°C for 5 min and then loaded onto a SDS gel. For the ATPase assay, 5–10 μg of plasma membrane with 5 μg/ml of the protease inhibitor mixture and 1 mM PMSF were diluted with sample buffer and solubilised on ice for 5 min and then loaded onto a SDS gel. The total monomer concentration was 5% for the stacking gel and 10% for the running gel. Electrophoresis was performed essentially according to the method of Laemmli.

Membrane peptides from the SDS-PAGE gel were electrophoretically transferred to a Hybond-C Super transfer membrane (Amersham) with a Multiphor II Electrophoresis Unit (LKB) according to the manufacturer's instructions. The polyclonal antiserum against plasma membrane H^+-ATPase was kindly provided by Prof. R. Serrano (Institute of Plant Molecular and Cellular Biology, Spain). Polyclonal antibodies raised against yeast BMH-1 were kindly provided by Dr. G.P.H. van Heusden (Leiden University, The Netherlands). The second antibody, goat anti-rabbit IgG conjugated with horseradish peroxidase, was purchased from Sigma. Immunodetection of 14-3-3 was carried out using the ECL system (Amersham), visualisation of ATPase was performed using anti-rabbit IgG conjugated with horseradish peroxidase and *o*-dianisidine as a substrate.

2.7. Reagents

MES, BTP, Tris, DTT, PMSF, SDS, ethylene glycol and sorbitol were purchased from Serva. Polyethyleneimine P and 5(6)-carboxyfluorescein were purchased from Fluka. Ascorbic acid, glycine, EDTA, EGTA, β-glycerophosphate, PVPP, PEG 3350, leupeptin,

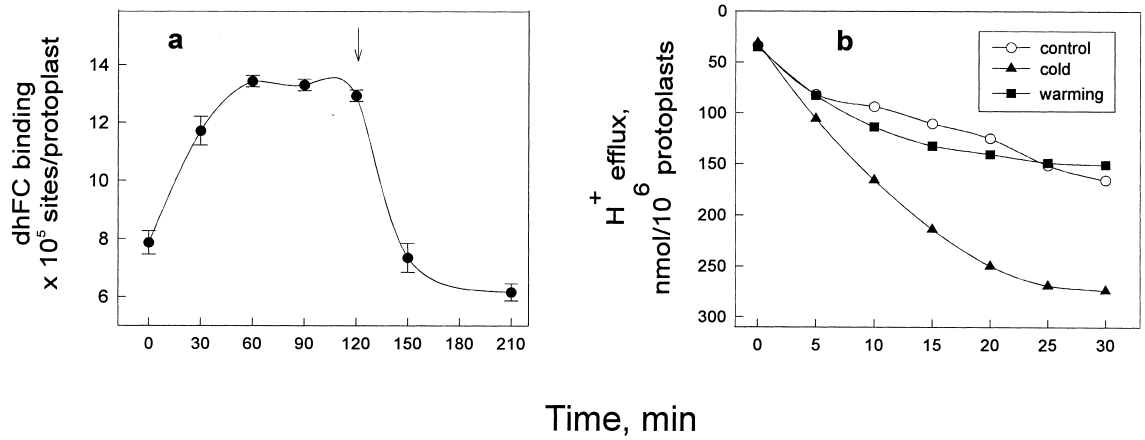

Fig. 1. Effect of cold stress on dhFC binding to protoplasts (a, arrow marks return to room temperature) and on acidification of the incubation medium (b, start corresponds to placing 10^6 protoplasts into fresh assay medium). (○) Control, (▲) protoplasts pre-incubated for 1 h at 0°C, (■) same protoplasts 15 min after returning them to room temperature.

aprotinin, antipain, sucrose and ATP were purchased from Sigma. Dextran T500 was purchased from Pharmacia. The enzymes were driselase (Kyowa Hakko Kogyo, Tokyo, Japan), cellulysin (Calbiochem, San Diego, CA, USA), cellulase R-10 and macerozyme (Serva, Heidelberg, Germany). Other reagents were from domestic sources of the highest purity available. dhFC (specific activity 2 TBq/mmol) was from the Institute of Molecular Genetics (Moscow).

3. Results

Fig. 1a shows that the number of FCBSs in protoplasts gradually increases under cold exposure. This effect is well seen in 30 min and persists for at least 2 h of incubation at 0°C (longer times were not tested). Concurrently, there is a rise in the H^+ pump activity as revealed by acidification of the incubation medium (Fig. 1b). The effect is reversible: in 15 min after returning the protoplasts to room temperature, the number of FCBSs and the rate of H^+ efflux revert to the initial values.

As follows from Table 1, the increase in FCBSs is also observed in the plasma membranes isolated from the protoplasts exposed to cold. In parallel, the amount of 14-3-3 proteins rises in the plasma membranes and declines in the soluble fraction.

There is a correspondence between the increase in the amount of FCBSs in the protoplasts under cold stress and in their microsomal fraction and plasma membrane. Also, relative changes occur in the densities of FCBSs and 14-3-3 proteins in the plasma membrane, whereas in the total microsomal fraction, the amount of 14-3-3 proteins remains the same.

Very similar results were obtained for intact suspension-cultured cells (Table 2), with the only difference that at least a 4 h cold exposure was required to elicit a distinct change in the amount of FCBSs (measured in isolated plasma membrane).

Fig. 2 shows that both the H^+ efflux from cold-exposed cells and the H^+-ATPase activity in the plasma membranes isolated from the same cells are higher than the control activity. Western blot analysis showed that the content of the enzyme in the plasma membrane isolated both from protoplasts and from intact cells did not increase (data not shown).

4. Discussion

Rapid cooling of protoplasts derived from suspension-cultured sugar beet cells results in activation of the H^+ pump and an increase in the number of FCBSs in the plasma membrane. The difference between protoplasts and intact cells is the time of cold exposure required to elicit a distinct effect. We could not isolate the protoplast plasma membranes in amounts sufficient to measure the ATPase activity, but the plasma membranes from cold-stressed cells exhibit a H^+-ATPase activity about 30% higher than the control activity. On the other hand, the number of H^+-ATPase molecules in the plasma membranes does not change, hence the rise in activity is most probably due to an altered K_M and V_{max}.

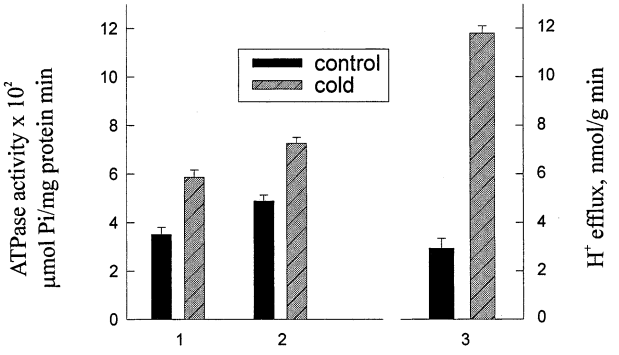

Fig. 2. H^+-ATPase activity of plasma membranes isolated from control cells and cells subjected to cold stress measured at (1) pH 7.0 and (2) pH 6.4 and (3) H^+ efflux from the cells.

The quantitative discrepancy between the increments in the H^+ efflux from the cells and in the ATPase activity of isolated plasma membranes that we observed in cold stress (Fig. 2) has more than once been reported for other kinds of exposures, e.g. plant treatment with FC [21,22]. The proton pumping activity is stimulated much more than the ATPase activity in isolated plasma membranes upon proteolytic removal of the C-terminal domain of plant H^+-ATPase [23]. The same effect is produced by mutational truncation of the yeast H^+-ATPase by 11 C-terminal residues [24]. Quite a few point mutations in the C-terminal part of plant H^+-ATPase are known to enhance the coupling between ATP hydrolysis and proton pumping [25]. All these data gave grounds for suggesting that in a substantial portion of H^+-ATPase in the plant cell, these processes are uncoupled because of the altered position of the C-terminal domain relative to the rest of the molecule [2]. Probably, upon association of the 14-3-3 dimer with the C-terminal part of H^+-ATPase, which gives rise to the FCBSs, the C-terminus is shifted to increase the coupling.

Our results indicate that cold stress stimulates such association and the latter appears to be the main cause of the observed increase in the H^+ efflux. The increment in the 14-3-3 proteins in the plasma membrane with a concurrent decline in the cytoplasm argue in favour for that additional

Table 1
Changes in the amount of FCBSs and 14-3-3 proteins in protoplasts under cold stress

Parameters		Microsomal membrane	Soluble protein (100 000 × *g* supernatant)	Plasma membrane
FCBS pmol/mg protein	Control	2.9 ± 0.5		11.6 ± 3.6
	Cold	6.9 ± 0.7	ND	20.1 ± 6.4
	Ratio (cold/control)	2.4 ± 0.4		1.8 ± 0.3
14-3-3 arbitrary units/mg protein	Control	100 ± 10	330 ± 40	250 ± 30
	Cold	98 ± 8	160 ± 10	420 ± 30
	Ratio (cold/control)	1.0 ± 0.1	0.51 ± 0.07	1.7 ± 0.2

Data were averaged for four independent membrane preparations. The arbitrary unit for 14-3-3 proteins is the area under the densitometric peak per 1 μg plasmalemmal protein. The peak area was linearly proportional to the amount of protein up to 10 μg.

Table 2
Changes in the amount of FCBSs and 14-3-3 proteins in intact cells under cold stress

Parameters		Microsomal membrane	Soluble protein (100 000 × *g* supernatant)	Plasma membrane
FCBS pmol/mg protein	Control	1.3 ± 0.2		3.9 ± 0.3
	Cold	2.5 ± 0.3	ND	6.2 ± 0.5
	Ratio (cold/control)	1.9 ± 0.3		1.6 ± 0.4
14-3-3 arbitrary units/mg protein	Control	135 ± 15	160 ± 20	240 ± 30
	Cold	140 ± 20	97 ± 7	330 ± 50
	Ratio (cold/control)	1.1 ± 0.1	0.62 ± 0.08	1.4 ± 0.2

The values are the means ($n = 3$) with S.E.M.

FCBSs form through association of the cytosolic 14-3-3 with 'vacant' H^+-ATPase molecules that appear to be ample in the plasma membrane. The protoplasts probably maintain an equilibrium between the membrane-bound and soluble 14-3-3, as evidenced by the rapid decrease in the number of FCBSs to the initial value when the protoplasts are returned to room temperature. On the strength of the coincident data in Table 1 and 2, this conclusion can be applied to the intact cells.

It is noteworthy that the redistribution of 14-3-3 under cold stress takes place mainly between the cytoplasm and the plasma membrane but is not observed for other membranes. Indeed, in the plasma membrane, the increase in 14-3-3 closely parallels that in the FCBSs (which are known to be present only in this membrane [13]) but is masked in the overall microsomal fraction (see Table 1 and 2). Plants as well as animals have various 14-3-3 isoforms [9]. According to our unpublished data, there are at least seven isoforms in cultured sugar beet cells. It may well be that the observed redistribution involves only certain 14-3-3 isoforms, but there is yet not enough ground for making such a conclusion.

Our results suggest that under cold stress, the soluble cytoplasmic 14-3-3 proteins associate as dimers with the plasmalemmal H^+-ATPase, thereby activating the H^+ pumping. A natural question is what causes the transition of the soluble 14-3-3 into the membrane-bound state. The cold-induced increase in 14-3-3 is observed only in the plasma membrane, hence it can be thought that alterations in the H^+-ATPase, rather than in the 14-3-3, cause them to associate.

We think there may be two kinds of such modifications. One is the Ca^{2+}-dependent phosphorylation of the H^+-ATPase C-terminal domain and the other is a conformational change in the ATPase complex that makes the C-terminal domain accessible for the 14-3-3 proteins. The first idea is supported by the increasing affinity between the 14-3-3 dimer and its protein target upon phosphorylation of the latter [26] and by the entry of Ca^{2+} ions into plant cells under cold stress [27]. The second idea is supported by the attenuation of membrane fluidity with a decreasing temperature [28] and by the significant lag (4 h) in the response of intact cells to cooling. We failed to observe any effect of Ca^{2+} channel blockers verapamil and La^{3+} or phosphorylation/dephosphorylation inhibitors staurosporin and okadaic acid on the cold induction of FCBSs (data not shown). It is, however, premature to choose between these two alternatives.

Modulation of H^+ pumping through adjusting the number of H^+-ATPase complexes with 14-3-3 proteins suggested by the results of this work may also underlie the regulation of its activity in plant cells under other external stimuli, such as changes in humidity, illumination etc. This is a goal of our further studies.

Acknowledgements: This work was supported by the Russian Foundation for Basic Research (Grant 98-04-48655) and INTAS (Grant 94-4358). We are grateful to Prof. R. Serrano for the generous gift of antibodies against specific domains of the plasma membrane H^+-ATPase and to Dr. G.P.H. van Heusden for the generous gift of antibodies against yeast 14-3-3 protein (BMH-1). The authors wish to thank Dr. A.V. Galkin for fruitful discussion and valuable comments.

References

[1] Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94.
[2] Palmgren, M.G. (1998) Adv. Bot. Res. 28, 2–70.
[3] De Boer, A.H. and Korthout, H.A.A.J. (1996) J. Plant Growth Regul. 18, 99–105.
[4] Oecking, C., Piotrowski, M., Hagemeier, J. and Hagemann, K. (1997) Plant J. 12, 441–453.
[5] Pallmgren, M.G., Sommarin, M., Serrano, R. and Larsson, C. (1991) J. Biol. Chem. 266, 20470–20475.
[6] Jahn, T., Fuglsang, A.T., Olsson, A., Bruntrup, I.M., Collinge, D.B., Volkman, D., Sommarin, M., Palmgren, M.G. and Larsson, C. (1997) Plant Cell 9, 1805–1814.
[7] Fullone, M.R., Visconti, S., Marra, M., Fogliano, V. and Aducci, P. (1998) J. Biol. Chem. 273, 7698–7702.
[8] Oecking, C. and Hagemann, K. (1999) Planta 207, 480–482.
[9] Ferl, R. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 49–73.
[10] Aitken, A. (1996) Trends Cell Biol. 6, 341–347.
[11] Baunsgaard, L., Fuglsang, A.T., Jahn, T., Korthout, H.A.A.J., De Boer, A.H. and Palmgren, M.G. (1998) Plant J. 13, 661–671.
[12] Piotrovski, M., Morsomme, P., Boutry, M. and Oecking, C. (1998) J. Biol. Chem. 273, 30018–30023.
[13] Aducci, P., Marra, M., Fogliano, V. and Fullone, M.R. (1995) J. Exp. Bot. 46, 1463–1479.
[14] De Boer, A.H. (1997) Trends Plant Sci. 2, 60–66.
[15] Korthout, H.A.A.J. and De Boer, A.H. (1994) Plant Cell 6, 1681–1692.
[16] Basel, L.E., Zukovski, A.T. and Cleland, R.E. (1994) Plant Physiol. 104, 691–697.
[17] Drabkin, A.V., Trofimova, M.S., Smolenskaya, I.N., Klychnikov, O.I., Chelysheva, V.V. and Babakov, A.V. (1997) FEBS Lett. 405, 145–147.
[18] Trofimova, M.S., Klychnikov, O.I., Nosov, A.V. and Babakov, A.V. (1999) Russ. J. Plant Physiol. 46, 5–10.
[19] Larsson, C., Sommarin, M. and Widell, S. (1994) Methods Enzymol. 226, 451–469.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Johansson, F., Sommarin, M. and Larsson, C. (1993) Plant Cell 5, 321–327.
[22] Jahn, T., Johansson, F., Luthen, H., Volkman, D. and Larsson, C. (1996) Planta 199, 359–365.

[23] Palmgren, M.G., Larsson, C. and Sommarin, M. (1990) J. Biol. Chem. 266, 20470–20475.
[24] Portillo, F., Larrinoa, I.F. and Serrano, R. (1989) FEBS Lett. 247, 381–385.
[25] Morsomme, P., de Kerchove d'Exaerde, A., De Meester, S., Thines, D., Goffeau, A. and Boutry, M. (1996) EMBO J. 15, 5513–5526.
[26] Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Cell 84, 889–897.
[27] Knight, H., Trewavas, A.J. and Knight, M.R. (1996) Plant Cell 8, 489–503.
[28] Harwood, J.L., Jones, A.L., Perry, H.J., Rutter, A.J., Smith, K.L. and Williams, M. (1994) in: Temperature Adaptation of Biological Membranes (Cossins, A.R., Ed.), pp. 107–118, Portland Press Proceedings, London.