Abscisic acid plasmalemma perception triggers a calcium influx essential for *RAB18* gene expression in *Arabidopsis thaliana* suspension cells

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Abstract Pretreatment of *Arabidopsis thaliana* suspension cells with impermeant calcium chelator EGTA inhibited the ABA-induced *RAB18* gene expression. However, extracellular calcium alone, up to 10 mM, did not trigger *RAB18* expression. Spectrofluorimetric extracellular Ca^{2+} measurement with Fluo-3 showed a fast, within 1 min, Ca^{2+} influx associated with outer plasmalemma ABA perception. In the presence of the Ca^{2+} blockers Cd^{2+} and Ni^{2+}, *RAB18* expression was suppressed. Pimozide and fluspirilene inhibited Ca^{2+} influx and ABA-induced *RAB18* expression. Thus we demonstrated the involvement of specific Ca^{2+} influx in the ABA signaling sequence leading to *RAB18* expression.

Key words: Abscisic acid transduction; Calcium channel inhibitor; *Arabidopsis thaliana* suspension cells

1. Introduction

Calcium is a universal second messenger in plant signaling processes [1,2]. Increase in $[Ca^{2+}]_{cyt}$ was recorded in cellular responses to several stimuli [3]. For example, the stomatal closure triggered by the hormone abscisic acid (ABA) is mediated by Ca^{2+}. The rapid ABA-induced increase in $[Ca^{2+}]_{cyt}$ was first demonstrated on microinjected intact guard cells with Ca^{2+} fluorescent dye [4,5], and was confirmed on guard cell protoplasts loaded from the patch-clamp pipette with Fura-2 [6]. Artificial increase in $[Ca^{2+}]_{cyt}$ obtained by permeation of the plasma membrane with a Ca^{2+} ionophore or by increasing extracellular $[Ca^{2+}]_o$ were also effective, in absence of ABA, to close the stomatal aperture [7]. Conversely, Ca^{2+} chelation was shown to abolish ABA induction of stomatal closure in *Commelina communis* [8]. The increase in $[Ca^{2+}]_{cyt}$ may be due to a redistribution of Ca^{2+} from intracellular compartments [7,9,10]. However it was also demonstrated, by patch-clamp analyses, that ABA could activate Ca^{2+} plasma membrane channels [6]. Thus a direct influx of extracellular Ca^{2+} may exert a role in ABA signaling pathway.

In guard cells, Ca^{2+} modulates the activity of many targets especially ion channels: K^+ inward rectifier currents are inhibited [10], and anion channels are activated [11,12]. In dedifferentiated *Arabidopsis thaliana* suspension cells, we obtained evidence that the activation of anion channels and the inhibition of K^+ inward rectifier currents resulted from the outer plasmalemma perception of ABA which triggers the ABA inducible *RAB18* gene expression [13,14]. Here, we establish that the ABA plasmalemma perception triggers, through specific channels, a calcium influx which is essential for *RAB18* expression.

2. Materials and methods

2.1. Plant material

A. thaliana L. ec. Columbia suspension cells were obtained by Axelos et al. [15]. Cells were cultured at 24°C, under continuous white light (40 μmol m^{-2} s^{-1}) with an orbital agitation at 130 rpm, in 500 ml Erlenmeyer flasks containing 200 ml Jouanneau and Péaud-Lenoël (JPL) culture medium [16], pH 6.8. The suspension was subcultured weekly and all the experiments were conducted on 4 days old cells after subculture. The viability of the cells during the 3 h experimental time course was systematically checked with trypan blue tests (not shown).

2.2. Chemicals

(+/−) Cis-trans-ABA, Fluo-3, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetyl acid (EGTA), specific Ca^{2+} effectors (nifedipine, diltiazem, verapamil, fluspirilene, pimozide, Bay K8644 and A23187) were from Sigma-Fluka Chemical Corp.

2.3. RAB18 responsive test and Northern blot analysis

A 5 ml suspension was incubated for 3 h under the conditions of culture. ABA–BSA purified conjugate was added in 50 mM Na_2SO_4, 50 mM pH 6.8 phosphate buffer [13]. The Ca^{2+} effectors were added with ABA–BSA simultaneously. The concentration of ABA linked to BSA was expressed as ABA-equivalent concentration [13]. Northern blot analyses were performed according to the protocol previously described [13]. The regularity of the deposits (equal loading) was checked by ethidium bromide staining or cDNA *18S* probe hybridization. All experiments were performed at least in triplicate.

2.4. Extracellular Ca^{2+} measurement

Free calcium level in the culture medium, $[Ca^{2+}]_o$, was measured with Fluo-3 according to the protocol described by Tepikin et al. [17]. Four days old cells were transferred in a Mg^{2+} free and low Ca^{2+} concentrated (50 μM) JPL culture medium for 4 h prior to treatment. Experiments were done on 5 ml suspension cells, i.e. 1 vol sedimented cells suspended in 4 vol culture medium. Then, the culture medium was filtrated on 40 μm mesh nylon membrane and aliquots (400 μl) of cell culture medium collected were mixed with Fluo-3 (10 μM). Fluorescence was measured at room temperature with an LS-5 spectrofluorimeter (Perkin-Elmer). Excitation and emission wavelengths were

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490 and 530 nm, respectively. Standard Ca^{2+} curve was established with fluorescence measured for dilutions of $CaCl_2$ stock solution in 10 mM EGTA diluted in JPL modified medium. Free Ca^{2+} concentrations were calculated according to the BAD4 software [18].

3. Results

3.1. Extracellular Ca^{2+} is a requisite for ABA–BSA-induced *RAB18* expression

RAB18 expression was activated by ABA, either impermeant (Fig. 1A) or free (not shown). Preincubation of cells in the culture medium (0.9 mM $CaCl_2$) with the specific Ca^{2+} impermeant chelator EGTA, for 30 min, before ABA–BSA (10^{-5} M equivalent ABA) addition, inhibited *RAB18* expression. A slight *RAB18* expression was observed in cells treated with EGTA alone but a dramatic inhibition of the ABA–BSA-induced *RAB18* expression was observed with 5 mM EGTA added to the medium of the treated cells (Fig. 1A). In the presence of ABA–BSA, 50 μM $CaCl_2$ in the culture medium was sufficient to allow ABA specific expression of *RAB18*. Increasing extracellular calcium concentration, up to 10 mM, had no effect on the intensity of *RAB18* expression. $CaCl_2$ alone was unable to induce *RAB18* expression. Actually, whatever the amount of $CaCl_2$ added, *RAB18* expression was never observed without free (not shown) or impermeant ABA (Fig. 1B).

3.2. Activity of Ca^{2+} channels is involved in ABA transduction

Ca^{2+} blockers and channel inhibitors were co-incubated with ABA–BSA. The specific Ca^{2+} blockers, Ni^{2+} and Cd^{2+}, inhibited *RAB18* expression. During the time course of experiment (3 h), the viability of the cells was assessed by the trypan blue test and their unmodified capacity to synthesize *18S* RNA. Total inhibition was observed at 0.5 mM Cd^{2+} whereas

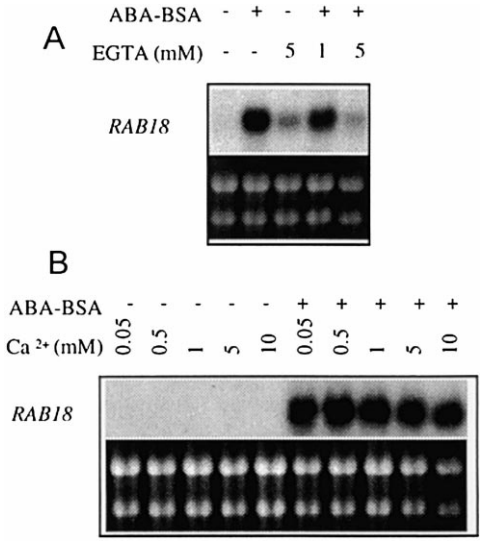

Fig. 1. ABA-induced expression of *RAB18* in *A. thaliana* suspension cells is calcium dependent. A: Cells were preincubated with EGTA (1 and 5 mM) for 30 min prior to addition of ABA–BSA impermeant conjugate (10^{-5} M equivalent ABA) in a 0.9 mM Ca^{2+} JPL medium. B: Cells were incubated with or without ABA–BSA in JPL media containing 0.05 mM, 0.5 mM, 1 mM, 5 mM and 10 mM Ca^{2+} respectively. Northern blot analysis was done from 10 μg total RNA. Ethidium bromide staining of *25S* and *18S* rRNAs is shown as control.

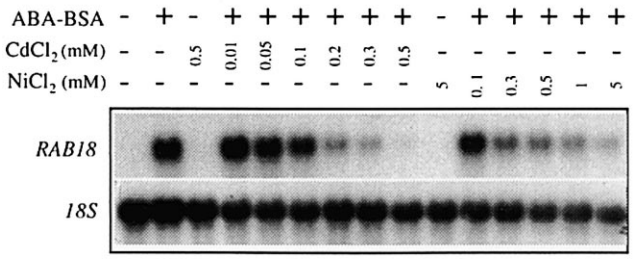

Fig. 2. Effect of $CdCl_2$ and $NiCl_2$ on *RAB18* expression in *A. thaliana* suspension cells. Northern blot analysis of total RNA (10 μg) from cells co-incubated for 3 h with ABA–BSA conjugate (10^{-5} M equivalent ABA) and cadmium or nickel respectively. Similar RNA loading was checked by hybridization with an *Arabidopsis 18S* ribosomal probe.

a faint expression was still observed with 5 mM Ni^{2+} (Fig. 2) or 5 mM Co^{2+} (not shown). Similar features were reported in animal cells especially for L- and N-type Ca^{2+} channels. Therefore, several specific organic inhibitors of L-type Ca^{2+} channels were tested on *RAB18* expression. Up to 100 μM diltiazem, 50 μM nifedipine or 100 μM verapamil did not modify *RAB18* expression. The L-type channel activator Bay K8644 (100 μM) was also inefficient (Fig. 3A). By contrast, inhibitors of L-, N- and T-type Ca^{2+} channels, fluspirilene (50 μM), and pimozide (50 μM) considerably altered *RAB18* expression (Fig. 3B).

3.3. ABA–BSA triggers Ca^{2+} uptake by cells

Spectrofluorimetric extracellular Ca^{2+} ($[Ca^{2+}]_o$) measurement, with the fluorescent dye Fluo-3 shows that 10^{-5} M ABA induced a fast decrease in $[Ca^{2+}]_o$. However, due to interaction of divalent cations with Fluo-3, the method could not be used for $[Ca^{2+}]_o$ measurement in presence of Ni^{2+} or Cd^{2+}. In less than 1 min, the $[Ca^{2+}]_o$ fell down to 25 μM, at 22°C, whereas no decrease in $[Ca^{2+}]_o$ occurred at 2°C (Fig. 4A). Thus, the ABA-induced $[Ca^{2+}]_o$ decrease depends on a cellular active phenomenon. The same feature was observed with impermeant ABA, albeit with a higher ABA–BSA concentration (3.3 10^{-5} M equivalent ABA). At 10^{-5} M ABA–BSA (equivalent ABA), a significant but lower diminution in $[Ca^{2+}]_o$ was recorded (Fig. 4B). BSA alone triggered no variation in $[Ca^{2+}]_o$ (not shown), thus the ABA specificity of the calcium influx was demonstrated. The influx of Ca^{2+} was followed with a two-step Ca^{2+} efflux: first, an efflux of about 50% of the Ca^{2+} absorbed within 20 min, then $[Ca^{2+}]_o$ remained unchanged. One hour after the application of ABA, initial $[Ca^{2+}]_o$ was not fully recovered (Fig. 4A). With ABA–BSA, Ca^{2+} taken up remained in the cells, since no efflux of Ca^{2+} was observed, within 1 h, whatever the concentration of the conjugate used (Fig. 4B). Verapamil (200 μM) had no effect on the ABA-induced absorption of Ca^{2+} by the cells while both pimozide (50 μM) and fluspirilene (50 μM) inhibited the ABA-induced Ca^{2+} influx. Furthermore, verapamil inhibited the subsequent Ca^{2+} efflux (Fig. 4C).

4. Discussion

We present experiments which associate expression of an ABA inducible gene with measure of changes in extracellular Ca^{2+} level in *A. thaliana* suspension cells. Addition of EGTA and Ca^{2+} channel inhibitors allows us to establish that Ca^{2+}

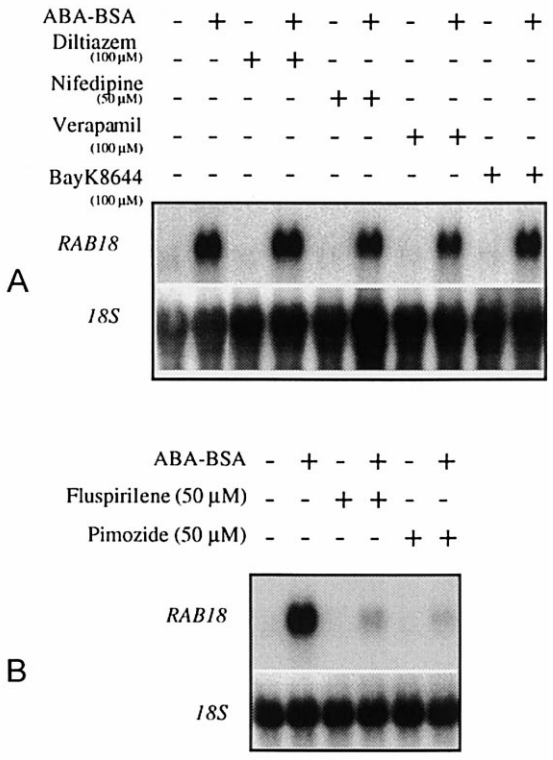

Fig. 3. Effect of specific L- and N-type Ca^{2+} channel effectors on *RAB18* expression in *A. thaliana* suspension cells. Northern blot analysis of total RNA (10 μg) from cells incubated simultaneously, for 3 h, with ABA–BSA conjugate (10^{-5} M equivalent ABA) and specific Ca^{2+} channel effectors. A: L-type Ca^{2+} channel effectors. B: L- and N-type Ca^{2+} channel inhibitors. Similar RNA loading was checked by hybridization with an *Arabidopsis 18S* ribosomal probe.

influx through specific plasmalemma channels is a major requirement in the ABA signaling chain leading to *RAB18* expression.

In *A. thaliana* suspension cells, we have previously demonstrated that the outer plasmalemma perception of ABA triggers a chain of events leading to the expression of *RAB18* [13]. A fast depolarization of the plasmalemma, due to anion channel activation, is involved in this signaling cascade [14]. Here, we show that extracellular Ca^{2+} is necessary to the ABA-induced *RAB18* expression in *A. thaliana* suspension cells. When $[Ca^{2+}]_o$ was decreased from 0.9 mM to 50 μM, the ABA-induced *RAB18* expression was unchanged. Chelating extracellular Ca^{2+} with EGTA impaired *RAB18* expression (Fig. 1A). Similarly, the ABA-induced stomatal closure was abolished by EGTA in *C. communis* [8]. However, in absence of ABA, an increase in $[Ca^{2+}]_o$ up to 10 mM was inefficient to trigger the *RAB18* expression. In *A. thaliana* guard cells, an elevation in $[Ca^{2+}]_o$ could provoke a $[Ca^{2+}]_{cyt}$ increase, but in 50% of the tested cells only [19]. In those cells, Ca^{2+} ionophores failed to consistantly elevate $[Ca^{2+}]_{cyt}$. Similarly, in absence of ABA, the ionophore A23187 (up to 80 μM) was unable to induce *RAB18* expression in *A. thaliana* suspension cells (data not shown). Therefore, *A. thaliana* cells differ from *C. communis* guard cells which accumulate Ca^{2+} when treated with an ionophore [7]. In the presence of ABA, 50 μM $[Ca^{2+}]_o$ was sufficient to allow the induction of *RAB18* expression (Fig. 1B). In *Vicia faba* guard cells, direct measurements of ABA activated ion currents highlighted the participation of external Ca^{2+} [6,20]. It was also recently reported that the ABA opened plasmalemma Ca^{2+} channels were responsible for Ca^{2+} uptake in *V. faba* guard cells [21]. In the present study, the cytosolic Ca^{2+} was not measured but the participation of Ca^{2+} influx through plasmalemma channels in the ABA-induced *RAB18* transduction pathway was demonstrated.

The pattern of changes in Ca^{2+} level in the culture medium of free ABA treated cells (Fig. 4A) differs from that of cells treated with impermeant ABA (Fig. 4B). In both cases, a fast

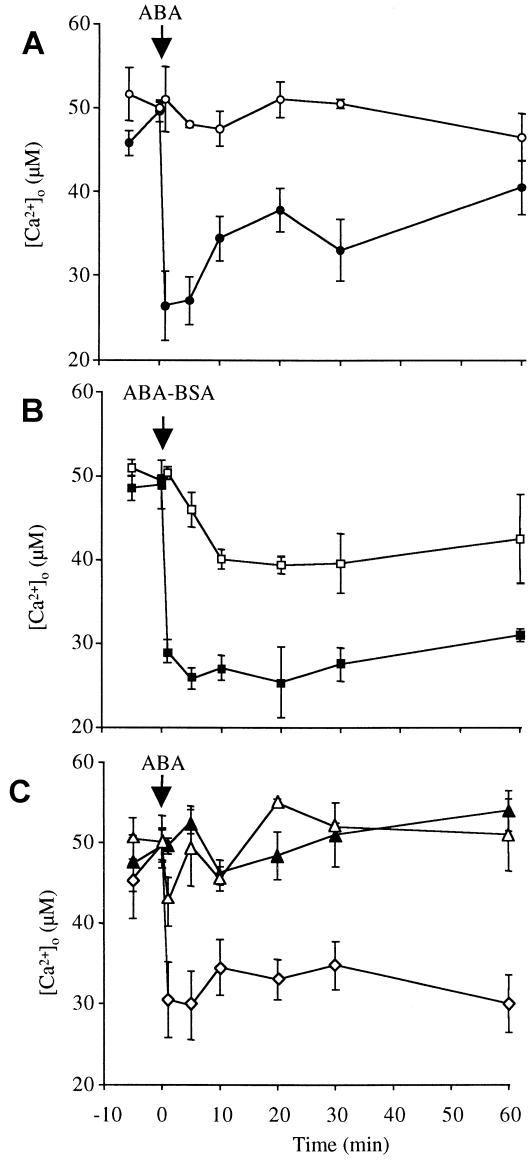

Fig. 4. ABA-induced Ca^{2+} uptake by *A. thaliana* suspension cells. Cells were first equilibrated for 4 h in a low (50 μM) Ca^{2+} level medium, then ABA or ABA–BSA was added (t_0: arrow). Ca^{2+} level was measured in filtrated aliquots of culture medium with Fluo-3 (10 μM). A: The addition of ABA (10^{-5} M) was performed either at 2°C (open circles) or at 22°C (dark circles). B: Addition of ABA–BSA at 22°C at 10^{-5} M equivalent ABA (open squares) or 3.3×10^{-5} M equivalent ABA (dark squares). C: Simultaneous addition of ABA (10^{-5} M) and pimozide (50 μM, open triangles), fluspirilene (50 μM, dark triangles) or verapamil (200 μM, diamond). Each curve is representative of three independent experiments.

uptake of Ca^{2+} by the cells was observed, but a higher ABA concentration was required with the conjugate (3.3×10^{-5} M equivalent ABA) than with free ABA (10^{-5} M) to reach similar $[Ca^{2+}]_o$ decrease. The requirement of a higher conjugate concentration, versus free ABA, was always observed with *RAB18* expression and we proposed previously that it was due to a steric hindrance of ABA in ABA–BSA [13]. It is also noticeable that no release of Ca^{2+} could be observed in ABA–BSA treated cells (Fig. 4B) while an efflux of Ca^{2+} appeared in free ABA treated cells (Fig. 4A). This efflux was almost abolished when verapamil was added with ABA (Fig. 4C). Therefore, one can consider that a fast and active transient influx of Ca^{2+} is strictly required for the induction of *RAB18* and must be distinguished from the efflux of Ca^{2+} which does not belong to the ABA transduction pathway leading to *RAB18* expression. The Ca^{2+} efflux, achieved through verapamil-sensitive Ca^{2+} transporters, is probably under the control of ABA which entered the cells, since it did not occur in impermeant ABA treated cells. This observation provides argument in favor of the existence of intracellular sites of ABA perception and different ABA signaling pathways [22,23].

Pharmacological attempts were made to characterize the Ca^{2+} plasmalemma transporters specifically activated by ABA. Cd^{2+} and Ni^{2+}, known to decrease Ca^{2+} fluxes in plasma membrane of animal and plant cells [24,25], inhibited the ABA–BSA-induced *RAB18* expression (Fig. 2). The higher efficiency of Cd^{2+} over Ni^{2+} suggests that Ca^{2+} channels related to the L- or N-type Ca^{2+} channels described in animal cells are involved [26]. Several types of organic compounds modulate the activity of L-type Ca^{2+} channels in animal and plant cells [27]. Three groups of compounds have been the most widely employed: phenylalkylamines (verapamil), 1,4-dihydropyridines (nifedipine and Bay K8644) and benzothiazepine (diltiazem). However, none of the organic specific L-type Ca^{2+} channel inhibitors tested, diltiazem, nifedipine and verapamil, were efficient to block the ABA signal transduction (Fig. 3A). The L-type Ca^{2+} channel activator (Bay K8644) was unable to induce the ABA specific expression of *RAB18* (Fig. 3A). The specific N-type Ca^{2+} channel inhibitor ω-conotoxin GVIA was also inefficient (data not shown). But, in presence of the diphenylbutylpiperidines, pimozide or fluspirilene (50 μM), that are known to block L-, N- or T-type Ca^{2+} channel, the ABA-induced expression of *RAB18* was inhibited (Fig. 3B). In carrot protoplasts, Ca^{2+} influx was inhibited by fluspirilene in the same range of concentration [28]. Interestingly, simultaneous adding of verapamil (Fig. 4C), diltiazem or nifedipine (not shown) with ABA did not modify the ABA-induced Ca^{2+} uptake. By contrast, pimozide and fluspirilene markedly inhibited the ABA triggered Ca^{2+} influx (Fig. 4C). Further characterization by electrophysiological studies of the ABA triggered Ca^{2+} plasmalemma transporters involved in the *RAB18* transduction chain is now required.

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References

[1] Bush, D.S. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122.
[2] Trewavas, A.J. and Malho, R. (1998) Curr. Opin. Plant Biol. 1, 428–433.
[3] Sanders, D., Brownlee, C. and Harper, J.F. (1999) Plant Cell 11, 691–706.
[4] McAinsh, M.R., Brownlee, C. and Hetherington, A.M. (1990) Nature 343, 186–188.
[5] McAinsh, M.R., Brownlee, C. and Hetherington, A.M. (1992) Plant Cell 4, 1113–1122.
[6] Schroeder, J.I. and Hagiwara, S. (1990) Proc. Natl. Acad. Sci. USA 87, 9305–9309.
[7] Gilroy, S., Fricker, M.D., Read, N.D. and Trewavas, A.J. (1991) Plant Cell 3, 333–344.
[8] de Silva, D.L.R., Hetherington, A.M. and Mansfield, T.A. (1985) N. Phytol. 100, 473–482.
[9] Gilroy, S., Read, N.D. and Trewavas, A.J. (1990) Nature 346, 769–771.
[10] Lemtiri-Chlieh, F. and MacRobbie, E.A.C. (1994) J. Membr. Biol. 20, 951–962.
[11] McAinsh, M.R. and Hetherington, A.M. (1998) Trends Plant Sci. 3, 32–36.
[12] Schroeder, J.I. and Hagiwara, S. (1989) Nature 338, 427–430.
[13] Jeannette, E., Rona, J.P., Bardat, F., Cornel, D., Sotta, B. and Miginiac, E. (1999) Plant J. 18, 13–22.
[14] Ghelis, T., Dellis, O., Jeannette, E., Bardat, F., Cornel, D., Miginiac, E., Rona, J.P. and Sotta, B. (2000) FEBS Lett. 474, 43–47.
[15] Axelos, M., Curie, C., Mazzolini, L., Bardet, C. and Lescure, B. (1992) Plant Physiol. Biochem. 30, 123–128.
[16] Jouanneau, J.P. and Péaud-Lenoël, C. (1967) Physiol. Plant 20, 834–850.
[17] Tepikin, A.V., Voronina, S.G., Gallacher, D.V. and Petersen, O.H. (1992) J. Biol. Chem. 267, 3569–3572.
[18] Brooks, S.P. and Storey, K.B. (1992) Anal. Biochem. 201, 119–126.
[19] Allen, G.J., Kuchitsu, K., Chu, S.P., Murata, Y. and Schroeder, J.I. (1999) Plant Cell 11, 1785–1798.
[20] Grabov, A. (1998) Proc. Natl. Acad. Sci. USA 95, 4778–4783.
[21] Hamilton, D.W.A., Hills, A. and Köhler, B. (2000) Proc. Natl. Acad. Sci. USA 97, 4967–4972.
[22] Schwartz, A., Wu, W.H., Tucker, E.B. and Assmann, S. (1994) Proc. Natl. Acad. Sci. USA 91, 4019–4023.
[23] Allan, A.C., Fricker, M.D., Ward, J.L., Beale, M.H. and Trewavas, A.J. (1994) Plant Cell 6, 1319–1328.
[24] Ping, Z., Yabe, I. and Muto, S. (1992) Protoplasma 171, 7–18.
[25] Marshall, J., Corzo, A., Leigh, R.A. and Sanders, D. (1994) Plant J. 5, 683–694.
[26] Randall, A.D. (1998) J. Membr. Biol. 161, 207–213.
[27] White, P.J. (2000) Biochim. Biophys. Acta 1465, 171–189.
[28] Graziana, A., Fosset, M., Ranjeva, R., Hetherington, A.M. and Lazdunski, M. (1998) Biochemistry 27, 764–768.