Two calcium mobilizing pathways implicated within abscisic acid-induced stomatal closing in *Arabidopsis thaliana*

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Abstract

The present study investigated whether, depending on the abscisic acid (ABA) concentration, phospholipase C (PLC) would be implicated within a Ca^{2+} mobilizing pathway that would regulate stomatal aperture under standard watering conditions. Among Al sensitive mutants the *als1*-1 mutant of *Arabidopsis thaliana* (L.) Heynh. (Columbia-4 ecotype) was selected for a pharmacological approach of stomatal closing in leaf epidermal peels induced by 3, 20 or 30 μM ABA. Comparison with the wild type (WT) revealed that, exclusively in the *als1*-1 mutant, the stomatal response to 3 or 20 μM ABA was inhibited by about 40 %, whereas the stomatal response to 30 μM ABA and the wilting response to drought were unaffected. In WT, the Ca^{2+} buffer EGTA and the PLC inhibitor, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), specifically inhibited by about 70 and 40 %, respectively, the response to 3 or 20 μM ABA, while the Ca^{2+} buffer 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) inhibited by about 70 % the response to 3, 20 or 30 μM ABA. EGTA, BAPTA and U73122 did not inhibit the part of the response to 3 or 20 μM ABA that was unaffected by the *als1*-1 mutation. Together, these results showed that ABA closes the stomata through two different Ca^{2+} mobilizing pathways. Since PLC could be indirectly deactivated in the *als1*-1 mutant, these results might suggest that, under sufficient water supply, PLC-mediated Ca^{2+} mobilization is needed for the regulation of stomatal aperture by endogenous ABA resting at concentrations below a drought-specific threshold value.

Additional key words: ABA concentration, *als1*-1 mutation, drought, phospholipase C.

Introduction

In *Arabidopsis thaliana* (L.) Heynh. cv. Columbia, Al induced many genes, among which were genes that combat oxidative stress such as peroxidase gene (Richards *et al*. 1998). These genes would be organized and regulated in a complex fashion, as suggested by quantitative traits loci analysis of Al tolerance (Kobayashi and Koyama 2002). Al resistant (*alr*) mutants could overexpress peroxidase gene and, thereby, stimulate peroxidase activity in favour of a higher tolerance to Al-induced oxidative stress. On the contrary, Al sensitive (*als*) mutants could affect Al tolerance through repressing peroxidase gene and, thereby, decreasing peroxidase activity. Since H_2O_2 mediates opening of hyperpolarization dependent inward Ca^{2+} channels in the plasma membrane (Grabov and Blatt 1998, Pei *et al*. 2000), variations in the H_2O_2 pool might characterize *alr* (Degenhardt *et al*. 1998, Larsen *et al*. 1998) and *als* (Larsen *et al*. 1996) mutants of *A. thaliana* (L.) Heynh. (Columbia-4 ecotype) isolated in the Kochian's Laboratory (Cornell University, Ithaca, USA). In *als* mutants of this laboratory, indeed, Al mimicked H_2O_2 in triggering Ca^{2+} entry into root hairs (Jones *et al.* 1998), which did not happen in *alr* mutants for which in no case was a cytosolic Ca^{2+} increase observed. Likewise, exclusively in *als* mutants, hydrolysis of phosphoinositide (PI) peroxides could be reduced, which should reduce PI turnover.

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Abbreviations: ABA - abscisic acid; *alr* - Al resistant ; *als* - Al sensitive; BAPTA - 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; DAG - diacylglycerol; GP Ant-2 - pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2; G protein - GTP-binding protein; IP_3 - inositol triphosphate; PI - phosphoinositide; PLC - phospholipase C; 7TMS - seven-transmembrane-span; U73122 - 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione; U73343 - 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; WT - wild type.

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In the guard cell, it had been shown that abscisic acid (ABA) stimulated PI turnover (Lee *et al*. 1996), which would have resulted in stomatal closing. Indeed, ABA-stimulated PI turnover activates PI-specific phospholipase C (PLC) (Lee *et al*. 1996), which enhances the production of inositol triphosphate (IP_3) that induces stomatal closing (Gilroy *et al*. 1990). Therefore, if ABA stomatal closing was affected in *als* mutants, this would have occurred through a possible increase in the pool of PI peroxides.

In bioassay of leaf epidermis peeled from both *A. thaliana* (Cousson 2003) and the monocot plant *Commelina communis* (L.) (Cousson and Vavasseur 1998), we had shown that two potential Ca^{2+} mobilizing pathways are implicated in the induction of gradual stomatal closing by increasing the concentration of applied ABA. At low or high ABA concentrations, respectively, guard cell Ca^{2+} mobilization would implicate or not PLC whose activity seems to depend not only on PI turnover (Lee *et al*. 1996) and cytosolic Ca^{2+} (Franklin-Tong *et al*. 1996) but also on G proteins (Legendre *et al*. 1993). But, sometimes, our demonstration had proceeded from analogies with animal features and, thereby, must have been considered cautiously. It had been shown that kinetics of guard cell Ca^{2+} oscillations would encode information, which would control the magnitude of ABA-induced stomatal closures (Staxen *et al*. 1999, Allen *et al*. 2001). In this context, the finding that different ABA concentrations induced different kinetic patterns of guard cell Ca^{2+} oscillations (Staxen *et al*. 1999) might have corroborated our demonstration. However, one should not have excluded that such kinetic changes would have resulted from a possible gradual stimulation of a unique Ca^{2+} mobilizing pathway by increasing the ABA concentration.

The fact that ABA-insensitive mutants of *A. thaliana* withered in standard watering conditions (Koornneef *et al*. 1984) had suggested that endogenous ABA regulates stomatal aperture in conditions other than drought. Assuming that drought would greatly enhance the ABA concentration in the leaf epidermal apoplast, we hypothesized that PLC is a key enzyme of Ca^{2+} mobilization implicated within ABA stomatal closing under sufficient water supply. In order to get clear cut evidence supporting this hypothesis, the present study compared stomatal closing response in leaf epidermal peels of *als* mutants of *A. thaliana* (L.) Heynh. (Columbia-4 ecotype) with the wild type (WT) to 3, 20 or 30 μM ABA.

Materials and methods

Plants and treatments: Provided by the Kochian's Laboratory (Cornell University, Ithaca, USA), seeds of the *A. thaliana* (L.) Heynh. (Columbia-4 ecotype) WT and *als1*-1, *als3*, *als4* and *als5* mutants were germinated on a 8 g dm^{-3} agar HP697 (*Kalys*, Roubaix, France)-solidified medium, which was composed of 10 g dm^{-3} sucrose (*Sigma Chemical Co*., St Louis, USA) and the medium containing 2.0 mM KNO_3, 1.1 mM $MgSO_4$, 805.0 μM $Ca(NO_3)_2$, 695.0 μM KH_2PO_4, 60.0 μM K_2HPO_4, 20.0 μM Na_2EDTA, 20.0 μM $FeSO_4$, 9.25 μM H_3BO_3, 3.60 μM $MnSO_4$, 3.00 μM $ZnSO_4$, 0.78 μM $CuSO_4$ and 74 nM $(NH_4)_6Mo_7O_{24}$. The four *als* mutants were homozygous mutant lines that Larsen *et al*. (1996) had obtained by self fertilization of M_3 progeny originated from ethyl methylsulfonate-mutagenized WT seeds whose germination and radicule elongation were hypersensitive to Al. Ten days after germination, the seedlings were grown in pots (65 × 65 × 70 mm) with moistened coarse sand and watered three times a day with the nutrient solution at temperature of 22 °C, relative humidity of 70 %, 8-h photoperiod and irradiance of 250 μmol m^{-2} s^{-1} (supplied by 150 W mercury lamps *HQI-TS*, *Osram*, München, Germany). Four- to five-week old plants were providing leaf epidermal peels for stomatal closing bioassays or were stressed by withholding water for 2 to 8 d.

Bioassays with epidermal peels: Leaf epidermis was peeled from WT and the *als1*-1, *als3*, *als4* and *als5* mutants at the end of the night period. For each comparative experiment, epidermal strips (up to 10 × 5 mm) were obtained from the same leaf by placing the abaxial epidermis cuticule side-down on microscope slides covered with the *Dow Corning 355* medical adhesive silicone (*Vermed Laboratory*, Neuilly-en-Thelle, France). Then, most of the green tissues were gently removed from each epidermal strip by using another microscope slide. Two epidermal peels per treatment were immersed in 10 cm^3 incubation medium, except for experiments related to the incubation time (then, one peel per incubation time was immersed).

Stomatal closing in response to ABA was assayed starting with high stomatal apertures (approx. 5 μm). These apertures were obtained by incubating the peels for 2 h at 20 °C under white light in 30 mM potassium iminodiacetate (K_2IDA, *Sigma*), 10 mM 2-(N-morpholino)ethane sulfonic acid (*Sigma*), pH 6 and CO_2-free air. Afterwards, light continued for at least 1 h 30 min in the presence of 3, 20 or 30 μM ABA. The impermeant anion IDA was used instead of chloride because KCl reduces sensitivity to ABA in *A. thaliana*. Since CO_2 in normal air has been shown to interfere on ABA-induced stomatal closing response in *A. thaliana* (Leymarie *et al*. 1998), the incubation medium was bubbled throughout the experiments with CO_2-free air at a rate of 33 cm^3 min^{-1}, which was obtained by passing dry air over sodalime (*Soda Asbestos*, *Prolabo*, Paris, France). It was verified that methanol, in which ABA was dissolved, did not change stomatal aperture.

To investigate the Ca^{2+} dependence of ABA stomatal

closing, cytosolic free Ca^{2+} of the guard cell was buffered by adding separately the Ca^{2+} chelators EGTA (*Sigma*) and 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA; *Sigma*) into the incubation medium throughout experiments conducted with ABA. EGTA and BAPTA were tested at 1.5 mM that has been shown to be the lowest concentration allowing maximum inhibition of ABA stomatal closing in *Arabidopsis* (Cousson 2003). The EGTA (50 mM) and BAPTA (50 mM) stock solutions contained a significant amount of K^+. The control incubation medium contained K_2IDA to adjust its final K^+ concentration to the same value as that of the EGTA- or BAPTA-containing incubation medium.

To investigate the possible implication of PLC within ABA stomatal closing, the PLC inhibitor, 1-[6-[[17-β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and its inactive analog, 1-[6-[{17-β-3-methoxyestra-1,3,5(10)-trien-17-yl}amino] hexyl]-2,5-pyrrolidine-dione (U73343) (Smith *et al*. 1990) were separately added to the incubation medium, throughout experiments conducted with ABA. U73122 and U73343 were tested at 3 nM, which had allowed U73122 to specifically inhibit the stomatal closing response to 3 μM ABA in *Arabidopsis* (Cousson 2003).

To investigate the possible implication of G proteins within ABA stomatal closing, the G protein antagonist (Mukai *et al*. 1992), pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2 (GP Ant-2) and the inactive mastoparan analog, mas17 (Higashijima *et al*. 1990) were tested on the ABA response at concentrations 10 and 7 μM, respectively. Used at these concentrations, GP Ant-2 and mas17 had specifically caused maximum inhibition of the stomatal closing response to 30 μM ABA without affecting the viability of the guard cells in *Arabidopsis* (Cousson 2003). These modulators were added separately or in combination to the incubation medium throughout the experiments.

While mas17 was dissolved in sterile double distilled water, U73122, U73343 and GP Ant-2 were dissolved in DMSO, and control incubation media contained this solvent at the same concentrations (up to 1%, v/v) as these of the U73122-, U73343- and GP Ant-2-containing incubation media. By themselves, all these compounds did not significantly change the mean stomatal aperture (Cousson 2003). They were purchased from *Biomol Research Laboratories*, Plymouth, UK.

Data analysis: For the drought experiments, sets of twelve plants were tested for WT and each mutant. From day 2 to day 8 of the drought treatment, wilting of the plants was daily recorded and, for each set, the percentage of wilted plants was calculated. For the bioassays, the viability of the guard cells was verified by staining the epidermal peels with neutral red at the end of each treatment. Stomata without underlying mesophyll were used for measurement of the stomatal aperture. Only stomata, of which the ostiole length was higher than one-third of the stomatal length, were examined. For each epidermal peel, 100 stomatal apertures were measured. For each treatment, the stomatal response was evaluated by measuring two epidermel peels, one peel just before applying ABA and the second at least 1 h 30 min after adding ABA. Then, the ABA-induced extent in stomatal closing was calculated as the difference between the stomatal apertures measured just before and 1 h 30 min after applying ABA. Other treatments were analysed in relation to the incubation time of the epidermal peels over the 0 to 3 h 30 min range (Fig. 1). For each incubation time, one peel was taken into account. All the experiments were independently repeated at least three times.

Results and discussion

The *als1*-1 mutation specifically affects the stomatal closing response to 3 or 20 μM ABA: Compared to WT, withering of the *als3* mutant under water stress conditions occurred three days before that of WT and the other tested *als* mutants (Table 1). Accordingly, since ABA mediates drought tolerance (Davies and Mansfield 1983), one could have expected that the *als3* mutation affected ABA stomatal closing. However, the stomatal response of the *als3* mutant to 3, 20 or 30 μM ABA was similar in magnitude to that of WT (Table 2). Molecular cloning of the *als3* mutant had established that the *als3* mutation disrupts regulation of a gene encoding a phloem specific ATP-binding cassette transporter (Larsen, personal communication). Our results might have suggested that this phloem transporter would be implicated in an ABA-independent process that contributes to delay drought-induced withering.

In the *als1*-1 mutant, exclusively, ABA stomatal closing was affected: compared to WT, the extents in stomatal closing induced by 3 and 20 μM ABA decreased from 2.5 to 1.5 μm and from 3.2 to 1.9 μm, respectively, while the response to 30 μM ABA was unchanged (Table 2). Thus, in the *als1*-1 mutant, the stomatal response to 3 or 20 μM ABA was specifically and partially (by about 40 %) inhibited, whereas drought-induced wilting (Table 1) was unaffected. This suggested that 3 or 20 μM ABA, but not 30 μM ABA, would have induced stomatal closing through processes, which are implicated within regulation of stomatal aperture under other hydric conditions than drought. This possibility was consistent with the previous observation that ABA insensitive mutants of *A. thaliana* withered under standard watering conditions (Koornneef *et al*. 1984).

The *als1*-1 mutation suppresses the specific inhibitory effect of EGTA on the stomatal closing response to 3 or 20 μM ABA: Since the *als1*-1 mutation specifically

Table 1. Wilting in *A. thaliana* as affected by Al sensitive (*als*) mutations. The wild type (WT) and *als1*-1, *als3*, *als4* and *als5* mutants were stressed by withholding water for 2 to 8 d. Each experiment was repeated three times. Twelve 4 to 5-week-old plants were tested for WT and each mutant. Data represented means ± SE.

Stress [d]	Wilting plants [%] WT	*als1*-1	*als3*	*als4*	*als5*
2	0	0	0	0	0
3	0	0	16 ± 8	0	0
4	0	0	33 ± 8	0	0
5	0	0	75 ± 8	0	0
6	25 ± 8	25 ± 8	100	16 ± 8	25 ± 8
7	75 ± 8	50 ± 8	100	50 ± 8	66 ± 8
8	100	100	100	100	100

affected the stomatal response to 3 or 20 μM ABA, it was pharmacologically investigated whether or not this occurred through inhibition of Ca^{2+} signalling.

In WT, stomatal aperture approximately decreased from 5.0 to 2.5, 1.8 or 1.7 μm in response to 3, 20 or 30 μM ABA, respectively (Fig. 1). In the presence of BAPTA, stomatal aperture approximately decreased from 4.2 to 3.5, 3.1 or 3.2 μm in response to 3, 20 or 30 μM ABA, respectively (Fig. 1). In the presence of EGTA, stomatal aperture approximately decreased from 5.7 to 4.9, 4.8 or 2.5 μm in response to 3, 20 or 30 μM ABA, respectively (Fig. 1). Thus, in WT, BAPTA inhibited by about 70 % the response to 3, 20 or 30 μM ABA, whereas EGTA specifically inhibited by about 70 % the response to 3 or 20 μM ABA. Furthermore, in the *als1*-1 mutant, BAPTA, but not EGTA, inhibited by about 70 % the response to 30 μM ABA. By contrast, BAPTA or EGTA did not inhibit the part of the response to 3 or 20 μM ABA that was unaffected by the *als1*-1 mutation (Fig. 1).

Table 2. ABA-induced stomatal closing in *A. thaliana* as affected by Al sensitive (*als*) mutations. Leaf epidermal peels of the wild type (WT) and *als1*-1, *als3*, *als4* and *als5* mutants were incubated under light and CO_2-free air throughout the experiments. Two hours after the start of the experiments 3, 20 or 30 μM ABA was applied. Each experiment was repeated at least three times. For each experiment, the stomatal response was established by measuring two epidermal peels (100 stomatal apertures measured for each peel), one peel just before applying ABA and the other at least 1 h 30 min after applying ABA. ABA-induced stomatal closing was taken as the difference between the stomatal apertures measured just before and 1 h 30 min after applying ABA. Data represented means ± SE.

ABA [μM]	Stomatal closing [μm] WT	*als1*-1	*als3*	*als4*	*als5*
3	2.5 ± 0.1	1.5 ± 0.1	2.4 ± 0.2	2.4 ± 0.2	2.5 ± 0.1
20	3.2 ± 0.2	1.9 ± 0.2	3.2 ± 0.1	3.3 ± 0.2	3.3 ± 0.2
30	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.2	3.2 ± 0.2	3.2 ± 0.2

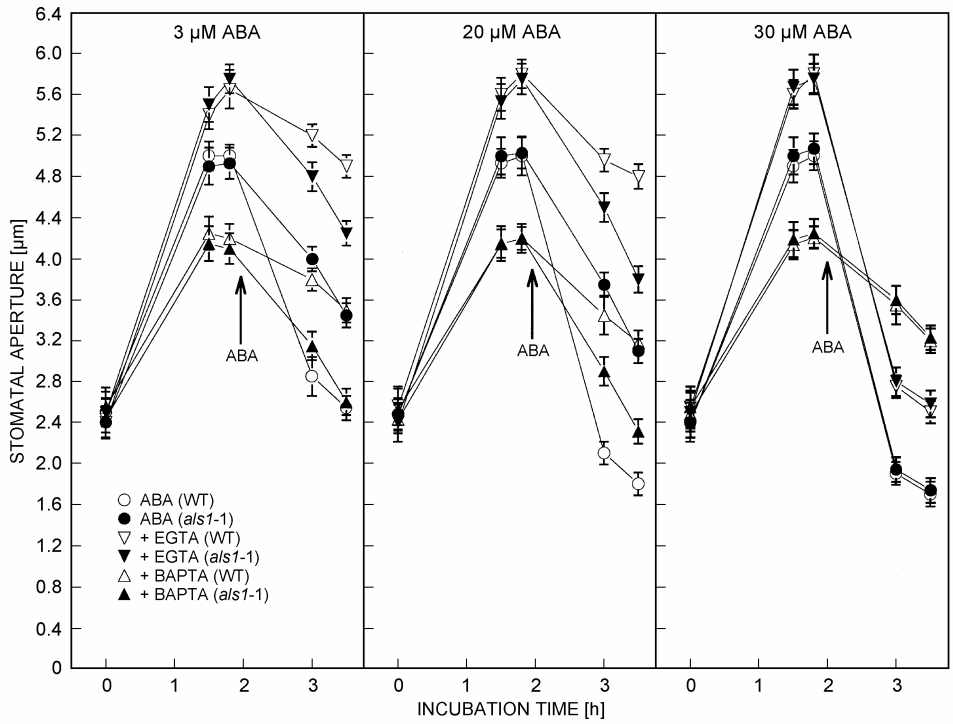

Fig. 1. Compared effects of Ca^{2+} buffers on ABA-induced stomatal closing in the wild type (WT) and *als1*-1 mutant of *A. thaliana*. Leaf epidermal strips were incubated with the Ca^{2+} buffer EGTA (1.5 mM) or BAPTA (1.5 mM) under light and CO_2-free air throughout the experiments. ABA at concentration 3 μM (A), 20 μM (B) or 30 μM (C) was added 2 h after the start of the experiments. The results were means ± SE.

Table 3. ABA-induced stomatal closing in *A. thaliana* as affected by different inhibitors in WT and *als1*-1 mutant. Leaf epidermal peels were incubated with different compounds under light and CO_2-free air throughout the experiments. Two hours after the start of the experiments 3, 20 or 30 μM ABA was applied. For detail see Table 2.

Compound	Stomatal closing [μm]					
	WT			*als1*-1		
	3 μM	20 μM	30 μM	3 μM	20 μM	30 μM
Control	2.5 ± 0.1	3.2 ± 0.2	3.3 ± 0.2	1.5 ± 0.1	1.9 ± 0.1	3.4 ± 0.2
3 nM U73122	1.5 ± 0.1	1.9 ± 0.1	3.1 ± 0.2	1.4 ± 0.1	2.0 ± 0.1	3.3 ± 0.1
3 nM U73343	2.4 ± 0.1	3.2 ± 0.2	3.2 ± 0.1	1.5 ± 0.1	2.0 ± 0.2	3.2 ± 0.2
7 μM mas17	2.5 ± 0.2	3.3 ± 0.1	2.2 ± 0.1	1.3 ± 0.2	1.9 ± 0.1	2.0 ± 0.1
10 μM GP Ant-2	2.3 ± 0.2	3.4 ± 0.2	2.1 ± 0.2	1.4 ± 0.1	2.1 ± 0.2	2.1 ± 0.2
7 μM mas17 +10 μM GP Ant-2	2.4 ± 0.1	3.2 ± 0.2	1.0 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	1.0 ± 0.1

Our results showed that the response to 3, 20 or 30 μM ABA depended on the cytosolic free Ca^{2+} concentration and that the *als1*-1 mutation affected the stomatal response to 3 or 20 μM ABA by inhibiting Ca^{2+} signalling. Moreover, they confirmed that a Ca^{2+}-independent pathway was implicated within ABA stomatal closing (Cousson 2003), which accounted for 30 % of the ABA response in WT. In that regards, studying further the stomatal response of the *als1*-1 mutant to 3 or 20 μM ABA should provide new insights into mechanisms by which ABA induced stomata to close in a Ca^{2+}-independent fashion. As for Ca^{2+}-dependent ABA-induced stomatal closure, it would have accounted for 70 % of a response whose magnitude was gradually increased only for exogenous ABA concentrations increasing from 3 to 20 μM (Fig. 1). It was known that BAPTA, but not EGTA, buffered efficiently rapid increases in the cytosolic free Ca^{2+} concentration (Armstrong and Blatt 1995). Therefore, since EGTA, but not BAPTA, specifically inhibited the response to 3 or 20 μM ABA, one could have supposed that, within this gradual response, slow increases in the cytosolic free Ca^{2+} concentration of the guard cell specifically characterized Ca^{2+} signalling. This might have resulted from a gradual stimulation of a unique Ca^{2+} mobilizing pathway that could have been specifically affected by the *als1*-1 mutation.

The *als1*-1 mutation suppresses the specific inhibitory effect of U73122 on the stomatal closing response to 3 or 20 μM ABA: The stomatal closing response to 3, 20 or 30 μM ABA was tested with the PLC inhibitor, U73122 in WT and the *als1*-1 mutant. In WT, U73122 inhibited by about 40 % the response to 3 or 20 μM ABA: the extent in stomatal closing approximately decreased from 2.5 to 1.5 μm and from 3.2 to 1.9 μm due to 3 and 20 μM ABA, respectively (Table 3). By contrast, U73122 did not inhibit the response to 30 μM ABA in WT (Table 3). Used at a concentration 3 nM similar to that of U73122 (Table 3) or increasing up to the micromolar range (data not shown), the U73122 analog, U73343 did not inhibit ABA stomatal closing in WT. Furthermore, U73122 did not inhibit the part of the response to 3 or 20 μM ABA that was unaffected by the *als1*-1 mutation: in the *als1*-1 mutant, U73122 did not change extents in stomatal closing that 3 and 20 μM ABA adjusted at 1.5 and 1.9 μm, respectively (Table 3), which accounted for 60 % of the WT response to 3 or 20 μM ABA (Table 2).

In mammalian cells, it had been shown that U73122, but not U73343, inhibited receptor-coupled PLC-dependent processes such as Ca^{2+} mobilization and the production of both IP_3 and diacylglycerol (DAG). Likewise, U73122 could have inhibited selectively similar processes in WT guard cells, since U73122, but not U73343, had been shown to inhibit PLC of *Nicotiana rustica* guard cells (Staxen *et al*. 1999). Therefore, our results suggested that U73122 specifically affected the response to 3 or 20 μM ABA through inhibiting the PLC activity. This possibility, together with the fact that IP_3 (via Ca^{2+} mobilization) and DAG, respectively, induced stomatal closing (Gilroy *et al*. 1990) and opening (Lee and Assmann 1991), allowed to explain why in WT, there was a discrepancy between the percentages of inhibition of the ABA response by U73122 (40 %) and the Ca^{2+} buffer EGTA (70 %). In WT guard cells, indeed, U73122 would have decreased both the IP_3 and DAG concentrations: the drop in DAG concentration would have partially closed the stomata within a process independent of IP_3-mediated Ca^{2+} mobilization.

The specific EGTA and U73122 inhibitory effects suggested that, contrary to 30 μM ABA, 3 or 20 μM ABA induced stomatal closing through PLC-mediated Ca^{2+} mobilization. This was reinforced by the fact that the EGTA and U73122 inhibitory effects were suppressed in the *als1*-1 mutant because the *als1*-1 mutation would have deactivated PI-specific PLC. As discussed in the introduction, indeed, the *als1*-1 mutant had behaved similarly to an *als* mutant that affects ABA stomatal closing likely through decreasing hydrolysis of PI peroxides, thereby reducing the PLC activity *via* the reduction of PI turnover (Lee *et al*. 1996).

The potential role of PLC in Ca^{2+} mobilization of ABA stomatal closing urged us to examine the putative

link of PLC to a G protein-coupled 7-transmembrane-span (7TMS)-like receptor, which would perceive ABA from the apoplast. Indeed, regulation of the PLC activity would implicate G proteins in higher plants (Legendre *et al*. 1993). Furthermore, in mammalian cells, U73122 would have acted on G_q proteins coupled to PLCβ (Taylor *et al*. 1991, Yule and Williams 1992) and linked to 7TMS receptors (Berridge 1993) within surface receptor-G protein-PLC transduction units.

In both WT (Table 3) and the *als1*-1 mutant (Table 3), the G protein modulators, mas17 and GP Ant-2 specifically inhibited the stomatal response to 30 μM ABA by about 35 % and their effects were additive: when applied separately, they induced the extent in stomatal closing to decrease approximately from 3.3 to 2.1 μm, whereas, when they were applied in combination, the extent in stomatal closing approximately decreased from 3.3 to 1.0 μm. These results were not due to side effects because mas17 and GP Ant-2 did not change the mean stomatal aperture in the absence of ABA (Cousson 2003, results not shown). Furthermore, when mas17 and GP Ant-2 were tested together, substituting BAPTA for mas17 or GP Ant-2 did not change the percentage (70 %) of inhibition of the ABA response (results not shown), which corresponded to the BAPTA inhibitory effect (Fig. 1).

In animal cells, the hydrophobic peptide, GP Ant-2 competed with the receptor for G protein binding (Mukai *et al*. 1992) and the inactive mastoparan analog, mas17 bound to 7TMS receptors without mimicking their G protein activation (Higashijima *et al*. 1990). By analogy with these animal features, the fact that, in WT, mas17 and GP Ant-2 did not inhibit the stomatal response to 3 or 20 μM ABA (Table 3) suggested that, if any, mas17 and GP Ant-2 inhibited G protein functioning other than the putative one, which would regulate the PLC activity.

Together, our results suggested that ABA is perceived extracellularly by a G protein-coupled 7TMS-like receptor, which is implicated within two Ca^{2+} mobilizing pathways. In addition to the U73122 effect, the selective, partial and additive effects of mas17 and GP Ant-2 suggested that this putative 7TMS-like receptor is coupled to three G proteins. Each Ca^{2+} mobilization would be mediated by specific interactions between these G proteins and the 7TMS-like ABA receptor. In that regards, it must have been noted that a 7TMS receptor has been identified in *A. thaliana* (Plakidou-Dymock *et al*. 1998, Pandey and Assmann 2004), which interacts with a G protein to regulate ABA signalling in the guard cells (Pandey and Assmann 2004). The present study improved a previous hypothesis (Cousson 2003) through the suggestion that increasing the exogenous ABA concentration from 20 to 30 μM was sufficient to modulate the coupling of several G proteins to the same receptor within the ABA perception complex. This was supported by Birnbaumer and Birnbaumer (1995) who had reported a somewhat similar functioning of G protein-coupled receptors in animal cellular systems. Furthermore, our results showed that the magnitude of the stomatal closing response was not changed by such a putative modulation of G protein-ABA receptor coupling, although this ABA concentration-dependent modulation would have changed the Ca^{2+} mobilizing pathway.

In conclusion, drought-induced wilting of the *als1*-1 mutant did not precede that of WT. Thus, PLC-mediated Ca^{2+} mobilization could characterize the regulation of stomatal aperture by endogenous ABA whose concentration would not exceed a threshold value under sufficient water supply. Furthermore, when grown at the 15 - 17 °C, *A. thaliana* (Columbia ecotype) plants wilted faster than control plants grown at the 23 - 25 °C (unpublished data). Compared to the control plants, the plants grown at the 15 - 17 °C exhibited, at each ABA concentration tested up to 30 μM in bioassays with epidermal peels, a stomatal closing response of smaller amplitude, which had seemed to be independent of the cytosolic free Ca^{2+} concentration (Cousson 2003). Therefore, it was possible that Ca^{2+} signalling of the stomatal closing response of epidermal peels to 30 μM ABA would be somewhat similar to Ca^{2+} signalling that might be implicated within ABA regulation of stomatal aperture in undetached leaves under drought. However, the fact that the stomatal closing response to 30 μM ABA and the wilting response to drought were exclusively unchanged in the *als1*-1 mutant did not provide a clear cut evidence for such a possibility. In that regards, it might be fruitfull to test *alr* mutants in experiments similar to those of the present study. Indeed, *alr* mutations that had been shown to abolish H_2O_2-induced Ca^{2+} entry in root hairs (Jones *et al*. 1998) should prevent the hyperpolarization-dependent Ca^{2+} increase under high ABA concentrations that Grabov and Blatt (1998) had previously described in the guard cells.

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