Involvement of phospholipase C-independent calcium-mediated abscisic acid signalling during *Arabidopsis* response to drought

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Abstract

The present study investigated whether Ca^{2+} mobilization independent of phosphoinositide-specific phospholipase C (PI-PLC) would delay wilting in *Arabidopsis thaliana* (L.) Heynh. cv. Columbia through mediating stomatal closure at abscisic acid (ABA) concentrations rising beyond a drought-specific threshold value. In wild type (WT) epidermis, the PI-PLC inhibitor (U73122) affected the stomatal response to 20 μM ABA but not to 30 μM ABA. Disruption in GTP-binding protein α subunit 1 (GPA1) affected the stomatal response to 30 μM ABA, but not to 20 μM ABA. In the *gpa1-4* mutant, the inhibitory effects of the Ca^{2+} buffer, 1,2-bis(*0*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA), the inactive mastoparan analogue, mas17 and the antagonist of cyclic ADP-ribose synthesis, nicotinamide, were differentially attenuated on 30 μM ABA-induced stomatal closure. By contrast, the NADPH oxidase *atrbohD/F* double mutation fully suppressed inhibition of 20 μM ABA-induced stomatal closure by BAPTA or U73122 as well as inhibition of 30 μM ABA-induced stomatal closure by BAPTA, mas17 or nicotinamide. On the contrary, The Al resistant *alr*-104 mutation modulated ABA-induced stomatal closure by a stimulatory effect of U73122 and an increased sensitivity to mas17, nicotinamide and BAPTA. Compared to WT, the *atrbohD/F* double mutant was more hypersensitive than the *gpa1*-4 mutant to wilting under the tested water stress conditions, whereas wilting was delayed in the *alr*-104 mutant. Since the *atrbohD/F* mutation breaks down ABA-induced Ca^{2+} signalling through fully preventing apoplastic Ca^{2+} to enter into the guard cells, these results showed that a putative guard cell GPA1-dependent ADP-ribosyl cyclase activity should contribute to drought tolerance within PI-PLC-independent-Ca^{2+}-mediated ABA signalling.

Additional key words: ADP-ribosyl cyclase, *alr*-104 mutation, *atrbohD/F* double mutation, *gpa1*-4 mutation, stomatal closure.

Introduction

In *Arabidopsis thaliana* (L.) Heynh. cv. Columbia, phosphoinositide-specific phospholipase C (PI-PLC) would be the sole Ca^{2+} mobilizing mediator within stomatal regulation by endogenous abscisic acid (ABA) resting at concentrations below a drought-specific threshold value that approximates to 30 μM (Cousson 2003a, 2007, 2008). Beyond this ABA concentration threshold, however, stomatal regulation could require specifically PI-PLC-independent Ca^{2+} mobilization. This has been suggested by bioassays with both abaxial leaf epidermis and the whole plant. Under particular drought conditions, indeed, the Al sensitive *als1*-1 mutant and the wild type (WT) wilted following the same kinetics and magnitude, whereas the *als1*-1 mutation and the PI-PLC inhibitor, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) paralled each other to specifically affect Ca^{2+} buffer-sensitive stomatal closure in response to 20 μM ABA, but not to

Received 21 March 2008, *accepted* 17 December 2008.

Abbreviations: ABA - abscisic acid; ARC - ADP-ribosyl cyclase; BAPTA - 1,2-bis(*0*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; cADPR - cyclic ADP-ribose; DAG - diacylglycerol; Dex - dexamethasone; GPA1 - GTP-binding protein α subunit 1; G protein - GTP-binding protein; Gα - GTP-binding protein α subunit ; NAADP - nicotinic acid adenine dinucleotide phosphate; PI-PLC - phosphoinositide-specific phospholipase C; U73122 - 1,6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] aminohexyl-1H-pyrrole-2,5-dione; U73343 - 1,6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] aminohexyl-2,5-pyrrolidine-dione; WT - wild type; 7TMS - seven-transmembrane-spanning.

Acknowledgements: This work was supported by an IFCPAR grant. The author gratefully thanks Dr. J. Liang (Yangzhou University, Yangzhou, P.R. China), Prof. L. Kochian (Cornell University, Ithaca, USA) and Prof. J.I. Schroeder (University of California, La Jolla, USA) for seeds of homozygous *gpa1*-4, *alr*-104 and *atrbohD/F* mutant lines of *Arabidopsis thaliana* cv. Columbia.

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30 µM ABA (Cousson 2007). In WT, moreover, the plants grown at 22 °C delayed drought-induced wilting, when compared to plants grown at 15 - 17 °C (unpublished data) whose abaxial leaf epidermis specifically responded to 20 or 30 µM ABA by a limited Ca^{2+} buffer-insensitive stomatal closure (Cousson 2003a). However, any direct evidence was still lacking to coroborate or not the above mentioned hypotheses. Furthermore, bioassays with whole plants and suspension cell cultures had shown that, under stress conditions (Hirayama *et al*. 1995, Takahashi *et al*. 2001) differing from our previously reported drought test (Cousson 2007), dehydration combined to high salinity activates PI-PLC in both ABA-dependent and ABA-independent fashions. These last data likely overlapped a secondary involvement of PI-PLC (Wu *et al*. 1997, Sanchez and Chua 2001) that might characterize only adaptability to particular water stress conditions.

It is known that guard cell osmoregulation leads to stomatal closure *via* loss of cell pressure potential, which occurs through modulating cytoskeleton dynamics and coordinating inhibition of salt uptake with stimulation of salt release from the guard cell. Cytosolic Ca^{2+} and pH would act separately within regulation of the K^+-inward and K^+-outward rectifying channels (Blatt and Armstrong 1993, Grabov and Blatt 1997). At the plasma membrane, however, activation of these channels tightly depend on the degree of polarization, and cross-talk between cytosolic Ca^{2+} and pH could not be excluded from such a voltage control for the following reasons. First, the cytosolic Ca^{2+} and proton concentrations would regulate the electrogenic proton pump and the anion-outward rectifying channel not only in parallel but also in a complex counteracting cross-talk (Kinoshita *et al*. 1995, Schulz-Lessdorf *et al*. 1996, Cousson 2002). Next, the electrogenic proton pump and the anion-outward rectifier control polarization of the guard cell plasma membrane through antagonistic voltage activities. To investigate further such possibilities, Columbia mutants could be relevant as the following ones. First, the Al resistant *alr*-104 mutant that has been characterized by a net proton influx into the root apex (Degenhardt *et al*. 1998), which, in the case of a constitutive mutational expression, might interfere with cytosolic pH regulation of the guard cell and, in turn, with cytosolic alkalinization underlying ABA stomatal closure. Second, the NADPH oxidase *atrbohD/F* double mutant that has been shown to fully inhibit guard cell Ca^{2+} signalling in response to ABA (Kwak *et al*. 2003). At last, an intracellular acidic pH clamp procedure had specifically allowed Wang *et al*. (2001) to report mediation of GTP-binding protein (G protein) α subunit (Gα) 1 (GPA1) within depolarization-mediated activation by ABA of the anion-outward rectifier at the plasma membrane of *Arabidopsis thaliana* cv. Wassilewskija guard cell protoplasts. Therefore, the allelic Columbia mutant knockout for GPA1, *gpa1*-4 (Chen *et al*. 2006) was expected to impair ABA-induced plasma membrane depolarization only when intracellular pH of the guard cells would be clamped by incubating peeled leaf epidermis with a membrane-permeant weak acid.

Here, we aimed to directly evidence involvement of PI-PLC-independent Ca^{2+}-mediated ABA signalling within delayed wilting to drought. Bioassay procedures of water stress and ABA concentration-dependent stomatal closure were conducted as previously reported (Cousson 2007, 2008) with abaxial leaf epidermal peels and whole plants of the *alr*-104, *atrbohD/F* and *gpa1*-4 Columbia mutants in comparison to WT. Given the above evidenced or expected features of these different mutants, such experiments were designed in an attempt to select a Columbia mutant whose leaf epidermis responds to 30 µM ABA, but not to 20 µM ABA, by Ca^{2+}-independent stomatal closure and the whole plant responds to drought by hypersensitive wilting.

Materials and methods

Plants and treatments: WT and the homozygous *atrbohD/F*, *alr*-104 and *gpa1*-4 mutants were in the *Arabidopsis thaliana* (L.) Heynh. cv. Columbia genetic background. The *atrbohD/F* double mutant had been obtained at the Schroeder's laboratory (University of California, La Jolla, USA) from crosses between homozygous T-DNA insertional mutants of two guard cell-expressed NADPH oxidase genes, *AtrbohD* and *AtrbohF* (Kwak *et al*. 2003). The *alr*-104 mutant had been obtained by self fertilization of M_3 progeny originated from ethyl methylsulfonate-mutagenized WT seeds screened at the Kochian's laboratory (Cornell University, Ithaca, USA), as previously described (Larsen *et al*. 1998). Given by Dr J. Liang (Yangzhou University, Yangzhou, People's Republic of China), the *gpa1*-4 mutant was disrupted by T-DNA insertion in the sole canonical Gα gene, *GPA1* (Chen *et al*. 2006). All the seeds were germinated and the seedlings were grown for 10 days 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), 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 $(NH_4)_6Mo_7O_{24}$. Then, the seedlings were grown under normal air in pots with moistened coarse sand and watered 3 times a day with the nutrient solution. The plants were cultured at 22 °C, relative humidity of 70 %, 8-h photoperiod and irradiance of 250 µmol m^{-2} s^{-1} (150 W mercury lamps - *HQI-TS, Osram*, München, Germany). Four- to five-week-old plants were providing abaxial leaf epidermal peels for stomatal closure bioassays or stressed by withholding water for 1 to 8 d.

Bioassays with epidermal peels: Abaxial leaf epidermis with stomatal guard cells was peeled from WT and the *atrbohD/F*, *alr*-104 and *gpa1*-4 mutants at the end of the night period. For each comparative experiment, abaxial epidermal strips (up to 10 × 5 mm) were obtained from the same fully expended leaf and placed 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 throughout the experiments.

Stomatal closure in response to ABA was assayed starting with high stomatal apertures that approximately varied between 4 and 5 μm. These apertures were obtained by incubating the peels for 3 h at 20 °C under white light in 40 mM KCl, 10 mM MES (*Sigma*), pH 6 and CO_2-free air. Afterwards, light continued for 2 h in the absence or presence of 20 or 30 μM ABA. Since CO_2 in normal air has been shown to interfere with the ABA-induced stomatal closure response of *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).

To investigate the Ca^{2+} dependence of ABA stomatal closure, it was attempted to buffer apoplastic and cytosolic free Ca^{2+} of the guard cell by adding the plant Ca^{2+} chelator, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA; *Sigma*) (Armstrong and Blatt 1995) to the incubation medium throughout the experiments. BAPTA was prepared from a 50 mM stock solution that contained a significant amount of K^+ and added at 1.5 mM, which had previously allowed maximum inhibition of ABA stomatal closure in cv. Columbia (Cousson 2003a). The control incubation medium contained potassium iminodiacetate (*Sigma*) to adjust its final K^+ concentration to the same value as that of the BAPTA-containing incubation medium.

To investigate the possible implication of G protein-regulated PI-PLC activity within Ca^{2+}-dependent ABA stomatal closure, the PI-PLC inhibitor, U73122 (*Biomol Research Laboratories*, Plymouth, UK) (Thompson *et al*. 1991) was added to the incubation medium in the presence or absence of BAPTA. This PI-PLC inhibitor was tested at 3 nM, which had previously allowed a specific inhibition of ABA stomatal closure in the Columbia ecotype (Cousson 2003a, 2007, 2008). It was verified that DMSO, in which U73122 was dissolved, did not interfere by itself with the stomatal U73122 effect.

To investigate the possible implication of G protein-regulated ADP-ribosyl cyclase (ARC) activity within Ca^{2+}-dependent ABA stomatal closure, the antagonist of cyclic ADP-ribose (cADPR) synthesis, nicotinamide (*Sigma*) (Sethi *et al*. 1996) and the inhibitor of G protein functioning, mas17 (*Biomol*) (Higashijima *et al*. 1990) were respectively added at 50 mM and 7 μM separately or in combination to the incubation medium in the presence or absence of BAPTA, which previously exhibited maximum inhibitory effects on ABA stomatal closure (Leckie *et al*. 1998, Cousson 2003a, 2007, 2008).

To investigate the cytosolic pH dependence of ABA stomatal closure, the membrane-permeant weak acid, butyric acid was added at 1 mM to the incubation medium containing 40 mM KCl and buffered at pH 6 with 10 mM MES throughout the experiments.

Data analysis: For the drought experiments, sets of twelve plants were tested for WT and each mutant. From day 1 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 stomatal closure bioassays, the viability of the guard cells was verified by staining the epidermel 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 at least were measured. For each treatment, the stomatal response was evaluated by comparing two epidermal peels, one peel being measured just before applying ABA, and the other peel being measured 2 h after applying ABA. Then, ABA stomatal closure was calculated as the difference between the stomatal apertures measured just before and 2 h after applying ABA. All the experiments were independently repeated at least three times.

Results and discussion

BAPTA sensitivity of ABA-induced stomatal closure: Compared to WT, the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants exhibited differential ABA stomatal closure from the abaxial leaf epidermis. Calculated as the mean difference between the stomatal apertures reached after 3 h incubation under white light in 40 mM KCl, 10 mM MES, pH 6 and CO_2-free air and those measured 2 h after exogenously applying ABA, 20 μM ABA-induced stomatal closure was similar (approx. 3.0 μm) in WT and the *gpa1*-4 mutant, whereas it decreased to 1.1 and 1.8 μm, respectively, in the *alr*-104 and *atrbohD/F* mutants. By contrast, 30 μM ABA-induced stomatal closure was roughly similar in WT (3.1 μm) and the *alr*-104 mutant (2.9 μm), whereas it decreased to 0.9 and 2.1 μm, respectively, in the *atrbohD/F* and *gpa1*-4 mutants (Table 1).

To investigate whether or not these effects were at least partially resulted from modulating Ca^{2+} dependency of the ABA stomatal response, treatments with 1.5 mM efficient Ca^{2+} buffer BAPTA (Armstrong and Blatt 1995)

added to the incubation medium for 5 h were performed (Table 1). In the *atrbohD/F* mutant, BAPTA did not change stomatal closure induced by 20 or 30 μM ABA, whereas it roughly decreased ABA stomatal closure from 3.0 to 0.9 μm in WT (about 70 % inhibition), as previously reported (Cousson 2003a, 2007, 2008). By contrast, BAPTA roughly annulled stomatal closure induced by 20 or 30 μM ABA in the *alr*-104 mutant. Compared to WT, in the *gpa1*-4 mutant BAPTA decreased stomatal closure induced by 20 or 30 μM ABA from 3.1 or 2.1 μm to 0.9 or 1.4 μm, respectively. Therefore, 30 μM ABA-induced stomatal closure was suggested to specifically involve GPA1 within Ca^{2+} mediation.

Effects of mas17 and nicotinamide on ABA-induced stomatal closure: Working on animals, Sethi *et al*. (1996) had established that nicotinamide was a plasma membrane-permeant antagonist of cADPR synthesis. Applying this compound to *Commelina communis* abaxial leaf epidermis had inhibited Ca^{2+}-mediated ABA-induced stomatal closure (Leckie *et al*. 1998) in a fashion somewhat similar to that of the inactive mastoparan analogue, mas17 (Cousson and Vavasseur 1998). In the cv. Columbia, mas17 had specifically and partially inhibited 30 μM ABA-induced stomatal closure (Cousson 2003a, 2007, 2008). Here, nicotinamide had in WT the same inhibitory effect (about 35 % inhibition) that was not additive to the inhibition of 30 μM ABA-induced stomatal closure by BAPTA (about 70 % inhibition), as recently reported (Cousson 2008). Compared to WT, mas17 and nicotinamide similarly decreased 30 μM ABA-induced stomatal closure from 3.0 to about 0.8 μm in the *alr*-104 mutant, which corresponded to an increasing inhibitory effect (about 70 % inhibition) and was concommitant with full inhibition of 30 μM ABA-induced stomatal closure by BAPTA. In the *atrbohD/F* mutant, by contrast, 30 μM ABA-induced stomatal closure was insensitive to mas17, nicotinamide and BAPTA, whether these compounds were applied separately or in combinations. These last features were conserved in the *gpa1*-4 mutant, except for the BAPTA inhibitory effect that was only decreased in comparison to WT (Table 2). In previous experiments, a substantial inhibition of auxin-induced stomatal opening (Cousson 2003b) or ABA-induced stomata closure (Cousson 2008) by nicotinamide and mas17 was observed in cv. Columbia. In parallel, mastoparan and its active analogue mas7 affected stomatal aperture only in a very limited fashion that Kelly *et al*. (1995) interpreted as reflecting a stimulatory effect of these compounds on opposite stomatal movements. This possibility has been confirmed by the finding that, in a concentration-dependent fashion, mas7 has fully counteracted mas17 within inhibition of both ABA-induced stomatal closure (Cousson, unpublished data) and auxin-induced stomatal opening (Cousson 2003b), provided that the active and inactive mastoparan analogues were simultanously added at least 1 h before applying the hormonal signal. Therefore, the effects of mas17 might have resulted from uncoupling at least one seven-transmembrane-spanning (7TMS)-like receptor to GPA1 or unconventional Gαs. Indeed, mas17 inhibits functioning of particular G proteins coupled to 7TMS receptors through binding to the receptor without mimicking the targeted activation by mastoparan or mas7 (Higashijima *et al*. 1990). Consequently, mas17 should prevent agonist-binding 7TMS receptors from activating their coupled G proteins. Inhibition of G protein-dependent stomatal movements by mas17 was plausible as much as an experimental consensus has indicated that mastoparan rapidly activated Ca^{2+}- and reactive oxygen species-mediated plant MAP kinase signalling that did not depend on GPA1 and was insensitive to mas17 (Miles *et al*. 2004). Although this consensus has been established from manipulating other plant cell types than the guard cell, indeed, it would have agreed with hypothesis that mas17 interferes with guard cell Ca^{2+}- and H_2O_2-dependent ABA signalling only because this transduction pathway involves Gα functioning.

The NADPH oxidase *atrbohD/F* double mutation breaks down ABA-induced Ca^{2+} signalling through fully preventing apoplastic Ca^{2+} to enter into the guard cells (Kwak *et al*. 2003). Therefore, the fact that this mutation fully suppressed inhibition of 30 μM ABA-induced stomatal closure by BAPTA, mas17 or nicotinamide strongly suggested that these compounds interfered with Ca^{2+} signalling of this ABA response. Accordingly, observing uneffectiveness of nicotinamide and mas17 in the *gpa1*-4 mutant as well as in the *atrbohD/F* mutant (Table 2) showed that putative GPA1-up-regulated ARC activity is involved within Ca^{2+} mediation of 30 μM ABA-induced *Arabidopsis* stomatal closure. Evidence for coupling heterotrimeric G proteins to ARC had remained elusive in the case of animal cellular systems because

Table 1. BAPTA-responsive ABA stomatal closure in cv. Columbia as differentially modulated by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations. Abaxial leaf epidermal peels of the wild type (WT) and each mutant were incubated with or without the Ca^{2+} buffer, BAPTA (1.5 mM) under light and CO_2-free air and 3 h after starting the experiments, 20 or 30 μM ABA was added. Each experiment was repeated at least three times and the stomatal response was established by measuring two epidermal peels (100 stomatal apertures measured for each peel). ABA-induced stomatal closure was taken as the difference between the stomatal apertures measured just before and 2 h after applying ABA. Data represent means ± SE.

Plants	ABA-induced stomatal closure [μm]			
	-BAPTA		+BAPTA	
	20 μM	30 μM	20 μM	30 μM
WT	3.0 ± 0.1	3.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.2
alr-104	1.2 ± 0.2	2.9 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
atrbohD/F	1.8 ± 0.1	0.9 ± 0.2	1.9 ± 0.2	0.9 ± 0.2
gpa1-4	3.1 ± 0.1	2.1 ± 0.1	0.9 ± 0.1	1.4 ± 0.1

Table 2. Mimicking between the inactive mastoparan analogue, mas17 and the antagonist of cADPR synthesis, nicotinamide as differentially modulated by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations. Nicotinamide (50 mM) and mas17 (7 μM) were added separately or in combination to abaxial leaf epidermal peels of WT and the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants incubated under light and CO_2-free air in the absence or presence of the Ca^{2+} buffer 1.5 mM BAPTA and 3 h after starting the experiments 30 μM ABA was added. BAPTA test was recorded in comparison. For detail see Table 1.

Treatments	30 μM ABA-induced stomatal closure [μm]			
	WT	*alr*-104	*atrbohD/F*	*gpa*1-4
No treatment	3.2 ± 0.2	3.0 ± 0.2	1.0 ± 0.2	2.1 ± 0.2
BAPTA	1.0 ± 0.1	0.2 ± 0.1	0.9 ± 0.2	1.4 ± 0.1
mas17	2.1 ± 0.1	0.8 ± 0.2	0.9 ± 0.2	2.0 ± 0.2
mas17 + BAPTA	1.0 ± 0.2	0.2 ± 0.2	0.9 ± 0.1	1.4 ± 0.1
nicotinamide	2.2 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	2.1 ± 0.2
nicotinamide +BAPTA	0.9 ± 0.1	0.2 ± 0.1	0.9 ± 0.2	1.4 ± 0.2
nicotinamide + mas17	2.0 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	2.2 ± 0.1
nicotinamide + mas17 + BAPTA	1.0 ± 0.2	0.1 ± 0.1	1.2 ± 0.1	1.5 ± 0.1

their studies (Ceni *et al*. 2003) had not used G protein-defective mutants. Although G protein analysis of the up-to-date identified cyclic GMP-dependent or-independent soluble ARC had been lacking, these studies had suggested that, if any, G protein-regulated ARC activity would be rather independent of cyclic GMP and bound to the membrane fraction. In cv. Columbia, inhibiting cyclic GMP synthesis (Cousson 2003b) had not affected 30 μM ABA-induced stomatal closure (Cousson, unpublished results cited in Cousson 2003a). Therefore, this ABA response might have implicated a plasma membrane- or endomembrane-bound G protein-coupled ARC. In cv. Columbia, furthermore, Sanchez *et al*. (2004) had shown that applying 50 μM ABA to whole plants resulted in early ARC activation leading to gene expression that was only partially overlapped by dexamethasone (Dex)-induced *Aplysia ARC* gene expression. Since the *Aplysia ARC* gene codes for a soluble G protein-uncoupled ARC, failing to clone *Arabidopsis ARC* gene(s) from this *Aplysia* gene (Prof. N-H Chua, personal communication) would have been consistent with the present evidence for a putative membrane-bound GPA1-up-regulated ARC.

ABA-induced stomatal closure performed with an homozygous Columbia line carrying the Dex-inducible *AtPLC1* antisense transgene has just strongly suggested – *via* mimetic stomatal inhibitory effects of mas17 and nicotinamide – that 30 μM ABA-induced Ca^{2+}-mediated stomatal closure specifically involves ARC in a G protein-regulated fashion independent of guard cell AtPLC1 activity (Cousson 2008). These evidences were not contradicted by the data obtained with the *atrbohD/F* and *gpa1*-4 mutants (Table 2). In cv. Columbia, furthermore, BAPTA did not increase inhibition of 20 μM ABA-induced stomatal closure by U73122 (Cousson 2008), which likely resulted from inhibiting PI-PLC-mediated Ca^{2+} mobilization (Cousson 2007, 2008). Given the fact that the *gpa1*-4 mutant did affect neither the magnitude nor the BAPTA sensitivity of 20 μM ABA-induced stomatal closure (Table 1), observing that the *gpa1*-4 mutation does not interfere with the U73122, stomatal effect would show that a PI-PLC-independent and GPA1-up-regulated process should contribute – *via* putative guard cell ARC – to Ca^{2+} mediation within 30 μM ABA-induced stomatal closure.

Effect of U73122 on ABA-induced stomatal closure: In animals, the aminosteroid U73122, but not its close analogue, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-2,5-pyrrolidine-dione (U73343), inhibits receptor-coupled PI-PLC-dependent Ca^{2+} mobilization likely through interfering with Gα-dependent regulation (Thompson *et al*. 1991). To investigate whether or not the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations could have differentially modulated Ca^{2+} dependency of ABA stomatal closure through interfering at least partially with guard cell PI-PLC, 3 nM U73122 was tested, because, contrary to applying 3 nM U73343, this treatment had previously allowed substantial and specific inhibition of 20 μM ABA-induced stomatal closure in cv. Columbia (Cousson 2003a, 2007, 2008). Applying 3 nM U73122 throughout the experiments did not inhibit 30 μM ABA-induced stomatal closure in any of the tested plants (Table 3), and the same uneffectiveness was obtained with 3 nM U73343 for testing 20 μM ABA (data not shown). In WT, U73122 specifically decreased from 3.1 to 1.9 μm the stomatal closure induced by 20 μM ABA (about 40 % inhibition), as previously reported (Cousson 2003a, 2007, 2008) and this feature was conserved in the *gpa1*-4 mutant (Table 3). BAPTA did not increase the U73122 inhibitory effect in each of these two plants (Table 4). In the *atrbohD/F* mutant, by contrast, 20 μM ABA-induced stomatal closure was insensitive to separately or simultanously applied U73122 and BAPTA (Tables 3 and 4). Strickingly, U73122 specifically increased 20 μM ABA-induced stomatal closure from 1.1 to 1.8 μm in the *alr*-104 mutant (stimulation by about 65 %) (Table 3).

The mechanism of U73122 action has just been

Table 3. Effect of U73122 on ABA-induced stomatal closure as differentially modulated by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations. Abaxial leaf epidermal peels of WT and the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants were incubated with or without the PLC inhibitor, U73122 (3 nM) under light and CO_2-free and 3 h after starting the experiments 20 or 30 μM ABA was added. For detail see Table 1.

Plants	ABA-induced stomatal closure [μm] -U73122		+U73122	
	20 μM	30 μM	20 μM	30 μM
WT	3.1 ± 0.1	3.1 ± 0.2	1.9 ± 0.2	3.2 ± 0.1
alr-104	1.1 ± 0.2	2.9 ± 0.2	1.8 ± 0.2	2.8 ± 0.1
atrbohD/F	1.8 ± 0.2	0.9 ± 0.2	1.9 ± 0.2	0.9 ± 0.2
gpa1-4	3.0 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	1.8 ± 0.1

Table 4. Combined effects of BAPTA and U73122 on ABA-induced stomatal closure. BAPTA (1.5 mM) and U73122 (3 nM) were applied separately or in combination to abaxial leaf epidermal peels of WT and the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants incubated under light and CO_2-free air and 3 h after starting the experiments 20 μM ABA was added. For detail see Table 1.

Treatments	ABA-induced stomatal closure [μm] WT	*alr*-104	*atrbohD/F*	*gpa1*-4
No treatment	3.0 ± 0.1	1.1 ± 0.2	1.9 ± 0.1	3.0 ± 0.2
U73122	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.1
BAPTA	0.9 ± 0.2	0.2 ± 0.2	1.9 ± 0.2	1.0 ± 0.2
U73122 + BAPTA	1.9 ± 0.2	1.7 ± 0.2	2.0 ± 0.1	1.8 ± 0.1

approached by showing that Dex mimicked U73122 to specifically affect 20 μM ABA-induced stomatal closure through suppressing its Ca^{2+} buffer sensitivity in an homozygous Columbia line carrying the Dex-inducible *AtPLC1* antisense transgene (Cousson 2008). Since Dex did not affect ABA-induced stomatal closure of the untransformed Columbia line (Cousson 2008), these features likely implicated U73122 as inhibiting the PI-PLC isoform, AtPLC1. Therefore, mimicking between the *gpa1*-4 mutant and WT within inhibition by BAPTA (Table 1) and/or U73122 (Tables 3, 4) of stomatal closure in response to 20 μM ABA but not to 30 μM ABA was relevant to the following features. A PI-PLC-independent and GPA1-up-regulated Ca^{2+} mediation characterizes – *via* putative ARC – the stomatal closure response to 30 μM ABA, whereas a putative PI-PLC-dependent Ca^{2+} mediation characterizes the stomatal closure response to 20 μM ABA. Compared to WT, therefore, the *gpa1*-4 mutant should reduce the BAPTA sensitivity of 30 μM ABA-induced stomatal closure (Table 1) only through interfering with PI-PLC-independent Ca^{2+} mediation. This, indeed, was coroborated by the observed U73122 insensitivity of 30 μM ABA-induced WT stomatal closure (Table 3). However, the *alr*-104 mutant surprisingly modulated 20 μM ABA-induced stomatal closure since it was both fully inhibited by BAPTA (Tables 1, 4) and strongly stimulated by U73122 even in the presence of BAPTA (Tables 3, 4). Although stimulatory, furthermore, applying U73122 with or without BAPTA did not restore 20 μM ABA-induced WT stomatal closure in the *alr*-104 mutant (Tables 3, 4). In the *alr*-104 mutant, accordingly, PI-PLC would have been involved within the response to 20 μM ABA not only through BAPTA-sensitive up-regulation but also through BAPTA-insensitive down-regulation. Although the U73122 effect was not stimu-latory on 20 μM ABA-induced WT stomatal closure, it was less inhibitory (40 % inhibition; Table 3) than that of BAPTA (70 % inhibition; Tables 1, 4). Therefore, BAPTA-insensitive down-regulation by PI-PLC of 20 μM ABA-induced stomatal closure would have occurred also in WT but at a lesser extent compared to the *alr*-104 mutant. Furthermore, although BAPTA efficiently chelates both apoplastic and cytosolic Ca^{2+} (Armstrong and Blatt 1995), the cytosolic free Ca^{2+} oscillatory signal-disrupting *atrbohD/F* mutation (Kwak *et al*. 2003) mimicked U73122 to inhibit 20 μM ABA-induced stomatal closure by about 40 % (Table 3) in WT in a fashion that was insensitive to the BAPTA inhibitory effect (70 % inhibition; Table 4). Consequently, the process, by which PI-PLC would have down-regulated
20 μM ABA-induced stomatal closure, should have not excluded a rise in cytosolic free Ca^{2+} that would have not been sufficiently buffered by BAPTA.

Effects of butyric acid: Testing 1 mM butyric acid application on ABA-induced stomatal closure of abaxial leaf epidermis investigated whether or not guard cell pH would have been involved in differential modulation of the ABA response by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants. Indeed, a comparable weak acid treatment had been evaluated to decrease cytosolic pH by about 0.4 pH units in the *Vicia* guard cell (Blatt and Armstrong 1993). Compared to WT, the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants differentially modulated responsiveness of ABA-induced stomatal closure to butyric acid (Table 5). Butyric acid and the *alr*-104 mutation similarly decreased 20 μM ABA-induced stomatal closure from 3.0 to about 1.0 μm in comparison with WT, whereas none of them had a significant effect on 30 μM ABA-induced stomatal closure. Moreover, butyric acid did not change the *alr*-104 mutant behaviour in its ABA stomatal closure response. Therefore, butyric acid and the *alr*-104 mutation mimicked each other. This mimicking, furthermore, revealed that nicotinamide and mas17 similarly decreased 20 μM ABA-induced stomatal closure by about 0.5 μm (Table 6). This last inhibitory effect was rather unexpected and would have affected another putative G protein-upregulated ARC-mediated process than the above considered one, which was specifically involved within Ca^{2+} mediation of 30 μM ABA-induced stomatal closure. Since BAPTA sensitivity of this butyric acid-dependent process lacked to be evaluated here, such a process, however, did not yet exclude that AtPLC1

would have been the sole Ca^{2+}-mobilizing mediator of 20 μM ABA-induced stomatal closure whether peeled WT epidermis was incubated with butyric acid (present study) or not (Cousson 2008). By contrast, the *atrbohD/F* mutation decreased 30 μM ABA-induced stomatal closure from 3.1 to 1.0 μm, whereas, together with butyric acid, it decreased the same ABA response from 2.9 to 0.1 μm. Likewise, the *gpa1*-4 mutation decreased this stomatal response from 2.9 to 0.8 μm in the presence of butyric acid, whereas it only decreased from 3.1 to 2.0 μm in the absence of butyric acid. Therefore, butyric acid enhanced the *atrbohD/F* and *gpa1*-4 mutational effects on 30 μM ABA-induced stomatal closure. In a close parallel to the *alr*-104 mutation, furthermore, butyric acid enhanced the inhibition of 30 μM ABA-induced stomatal closure by mas17 or nicotinamide (Table 6) to the same extent as it enhanced the *gpa1*-4 mutational effect (Table 5). Taken together, all these results suggested that, compared to WT, guard cell cytosol is acidified in the *alr*-104 mutant, and that at least one putative ARC isoform is activated not only by GPA1 but also by cytosolic acidification within the stomatal closure response not only to 30 μM ABA but also to 20 μM ABA.

In animals, decreasing cytosolic pH from 7 to 6 modulates multifunctional membrane-bound ARC activity in favour of the cyclic GMP-independent base-exchange mechanism leading to biosynthesis of nicotinic acid adenine dinucleotide phosphate (NAADP) (Graeff *et al*. 1998, Ceni *et al*. 2003). Here, butyric acid mimicked the *alr*-104 mutation to enhance sensitivity of 30 μM ABA-induced stomatal closure to BAPTA, mas17 and nicotinamide. Taking into account cytosolic acidification induced by applying butyric acid to *Vicia* guard cells incubated under the pH buffering conditions (Blatt and Armstrong 1993), a drop in cytosolic pH by about 0.4 pH unit could have resulted from loading with 1 mM butyric acid in cv. Columbia epidermal guard cells bathing with 40 mM KCl, pH 6, 10 mM MES-buffered medium. Therefore, the observed mimetic effects of butyric acid and the *alr*-104 mutation would have implicated such an acidification, which could be relevant of the rhizosphere pH rise by about 0.15 pH unit that had been recorded at the root tip of the *alr*-104 mutant (Degenhardt *et al*. 1998) in the case of a constitutive mutant pH trait. Degenhardt *et al*. (1998), indeed, had evaluated the magnitude of the pH differences at the plasma membrane surface as likely higher than that measured at the root surface because the electrically charged cell wall would act as a barrier for putative hydroxyl ion release into the root bathing medium.

Assuming that the *alr*-104 mutation acidifies the cytosol and nicotinamide antagonizes both cADPR and NAADP synthesis, the mimetic enhancing effect that was observed on the BAPTA, mas17 and nicotinamide sensitivities of stomatal closure in response to 30 μM ABA in WT suggested next hypothesis. Slightly increasing cytosolic proton concentration from pH 7

Table 5. Butyric acid responsiveness of ABA-induced stomatal closure as differentially modulated by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations. Abaxial leaf epidermal peels of the wild type (WT) and the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants were incubated under light and CO_2-free air with or without 1 mM butyric acid and 3 h after starting the experiments 20 or 30 μM ABA was added. For detail see Table 1.

Plants	ABA-induced stomatal closure [μm]			
	-butyric acid		+butyric acid	
	20 μM	30 μM	20 μM	30 μM
WT	3.0 ± 0.1	3.1 ± 0.1	1.0 ± 0.1	2.9 ± 0.2
alr-104	1.2 ± 0.2	2.9 ± 0.2	1.0 ± 0.2	2.9 ± 0.1
atrbohD/F	1.8 ± 0.1	1.0 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
gpa1-4	2.9 ± 0.1	2.0 ± 0.1	0.5 ± 0.1	0.8 ± 0.1

Table 6. Mimetic effect of butyric acid and the *alr*-104 mutation on ABA-induced stomatal closure. The modulators BAPTA (1.5 mM), U73122 (3 nM), nicotinamide (50 mM) and mas17 (7 μM) were applied separately to abaxial leaf epidermal peels of the *alr*-104 mutant and WT incubated under light and CO_2-free air with or without 1 mM butyric acid and 3 h after starting the experiments 20 or 30 μM ABA was added. For detail see Table 1.

Treatment	Stomatal closure [μm]			
	-butyric acid		+butyric acid	
	WT	*alr*-104	WT	*alr*-104
20 μM ABA	3.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
20 μM ABA + BAPTA	0.8 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
20 μM ABA + U73122	1.8 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.1
20 μM ABA + nicotinamide	3.0 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.1
20 μM ABA + mas17	2.9 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
30 μM ABA	3.1 ± 0.1	2.8 ± 0.1	2.9 ± 0.2	2.9 ± 0.1
30 μM ABA + BAPTA	0.9 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1
30 μM ABA + U73122	3.1 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.9 ± 0.2
30 μM ABA + nicotinamide	2.1 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.1
30 μM ABA + mas17	2.0 ± 0.1	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1

might influence a putative membrane-bound GPA1-up-regulated ARC activity through differentially modulating biosynthesis and hydrolysis of the intracellular Ca^{2+} mobilizing inducers, cADPR and NAADP (Sanders *et al*. 2002) within 30 μM ABA-induced stomatal closure. In turn, guard cell Ca^{2+} would be mobilized so that Ca^{2+}-induced activation of the anion outward-rectifying channel (Schulz-Lessdorf *et al*. 1996) sufficiently depolarizes the plasma membrane. By itself, indeed, cytosolic acidification up-regulates the K^+ inward-rectifying channel (Grabov and Blatt 1997) and down-regulates the K^+ outward-rectifying channel (Blatt and Armstrong 1993), which counteracts depolarization of the plasma membrane. This interpretation outlined that plasma membrane depolarization allowing significant stomatal closure would have been ensured without ABA-induced alkalinization of the guard cell cytosol. This last point of view had been already argued by Wang *et al*. (2001). They had evidenced GPA1-up-regulation of the anion outward-rectifying channel at more acidic guard cell cytosolic pH. However, their results had been only related to 20 μM ABA-induced stomatal closure and rather unexplained. From our study, emerged the possibility that acidic up-regulation of the anion-outward rectifier would have implicated two different processes requiring both ARC and GPA1, one of them characterizing Ca^{2+} mediation of 30 μM ABA-induced stomatal closure and the other one – may be different from Ca^{2+} mediation – participating to 20 or 30 μM ABA-induced stomatal closure. Indeed, under conditions that should have led to slight cytosolic alkalinization (epidermis incubated without butyric acid), first, stomatal closure to 30 μM ABA, but not to 20 μM ABA, was similarly affected by nicotinamide (Table 6) and the *gpa1*-4 mutation (Table 5), and this mimetic inhibitory effect was only amplified to the same extent by butyric acid and/or the *alr*-104 mutation (Table 6). Secondly, 20 μM ABA-induced stomatal closure was significantly affected by nicotinamide or mas17 (Table 6) as well as by the *gpa1*-4 mutation (Table 5) only when epidermis was incubated with butyric acid. Thirdly, nicotinamide and mas17 similarly inhibited 20 μM ABA-induced stomatal closure in the *alr*-104 mutant whether epidermis was incubated with butyric acid or not (Table 6).

Applying butyric acid mimicked the *alr*-104 mutation to significantly decrease 20 μM ABA-induced stomatal closure in its magnitude but not in its BAPTA sensitivity (Table 6). Furthermore, this mimicking included a stimulatory effect of the PI-PLC inhibitor, U73122, since 3 nM U73122 approximately increased 20 μM ABA-induced stomatal closure from 1.0 to 1.9 μm when epidermis of WT or the *alr*-104 mutant was bathing with 1 mM butyric acid (Table 6). Therefore, all these features could be due to cytosolic acidification and would implicate PI-PLC. From above mentioned considerations, one can suggest that this mimicking would have resulted mainly from a process that cytosolic acidification would have promoted such as BAPTA-insensitive PI-PLC-mediated down-regulation of the stomatal response to 20 μM ABA. This down-regulating process could have specifically involved endogenous diacylglycerol (DAG). Indeed, DAG is produced by PI-PLC as it is in the case of the intracellular Ca^{2+}-mobilizing and stomatal closure inducer inositol-triphosphate (Gilroy *et al*. 1990), which opens stomata likely through activating the electrogenic proton pump at the guard cell plasma membrane (Lee and Assmann 1991). Furthermore, a positive interaction between DAG and the cytosolic proton concentration within BAPTA-insensitive PI-PLC-mediated down regulation of 20 μM ABA-induced stomatal closure should have been considered for the following reasons. Firstly, a particular cytosolic free Ca^{2+} rise is needed for activating DAG-producing PI-PLC, especially the Columbia AtPLC1 isoform (Sanchez and Chua 2001) that would be primarily involved within this ABA stomatal response (Cousson 2008). Secondly, at the guard cell plasma membrane, this Ca^{2+} rise could deactivate the electrogenic proton pump (Kinoshita *et al*. 1995) if the cytosolic concentration of its substrate would not be sufficient to sustain the pumping activity (Cousson 2002). Thirdly, increasing the cytosolic proton concentration hyperpolarizes the plasma membrane as much as it up-regulates the K^+ inward-rectifying channel and down-regulates the K^+ outward-rectifying channel (Blatt and Armstrong 1993, Grabov and Blatt 1997). Consequently, the inferred cytosolic free Ca^{2+} rise would have not been efficiently buffered by BAPTA (Tables 3, 4).

Drought-induced wilting: The NADPH oxidase *atrbohD/F* double mutation disrupts ABA-induced Ca^{2+} signalling through fully preventing apoplastic Ca^{2+} to enter into the guard cells (Kwak *et al*. 2003). Therefore, the fact that this mutation fully suppressed inhibition of 30 μM ABA-induced stomatal closure by BAPTA, mas17 or nicotinamide (Table 4) strongly suggested that these compounds interfered with Ca^{2+} signalling of the stomatal closure response to 30 μM ABA. Moreover, the *gpa1*-4 mutant annulled the mimetic mas17 and nicotinamide

Table 7. Wilting as differentially modulated by the *alr*-104, *atrbohD/F* and *gpa1*-4 mutations. WT and the *alr*-104, *atrbohD/F* and *gpa1*-4 mutants were stressed by withholding water for 1 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	*alr*-104	*atrbohD/F*	*gpa1*-4
1	0	0	0	0
2	0	0	33 ± 8	0
3	0	0	66 ± 8	25 ± 16
4	0	0	100	50 ± 8
5	25 ± 8	0	100	100
6	66 ± 16	25 ± 16	100	100
7	100	50 ± 8	100	100
8	100	84 ± 16	100	100

sensitivities (Table 2) and reduced the BAPTA sensitivity (Tables 1, 2) of 30 μM ABA-induced stomatal closure, whereas this GPA1 null mutant (Chen *et al*. 2006) mimicked WT within inhibition of 20 μM ABA-induced stomatal closure by BAPTA (Table 1) and U73122 (Table 3). Since U73122 likely inhibits PI-PLC activity (Cousson 2007, 2008), the latter trait should have implicated GPA1-independent and PI-PLC-dependent Ca^{2+} mediation of 20 μM ABA-induced stomatal closure. Therefore, the former *gpa1*-4 mutational trait would have revealed that a PI-PLC-independent and GPA1-up-regulated process contributes - *via* putative guard cell ARC - to Ca^{2+} mediation within 30 μM ABA-induced stomatal closure.

Compared to WT, the *atrbohD/F* double mutant was more hypersensitive than the *gpa1*-4 mutant to the tested water stress conditions, whereas drought-induced wilting was delayed in the *alr*-104 mutant (Table 7). Taken with the above mentioned results, these wilting phenotypes showed a tight correlation between more or less delayed wilting and the degree of promotion or inhibition of BAPTA-, mas17- and nicotinamide-sensitive 30 μM ABA-induced stomatal closure, respectively.

Consequently, a putative guard cell GPA1-dependent ARC activity was shown to contribute to drought tolerance within PI-PLC-independent Ca^{2+}-mediated ABA signalling under the present drought test. Under stress conditions combining dehydration with high salinity, this Ca^{2+} mediation, induced by apoplastic ABA concentrations largely above 30 μM, could be crucial and involve cADPR and NAADP within a signalling interplay, from which efficient stomatal closure could be ensured at the guard cell plasma membrane not only by stimulating the anion-outward rectifier but also by preventing enough the coordinated hyperpolarizing activities of the electrogenic proton pump and the K^+ inward-rectifying channel. This last possibility should be outlined as much as at least one PI-PLC isoform had been shown, under dehydration and salt stress, to be implicated within both ABA-dependent and ABA-independent drought tolerance (Hirayama *et al*. 1995, Shinozaki and Yamaguchi-Shinozaki 2000, Takahashi *et al*. 2001), which likely overlaps a secondary involvement of PI-PLC (Wu *et al*. 1997, Sanchez and Chua 2001). Indeed, the present study suggested that such a secondary PI-PLC involvement does not exclude substantial production of DAG whose potential plasma membrane hyperpolarizing action should be then efficiently counterbalanced.

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