Putative primary involvement of *Arabidopsis* phosphoinositide-specific phospholipase C1 within abscisic acid-induced stomatal closing

A. COUSSON[1,2,3]

CEA, DSV, IBEB, Lab. Echanges Membran & Signalisation, Saint-Paul-lez-Durance, F-13108, France[1]
CNRS, UMR Biol. Veget. & Microbiol. Environ., Saint-Paul-lez-Durance, F-13108, France[2]
Aix-Marseille Université, Saint-Paul-lez-Durance, F-13108, France; Alain Cousson[3]

Abstract

Stomatal closing to abscisic acid (ABA) was studied in leaf epidermal peels of a dexamethasone (Dex)-inducible transgenic line expressing the phospholipase C *AtPLC1* antisense in the Columbia genetic background. In the absence of Dex, the Ca^{2+} buffer, ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) and the phopholipase C inhibitor, 1-[6-{[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino}hexyl]-1H-pyrrole-2,5-dione (U73122) specifically inhibited the response to 20 μM ABA, whereas the Ca^{2+} buffer, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (BAPTA) inhibited the response to 20 or 30 μM ABA. Neither EGTA nor BAPTA increased the U73122 effect. Applying 30 μM Dex specifically affected 20 μM ABA-induced stomatal closing through reducing its magnitude as well as suppressing the EGTA, BAPTA and U73122 inhibitory effects. Neither Dex nor U73122 changed the specific inhibitory effects of both the antagonist of cyclic ADP-ribose synthesis, nicotinamide and the GTP-binding protein (G protein) modulators, pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2 (GP Ant-2) and mas17 on 30 μM ABA-induced stomatal closing. When tested in combination, substituting nicotinamide for mas17, but not for GP Ant-2, enhanced their inhibitory effect to an extent that BAPTA did not increase. These results supported that AtPLC1 primarily mediates the Ca^{2+}-dependent stomatal closing response to 20 μM ABA as much as 30 μM Dex did not affect 20 μM ABA-induced stomatal closing when tested on the wild type Columbia-4 ecotype. Furthermore, the present study suggested that Ca^{2+} mobilization did not involve any dependency between AtPLC1 and a putative G protein-coupled ADP-ribosyl cyclase at the tested ABA concentrations.

Additional key words: *Arabidopsis thaliana* (L.) Heynh. cv. Columbia, *AtPLC1* antisense, Ca^{2+} buffer, dexamethasone, GTP-binding protein modulators, phopholipase C inhibitor.

Introduction

Stomatal movement bioassays with abaxial leaf epidermal peels of both the monocot plant, *Commelina communis* (Cousson and Vavasseur 1998) and the dicot plant, *Arabidopsis thaliana* (Cousson 2003a) had previously shown that putative involvement of phosphoinositide-specific phospholipase C (PI-PLC) activity within guard cell Ca^{2+} mobilization depends on the ABA concentration. An Al sensitive *A. thaliana* (Columbia-4 ecotype) mutant isolated by Larsen *et al.* (1996), *als1*-1, has just provided clear cut evidence for the existence of two Ca^{2+} mobilizing pathways within ABA stomatal closing, one of them being involved in the ABA regulation of stomatal aperture under sufficient water supply (Cousson 2007). Since PI-PLC activity could be repressed in the *als1*-1 mutant, pharmacological dissection of its ABA stomatal closing response has

Received 21 September 2007, *accepted* 3 March 2008
Abbreviations: ABA - abscisic acid; ARC - ADP-ribosyl cyclase; BAPTA - 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid; cADPR - cyclic ADP-ribose; Dex - dexamethasone; EGTA - ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; GP Ant-2 - pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2; G protein - GTP-binding protein; Gα - G protein α subunit; IP_3 - inositol 1,4,5-triphosphate; PI-PLC - phosphoinositide-specific phospholipase C; 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; 7TMS - seven-transmembrane-span.
Acknowledgements: This work was supported by an IFCPAR grant. The author gratefully thanks Pr. Nam-Haï Chua (The Rockefeller University, New York, USA) for having given seeds of a Dex-inducible transgenic *AtPLC1* antisense line of *A. thaliana* (L.) Heynh. cv. Columbia.
Fax: (+33) 4 42 254656; e-mail: alain.cousson@cea.fr

suggested that, under sufficient water supply, PI-PLC-mediated Ca^{2+} mobilization is primarily needed for the regulation of *Arabidopsis* stomatal aperture by endogenous ABA resting at concentrations below a drought-specific threshold value (Cousson 2007).

Such a possibility had to be studied further because it was apparently in conflict with two features characterizing PI-PLC activity of *A. thaliana* cv. Columbia. First, the PI-PLC isoform, AtPLC1 is induced by dehydration and high ABA concentrations (Hirayama *et al*. 1995). Second, AtPLC1 is only needed for secondary responses to ABA signals (Sanchez and Chua 2001). The present study questioned whether and, if so, how AtPLC1 activity is involved within ABA-dependent stomatal regulation by applying the post transcriptional gene silencing technology to an *A. thaliana* cv. Columbia homologous system expressing the *AtPLC1* antisense transgene only in the presence of Dex. In this system, treating transgenic plants with 30 μM Dex for at least 1 h had been previously needed to considerably lower their ABA-dependent inositol 1,4,5-triphosphate (IP_3) content (Sanchez and Chua 2001), which had indicated that, under these conditions, IP_3 producing AtPLC1 activity had been repressed through either degrading mRNA coded by *AtPLC1* or blocking its traduction into AtPLC1. Here, the stomatal movement bioassay procedure that has been already used for analysis of ABA stomatal closing in the *als1*-1 mutant (Cousson 2007) was conducted in the presence or absence of 30 μM Dex throughout 5 h of experiments with a Dex-inducible transgenic line carrying the *AtPLC1* antisense in the Columbia genetic background.

Materials and methods

Plants: Seeds of a Dex-inducible transgenic line of *Arabidopsis thaliana* (L.) Heynh. carrying the *AtPLC1* antisense in the cv. Columbia genetic background (T) were a generous gift of Prof. Chua (The Rockefeller University, New York, USA). These seeds had been produced from transgenic plants obtained from a T3 homozygous line selected as previously reported (Sanchez and Chua 2001). The *AtPLC1* antisense transgene was derived from cloning of the Columbia *AtPLC1* cDNA (Hirayama *et al*. 1995) and incorporated into the pPZP vector (Hajdukiewicz *et al*. 1994)-derived plasmid pTA211 (Sanchez and Chua 2001), which encoded a glucocorticoid-regulated factor mediating Dex-inducible transcription of promotors containing Gal4 upstream activation sequence (Aoyama and Chua 1997) upon expression from the constitutive G10-90 promoter (Ishige *et al*. 1999). Seeds of both wild type (WT) Columbia-4 ecotype and T were germinated and the seedlings were grown for 10 d 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 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 in pots with a coarse sand watered three 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} (supplied by 150 W mercury lamps (*HQI-TS, Osram*, München, Germany).

Bioassay with epidermal peels: Abaxial leaf epidermis with stomatal guard cells was peeled from four- to five-week old WT and T plants at the end of the night period. For each comparative experiment, epidermal strips (up to 10 × 5 mm) were obtained from the same fully expended 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 in the presence or absence of 30 μM Dex (*Calbiochem-Novabiochem Co*, La Jolla, USA), throughout the experiments. It was verified that DMSO (*Sigma*), in which Dex was dissolved, did not change stomatal aperture.

Stomatal closing in response to ABA was assayed starting with high stomatal apertures (approx. 5 μm). These apertures were obtained in the presence or absence of Dex by incubating the peels for 3 h at 20 °C under white light in 30 mM potassium iminodiacetate (*Sigma*), 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. The impermeant anion iminodiacetate 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 the ABA-induced stomatal closing 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). 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 the plant Ca^{2+} chelator, EGTA BAPTA (*Sigma*) to the incubation medium, throughout the experiments. Each of these buffers was added at 1.5 mM, which had previously caused maximum inhibition of ABA stomatal closing in *A. thaliana* (Cousson 2003a). The EGTA (50 mM) and BAPTA (50 mM) stock solutions contained a significant amount of K^+. The control incubation medium contained

potassium iminodiacetate 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 G protein-regulated PI-PLC activity within Ca^{2+}-dependent ABA stomatal closing, the PI-PLC inhibitor, U73122 (*Biomol Research Laboratories*, Plymouth, UK) and its inactive analogue, 1-[6-{[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino} hexyl]-2,5-pyrrolidine-dione (U73343; *Biomol Res. Lab.*) (Thompson *et al*. 1991) were separately added to the incubation medium in the presence or absence of either EGTA or BAPTA, throughout the experiments. U73122 and U73343 were tested at 3 nM, which had previously shown a specific and substantial inhibition of ABA stomatal closing by U73122 in both *C. communis* (Cousson and Vavasseur 1998) and *A. thaliana* (Cousson 2003a, 2007).

To investigate the possible implication of G protein-regulated ADP-ribosyl cyclase (ARC) activity within Ca^{2+}-dependent ABA stomatal closing, the antagonist of cyclic ADP-ribose (cADPR) synthesis, nicotinamide (*Sigma*) (Sethi *et al*. 1996) and the G protein modulators, GP Ant-2; *Biomol*) (Mukai *et al*. 1992) and mas17 (Higashijima *et al*. 1990) (*Biomol Res. Lab.*) were added separately or in combination to the incubation medium in the presence or absence of either U73122, EGTA or BAPTA, throughout the experiments. Nicotinamide (50 mM), GP Ant-2 (10 μM) and mas17 (7 μM) were tested at concentrations that previously exhibited maximum inhibitory effects on ABA-induced stomatal closing (Leckie *et al*. 1998, Cousson 2003a).

Except for EGTA and BAPTA, all these compounds as well as DMSO, in which U73122, U73343 and GP Ant-2 were dissolved, did not significantly change the stomatal aperture in the absence of exogenous ABA.

Data analysis: 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 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 adding 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. Each datum point represented mean ± SE.

Results and discussion

The inhibitory effect of Dex on stomatal closing depends on the Dex-inducible *AtPLC1* antisense transgene and on the exogenous ABA concentration: Calculated as the difference between the apertures measured just before and 2 h after adding ABA, 20 μM ABA-induced stomatal closure was decreased from 3.1 to 1.8 μm by applying throughout the experiment 30 μM Dex to abaxial leaf epidermis peeled from T plants (Table 1). Contrasting with this partial inhibitory effect (about 40 % inhibition), 30 μM Dex did not affect 30 μM ABA-induced stomatal closure (Table 1). Moreover, control experiments performed on WT plants showed that, by itself, applying 30 μM Dex during 5 h did not change the magnitude of the stomatal closing responses to 20 and 30 μM ABA (Table 1).

Table 1. Dex affects ABA stomatal closing in Dex-inducible *AtPLC1* antisense transgenic plants (T) but not in the wild type (WT) *A. thaliana* cv. Columbia. Abaxial leaf epidermal peels were incubated under light and CO_2-free air with or without 30 μM Dex throughout the experiments. Three hours after starting the experiments, 20 or 30 μM ABA was added. Stomatal closing was taken as the difference between the stomatal apertures measured just before and 2 h after adding ABA. Means ± SE calculated from at least three independent experiments.

Plant	ABA [μM]	Stomatal closing [μm] -Dex	+Dex
WT	20	3.0 ± 0.1	3.1 ± 0.2
T	20	3.1 ± 0.2	1.8 ± 0.1
WT	30	3.1 ± 0.2	3.1 ± 0.2
T	30	3.2 ± 0.1	3.3 ± 0.2

When Dex was applied to the epidermal peels in the absence of ABA, it did not change the mean stomatal aperture throughout 5 h of experiment (results not shown). Thus, the inhibition of ABA stomatal closing specifically observed from T plants was not a side effect of Dex. Rather, this inhibition would have resulted from deactivation of the IP_3 producing enzyme, AtPLC1 through Dex-inducible post transcriptional gene silencing that degrades mRNA coded by *AtPLC1* or blocks its traduction into AtPLC1. Indeed, applying 30 μM Dex for 1 h in hydroponic culture of Dex-inducible transgenic plants similar to those of the present study had been sufficient to considerably lower their ABA-dependent IP_3 levels (Sanchez and Chua 2001). Since IP_3 is known not only to mediate ABA signalling (Burnette *et al*. 2003) but also to mobilize Ca^{2+} from internal stores (Gilroy *et al*. 1990), it was investigated whether or not Dex interfered with the Ca^{2+} dependence of ABA stomatal closing.

Dex suppresses the inhibitory effect of Ca^{2+} buffers on ABA stomatal closing only in the Dex-inducible *AtPLC1* antisense transgenic plants: It was pharmacologically questioned whether or not 30 μM Dex

inhibited the stomatal closing response of T plants to 20 µM ABA through inhibiting Ca^{2+} signalling. When tested at 1.5 mM, each of the plant Ca^{2+} buffers, EGTA and BAPTA (Armstrong and Blatt 1995) approximately decreased 20 µM ABA-induced stomatal closure from 3.2 to 0.9 µm in both WT and T plants (about 70 % inhibition) in the absence of Dex (Table 2). The results obtained in WT agreed with a previous study (Cousson 2007) and corresponded to maximum inhibitory effect of Ca^{2+} buffering on such a stomatal response in the cv. Columbia (Cousson 2003a). Applying 30 µM Dex during 5 h prevented this Ca^{2+} buffering inhibitory effect in T but not in WT plants (Table 2). Furthermore, 30 µM Dex did not change the effect of 1.5 mM BAPTA on the closing response to 30 µM ABA in both T and WT plants with or without Dex. This Ca^{2+} buffering treatment decreased 30 µM ABA-induced stomatal closure from 3.3 to 1.0 µm (about 70 % inhibition), whereas 1.5 mM EGTA had no effect (Table 2), in agreement with the differential BAPTA and EGTA effects on 30 µM ABA-induced stomatal closing previously shown in *A. thaliana* cv. Columbia (Cousson 2003a).

Table 2. Dex removes Ca^{2+} buffer-induced inhibition of the stomatal closing response to 20 µM ABA in Dex-inducible *AtPLC1* antisense transgenic plants (T) but not in the wild type (WT). The Ca^{2+} buffer, EGTA or BAPTA was applied at 1.5 mM to abaxial leaf epidermal peels incubated under light and CO_2-free air with or without 30 µM Dex throughout the experiments. Three hours after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1.

Plant	Buffer	ABA-induced stomatal closing [µm] -Dex 20 µM	-Dex 30 µM	+Dex 20 µM	+Dex 30 µM
WT	-	3.1 ± 0.1	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2
T	-	3.2 ± 0.1	3.2 ± 0.2	1.9 ± 0.1	3.3 ± 0.2
WT	EGTA	0.9 ± 0.1	3.2 ± 0.1	0.9 ± 0.1	3.3 ± 0.2
T	EGTA	0.8 ± 0.1	3.3 ± 0.1	1.8 ± 0.2	3.3 ± 0.2
WT	BAPTA	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.1
T	BAPTA	0.9 ± 0.2	0.9 ± 0.2	1.8 ± 0.1	1.0 ± 0.1

Together, these results revealed that Dex could mimick the aluminum sensitive *als1*-1 mutation (Cousson 2007) only in the Dex-inducible *AtPLC1* antisense transgenic plants through interfering with the differential inhibitory effects of the Ca^{2+} buffers, EGTA and BAPTA on ABA stomatal closing. Using the *als1*-1 mutant has just shown that decreasing exogenously applied ABA from 30 to 20 µM changed guard cell Ca^{2+} mobilization. It is known that BAPTA, but not EGTA, buffers efficiently rapid increases in cytosolic free Ca^{2+} (Armstrong and Blatt 1995). Therefore, since EGTA, but not BAPTA, specifically inhibited 20 µM ABA-induced stomatal closure, it was possible that slow increases in guard cell cytosolic free Ca^{2+} specifically resulted from such a change in Ca^{2+} mobilization. To question further whether or not the above mentioned stomatal effects of Dex resulted from AtPLC1 deactivation, the PI-PLC inhibitor, U73122 as well as its close inactive analogue, U73343 were tested with or without 30 µM Dex on ABA stomatal closing of abaxial leaf epidermis peeled from T plants.

Dex suppresses the specific inhibitory effect of the PI-PLC inhibitor, U73122 on ABA stomatal closing only in the Dex-inducible *AtPLC1* antisense transgenic plants: In the absence of Dex, 3 nM U73122 decreased 20 µM ABA-induced stomatal closure from 3.2 to 1.9 µm, approximately, whereas 3 nM U73343 had no effect (Table 3). This U73122 effect (about 40 % inhibition) was suppressed by Dex in T but not in WT plants. In the T plants, indeed, 30 µM Dex kept stomatal closure at about 1.8 µm whether U73122 was applied or not (Table 3). None of these treatments affected 30 µM ABA-induced stomatal closure (Table 3) in both T and WT plants. The specific U73122 effect was similar to U73122-induced inhibition of ABA stomatal closing that had been previously shown in cv. Columbia (Cousson 2003a).

Table 3. Dex differentially removes inhibition of ABA-induced stomatal closing by inhibitors of GTP-binding protein functioning in Dex-inducible *AtPLC1* antisense transgenic plants (T) but does not change this inhibition in the wild type (WT). The inhibitors, U73122 (3 nM ; U), U73343 (3 nM), mas17 (7 µM ; mas) and GP Ant-2 (10 µM ; GP) were applied separately or in combination to abaxial leaf epidermal peels incubated under light and CO_2-free air with or without 30 µM Dex throughout the experiments. 3 h after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1.

Plant	Inhibitors	ABA-induced stomatal closing [µm] -Dex 20 µM	-Dex 30 µM	+Dex 20 µM	+Dex 30 µM
WT	-	3.2 ± 0.1	3.2 ± 0.2	3.1 ± 0.1	3.2 ± 0.1
T	-	3.2 ± 0.2	3.2 ± 0.1	1.8 ± 0.2	3.3 ± 0.2
WT	U	1.8 ± 0.1	3.1 ± 0.2	1.8 ± 0.2	3.3 ± 0.1
T	U	1.9 ± 0.1	3.2 ± 0.2	1.8 ± 0.1	3.2 ± 0.2
WT	U73343	3.2 ± 0.1	3.1 ± 0.2	3.0 ± 0.1	3.3 ± 0.2
T	U73343	3.1 ± 0.1	3.3 ± 0.1	1.9 ± 0.1	3.3 ± 0.1
WT	mas17	3.2 ± 0.1	2.0 ± 0.1	3.2 ± 0.2	2.1 ± 0.2
T	mas17	3.2 ± 0.2	2.1 ± 0.1	1.9 ± 0.2	2.1 ± 0.1
WT	GP Ant-2	3.2 ± 0.2	2.0 ± 0.2	3.2 ± 0.1	2.0 ± 0.2
T	GP Ant-2	3.1 ± 0.1	2.1 ± 0.1	1.8 ± 0.2	2.2 ± 0.1
WT	mas+GP	3.0 ± 0.2	1.0 ± 0.2	3.1 ± 0.2	0.9 ± 0.2
T	mas+GP	3.1 ± 0.1	1.0 ± 0.1	1.9 ± 0.2	1.1 ± 0.1
WT	U+mas	1.9 ± 0.2	2.1 ± 0.1	1.8 ± 0.1	2.1 ± 0.2
T	U+mas	1.8 ± 0.1	2.2 ± 0.1	1.8 ± 0.1	2.1 ± 0.1
WT	U+GP	1.9 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	2.1 ± 0.2
T	U+GP	1.9 ± 0.2	2.1 ± 0.1	1.8 ± 0.1	2.1 ± 0.2
WT	U+mas+GP	1.9 ± 0.2	1.1 ± 0.2	1.8 ± 0.2	1.1 ± 0.1
T	U+mas+GP	1.9 ± 0.1	1.1 ± 0.1	1.8 ± 0.2	1.0 ± 0.1

As previously observed (Cousson and Vavasseur 1998, Cousson 2003a), U73122 had no stomatal effect in the absence of ABA, indicating that its inhibitory effect was not a side effect. When separately applied in the absence of Dex, EGTA, BAPTA and U73122 inhibited ABA stomatal closing (Tables 2, 3) as previously reported in cv. Columbia (Cousson 2003a). Neither EGTA nor BAPTA increased the U73122 inhibitory effect (Table 4). As in the case of EGTA and BAPTA, applying 30 µM Dex prevented the U73122 inhibitory effect in T but not in WT plants (Table 3). All these features supported that Dex acted only through deactivating AtPLC1 within a Dex-inducible homologous expression system. Consequently, these results strongly supported that, *1*) AtPLC1 mediates the Ca^{2+} dependence of 20 µM ABA-induced stomatal closing and *2*) 3 nM U73122 inhibits AtPLC1 activity.

This U73122 effect confirmed the specific half inhibition of 10 nM ABA-induced stomatal closure by 3 nM U73122 shown in *Commelina communis* (Cousson and Vavasseur 1998) but differed from the U73122 effect previously reported by Staxen *et al*. (1999). They had used *C. communis* epidermal peels and tested U73122 at 1 µM: then maximum effects had been 20 % inhibition of 1 µM ABA-induced stomatal closure and alteration in 1 µM ABA-induced cytosolic free Ca^{2+} oscillations of guard cells. Their stomatal effect had no significance because, when tested in the 100 nM - 10 µM range, U73122 and U73343 had similarly influenced *Commelina* and *Arabidopsis* ABA stomatal closing from no detectable effect up to 20 % inhibition (Cousson 2003a). As for alteration in the cytosolic free Ca^{2+} oscillatory pattern, it could have resulted from inhibition of Ca^{2+} influx through blocking voltage-dependent plasma membrane Ca^{2+} channels. When tested in the micromolar range, indeed, slightly higher U73343 concentrations had mimicked U73122 to interfere with the Ca^{2+} status of diverse animal cellular systems in a PI-PLC-independent fashion and, in the case of rat portal vein myocytes, to inhibit the L-type Ca^{2+} channel (Macrez-Leprêtre *et al*. 1996, Takenouchi *et al*. 2005). This should be considered, as much as 100 nM ABA-induced *C. communis* stomatal closing had been pharmacologically shown to involve a putative voltage-dependent plasma membrane Ca^{2+} channel that approximates the L-type (Cousson 1999). Whether U73343 concentrations slightly higher than 1 µM mimick or not 1 µM U73122 to alterate 1 µM ABA-induced cytosolic free Ca^{2+} oscillations in the *Commelina* guard cell should be tested to confirm or not our present interpretation.

Tested in the 1 - 10 µM range, U73122, but not U73343, had been shown in animal systems to interfere with PI-PLC activity through inhibiting its positive regulation by G protein α subunits (Gαs) (Thompson *et al*. 1991, Lin *et al*. 2006). Coupling of $G\alpha_q$ or $G\alpha_h$ to the PLCβ or PLCδ1 isoforms, respectively, had been demonstrated. First, upon light stimulation of the seven-transmembrane-span (7TMS) receptor, rhodopsin, $G\alpha_q$ is activated and directly binds to PLCβ in the compound eye of *Drosophila* (Bähner *et al*. 2000). Second, follicle-stimulating hormone induces rat sertoli cell Ca^{2+} influx through a signalling pathway that implicates both the activation of $G\alpha_h$, the translocation of PLCδ1 from the cytosol to the plasma membrane and the formation of a $G\alpha_h$/PLCδ1 complexe (Lin *et al*. 2006). Likewise, although our study tested a thousand times lower concentration of U73122, the fact that U73122, but not U73343, substantially inhibited ABA stomatal closing only in the absence of Dex suggested G protein-coupled AtPLC1 activity within guard cell ABA signalling.

The *Arabidopsis* 7TMS-like receptor GCR1 had been suggested (Apone *et al*. 2003) to activate *Nicotiana* Pl-PLC through coupling to the canonical *Arabidopsis* Gα, GPA1. But Apone *et al*. (2003) had worked on an heterologous expression system controlled by the constitutive CaMV35S promoter (Colucci *et al*. 2002) and the positive correlation found between the overexpression of either GCR1 or GPA1 and PI-PLC activation within thymidine incorporation into DNA could reflect indirect or artificial links between these proteins. Such a system had likely exhibited neomorphic phenotypes as much as its inhibition of PI-PLC activity by 100 µM U73122 had been only reported in *in vitro* assays and could not result from blocking a putative G protein coupling. Indeed, 100 µM U73122 had been shown to inhibit only *in vitro* activity of a recombinant *Nicotiana rustica* PI-PLC that was not coupled to G protein (Staxen *et al*. 1999). Therefore, coupling of a canonical or unconventional Gα, but not GPA1, to AtPLC1 is possible. Up to now, *GPA1* was the unique *Arabidopsis* gene coding for a canonical Gα, but several groups of Gα-like proteins were identified too (Assmann 2002). Despite some differences, the molecular structures of mammalian PLCδ1 (Essen *et al*. 1996) and AtPLC1 (Mueller-Roeber and Pical 2002) are related: in particular, they do not contain a regulatory carboxy-terminal region thought to be needed for coupling PI-PLC to G protein. However, PLCδ1 is tightly coupled to $G\alpha_h$ (Lin *et al*. 2006). Then, assuming that the inhibitory property of U73122 depends on the molecular mechanisms that couple G proteins to PI-PLC isoforms, the fact that inhibition of AtPLC1 would be optimal at a considerably lower U73122 concentration than those reported for blocking $G\alpha_h$-PLCδ1 coupling (Lin *et al*. 2006) might corroborate that a putative Gα-like protein-AtPLC1 coupling exists, which differs from $G\alpha_h$-PLCδ1 coupling.

A possible GPA1-AtPLC1 coupling could not be definitively ruled out since Wang *et al*. (2001) had shown that an acidic pH-clamp of *Arabidopsis* guard cells revealed an inhibitory effect of the GPA1 null mutants, *gpa1*-1 and *gpa1*-2 on 20 µM ABA-induced stomatal closure, suggesting that a redundant GPA1-independent pathway activates the slow anion channel of the guard cell plasma membrane at alkaline pH. In the present study, however, 3 nM U73122 inhibited 20 µM ABA-

induced closure of WT stomata incubated without any acidic pH clamp, which might exclude a possible interference of U73122 with GPA1 functioning and, consequently, corroborate further a putative Gα-like protein-AtPLC1 coupling. Nethertheless, hypothesis that a redundant GPA1-AtPLC1 coupling mediates 20 µM ABA-induced stomatal closing was examined further by testing two other inhibitors of G protein functioning, GP Ant-2 and mas17 in relation to Dex.

Applying 30 µM Dex did not change inhibition of ABA stomatal closing by 10 µM GP Ant-2 and 7 µM mas17 (Table 3). Whether Dex was applied or not, GP Ant-2 and mas17 decreased specifically and similarly 30 µM ABA-induced stomatal closure from 3.2 to 2.1 µm, approximately (inhibition by about 35 %), and these effects were additive. Moreover, 3 nM U73122 mimicked 30 µM Dex in having no influence on these inhibitory effects, and, reciprocally, neither GP Ant-2 nor mas17 changed the specific inhibitory effect of 3 nM U73122 on 20 µM ABA-induced stomatal closure (Table 3).

Table 4. The Ca^{2+} buffers, EGTA and BAPTA do not increase U73122-induced inhibition of the stomatal closing response to 20 µM ABA. Each of the Ca^{2+} buffers was applied separately at 1.5 mM to abaxial leaf epidermis peeled from Dex-inducible *AtPLC1* antisense transgenic plants of *A. thaliana* (L.) Heynh. cv. Columbia and incubated under light and CO_2-free air with 3 nM U73122 and without 30 µM Dex throughout the experiments. Three hours after starting the experiments, 20 µM ABA was added. For detail see Table 1.

Treatment	Stomatal closing [µm]
-	3.2 ± 0.2
U73122	1.8 ± 0.2
U73122 + EGTA	1.9 ± 0.2
U73122 + BAPTA	1.8 ± 0.2

Since mastoparan and its active analogue, mas7 mimick 7TMS receptors within G protein activation at least in a few animal systems (Higashijima *et al*. 1990), the inhibitory stomatal effect exhibited here by its inactive analogue, mas17 could result from an interference with coupling of Gαs to 7TMS receptors. Our observed mas17 effect contrasted with the limited, possible dual, stomatal effects of both mastoparan and mas7 (Kelly *et al*. 1995). These contrasting results likely suggested that both G protein-dependent and -independent processes are needed for ABA stomatal closing. Thus, until now, GPA1 implication has been shown only for ABA inhibition of guard cell inward K^+ channels and pH-related ABA activation of anion channels (Wang *et al*. 2001). In that regards, the fact that mas7 had inactivated guard cell inward K^+ channels (Armstrong and Blatt 1995) together with the fact that both the *gpa1*-1 and *gpa1*-2 mutations had removed ABA inhibition of guard cell inward K^+ channels (Wang *et al*. 2001) signified that mas7 likely mimicks at least one 7TMS-like receptor within GPA1 activation. Also, mas17 would have inhibited 30 µM ABA-induced stomatal closure through preventing at least one 7TMS-like receptor from coupling to GPA1. Accordingly, the fact that mas17 did not inhibit 20 µM ABA-induced stomatal closure could not exclude the above considered possibility for a redundant GPA1-AtPLC1 coupling.

Table 5. Neither Dex nor U73122 changes inhibition of ABA stomatal closing by nicotinamide in both Dex-inducible *AtPLC1* antisense transgenic plants (T) and the wild type (WT). The inhibitors, U73122 (3 nM ; U), nicotinamide (NA; 50 mM), mas17 (7 µM ; mas) and GP Ant-2 (10 µM ; GP) were applied separately or in combination to abaxial leaf epidermal peels incubated under light and CO_2-free air with or without 30 µM Dex throughout the experiments. 3 h after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1.

Plant	Inhibitors	ABA-induced stomatal closing [µm] -Dex 20 µM	-Dex 30 µM	+Dex 20 µM	+Dex 30 µM
WT	-	3.1 ± 0.1	3.2 ± 0.1	3.0 ± 0.1	3.1 ± 0.1
T	-	3.2 ± 0.2	3.3 ± 0.1	1.8 ± 0.2	3.2 ± 0.2
WT	U	1.8 ± 0.2	3.2 ± 0.2	1.8 ± 0.1	3.2 ± 0.1
T	U	1.9 ± 0.1	3.2 ± 0.2	1.8 ± 0.2	3.3 ± 0.1
WT	NA	3.0 ± 0.1	2.0 ± 0.1	3.0 ± 0.2	2.0 ± 0.2
T	NA	3.1 ± 0.1	2.1 ± 0.1	1.9 ± 0.2	2.1 ± 0.2
WT	NA+U	1.9 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	2.0 ± 0.1
T	NA+U	1.8 ± 0.2	2.0 ± 0.1	1.9 ± 0.1	2.1 ± 0.2
WT	NA+mas	3.2 ± 0.2	2.1 ± 0.2	3.0 ± 0.2	2.2 ± 0.1
T	NA+mas	3.1 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.1 ± 0.1
WT	NA+GP	3.2 ± 0.1	1.0 ± 0.1	3.2 ± 0.2	1.1 ± 0.2
T	NA+GP	3.0 ± 0.2	0.9 ± 0.1	1.8 ± 0.2	1.0 ± 0.2
WT	NA+GP+mas	3.0 ± 0.1	1.0 ± 0.2	3.2 ± 0.1	1.1 ± 0.1
T	NA+GP+mas	3.2 ± 0.1	1.0 ± 0.2	2.0 ± 0.1	1.0 ± 0.1

Receptor coupling to G protein is differently inhibited by GP Ant-2 (Mukai *et al*. 1992) and mas 17 (Higashijima *et al*. 1990), which does not exclude that they could target the same Gαs. However, our study excluded that GP Ant-2 would target GPA1 because of the following features. First, the inhibitory GP Ant-2 effect was additive to the inhibitory mas17 effect. Second, mas7 counteracted the inhibitory mas17 effect but not that of GP Ant-2 as reported for the stomatal effects of mas17, mas7 and GP Ant-2 on auxin-induced *Arabidopsis* stomatal opening (Cousson 2003b). Therefore, since GPA1 is the unique canonical Gα, GP Ant-2 should have specifically inhibited stomatal closing to 30 µM ABA through targeting at least one Gα-like protein.

The two ligands, IP_3 (Burnette *et al*. 2003) and cADPR (Leckie *et al*. 1998), which gate plant endomembrane Ca^{2+} release channels (Sanders *et al*. 2002), are likely implicated as mediators within guard cell ABA signalling. Until now, it was not known whether IP_3 and cADPR act in the same or parallel guard

cell Ca^{2+}-dependent ABA signalling pathways (Hunt *et al*. 2003). Possibility that ABA-induced Ca^{2+} mobilization does not implicate any dependency between these mediators was questioned by testing the effect of the antagonist of cADPR synthesis, nicotinamide (Sethi *et al*. 1996) on ABA stomatal closing in relation to Dex. Dex did not change inhibition of ABA-induced stomatal closing by nicotinamide (Table 5). Whether 30 μM Dex was applied or not, the inhibitory effect of nicotinamide mimicked that of mas17: 50 mM nicotinamide specifically decreased 30 μM ABA-induced stomatal closure from approx 3.3 to 2.1 μm as did mas17 (Table 3) and this effect was additive to the GP Ant-2 inhibitory effect but not increased by mas17 (Table 5). Furthermore, U73122 did not change inhibition of ABA stomatal closing by nicotinamide, and reciprocally (Table 5). At last, neither nicotinamide, mas17, GP Ant-2 nor their combinations increased the BAPTA inhibitory effect on 30 μM ABA-induced stomatal closing (Table 6).

Since an inhibitor of guanylyl cyclase, LY83583 did not affect 30 μM ABA-induced stomatal closing (resuls not shown), this response would not be mediated by a cytosolic ARC isoform whose activation implicates cyclic GMP (Galione 1994). Rather, the mimetic mas17 and nicotinamide effects would have suggested that coupling GPA1 to a 7TMS-like receptor directly activates a plasma membrane-bound ARC isoform that contributes, *via* cADPR synthesis, to mobilize Ca^{2+} within 30 μM ABA-induced stomatal closing. This possibility might explain why the present study established an optimal inhibitory concentration of nicotinamide, 50 mM that differed from the optimal concentration that had mimicked LY83583 inhibition of auxin-induced *Arabidopsis* stomatal opening (Cousson 2003b). Plasma membrane-bound ARC whose activity depends on G protein had been reported in animal systems (Higashida *et al*. 1999). In cv. Columbia, ABA had been shown to activate ARC but cellular localization of this enzyme had not yet been determined (Sanchez *et al*. 2004).

The absence of a PH domain and the existence of both a putative Ca^{2+}-interacting EF-hand domain and a C2 domain within molecular structure of AtPLC1 (Mueller-Roeber and Pical 2002) had made possible that, compared to animal PI-PLCδ1 activity (Essen *et al*. 1996), AtPLC1 activity requires a somewhat similar cytosolic free Ca^{2+} environment and a different interaction with the plasma membrane. Accordingly, another *Arabidopsis* PI-PLC isoform closely related to PLCδ1, AtPLC2 is activated by low micromolar Ca^{2+} concentrations and predominantly localized in the plasma membrane (Otterhag *et al*. 2001). Moreover, a Ca^{2+}-activated PI-PLC is exclusively localized in the membrane fraction of tobacco guard cells (Hunt *et al*. 2003). Working on Dex-inducible transgenic *A. thaliana* lines carrying the *AtPLC1* sense transgene, Sanchez and Chua (2001) had proposed that the Ca^{2+} signal required for activation of the expressed latent AtPLC1 is induced by cADPR. However, the facts that neither Dex nor U73122 changed the nicotinamide effect on ABA stomatal closing in both T and WT plants and, reciprocally, that nicotinamide did not change the U73122 effect strongly suggested that, under our experimental conditions, AtPLC1-induced IP_3 production did not depend on ARC-induced cADPR production, and reciprocally.

In cv. Columbia, the aluminum sensitive *als1*-1 mutation has revealed a pharmacological profile of ABA stomatal closing (Cousson 2007) similar to that revealed by applying 30 μM Dex to epidermal peels of T plants. This reinforced that the *als1*-1 mutation would deactivate guard cell PI-PLC (Cousson 2007), in particular AtPLC1 that would be primarily needed for 20 μM ABA-induced stomatal closing. The *als1*-1 mutation, Dex and U73122 paralled each other to inhibit this stomatal response by about 40 %, whereas EGTA or BAPTA inhibited the same response by 70 %. The fact that neither EGTA nor BAPTA increased the inhibitory U73122 effect (Table 4) suggested that AtPLC1 is the sole mediator of Ca^{2+} mobilization in response to exogenously applied 20 μM ABA. Then, such a discrepancy in the above mentioned percentages of inhibition should be explained by a drop in cellular diacylglycerol that would result from inhibiting diacylglycerol-producing AtPLC1 but not from buffering cytosolic free Ca^{2+}. Indeed, diacylglycerol had been shown to open stomata (Lee and Assmann 1991). Consequently, our results suggested that a drop in cellular diacylglycerol induces Ca^{2+}-independent stomatal closing.

Our results strongly supported that, on the contrary, AtPLC1 is absolutely excluded from Ca^{2+} mobilization in response to exogenously applied 30 μM ABA. Since AtPLC1 is induced by dehydration and 100 μM ABA (Hirayama *et al*. 1995), one should question whether or

Table 6. Nicotinamide (NA) and the inhibitors of GTP-binding protein functioning, mas17 and GP Ant-2 inhibit the stomatal closing response to 30 μM ABA in fashions that are not additive to the inhibitory effect of the Ca^{2+} buffer, BAPTA. The Ca^{2+} buffer was applied at 1.5 mM to abaxial leaf epidermis peeled from Dex-inducible *AtPLC1* antisense transgenic plants and incubated without 30 μM Dex under light and CO_2-free air in the presence of NA (50 mM), mas17 (7 μM) and GP Ant-2 (10 μM) added separately or in combination throughout the experiments. Three hours after starting the experiments, 30 μM ABA was added. For detail see Table 1.

Treatments	Stomatal closing [μm] -BAPTA	+BAPTA
-	3.3 ± 0.2	0.9 ± 0.1
NA	2.0 ± 0.1	0.9 ± 0.1
mas17	2.1 ± 0.1	1.0 ± 0.2
GP Ant-2	2.0 ± 0.1	1.1 ± 0.1
mas17+GP Ant-2	1.0 ± 0.1	1.0 ± 0.1
NA+mas17	2.0 ± 0.1	1.0 ± 0.2
NA+GP Ant-2	1.0 ± 0.1	0.9 ± 0.2
NA+mas17+GP Ant-2	1.0 ± 0.1	1.0 ± 0.2

not applying 30 μM Dex to T plants mimick the *als1*-1 mutation within its uncapacity to cause an hypersensitive wilting response to drought (Cousson 2007). Unfortunately, Dex can be only applied to hydroponic cultures of such transgenic plants (Sanchez and Chua 2001) and, then, drought treatment cannot be performed. However, pharmacological analysis of ABA stomatal closing in stomatal movement bioassay revealed Dex effects that mimicked the stomatal effects of the *als1*-1 mutation (Cousson 2007). Therefore, it was possible that AtPLC1 deactivation does not affect the wilting response under the water stress conditions previously tested in the *als1*-1 mutant (Cousson 2007). Such a possibility, on one hand, was consistent with an efficient AtPLC1-independent stomatal closing response to ABA concentrations higher than a drought-specific threshold value that our results suggested. On the other hand, it was consistent with an ABA-independent involvement of the *cis*-acting element, TACCGACAT within the first rapid response of *rd29A* to drought (Yamaguchi-Shinozaki and Shinozaki 1994) that would greatly contribute, together with ABA stomatal closing, to prevent the previously tested wilting response of the *als1*-1 mutant from drought hypersensitivity.

In conclusion, pharmacological analysis of ABA stomatal closing in leaf epidermal peels of Dex-inducible *AtPLC1* antisense transgenic plants incubated without Dex revealed the features that have been previously established from untransformed plants of *A. thaliana* cv. Columbia (Cousson 2003a, 2007). Although silencing of the *AtPLC1* gene by Dex was not directly shown in the guard cells of the tested T plants, the following features strongly supported that the stomatal effects of Dex reported here were due to guard cell AtPLC1 deactivation: *1*) the Dex stomatal effects were observed in stomatal movement bioassays performed with epidermal peels composed mainly of guard cells and deprived of mesophyl cells; *2*) these stomatal effects were obtained by applying 30 μM Dex under light for more than 1 h that had been sufficient to considerably lower ABA-induced IP_3 leaf content of comparable *AtPLC1* antisense plants cultured in hydropony (Sanchez and Chua 2001); *3*) our Dex treatment did not affect the stomatal closing response not only to 30 μM ABA but also to 20 μM ABA when tested on WT leaf epidermis. Consequently, it was likely that AtPLC1 specifically and primarily mobilizes Ca^{2+} within the stomatal closing response to ABA resting at concentrations below a threshold value. The so far reported mimetic stomatal effects of Dex and the *als1*-1 mutation (Cousson 2007) suggested that this threshold is specifically induced by drought. Beyond this threshold, the present study established experimental conditions under which a putative ARC, but not AtPLC1, would contribute to Ca^{2+}-dependent ABA stomatal closing. From most of the above considerations, it was suggested that the *Arabidopsis* guard cell has an ABA perception and transduction complexe comprising at least GPA1 and two Gα-like proteins coupled to a 7TMS-like receptor and linked to at least two effector enzymes, among which are AtPLC1 and an ARC. Furthermore one can hypothesize that, beyond this drought-specific concentration threshold, endogenous ABA is sufficient to modulate coupling of both GPA1 and these Gα-like proteins to the ABA receptor and, consequently, to shift Ca^{2+} mobilization from AtPLC1-induced signal transduction into a pathway involving ARC as one of several putative mediators. This possibility would implicate the ABA receptor as either a unique dual affinity 7TMS ABA-binding protein or two 7TMS ABA-binding proteins having respectively high and low affinities to ABA. It is now required to look further for possible G protein-mediated activation of both AtPLC1 and a putative ARC at the guard cell plasma membrane, to focus on other predicted 7TMS proteins than GCR1 that had been identified as down-regulating guard cell ABA signalling (Pandey and Assmann 2004), and to clone the *Arabidopsis* genes that encode ARC and induce its activity in order to confirm or not these hypotheses.

References

Aoyama, T., Chua, N.-H.: A glucocorticoid-mediated transcriptional induction system in transgenic plants. - Plant J. **11**: 605-612, 1997.

Apone, F., Alyeshmerni, N., Wiens, K., Chalmers, D., Chrispeels, M.J., Colucci, G.: The G-protein-coupled receptor GCR1 regulates DNA synthesis through activation of phosphatidylinositol-specific phospholipase C. - Plant Physiol. **133**: 571-579, 2003.

Armstrong, F., Blatt, M.R.: Evidence for K^+ channel control in *Vicia* guard cells coupled by G-proteins to a 7TMS receptor mimetic. Plant J. **8**: 187-198, 1995.

Assmann, S.M.: Heterotrimeric and unconventional GTP binding proteins in plant cell signaling. - Plant Cell **14** (Suppl.): S355-S373, 2002.

Bähner, M., Sander, P., Paulsen, R., Huber, A.: The visual G protein of fly photoreceptors interacts with the PDZ domain assembled INAD signaling complex via direct binding of activated $G\alpha_q$ to phospholipase Cβ. - J. biol. Chem. **275**: 2901-2904, 2000.

Burnette, R.N., Gunesekera, B.M., Gillaspy, G.E.: An *Arabidopsis* inositol 5-phosphatase gain-of-function alters abscisic acid signaling. - Plant Physiol. **132**: 1011-1019, 2003.

Colucci, G., Apone, F., Alyeshmerni, N., Chalmers, D., Chrispeels, M.J.: *GCR1*, the putative *Arabidopsis* G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. - Proc. nat. Acad. Sci. USA **99**: 4736-4741, 2002.

Cousson, A.: Pharmacological study of two potential Ca^{2+} signalling pathways within stomatal closing in response to abscisic acid in *Commelina communis* L. - Plant Sci. **145**: 67-74, 1999.

Cousson, A.: Two potential Ca^{2+}-mobilising processes depend

on the abscisic acid concentration and growth temperature in the *Arabidopsis* stomatal guard cell. - J. Plant Physiol. **160**: 493-501, 2003a.

Cousson, A.: Pharmacological evidence for a positive influence of the cyclic GMP-independent transduction on the cyclic GMP-mediated Ca^{2+}-dependent pathway within *Arabidopsis* stomatal opening in response to auxin. - Plant Sci. **164**: 759-767, 2003b.

Cousson, A.: Two calcium mobilizing pathways implicated within abscisic acid-induced stomatal closing in *Arabidopsis thaliana*. - Biol. Plant. **51**: 285-291, 2007.

Cousson, A., Vavasseur, A.: Two potential Ca^{2+}-dependent transduction pathways in stomatal closing in response to abscisic acid. - Plant Physiol. Biochem. **36**: 257-262, 1998.

Essen, L-O., Perisic, O., Cheung, R., Katan, M., Williams, R.L.: Crystal structure of a mammalian phosphoinositide-specific phospholipase Cδ. - Nature **380**: 595-602, 1996.

Galione, A.: Cyclic ADP-ribose, the ADP-ribosyl cyclase and calcium signalling. - Mol. cell. Endocrinol. **98**: 125-131, 1994.

Gilroy, S., Read, N.D., Trewavas, A.J.: Elevation of cytoplasmic calcium by caged calcium or caged inositol triphosphate initiates stomatal closure. - Nature **346**: 769-771, 1990.

Hajdukiewicz, P., Svab, Z., Maliga, P.: The small, versatile *pPZP* family of *Agrobacterium* vectors for plant transformation. - Plant. mol. Biol. **25**: 989-994, 1994.

Higashida, H., Egorova, A., Higashida, C., Zhong, Z.-G., Yokoyama, S., Noda, M., Zhang, J.-S.: Sympathetic potentiation of cyclic ADP-ribose formation in rat cardiac myocytes. - J. biol. Chem. **274**: 33348-33354, 1999.

Higashijima, T., Burnier, J., Ross, E.M.: Regulation of G_i and G_o by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. - J. biol. Chem. **265**: 14176-14186, 1990.

Hirayama, T., Ohto, C., Mizoguchi, T., Shinozaki, K.: A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*. - Proc. nat. Acad. Sci. USA **92**: 3903-3907, 1995.

Hunt, L., Mills, L.N., Pical, C., Leckie, C.P., Aitken, F.L., Kopka, J., Mueller-Roeber, B., McAinsh, M.R., Hetherington, A.M., Gray, J.E.: Phospholipase C is required for the control of stomatal aperture by ABA. - Plant J. **34**: 47-55, 2003.

Ishige, F., Takaishi, M., Foster, R., Chua, N.-H., Oeda, K.: A G-box motif (GCCACGTGCC) tetramer confers high levels of constitutive expression in dicot and monocot. - Plant J. **18**: 443-448, 1999.

Kelly, W.B., Esser, J.E., Schroeder, J.I.: Effects of cytosolic calcium and limited, possible dual, effects of G protein modulators on guard cell inward potassium channels. - Plant J. **8**: 479-489, 1995.

Larsen, P.B., Tai, C.Y., Kochian, L.V., Howell, S.H.: *Arabidopsis* mutants with increased sensitivity to aluminum. - Plant Physiol. **110**: 743-751, 1996.

Leckie, C.P., McAinsh, M.R., Allen, G.J., Sanders, D., Hetherington, A.M.: Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. - Proc. nat. Acad. Sci. USA **95**: 15837-15842, 1998.

Lee, Y., Assmann, S.M.: Diacylglycerols induce both ion pumping in patch-clamped guard cell protoplasts and opening of intact stomata. - Proc. nat. Acad. Sci. USA **88**: 2127-2131, 1991.

Leymarie, J., Lascève, G., Vavasseur, A.: Interaction of stomatal responses to ABA and CO_2 in *Arabidopsis thaliana*. - Aust. J. Plant Physiol. **25**: 785-791, 1998.

Lin, Y.F., Tseng, M.J., Hsu, H.L., Wu, Y.W., Lee, Y.H., Tsai, Y.H.: A novel follicle-stimulating hormone-induced G alpha h/phospholipase C delta 1 signaling pathway mediating rat sertoli cell Ca^{2+}-influx. - Mol. Endocrinol. **20**: 2514-2527, 2006.

Macrez-Leprêtre, N., Morel, J.-L., Mironneau, J.: Effects of phospholipase C inhibitors on Ca^{2+} channel stimulation and Ca^{2+} release from intracellular stores evoked by α_{1A}- and α_{2A}-adrenoceptors in rat portal vein myocytes. - Biochem. biophys. Res. Commun. **218**: 30-34, 1996.

Mueller-Roeber, B., Pical, C.: Inositol phospholipid metabolism in *Arabidopsis*. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. - Plant Physiol. **130**: 22-46, 2002.

Mukai, H., Munekata, E., Higashijima, T.: G protein antagonists: a novel hydrophobic peptide competes with receptor for G protein binding. - J. biol. Chem. **267**: 16237-16243, 1992.

Otterhag, L., Sommarin, M., Pical, C.: N-terminal EF-hand-like domain is required for phosphoinositide-specific phospholipase C activity in *Arabidopsis thaliana*. - FEBS lett. **497**: 165-170, 2001.

Pandey, S., Assmann, S.M.: The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein α subunit GPA1 and regulates abscisic acid signaling. - Plant Cell **16**: 1616-1632, 2004.

Sanchez, J.-P., Chua, N.-H.: *Arabidopsis* PLC1 is required for secondary responses to abscisic acid signals. - Plant Cell **13**: 1143-1154, 2001.

Sanchez, J.-P., Duque, P., Chua, N.-H.: ABA activates ADPR cyclase and cADPR induces a subset of ABA-responsive genes in *Arabidopsis*. - Plant J. **38**: 381-395, 2004.

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F.: Calcium at the crossroads of signaling. - Plant Cell **14** (Suppl.): S401-S417, 2002.

Sethi, J.K., Empson, R.M., Galione, A.: Nicotinamide inhibits cyclic ADP-ribose-mediated calcium signalling in sea urchin eggs. - Biochem. J. **319**: 613-617, 1996.

Staxen, I., Pical, C., Montgomery, L.T., Gray, J.E., Hetherington, A.M., McAinsh, M.R.: Abscisic acid induces oscillations in guard cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. - Proc. nat. Acad. Sci. USA **96**: 1779-1784, 1999.

Takenouchi, T., Ogihara, K., Sato, M., Kitani, H.: Inhibitory effects of U73122 and U73343 on Ca^{2+} influx and pore formation induced by the activation of P2X7 nucleotide receptors in mouse microglial cell line. - Biochim. biophys. Acta. **1726**: 177-186, 2005.

Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E., Fisher, S.K.: The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for G_p in receptor compartmentation. - J. biol. Chem. **266**: 23856-23862, 1991.

Wang, X.-Q., Ullah, H., Jones, A.M., Assmann, S.M.: G protein regulation of ion channels and abscisic acid signaling in *Arabidopsis* guard cells. - Science **292**: 2070-2072, 2001.

Yamaguchi-Shinozaki, K., Shinozaki, K.: A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. - Plant Cell **6**: 251-264, 1994.