Arabidopsis Ca^{2+}-dependent protein kinase CPK3 mediates relationship of putative inositol triphosphate receptor with slow-type anion channel

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Abstract

It has been suggested in *Arabidopsis thaliana* (L.) Heynh. cv. Columbia that, contrary to 30 μM abscisic acid (ABA), 20 μM ABA induces guard cell Ca^{2+} mobilization through activating phosphoinositide-specific phospholipase C (PI-PLC)-dependent inositol 1,4,5-triphosphate (IP_3) production. Here, it was investigated whether Ca^{2+}-dependent protein kinase, CPK3 or CPK6 would mediate ABA-induced stomatal closure downstream of IP_3 production. In the knockout *cpk3*-1 mutant, the PLC inhibitor (U73122) adjusted 20 μM ABA-induced stomatal closure to the extent observed in the knockout *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants and the wild type, whereas, in the wild type, the inhibitor of IP_3-induced Ca^{2+} mobilization, xestospongin C (XeC), adjusted this closure to the extent observed in the *cpk3*-1 mutant. The Ca^{2+} buffer, EGTA and XeC positively interacted with the slow anion channel blocker, anthracene-9-carboxylic acid (9-AC) to inhibit 20 μM ABA-induced stomatal closure, which was suppressed in the dexamethasone-inducible *AtPLC1* antisense transgene or the knockout *cpk3*-1, *cpk6*-1, *cpk3*-1*cpk6*-1 and NADPH oxidase *atrbohD/F* mutants. Discrete concentrations of 9-AC or another slow anion channel blocker (probenecid) negatively interacted with the Ca^{2+} buffer, BAPTA or the inhibitor of cyclic ADP-ribose-induced Ca^{2+} mobilization, ruthenium red, to inhibit 30 μM ABA-induced stomatal closure in the wild type but not in the *cpk6*-1, *cpk3*-1*cpk6*-1 and *atrbohD/F* mutants. Based on so far revealed features of the tested compounds and plant materials, interpretation of the results confirmed that guard cell ABA concentration discriminates between two Ca^{2+} mediations and outlined that one of them sequentially implicates CPK6, PLC1, a putative IP_3 receptor homologue, CPK3, and the slow anion channel, whereas the other one excludes AtPLC1-dependent IP_3 production and CPK3.

Additional key words: abscisic acid concentration, anion channel blockers, Ca^{2+}-dependent protein phosphorylation, intracellular Ca^{2+} modulators, phospholipase C, stomatal closure.

Introduction

In higher plants, water loss and CO_2 assimilation are controlled by stomatal movements resulting from volume changes of the guard cells. Gain or loss of guard cell pressure potential, respectively, leads to opening or closing of the stomatal pore. Guard cell osmoregulation implicates plasma membrane voltage as controlling membrane exchanges of both cations and anions (Thiel *et al*. 1992). Stomatal movements are affected by vanadate, fusicoccin, light/dark and dark/light transitions (Assmann and Schwartz 1992, Cousson *et al*. 1995, Cousson 2002)

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Abbreviations: ABA - abscisic acid; AGI No. - *Arabidopsis* gene identifier number; ARC - ADP-ribosyl cyclase; cADPR - cyclic ADP-ribose; CDPK - Ca^{2+}-dependent protein kinase; CFTR - cystic fibrosis transmembrane conductance regulator; Dex - dexamethasone; Gα - GTP-binding protein α-subunit; G protein - GTP-binding protein; IP_3 - inositol 1,4,5-triphosphate; MRP - multidrug resistance-associated protein; MTX - methotrexate; PI-PLC - phosphoinositide-specific phospholipase C; Proben - probenecid; RRed - ruthenium red; R-type - rapid-type anion channel; S-type - slow-type anion channel; U73122 - 1,6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl-1H-pyrrole-2,5-dione; XeC - xestospongin C; 9-AC - anthracene-9-carboxylic acid.

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in fashions suggesting that auxin and abscisic acid (ABA) antagonize to regulate proton pumping activity within such voltage control (Davies and Mansfield 1987). Indeed, electrogenic transmembrane proton gradient controls the balance between the polarizing activity of the K^+-inward rectifying channel (Grabov and Blatt 1997) and the depolarizing activity of the anion-outward rectifying channel (Schulz-Lessdorf *et al*. 1996). Although auxin and ABA have opposite stomatal effects, cytosolic Ca^{2+} has long been suggested to mediate their guard cell transduction pathways (McAinsh *et al*. 1990, Cousson and Vavasseur 1998a), which has questioned specificity of such mediation. Studying animal systems has suggested that Ca^{2+} signalling specificity would result from amplitude and/or frequency modulation of cytosolic free Ca^{2+} oscillations (Dolmetsch *et al*. 1997), which has been confirmed in the case of stomatal movements (Allen *et al*. 2001). The pattern of guard cell Ca^{2+} oscillations depends on the ABA concentration (Staxen *et al*. 1999), which corroborates that endogenous ABA concentration discriminates between two guard cell Ca^{2+} mobilizations, one of them involving phosphoinositide-specific phospholipase C (PI-PLC) activity (Cousson and Vavasseur 1998b, Cousson 2003, 2007). Indeed, an aluminum-sensitive Columbia mutant, *als1*-1 (Larsen *et al*. 1996) has provided clear cut evidence for such discrimination (Cousson 2007). Accordingly, AtPLC1 (Hirayama *et al*. 1995) would be the sole Ca^{2+} mobilizing mediator at endogenous ABA concentrations resting below a drought-specific threshold value that should approximate 30 μM (Cousson 2008). Stomatal behaviour of the allelic Columbia mutant knockout for canonical GTP-binding protein (G protein) α-subunit (Gα) 1 (GPA1), *gpa1*-4 (Chen *et al*. 2006) has just shown that, beyond this threshold, putative GPA1-dependent ADP-ribosyl cyclase (ARC) activity should contribute to drought tolerance within PI-PLC-independent Ca^{2+}-mediated ABA signalling (Cousson 2009). Discriminating between two Ca^{2+} mobilizations has finally allowed to hypothesize that the *Arabidopsis* guard cell plasma membrane has an ABA perception and transduction complex comprising at least GPA1 and two Gα-like proteins coupled to a seven-transmembrane span-like receptors and linked to two effectors at least, among which are AtPLC1 and a yet to be identified ARC. Then, increasing exogenous ABA concentration from 20 to 30 μM would be sufficient to modulate coupling of both GPA1 and these Gα-like proteins to the ABA receptor and, consequently, to shift mediation of Ca^{2+} mobilization from AtPLC1 into putative ARC.

The stomatal closing mediator, inositol-1,4,5-triphosphate (IP_3) (Gilroy *et al*. 1990) originates from PI-PLC that hydrolyzes phosphatidylinositol-4,5-bisphosphate (Berridge 1993). Hunt *et al*. (2003) have immunolocalised a cytosolic free Ca^{2+}-activated PI-PLC in the tobacco guard cell. AtPLC1 is activated by Ca^{2+} (Sanchez and Chua 2001) and its molecular structure integrates a putative Ca^{2+}-interacting EF-hand domain (Mueller-Roeber and Pical 2002). Therefore, ABA-dependent AtPLC1 activity could depend not only on apoplastic Ca^{2+} entry but also on IP_3-mediated Ca^{2+} mobilization in the guard cell. Then, transduction of the ABA concentration signal could shape specific IP_3-mediated cytosolic free Ca^{2+} oscillations. Indeed, IP_3 gates plant endomembrane Ca^{2+} release channels (Allen *et al*. 1995, Sanders *et al*. 2002), which might result from binding to a coupled receptor that shares some homology with the animal IP_3 receptor (Berridge 1993). Accordingly, coordinated activities of inositol 5-phosphatase, PI-PLC and putative IP_3 receptor homologue could regulate kinetics of IP_3 turnover that is implicated in the control of ABA stomatal closing (Lee *et al*. 1996, Burnette *et al*. 2003). Another plant mediator of Ca^{2+} mobilization is the gene expression inducer, cyclic ADP-ribose (cADPR) that is synthesized by yet to be identified plant ARC isoform(s) (Allen *et al*. 1995, Wu *et al*. 1997, Leckie *et al*. 1998, Sanchez and Chua 2004). A Ca^{2+}-induced Ca^{2+} release process could implicate cADPR as an agonist for plant homologue(s) of the animal ryanodine receptor type II isoform (Galione *et al*. 1991, Galione 1994, Allen *et al*. 1995, Sethi *et al*. 1996, Muir *et al*. 1997, Cousson and Vavasseur 1998a, Leckie *et al*. 1998, Grabov and Blatt 1999, Sanders *et al*. 2002, Cousson 2004). Therefore, cADPR might interact positively with free Ca^{2+} of the guard cell cytosol as in the case of animal cells (Lee 1993) to shape cytosolic free Ca^{2+} oscillations whose frequency and amplitude, in turn, would be tightly regulated (Leckie *et al*. 1998).

Until recently, whether IP_3 and cADPR act in the same or parallel Ca^{2+}-dependent ABA signalling pathways has been unknown (Hunt *et al*. 2003). However, under simplified conditions of ABA stomatal closing bioassays (Cousson 2003, 2007, 2008, 2009), analysis has strongly suggested that Ca^{2+} mobilization does not involve any dependency between AtPLC1 and a putative ARC in the Columbia ecotype. Specific endomembrane receptors linked to guard cell Ca^{2+} release could be activated by IP_3 and cADPR. The present study questioned whether or not a yet to be identified IP_3 receptor and an homologue of the ryanodine receptor type II isoform induce separate ABA signalling steps downstream of Ca^{2+} mobilization. As previously suggested (Schroeder and Hagiwara 1989), implication of cytosolic free Ca^{2+} concentration within ABA-induced activation of the slow-type (S-type) anion channel has been finally shown at the plasma membrane of *Arabidopsis* guard cells (Wang *et al*. 2001). It was tempting to integrate the Ca^{2+}-dependent protein kinase (CDPK) isoforms, CPK3 and CPK6 within this Ca^{2+} mediation. Indeed, electrophysiology has shown that, unlike most of the guard cell Ca^{2+} sensors (Guo *et al*. 2002, Sanders *et al*. 2002, Albrecht *et al*. 2003, Harper *et al*. 2004, Pandey *et al*. 2004), these kinases mediate ABA signal transduction by activating the plasma

membrane Ca^{2+} inward- and anion outward-rectifying channels (Mori *et al*. 2006). However, this last study has reported stomatal movement bioassays whose data did not clearly fit in with any of the three models established from patch-clamp analyses of guard cell protoplasts. Indeed, partial and additive inhibition of 10 µM ABA-induced stomatal closure by *cpk3* and *cpk6* loss-of-function mutations (Mori *et al*. 2006) would suggest that CPK3 and CPK6 act in parallel Ca^{2+}-dependent branches of guard cell ABA signal transduction. Nevertheless, when voltage-clamped in the presence of 50 µM ABA, guard cell protoplasts of the *cpk3*, *cpk6* and *cpk3cpk6* mutants have shown complete inhibition of both the Ca^{2+} inward-rectifier and the S-type anion channel, suggesting that CPK3 and CPK6 act in the same Ca^{2+}-dependent ABA signalling cascades (Mori *et al*. 2006). These discrepancies urged us to question whether or not exogenous ABA concentration differentially implicates CPK3 and CPK6 to close the stomata.

It was attempted here to get stronger evidence for two Ca^{2+}-dependent mediations of ABA stomatal closing. Leaf abaxial epidermal peels deprived of mesopyll cells were routinely obtained in the Columbia genetic background from the wild type, the dexamethasone (Dex)-inducible *AtPLC1* antisense transgene, the *cpk3*-1 (SALK_107620), *cpk6*-1 (SALK_093308), *cpk3*-1*cpk6*-1 and *atrbohD/F* T-DNA insertional mutants. This last *Arabidopsis* mutant was included into the bioassays because the *atrbohD/F* loss-of-function double mutation completely impairs ABA promotion of H_2O_2 synthesis (Pei *et al*. 2000) and, in turn, apoplastic Ca^{2+} entry into the guard cell (Kwak *et al*. 2003). All these materials were tested in a pharmacological comparison of stomatal closing in response to exogenous 20 or 30 µM ABA that used compounds known to modulate either IP_3- or cADPR-induced Ca^{2+} mobilization, the intracellular free Ca^{2+} concentration, or membrane transport of organic and inorganic anions.

Materials and methods

Plants: Seeds of all plants were homozygous in the *Arabidopsis thaliana* (L.) Heynh. cv. Columbia genetic background. The *atrbohD/F* double mutant had been obtained from crosses between T-DNA insertional mutants of two guard cell-expressed *NADPH oxidase* genes, *AtrbohD* (*Arabidopsis* gene identifier number [AGI No.] At5g47910) and *AtrbohF* (AGI No. At1g64060) (Kwak *et al*. 2003). The allelic *cpk3*-1 and *cpk6*-1 mutants had been respectively obtained by T-DNA insertion into the guard cell-expressed *CDPK* genes, *CPK3* (AGI No. At4g23650) and *CPK6* (AGI No. At2g17290), and the *cpk3*-1*cpk6*-1 double mutant had been obtained from crosses between these single mutants (Mori *et al*. 2006). A Dex-inducible transgenic line carrying the *AtPLC1* antisense transgene had been 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 plasmid pTA211 (Sanchez and Chua 2001) derived from the pPZP vector (Hajdukiewicz *et al*. 1994), 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).

The seeds 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 of the nutrient solution containing 2.0 mM KNO_3, 1.1 mM $MgSO_4$, 805 µM $Ca(NO_3)_2$, 695 µM KH_2PO_4, 60 µM K_2HPO_4, 20 µM Na_2EDTA, 20 µM $FeSO_4$, 9.2 µM H_3BO_3, 3.6 µM $MnSO_4$, 3.0 µM $ZnSO_4$, 0.8 µM $CuSO_4$, and 74 nM $(NH_4)_6Mo_7O_{24}$. Then, the seedlings were grown under normal air in pots (65 × 65 × 70 mm) of moistened coarse sand and watered one time a day with the nutrient solution. The plant material was cultured at 22 °C and 70 % relative humidity under a 8-h photoperiod and irradiance of 250 µmol m^{-2} s^{-1} (150 W mercury lamps - *HQI-TS, Osram*, München, Germany).

Bioassays with epidermal peels: Leaf abaxial epidermis with stomatal guard cells was peeled from four- to five-week-old plants. The leaves were harvested at the end of the night period. For each comparative experiment, epidermal strips (up to 10 × 5 mm) were obtained from the same leaf by placing the abaxial epidermis cuticule side-down on microscope slides covered with the *Dow Corning 355* medical adhesive silicone (*Vermed laboratory*, Neuilly-en-Thelle, France). Then, most of the green tissues were gently removed from each epidermal strip by using another microscope slide. Two epidermal peels per treatment were immersed in 10 cm^3 incubation medium.

Stomatal closure in response to ABA was assayed starting with stomatal apertures ranging from 3.5 to 5.2 µm, approximatively. 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, irradiance continued for 2 h in the presence of 20 or 30 µM ABA (*Sigma*). Since CO_2 in normal air has been shown to interfere on ABA-induced stomatal closing response in *A. thaliana* (Leymarie *et al*. 1998), the incubation medium was bubbled throughout the experiments with CO_2-free air at a rate of 33 cm^3 min^{-1}, which was obtained by passing dry air over sodalime

(*Soda Asbestos, Prolabo,* Paris, France). It was verified that methanol, in which ABA was dissolved, did not change stomatal aperture.

To investigate the Ca^{2+} dependence of ABA stomatal closing, cytosolic free Ca^{2+} of the guard cell was buffered by adding separately the plant Ca^{2+} chelators EGTA and BAPTA (*Sigma*) (Armstrong and Blatt 1995) to the incubation medium throughout experiments. Depending on the experiments, EGTA and BAPTA were tested at 0.75 or 1.50 mM concentrations. The EGTA (50 mM) and BAPTA (50 mM) stock solutions contained a significant amount of K^+. The control incubation medium contained potassium iminodiacetate (*Sigma*) 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 PI-PLC-dependent IP_3 production within ABA stomatal closing, two compounds were tested: the aminosteroid, 1,6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl -1H-pyrrole-2,5-dione (U73122 ; *Biomol Research Laboratories*, Plymouth, UK) and the bis-1-oxaquinolizidine isolated from the sponge *Xestospongia*, xestospongin C (XeC; *Calbiochem*), known to inhibit the activity of PI-PLC (Thompson *et al*. 1991) and the endomembrane IP_3 receptor (Gafni *et al*. 1997) in animal cellular systems, respectively.

To investigate the possible implication of Ca^{2+}- and cADPR-induced intracellular Ca^{2+} release within ABA stomatal closing, ruthenium red (RRed ; *Sigma*) was tested, which is known to inhibit such a Ca^{2+} release that processes from animal ryanodine receptor type II isoform (Galione *et al*. 1991, Galione 1994) as it could be the case for plant cells (Allen *et al*. 1995, Muir *et al*. 1997, Cousson and Vavasseur 1998a).

To investigate possible implication of both the S-type anion channel and a multidrug resistance-associated protein (MRP) isoform within ABA stomatal closing, the following compounds were tested: anthracene-9-carboxylic acid (9-AC; *Sigma*), probenecid (Proben; *Sigma*) and methotrexate (MTX; *Sigma*), known to inhibit plant channel-mediated efflux currents of inorganic anions (Marten *et al*. 1992, Schwartz *et al*. 1995) and/or ATP-dependent MRP5-mediated uptake of 5'-fluoro-2'-deoxyuridine monophosphate in human cellular systems (Pratt *et al*. 2005).

All these compounds were added separately or in combination to the incubation medium, throughout the experiments. Among them, U73122, XeC and 9-AC were dissolved in DMSO (*Sigma*), and control incubation media contained this solvent at the same concentrations (up to 1 %, v/v) as these of the U73122-, XeC- or 9-AC-containing incubation media.

Data analysis: The viability of the guard cells was verified by staining the epidermal peels with neutral red at the end of each treatment. Stomata without underlying mesophyll were used for measurement of the stomatal aperture. Only stomata, of which the ostiole length was higher than one-third of the stomatal length, were examined. Stomatal apertures were measured with an optical microscope (*Optiphot, Nikon*, Tokyo, Japan) fitted with a camera and a digitizing table (*Houston Instrument*, Austin, Texas, USA). For each epidermal peel, 150 stomatal apertures were measured. In experiments conducted without exogenous ABA, each datum point represented the mean of 150 stomatal apertures with the confidence limits to the mean for $\alpha = 0.05$ [mean $\pm$ $t_{0.05}$ $\times$ SE (where $t_{0.05}$ is Student's *t*-value for $\alpha = 0.05$)]. In experiments conducted with exogenous ABA, the ABA stomatal closing response was evaluated by comparing two epidermal peels, one peel being examined just before adding ABA, and the other peel being examined 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 adding ABA. All the experiments were independently repeated at least three times.

Results and discussion

The *cpk3*-1 and *cpk6*-1 mutations differentially affect ABA stomatal closure: The 20 µM ABA-induced stomatal closure was decreased in *cpk3*-1 mutant from 2.95 to 0.88 µm (about 70 % inhibition) without changing stomatal closure in response to 30 µM ABA (Table 1). By contrast, in the *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants the stomatal closure induced by 20 µM ABA was approximately decreased from 2.95 to 1.80 µm (about 40 % inhibition), whereas 30 µM ABA-induced stomatal closure was decreased from 3.12 to 0.95 µm (about 70 % inhibition).

Therefore, under the tested experimental conditions, the exogenous ABA concentration discriminated between CPK3 and CPK6 within Ca^{2+}-mediated guard cell ABA signalling. However, when 20 µM ABA was applied, the partial inhibitory effects of the *cpk3*-1 and *cpk6*-1 loss-of-function mutations were not additive, which excluded that CPK3 and CPK6 act in parallel Ca^{2+}-dependent ABA signalling branches. Furthermore, disrupting CPK6 alone or together with CPK3 similarly decreased 20 µM ABA-induced stomatal closure, which suggested a CPK6 requirement upstream of CPK3 as much as disrupting CPK3 alone decreased this stomatal response to a greater extent. Interestingly, Cousson (2008) has shown that Ca^{2+}-chelating EGTA treatment does not increase inhibition of 20 µM ABA-induced stomatal closure obtained by applying 3 nM of the PLC inhibitor, U73122, although EGTA alone is more inhibitory. Two other

parallels were observed, which took into account previous studies. First, disrupting CPK6 roughly decreased 20 µM ABA-induced stomatal closure to the same extent (about 40 % inhibition) as silencing *AtPLC1* (Cousson 2008), applying 3 nM U73122 (Cousson 2007, 2008, 2009) or disrupting both the AtrbohD and AtrbohF NADPH oxidases (Cousson 2009). Second, disrupting CPK3 did not affect 30 µM ABA-induced stomatal closure but inhibited 20 µM ABA-induced stomatal closure by about 70 %, as previously reported for the EGTA inhibitory effect (Cousson 2007, 2008). Thus, emerged the possibility that Ca^{2+}-dependent stomatal response to 20 µM ABA sequentially integrates activation of CPK6, AtPLC1 and CPK3.

Table 1. The stomatal closure in response to 20 or 30 µM ABA was differentially inhibited in the *cpk3*-1 and *cpk6*-1 mutants, and *cpk3*-1*cpk6*-1 double mutant of *A. thaliana*. Abaxial epidermal peels were incubated under irradiance of 250 µmol m^{-2} s^{-1} and CO_2-free air and 3 h after starting the experiments, ABA was added. For each experiment, the stomatal response was established by examining two epidermal peels (150 stomatal apertures measured for each peel), one peel just before applying ABA and the other one 2 h after applying ABA. ABA-induced stomatal closure was taken as the difference between the stomatal apertures measured just before and 2 h after applying ABA. Results are means ± SE established from three independent replicates at least.

ABA [µM]	ABA-induced stomatal closure [µm] wild type	*cpk3*-1	*cpk6*-1	*cpk3*-1*cpk6*-1
20	2.95 ± 0.18	0.88 ± 0.15	1.75 ± 0.19	1.81 ± 0.20
30	3.12 ± 0.19	3.05 ± 0.16	0.98 ± 0.18	0.91 ± 0.21

The lack of additive inhibitory effects of the *cpk3*-1 and *cpk6*-1 mutations contrasted with effects of these mutations previously recorded (Mori *et al*. 2006). Procedure used then for examining stomatal movement bioassays could have been rather artefactual since stomatal aperture has been measured from guard cell fractions obtained by an abrasive treatment of leaves transiently bathed with both Ca^{2+} and ABA (Pei *et al*. 1997, Mori *et al*. 2006). By contrast, here was avoided most artefacts since leaf abaxial epidermis was peeled without underlying mesophyll and always bathed without exogenous Ca^{2+} until stomatal aperture was directly and *in situ* measured. As previously suggested (Mori *et al*. 2006), CPK6 could favour apoplastic Ca^{2+} entry into the guard cell (Kwak *et al*. 2003) through stimulating ABA promotion of H_2O_2 synthesis, which, in turn, could activate Ca^{2+}-dependent AtPLC1. This agreed with parallel inhibition of 20 µM ABA-induced stomatal closure by the *cpk6*-1 (Table 1) and *atrbohD/F* (Cousson 2009) loss-of-function mutations because disrupting both the AtrbohD and AtrbohF NADPH oxidases completely impairs ABA-induced H_2O_2 production (Kwak *et al*. 2003). Accordingly, the different inhibitory percentages obtained by EGTA (70 %) or each of these two mutations (40 %) outlined that the Ca^{2+} buffering treatment might have an efficiency different from that of these mutations to prevent apoplastic Ca^{2+} entry. As for CPK3, this CDPK could sense oscillations in cytosolic free Ca^{2+} concentration resulting from IP_3-induced Ca^{2+} mobilization. Accordingly, similar decrease in 20 µM ABA-induced stomatal closure obtained by the *cpk3*-1 mutation and the EGTA treatment (70 % inhibition) suggested that, under the tested bioassay conditions, applying 1.5 mM EGTA should have efficiently buffered IP_3-induced Ca^{2+} mobilization.

The exogenous ABA concentration influences overlapping effects of Ca^{2+} flux modulators: The inhibitors, XeC and RRed were tested to discriminate between putative guard cell homologues of the animal IP_3 receptor and ryanodine receptor type II isoform. In animals, XeC is, indeed, highly selective over ryanodine receptors, whereas other blockers of the IP_3 receptor, heparin and caffeine activate ryanodine receptors (De Smet *et al*. 1999). In animals and plants, RRed selectively blocks cADPR-induced Ca^{2+} mobilization, which proceeds from cADPR-activated ryanodine receptor type II isoform in animals (Galione *et al*. 1991, Galione 1994, Allen *et al*. 1995, Muir *et al*. 1997, Sanders *et al*. 2002).

Stomatal closure induced by 20 µM ABA was gradually decreased from 3.0 to 0.8 µm, approximately, by applying increased XeC concentrations from 1 to 50 nM (up to 70 % inhibition; Table 2). Accordingly, half inhibitory XeC concentration was about 5 nM that roughly inhibited 20 µM ABA-induced stomatal closure by 35 %. When 1.5 mM EGTA was applied alone or together with XeC, 20 µM ABA-induced stomatal closure kept constant at about 0.8 µm whether or not XeC was applied from 1 to 50 nM (Table 2). These data did not exclude *a priori* that the Ca^{2+} buffering treatment would prevent any of the tested XeC concentrations from inhibiting 20 µM ABA-induced stomatal closure. However, overlapping effects of EGTA and XeC should be considered since increasing XeC from 1 to 50 nM did not inhibit 30 µM ABA-induced stomatal closure (Table 2), as it has been shown for EGTA increasing up to 2 mM (Cousson 2003). On the contrary, stomatal closure induced by 30 µM ABA, but not by 20 µM ABA, was gradually affected up to 55 % inhibition by applying RRed from 10 to 50 µM: stomatal closure roughly decreased from 3.1 to 1.6 µm, which fitted in with a half inhibitory RRed concentration at about 30 µM corresponding to 30 % inhibition of 30 µM ABA-induced stomatal closure (Table 2). This partial inhibition could be overlapped by another Ca^{2+} chelator, BAPTA that, contrary to EGTA, had affected the stomatal response to 30 µM ABA (Cousson 2003). Indeed, when 1.5 mM BAPTA was applied alone or together with RRed, 30 µM ABA-induced stomatal closure kept constant at about

Table 2. ABA-induced stomatal closure of *A. thaliana* wild type as differentially affected by the intracellular Ca^{2+} release inhibitors, XeC and RRed. 1.5 mM of the Ca^{2+} buffer, EGTA or BAPTA was added or not to leaf abaxial epidermal peels incubated under irradiance of 250 µmol m^{-2} s^{-1} and CO_2-free air with or without increased concentrations of XeC or RRred and 3 h after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1.

XeC [nM]	RRed [µM]	ABA-induced stomatal closure [µm] 20 µM -EGTA	+EGTA	30 µM -BAPTA	+BAPTA
-	-	2.96 ± 0.18	0.85 ± 0.16	3.13 ± 0.16	0.95 ± 0.18
1	-	2.88 ± 0.21	0.89 ± 0.17	3.08 ± 0.15	1.01 ± 0.19
5	-	1.93 ± 0.22	0.82 ± 0.20	3.02 ± 0.17	0.99 ± 0.20
20	-	1.14 ± 0.20	0.87 ± 0.15	3.15 ± 0.15	0.97 ± 0.17
50	-	0.83 ± 0.17	0.86 ± 0.16	3.07 ± 0.14	0.92 ± 0.22
-	10	2.95 ± 0.18	0.83 ± 0.19	2.89 ± 0.17	0.95 ± 0.20
-	20	2.91 ± 0.17	0.85 ± 0.15	2.67 ± 0.19	0.98 ± 0.19
-	30	2.98 ± 0.16	0.79 ± 0.15	2.09 ± 0.20	1.02 ± 0.21
-	50	2.93 ± 0.18	0.77 ± 0.17	1.64 ± 0.22	0.91 ± 0.18

Table 3. Convergent effects of the intracellular Ca^{2+} modulators, EGTA and XeC on stomatal aperture are suppressed in T line (Dex-inducible *AtPLC1* antisense transgenic line incubated with 30 µM Dex throughout the experiments), *cpk3*-1 and *cpk6*-1 mutants of *A. thaliana*. 1.5 mM of the Ca^{2+} buffer, EGTA or BAPTA was added with or without 50 nM XeC to leaf abaxial epidermal peels and 3 h after starting the experiments, 150 stomatal apertures were measured for each peel. For detail see Table 1.

Pharmacological treatment [µM]	Stomatal aperture [µm] WT	T line	*cpk3*-1	*cpk6*-1
No treatment	4.39 ± 0.08	4.43 ± 0.11	5.09 ± 0.08	3.55 ± 0. 08
50 nM XeC	5.11 ± 0.13	4.40 ± 0.14	5.05 ± 0.12	3.60 ± 0. 11
1.5 mM EGTA	5.05 ± 0.12	4.39 ± 0.14	5.13 ± 0.11	3.57 ± 0. 11
1.5 mM BAPTA	3.83 ± 0.14	3.86 ± 0.15	3.82 ± 0.12	3.53 ± 0.11
50 nM XeC + 1.5 mM EGTA	5.19 ± 0.12	4.37 ± 0.15	5.10 ± 0.13	3.58 ± 0.12
50 nM XeC + 1.5 mM BAPTA	3.85 ± 0.13	3.80 ± 0.15	3.89 ± 0.13	3.50 ± 0.13

Table 4. Effects of the intracellular Ca^{2+} release inhibitors, XeC and RRed on ABA-induced stomatal closure in abaxial epidermal peels of T line, *cpk3*-1 and *cpk6*-1 mutants, and *atrbohD/F* double mutant of *A. thaliana*. Throughout the experiments, 50 nM XeC or 50 µM RRed was added or not and 3 h after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1.

Treatment	ABA-induced stomatal closure [µm] T line	*atrbohD/F*	*cpk3*-1	*cpk6*-1
20 µM ABA	1.91 ± 0.24	1.88 ± 0.17	0.90 ± 0.16	1.86 ± 0.19
20 µM ABA + 50 nM XeC	1.86 ± 0.26	1.92 ± 0.15	0.86 ± 0.20	1.89 ± 0.17
20 µM ABA + 50 µM RRed	1.85 ± 0.23	1.87 ± 0.18	0.86 ± 0.17	1.83 ± 0.20
30 µM ABA	3.10 ± 0.15	0.96 ± 0.18	3.12 ± 0.16	0.95 ± 0.16
30 µM ABA + 50 nM XeC	3.15 ± 0.18	0.93 ± 0.15	3.16 ± 0.19	0.97 ± 0.19
30 µM ABA + 50 µM RRed	1.63 ± 0.23	0.98 ± 0.20	1.67 ± 0.22	0.99 ± 0.17

0.9 µm (70 % inhibition) whether or not RRed was applied from 10 to 50 µM (Table 2). None of the tested materials discriminated between the XeC and EGTA effects, reinforcing that EGTA would overlap the stomatal XeC effect. Indeed, applying 30 µM Dex to epidermal peels of the Dex-inducible *AtPLC1* antisense Columbia transgene as well as mutating *CPK3* or *CPK6* suppressed the convergent XeC and EGTA effects shown by pharmacological manipulation of the wild type (Table 3). In particular, it was outlined that applying 50 nM XeC and/or 1.5 mM EGTA to wild type epidermis opened more the stomata to reach the aperture (about

5.1 µm) measured in the *cpk3*-1 mutant. By contrast, applying 1.5 mM BAPTA alone or together with XeC decreased stomatal aperture at about 3.8 µm in the wild type and the other tested plant materials except the *cpk6*-1 mutant, for which none of the above mentioned pharmacological treatments changed stomatal aperture kept constant at about 3.5 µm (Table 2). Such discriminating inhibitory effects were confirmed in the Dex-inducible *AtPLC1* antisense transgene and the *cpk3*-1 mutant that kept unchanged the RRed effect, whereas the XeC effect was suppressed in these materials (Table 4). However, the XeC and RRed effects were suppressed in the *atrbohD/F* and *cpk6*-1 mutants. Furthermore, these two mutants paralled each other to decrease 20 µM ABA- or 30 µM ABA-induced stomatal closure by about 1.1 µm (40 % inhibition) or 2.1 µm (70 % inhibition), respectively (Table 2 compared to Table 4), which confirmed data previously obtained (Cousson 2009) and above mentioned (Table 1).

It was assumed that the *atrbohD/F* and *cpk6*-1 mutations suppress ABA-induced Ca^{2+} mediation through blocking NADPH oxidase-dependent H_2O_2-mediated entry of apoplastic Ca^{2+} (Kwak *et al*. 2003, Mori *et al*. 2006). Accordingly, it was deduced that XeC and RRed inhibit two different processes depending on apoplastic Ca^{2+} entry into the guard cell. *A priori,* BAPTA and RRed might interfere with apoplastic Ca^{2+} entry itself since RRed could inhibit Ca^{2+} inward-rectifying channels at plant plasma membranes (Pineros and Tester 1997) and BAPTA has mimicked the *atrbohD/F* mutation to decrease 30 µM ABA-induced stomatal closure (Cousson 2009). However, BAPTA and EGTA have inhibited 20 µM ABA-induced stomatal closure by 70 % (Cousson 2007). In comparison, the *atrbohD/F* and *cpk6*-1 mutations inhibited the response to 20 µM ABA only by 40 % (Table 1 compared with Table 4). Therefore, although BAPTA should buffer cytosolic free Ca^{2+} more efficiently than EGTA (Armstrong and Blatt 1995), it would not suppress any Ca^{2+} rise that results from NADPH oxidase-dependent H_2O_2-mediated entry of apoplastic Ca^{2+}. Furthermore, nicotinamide would have partially overlapped BAPTA inhibition of the stomatal response to 30 µM ABA (Cousson 2008) as suggested here for RRed (Table 2), suggesting that each of these three compounds interferes with cADPR-induced Ca^{2+} mobilization of the guard cell. On one hand, indeed, nicotinamide competitively blocks ARC activity (Sethi *et al*. 1996) within cyclization of NAD into cADPR (Howard *et al*. 1993, Aarhus *et al*. 1995) and, on the other hand, applying 30 µM RRed to animal or plant systems half inhibits cADPR-induced Ca^{2+} mobilization (Galione 1994, Allen *et al*. 1995). This last feature corroborated the fact that 30 µM RRed inhibited 30 µM ABA-induced stomatal closure by about 35 %, whereas 1.5 mM BAPTA doubled the percentage of inhibition (Table 2).

Possible convergence of XeC, EGTA and the *cpk3*-1 mutation to inhibit 20 µM ABA-induced stomatal closure was likely relevant of inhibiting a transduction process that implicates AtPLC1 activation by a cytosolic free Ca^{2+} rise. Indeed, the XeC effect was suppressed in the *atrbohD/F* mutant or by manipulating the Dex-inducible *AtPLC1* antisense transgene so that *AtPLC1* silencing was likely achieved (Cousson 2008, Sanchez and Chua 2001) (Table 2 compared to Table 4). Likewise, these materials have suppressed the inhibitory effect of a Ca^{2+} buffering treatment (Cousson 2008, 2009). Moreover, they affected the stomatal response to 20 µM ABA by a lower percentage of inhibition in comparison with XeC or EGTA (Tables 2 and 4). To corroborate such possibilities, 50 nM XeC was applied to the *CPK3* and *CPK6* single and double mutants alone or together with 3 nM of the PI-PLC inhibitor, U73122 (Thompson *et al*. 1991) that has contributed to distinguish AtPLC1 as the Ca^{2+}-mobilizing mediator of the stomatal response to 20 µM ABA (Cousson 2008, 2009). Stricking features were revealed (Table 5). First, U73122 with or without XeC increased 20 µM ABA-induced stomatal closure of the *cpk3*-1 mutant from 0.85 to 1.90 µm (40 % stimulation) that approximated the stomatal responses of the *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants (U73122 uneffectiveness) and the wild type (40 % inhibition). Next, XeC decreased 20 µM ABA-induced stomatal closure of the wild type from 2.97 to 0.80 µm (70 % inhibition) that approximated the stomatal response of the *cpk3*-1 mutant (XeC uneffectiveness). At last, XeC did not change the stomatal response of the *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants.

As in the case of RRed, these results strongly suggested that XeC interferes with guard cell Ca^{2+} mobilization. However, XeC is a potent inhibitor of both the Ca^{2+}-pumping ATPases and IP_3 receptor at the endoplasmic reticulum membrane of animal cells (De Smet *et al*. 1999). Regarding to this, XeC uneffectiveness observed on 30 µM ABA-induced stomatal closure (Table 4) showed that any Ca^{2+} leak towards the cytosol would not have counteracted Ca^{2+} mobilization. Accordingly, parallel inhibitions of 20 µM ABA-induced stomatal closure by XeC and EGTA should be respectively explained by a blockage of an endomembrane IP_3 receptor-like and an efficient buffering of IP_3-induced Ca^{2+}mobilization. Therefore, the obtained results strongly suggested that CPK6 operates upstream of Ca^{2+}-activated AtPLC1 and IP_3 production, whereas CPK3 operates downstream of IP_3-induced Ca^{2+} mobilization whose triggering implicates an endo-membrane IP_3 receptor homologue within the stomatal response to 20 µM ABA.

The Ca^{2+} flux modulators, XeC and EGTA parallel each other to positively interact with the S-type anion channel blocker, 9-AC: Diverse approaches have suggested that the S-type anion channel of the guard cell is implicated within the ABA stomatal response as a

target for Ca^{2+} signalling (Schroeder and Hagiwara 1989, Grabov *et al*. 1997, Pei *et al*. 1997, Wang *et al*. 2001). In the monocot *Commelina communis*, Cousson (1999) has suggested that, according to the endogenous ABA concentration, different targets coexist within molecular structure of the S-type anion channel that specifically decode oscillatory patterns of cytosolic free Ca^{2+}. Then, it was possible that CPK3 mediates such a decoding process in response to exogenously applying 20 µM ABA, whereas a yet to be identified Ca^{2+} sensor would be implicated within another Ca^{2+} decoding at the S-type anion channel in response to 30 µM ABA. This was tested as following. In the wild type, it was investigated whether XeC or EGTA could interact positively with S-type anion channel blockers to prevent 20 µM ABA from closing the stomata. If any interaction was revealed, similar investigation would be carried out with the other materials to verify that no kind of possible interaction does occur then between these compounds, which should be expected since the different genetic materials were not sensitive to 50 nM XeC or 1.5 mM EGTA (Tables 3, 4 and 5). Probenecid (Proben) and anthracene-9-carboxylic acid (9-AC) were choosen as anion channel blockers since only 9-AC has inhibited anion efflux currents from *Arabidopsis thaliana* guard cells in electrophysiological experiments that have tested a large set of available blockers except Proben (Forestier *et al*. 1998).

When 9-AC was applied 3 h prior to 20 µM ABA in the wild type, two kinds of inhibitory effect were observed: increasing 9-AC in the 0.5 - 10.0 and 50 - 100 µM ranges respectively decreased ABA stomatal closure from 3.0 to 0.8 µm, and from 0.8 to 0.0 µm (Table 6). Increasing the applied ABA concentration from 20 to 30 µM changed the 9-AC effect since it was observed only beyond 10 µM 9-AC: then, increasing 9-AC in the 10 - 100 µM range roughly decreased ABA stomatal closure from 3.1 to 0.0 µm. Substituting Proben for 9-AC within the same protocol resulted in a somewhat different

Table 5. Effects of the inhibitors, U73122 and XeC on 20 µM ABA-induced stomatal closure in wild type and *cpk3*-1, *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants of *A. thaliana*. Leaf abaxial epidermal peels were incubated or not with the PLC inhibitor, U73122 and the intracellular Ca^{2+} release inhibitor, XeC added separately or in combination and 3 h after starting the experiments, ABA was added. For detail see Table 1.

Pharmacological treatment	20 µM ABA-induced stomatal closure [µm]			
	wild type	*cpk3*-1	*cpk6*-1	*cpk3*-1*cpk6*-1
No treatment	2.97 ± 0.16	0.85 ± 0.18	1.86 ± 0.19	1.90 ± 0.18
3 nM U73122	1.83 ± 0.21	1.90 ± 0.23	1.84 ± 0.23	1.85 ± 0.21
50 nM XeC	0.80 ± 0.19	0.92 ± 0.20	1.90 ± 0.21	1.92 ± 0.23
3 nM U73122+ 50 nM XeC	1.85 ± 0.23	1.89 ± 0.22	1.87 ± 0.23	1.88 ± 0.21

Table 6. Differential interactions between S-type anion channel blockers (9-AC and Proben) and Ca^{2+} flux modulators (XeC, EGTA, RRed and BAPTA) on inhibition of ABA-induced stomatal closure in *A. thaliana* (L.) Heynh. cv. Columbia. 9-AC or Proben were added or not to leaf abaxial epidermal peels incubated with either 5 nM XeC, 0.75 mM EGTA, 30 µM RRed or 0.75 mM BAPTA. Control was performed without any Ca^{2+} flux modulator. 3 h after starting the experiments, 20 or 30 µM ABA was added. For detail see Table 1. * - These compounds have the same effects.

9-AC [µM]	Proben [µM]	ABA-induced stomatal closure [µm] 20 µM control	+XeC (or EGTA)*	30 µM control	+RRed (or BAPTA)*
-	-	3.02 ± 0.18	1.95 ± 0.16	3.12 ± 0.21	2.09 ± 0.21
0.5	-	3.05 ± 0.21	2.02 ± 0.17	3.18 ± 0.19	2.12 ± 0.19
1	-	2.78 ± 0.20	1.30 ± 0.20	3.22 ± 0.17	2.07 ± 0.23
3	-	2.48 ± 0.23	0.92 ± 0.15	3.05 ± 0.20	2.04 ± 0.20
10	-	0.88 ± 0.19	0.83 ± 0.16	3.12 ± 0.23	2.07 ± 0.22
30	-	0.95 ± 0.21	0.85 ± 0.19	1.78 ± 0.25	0.90 ± 0.23
50	-	0.89 ± 0.22	0.87 ± 0.20	1.17 ± 0.20	0.87 ± 0.20
100	-	0.09 ± 0.19	0.02 ± 0.16	0.14 ± 0.19	0.06 ± 0.21
-	20	2.98 ± 0.18	1.94 ± 0.22	3.15 ± 0.19	2.13 ± 0.25
-	50	1.98 ± 0.21	0.93 ± 0.19	2.09 ± 0.23	1.05 ± 0.22
-	70	1.46 ± 0.24	0.95 ± 0.22	1.57 ± 0.17	1.07 ± 0.19
-	100	0.95 ± 0.23	0.99 ± 0.18	1.04 ± 0.24	1.08 ± 0.21
-	200	0.03 ± 0.18	0.17 ± 0.21	0.04 ± 0.15	0.11 ± 0.18

inhibition of ABA stomatal closure since the inhibitory effect was similar on the stomatal responses to 20 and 30 μM ABA, and observed only beyond 20 μM Proben. Increasing Proben concentration from 20 to 200 μM decreased ABA-induced stomatal closure from 3 to 0 μm (Table 6).

Whether XeC or EGTA could interact with 9-AC or Proben to decrease 20 μM ABA-induced stomatal closure was investigated by testing half inhibitory concentration of XeC (5 nM) or EGTA (0.75 mM) together with increasing concentrations of 9-AC or Proben at the start of the experiments. It was examined how suboptimal concentrations of 9-AC or Proben would influence the effect (35 % inhibition) of 5 nM XeC or 0.75 mM EGTA. In the presence of XeC or EGTA, increasing 9-AC from 0.5 to 1.0 or 3.0 μM decreased the stomatal response induced by 20 μM ABA from 2.0 to 1.3 or 0.9 μm, respectively (Table 6). When XeC or EGTA was not applied, similar enhancement in 9-AC concentration slightly decreased 20 μM ABA-induced stomatal closure from 3.0 to about 2.8 (8 % inhibition) or 2.5 μm (25 % inhibition), respectively (Table 6). Thus, applying 9-AC at 1 or 3 μM enhanced the XeC or EGTA effect in a stronger fashion than an additive one: these 9-AC concentrations increased the overall inhibition from 35 to 58 or 70 %. By contrast, the suboptimal Proben concentrations 20 and 50 μM enhanced the XeC or EGTA effect in an additive or weaker than additive fashion (Table 6).

Therefore, half inhibitory concentration of XeC or EGTA positively interacted with the suboptimal 9-AC concentrations, 1 and 3 μM to inhibit 20 μM ABA-induced stomatal closure. This interaction was suppressed in both the Dex-inducible *AtPLC1* antisense Columbia transgene and mutants knockout for CPK3 and/or CPK6 (Table 7). In the NADPH oxidase *atrbohD/F* mutant, 9-AC inhibited 20 μM ABA-induced stomatal closure only at concentrations increasing beyond 40 μM, as observed in the other materials (Table 8). Therefore, the effect of 1 and 3 μM 9-AC and its positive interaction with XeC or EGTA were relevant of inhibiting a Ca^{2+} signal transduction that would sequentially involve the AtrbohD and AtrbohF NADPH oxidases, CPK6, AtPLC1, a putative endomembrane IP_3 receptor homolog, CPK3 and the S-type anion channel. Also, it was assumed that IP_3-induced Ca^{2+} mobilization specifically activates CPK3 and, in turn, up-regulates the S-type anion channel. Thus, CPK3 could phosphorylate specific aminoacid (s) of the S-type channel protein and, in turn, induce a conformational change allowing large anion extrusion at the guard cell plasma membrane. In agreement, tight activation or deactivation of the S-type anion channel by phosphorylating or dephosphorylating processes would mediate or inhibit ABA stomatal closing in *Vicia faba* (Schmidt *et al*. 1995). Furthermore, the fact that, in *Arabidopsis*, the protein phosphatase inhibitor, okadaic acid has inhibited ABA stomatal closing (Pei *et al*. 1997) does not disagree with this possibility since a similar okadaic acid treatment has phenocopied disruption of a guard cell protein phosphatase 2A regulatory subunit, RCN1 that operates at early ABA signalling steps upstream of anion efflux activation (Kwak *et al*. 2002).

Using antagonists of the Ca^{2+}-calmodulin complex formation, parallel investigations in *Commelina communis* have not excluded that, according to the endogenous ABA concentration, one Ca^{2+}-calmodulin complex-dependent protein kinase or one CDPK at least would induce the S-type anion channel to decode specific oscillations in free cytosolic Ca^{2+} concentration (Cousson and Vavasseur 1998a, Cousson 1999). In *Arabidopsis thaliana* cv. Columbia, the present study gave evidence that, depending on the endogenous ABA concentration, guard cell IP_3- or cADPR-gated Ca^{2+} channels would mobilize Ca^{2+} - *via* separate activation of yet to be identified endomembrane homologues for, respectively, the IP_3 receptor and the ryanodine receptor type II isoform. As extensively argued above, this separate activation should shape two different oscillatory patterns in free cytosolic Ca^{2+} concentration. Accordingly, CPK3

Table 7. Uneffectiveness of the intracellular Ca^{2+} release inhibitor, XeC and the slow anion channel blocker, 9-AC on 20 μM ABA-induced stomatal closure in T line, *cpk3*-1, *cpk6*-1 and *cpk3*-1*cpk6*-1 mutants of *A. thaliana*. XeC and 9-AC were applied separately or in combination to leaf abaxial epidermal peels and after 3 h of experiment, 20 μM ABA was added. For detail see Table 1.

Pharmacological treatment	20 μM ABA-induced stomatal closure [μm]			
	T line	*cpk3*-1	*cpk6*-1	*cpk3*-1*cpk6*-1
No treatment	1.89 ± 0.24	0.83 ± 0.21	1.88 ± 0.17	1.85 ± 0.20
5 nM XeC	1.87 ± 0.27	0.91 ± 0.18	1.86 ± 0.18	1.91 ± 0.16
50 nM XeC	1.84 ± 0.25	0.85 ± 0.20	1.93 ± 0.21	1.83 ± 0.22
1 μM 9-AC	1.90 ± 0.22	0.89 ± 0.19	1.83 ± 0.16	1.87 ± 0.21
10 μM 9-AC	1.92 ± 0.24	0.86 ± 0.17	1.82 ± 0.19	1.83 ± 0.14
5 nM XeC + 1 μM 9-AC	1.89 ± 0.25	0.87 ± 0.19	1.95 ± 0.21	1.88 ± 0.22
50 nM XeC + 10 μM 9-AC	1.85 ± 0.23	0.81 ± 0.21	1.82 ± 0.20	1.85 ± 0.21

activation of S-type anion efflux currents within 20 µM ABA-induced stomatal closing does not exclude that another Ca^{2+}-dependent phosphorylation - *via* another possible CDPK - could mediate activation of S-type anion efflux currents when 30 µM ABA is applied. Indeed, the functional divergence in the EF hands of different calmodulin-like regulatory domains makes possible that different CDPKs sense and respond to different Ca^{2+} oscillatory patterns (Sanders *et al*. 2002, Harper *et al*. 2004). However, CPK6 does not phosphorylate the S-type anion channel in response to 30 µM ABA-dependent Ca^{2+} mobilization. Indeed, if such a phosphorylation occurred, it would sustain large depolarization of the plasma membrane, and, in turn, down-regulate apoplastic Ca^{2+} entry that is evoked only at hyperpolarized voltage, whereas the *cpk6*-1 mutation mimicked the *atrbohD/F* mutation to inhibit the ABA stomatal response - *via* abolishment of Ca^{2+} entry into guard cell protoplasts (Kwak *et al*. 2003, Mori *et al*. 2006). Since the phosphorylation substrate itself significantly changes Ca^{2+}sensing of several CDPKs (Lee *et al*. 1998), one could speculate that different oscillatory patterns in cytosolic free Ca^{2+} would induce some guard cell CDPKs to switch off phosphorylation of a protein and, in turn, switch on phosphorylation of another protein. Accordingly, it was not excluded that, beyond 30 µM, exogenous ABA could induce CPK3 to phosphorylate no more the S-type anion channel but another protein that up-regulates the hyperpolarization-evoked Ca^{2+}-inward rectifier, as suggested by whole-cell voltage clamping of *cpk3*-1 guard cell protoplasts bathed with 50 µM ABA (Mori *et al*. 2006).

The Ca^{2+} flux modulators, XeC, RRed, EGTA and BAPTA negatively interact with the S-type anion channel blockers, 9-AC and Proben: To analyze further the inhibitory effects of 9-AC and Proben, these compounds were tested in the wild type at suboptimal concentrations alone or with a suboptimal concentration of the Ca^{2+} flux modulators, RRed or BAPTA that inhibited *per se* the stomatal response to 30 µM ABA by about 35 %. Thus, it was examined how 9-AC or Proben would influence inhibition of 30 µM ABA-induced stomatal closure by 30 µM RRed or 0.75 mM BAPTA (Table 6). In the presence of RRed or BAPTA, increasing 9-AC from 10 to 30 µM roughly decreased 30 µM ABA-induced stomatal closure from 2.1 to 0.9 µm. When RRed or BAPTA was not applied, similar enhancement in 9-AC concentration roughly decreased 30 µM ABA-induced stomatal closure from 3.1 to 1.8 µm (about 43 % inhibition). Since applying 30 µM 9-AC increased the overall inhibition from 35 to 70 %, these 9-AC concentrations enhanced in a weaker than an additive fashion the RRed or BAPTA effect on 30 µM ABA-induced stomatal closure. Similar features were obtained with exogenous Proben concentrations increasing in the 20 - 70 µM range.

Thus, discrete 9-AC or Proben concentrations enhanced the RRed or BAPTA effect in a weaker than additive fashion leading overall inhibition of 30 µM ABA-induced stomatal closure to reach a plateau approximating 70 % inhibition. This negative interaction was equally observed when substituting, respectively, XeC or EGTA for RRed or BAPTA was tested on 20 µM ABA-induced stomatal closure (Table 6) and would suggest an indirect link between the cytosolic free Ca^{2+} modulator and the S-type anion channel blocker within the inhibitory process. Given the positive interaction between 9-AC and the Ca^{2+} modulator (XeC or EGTA) already shown, it was then assumed that, depending on its exogenous concentration, 9-AC, but not Proben, differentially prevents cytosolic free Ca^{2+} from activating the S-type anion channel during 20 µM ABA-induced stomatal closure. Furthermore, all the above mentioned effects were suppressed on 30 µM ABA-induced stomatal closure exclusively by the *atrbohD/F*, *cpk6*-1 and *cpk3*-1*cpk6*-1 mutations that inhibited *per se* the ABA response by about 70 % (Table 8 and 9 and results not shown). Accordingly, these pharmacological effects were relevant of inhibiting a Ca^{2+} mediation that would account for 70 % of the overall stomatal closure induced by 30 µM ABA and could involve sequentially the AtrbohD and AtrbohF NADPH oxidases, CPK6, cADPR-induced Ca^{2+} mobilization and the S-type anion channel.

Convergent inhibitory effects of 9-AC, Proben and MTX on ABA-induced stomatal closure: Proben, 9-AC and the amphiphatic organic anion, methotrexate (MTX) were compared on ABA stomatal closure to get more insights into the negative interaction between cytosolic free Ca^{2+} modulators and S-type anion channel blockers. Indeed, Proben and other amphiphatic organic anions that inhibit plant S-type anion channels (Marten *et al*. 1992, Schwartz *et al*. 1995) mimick MTX to competitively inhibit a multidrug resistance-associated protein (MRP) isoform, MRP5 within ATP-dependent uptake of 5'-fluoro-2'-deoxyuridine monophosphate in human cells (Pratt *et al*. 2005). Tables 8 and 9 showed that, although 9-AC, Proben and MTX had their own range of effective concentrations, they converged to inhibit ABA-induced stomatal closure in a fashion that depended both on the exogenous ABA concentration and the genetic material. Indeed, increasing 9-AC, Proben or MTX concentration, respectively, in the 20 - 100, 50 - 200 or 10 - 30 µM range resulted in the following features: 20 or 30 µM ABA-induced stomatal closure, respectively, decreased from about 1.9 or 1.0 to 0.0 µm in the *cpk6*-1 mutant, whereas it decreased from, approximately, 0.9 or 2.2 to 0.0 µm in the *cpk3*-1 mutant. Furthermore, 9-AC similarly inhibited 20 µM ABA-induced stomatal closure in the Dex-inducible *AtPLC1* antisense transgene, and the *cpk6*-1 and *atrbohD/F* mutants (Table 8).

Possible interpretation of this inhibitory convergence assumed that, at the guard cell plasma membrane, the

Table 8. Exogenous 9-AC concentration-dependent inhibition of ABA-induced stomatal closure in the T line, *cpk3*-1 and *cpk6*-1 mutants, and *atrbohD/F* double mutant of *A. thaliana*. Leaf abaxial epidermal peels were incubated with different 9-AC concentrations and 3 h after starting the experiments, 20 or 30 μM ABA was added. For detail see Table 1.

ABA [μM]	9-AC [μM]	ABA-induced stomatal closure [μm] T line	*cpk3*-1	*cpk6*-1	*atrbohD/F*
20	-	1.88 ± 0.21	0.96 ± 0.13	1.81 ± 0.13	1.85 ± 0.20
20	20	1.83 ± 0.22	0.85 ± 0.21	1.79 ± 0.21	1.83 ± 0.22
20	40	1.90 ± 0.20	0.89 ± 0.18	1.89 ± 0.16	1.87 ± 0.21
20	60	0.77 ± 0.26	0.67 ± 0.17	0.76 ± 0.22	0.63 ± 0.10
20	80	0.39 ± 0.23	0.34 ± 0.14	0.41 ± 0.18	0.30 ± 0.19
20	100	0.06 ± 0.17	0.10 ± 0.16	0.13 ± 0.16	0.04 ± 0.15
30	-	3.07 ± 0.21	3.15 ± 0.19	0.95 ± 0.21	0.92 ± 0.18
30	20	2.39 ± 0.23	2.44 ± 0.18	0.99 ± 0.24	0.96 ± 0.23
30	40	1.47 ± 0.25	1.42 ± 0.21	1.02 ± 0.21	1.06 ± 0.19
30	60	0.81 ± 0.21	0.76 ± 0.20	0.69 ± 0.19	0.76 ± 0.20
30	80	0.38 ± 0.24	0.37 ± 0.19	0.35 ± 0.21	0.43 ± 0.22
30	100	0.03 ± 0.18	0.11 ± 0.20	0.09 ± 0.16	0.06 ± 0.11

Table 9. Inhibition of ABA-induced stomatal closure in the *cpk3*-1 and *cpk6*-1 mutants of *A. thaliana* by Proben or MTX at different concentrations. Leaf abaxial epidermal peels were incubated with Proben or MTX and 3 h after starting the experiments, 20 or 30 μM ABA was added. For detail see Table 1.

Proben [μM]	MTX [μM]	ABA-induced stomatal closure [μm] 20 μM *cpk3*-1	*cpk6*-1	30 μM *cpk3*-1	*cpk6*-1
-	-	1.06 ± 0.21	1.85 ± 0.18	3.13 ± 0.16	0.99 ± 0.21
50	-	0.98 ± 0.21	1.90 ± 0.21	2.08 ± 0.15	1.04 ± 0.19
100	-	0.87 ± 0.22	0.92 ± 0.25	1.01 ± 0.17	1.09 ± 0.24
150	-	0.44 ± 0.25	0.37 ± 0.21	0.51 ± 0.15	0.57 ± 0.25
200	-	0.13 ± 0.17	0.06 ± 0.18	0.11 ± 0.18	0.02 ± 0.22
-	10	0.95 ± 0.22	1.83 ± 0.19	2.27 ± 0.17	0.95 ± 0.20
-	20	0.79 ± 0.24	0.75 ± 0.15	0.82 ± 0.21	0.76 ± 0.19
-	30	0.13 ± 0.18	0.17 ± 0.16	0.06 ± 0.19	0.09 ± 0.18

homolog of human MRPs, AtMRP5 down-regulates anion efflux currents through interfering with voltage control of anion channel activities. Indeed, 9-AC, Proben and MTX could be actively transported by AtMRP5 in agreement with previous MRP-related studies on human and *Arabidopsis* cells (Gaedeke *et al*. 2001, Pratt *et al*. 2005). Then, increasing their exogenous concentrations would allow larger organic anion influx in comparison with influx of anions generated from basal organic acid metabolism. Accordingly, the highest tested concentrations of 9-AC, Proben or MTX would affect stomatal closure by causing larger plasma membrane polarization, which should gradually inhibit depolarization-evoked anion efflux channels. Then, negative interaction observed between an amphiphatic organic anion and a Ca^{2+} flux modulator (XeC, RRed, EGTA or BAPTA) would reveal a limited plasma membrane depolarization allowing only weak Ca^{2+} activation of the S-type anion channel. This limited depolarization would account for 30 % of overall ABA stomatal closure and, consequently, delimit smaller or larger depolarizations that, respectively, should prevent or favour activation of the S-type anion channel by a signal transduction step arising from intracellular Ca^{2+} mobilization. Reinforcing that point of view, was the fact that a Ca^{2+}-independent step occurs in ABA activation of the S-type anion channel (Schwarz and Schroeder 1998).

In the *atrbohD/F* mutant, the reduced ABA stomatal closure has been annulled by incubating abaxial leaf epidermal peels with 1 mM of the membrane-permeant weak acid, butyric acid at a medium pH buffered to pH 6 (Cousson 2009). Since a comparable acid loading treatment had decreased cytosolic pH by about 0.4 pH units in the *Vicia* guard cell (Blatt and Armstrong 1993),

these findings have shown an overall cytosolic pH dependence of ABA stomatal closure in a mutant that suppress apoplastic Ca^{2+} entry into the guard cell (Kwak *et al*. 2003). Accordingly, it has been strongly suggested that a Ca^{2+}-independent pathway is implicated in ABA stomatal closing (Cousson 2003, 2007), as did the present study. Thus, emerged the possibility that cytosolic proton concentration would be sensed by a guard cell protein complex extruding a sufficient amount of inorganic anions to initiate significant plasma membrane depolarization. Below a putative threshold value of the electrical membrane potential, specific oscillations in cytosolic free Ca^{2+} would be subsequently sensed by the same anion-extruding complex to depolarize further the plasma membrane allowing, in turn, activation of the K^+-outward rectifier. Possibility that, according to the plasma membrane potential, each of these two parameters is separately and differentially sensed to activate the anion-outward rectifying channel is corroborated by the fact that cytosolic pH and Ca^{2+} separately regulate the K^+-inward and -outward rectifying channels (Blatt and Armstrong 1993, Grabov and Blatt 1997). Such a dual voltage-dependent sensing mechanism was equally supported by the different stomatal behaviours of the wild type and *atrbohD/F* mutant in response to butyric acid loading of leaf epidermal peels: 1 mM butyric acid inhibited 20 μM ABA-induced stomatal closure only by 60 % without significantly changing 30 μM ABA-induced stomatal closure in the wild type, whereas the same weak acid treatment annulled 20 or 30 μM ABA-induced stomatal closure in the *atrbohD/F* mutant (Cousson 2009). Indeed, this would result from a parallel and differential regulation of the electrogenic proton pump by the cytosolic proton and Ca^{2+} concentrations (Kinoshita *et al*. 1995, Cousson 2002) whose activity should be in tune with voltage-dependent alternation between the rapid (R)- and S-type anion channels (Raschke *et al*. 2003) to optimize plasma membrane depolarization (Roelfsema *et al*. 2004).

This paradigm was plausible as much as, contrary to the S-type anion channel, the R-type anion channel activity is not affected by disrupting CPK3 and CPK6 (Mori *et al*. 2006). Indeed, the R-type anion channel initiates plasma membrane depolarization in the *Vicia faba* guard cell (Raschke *et al*. 2003) and specifically senses both the cytosolic pH and the transmembrane gradient of proton concentation (Schulz-Lessdorf *et al*. 1996). In *Vicia faba*, the R-type anion channel likely functions as an homolog of the mammalian cystic fibrosis transmembrane conductance regulator (CFTR) (Schulz-Lessdorf *et al*. 1996). In this species, the S-type anion channel is positively controlled by a polypeptide exhibiting one epitope shared with the CFTR (Leonhardt *et al*. 2001), which could parallel up-regulation by CFTR of distinct outward rectifying chloride channels in mouse nasal epithelial cells (Gabriel *et al*. 1993). Comparable experiments should be undertaken in the Columbia ecotype disrupting AtMRP5 or a glucosidase, AtBG1 that hydrolyzes inactive glucosyl-ABA conjugates into active ABA (Lee *et al*. 2006). Indeed, present and previous convergent analyses of stomatal movement bioassays (Klein *et al*. 2003) and electrophysiology (Suh *et al*. 2007) were in a possible consensus suggesting that another ATP-binding cassette transporter, AtMRP5 down-regulates ABA-activated S-type anion efflux currents. These previous studies would be correlated with the present analysis by assuming that disruption in AtMRP5 highly increases guard cell responsiveness to the active ABA concentration resting in the leaf abaxial epidermis bathed without exogenous ABA. Then, such an increased responsiveness would result from removing polarization-mediated down-regulation of the Ca^{2+}-activated S-type anion channel. So, one could explain in a comparison with the wild type why stomatal aperture of *atmrp5*-1 epidermal peels incubated in the light is smaller and rather insensitive to exogenous ABA (Klein *et al*. 2003) and why exogenous ABA only slightly increases the S-type anion efflux current of *atmrp5*-1 guard cell protoplasts (Suh *et al*. 2007). Accordingly, significant impairment in Ca^{2+}-inward conductance of the *atmrp5*-1 mutant (Suh *et al*. 2007) would result from a plasma membrane polarization resting below the threshold voltage value required to activate the hyperpolarization-evoked Ca^{2+}-inward rectifier (Grabov and Blatt 1998, 1999, Pei *et al*. 2000).

In conclusion, the present study confirmed that ABA stomatal closing implicates not only two Ca^{2+} mediations at least but also a Ca^{2+}-independent pathway to transduce the exogenous ABA concentration signal whose a key target would be a protein complex that extrudes inorganic anions at the guard cell plasma membrane. Plasma membrane voltage control was considered as main regulator of this extruding activity whose relation to putative endomembrane IP_3 receptor homologue was shown to specifically require CPK3 functioning.

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