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Diacylglycerol pyrophosphate is a second messenger of abscisic acid signaling in *Arabidopsis thaliana* suspension cells

Christine Zalejski[1], Zongshen Zhang[2], Anne-Laure Quettier[1], Régis Maldiney[1], Magda Bonnet[1], Mathias Brault[2], Chantal Demandre[1], Emile Miginiac[1], Jean-Pierre Rona[2], Bruno Sotta[1] and Emmanuelle Jeannette[1,*]

[1]Physiologie Cellulaire et Moléculaire des Plantes, Unité Mixte de Recherche 7632-Centre National de la Recherche Scientifique, case 156, Université Pierre et Marie Curie, Le Raphaël, 3 rue Galilée 94200 Ivry sur Seine, France,

[2]Electrophysiologie des Membranes, Equipe d'Accueil 3514, case 7069, Université Denis Diderot, 2 place Jussieu 75251 Paris Cedex 05, France

Received 6 December 2004; accepted 23 December 2004.
*For correspondence (fax +33 0 1 44276232; e-mail ema@ccr.jussieu.fr).

Summary

In plants, the importance of phospholipid signaling in responses to environmental stresses is becoming well documented. The involvement of phospholipids in abscisic acid (ABA) responses is also established. In a previous study, we demonstrated that the stimulation of phospholipase D (PLD) activity and plasma membrane anion currents by ABA were both required for *RAB18* expression in *Arabidopsis thaliana* suspension cells. In this study, we show that the total lipids extracted from ABA-treated cells mimic ABA in activating plasmalemma anion currents and induction of *RAB18* expression. Moreover, ABA evokes within 5 min a transient 1.7-fold increase in phosphatidic acid (PA) followed by a sevenfold increase in diacylglycerol pyrophosphate (DGPP) at 20 min. PA activated plasmalemma anion currents but was incapable of triggering *RAB18* expression. By contrast, DGPP mimicked ABA on anion currents and was also able to stimulate *RAB18* expression. Here we show the role of DGPP as phospholipid second messenger in ABA signaling.

Keywords: diacylglycerol pyrophosphate, phosphatidic acid, abscisic acid, *RAB18*, anion currents, Arabidopsis suspension cells.

Introduction

In plants, the hormone abscisic acid (ABA) plays important roles in growth and development. Thus, in seeds, ABA is involved in maturation and dormancy of embryos, synthesis of storage proteins and desiccation tolerance (Finkelstein *et al.*, 2002). Moreover, ABA has a crucial role in the regulation of gas exchanges between leaves and atmosphere via the control of stomatal aperture (Schroeder *et al.*, 2001). ABA is also involved in resistance to different abiotic and biotic stresses (Knight and Knight, 2001).

ABA signaling has been extensively studied and numerous intermediates have been identified. Anion and potassium channels, protein phosphatases and kinases, calcium and cADP-ribose are major elements of the ABA transduction pathways (Leung and Giraudat, 1998; Schroeder *et al.*, 2001). Recently, phospholipids have also been identified as essential partners in ABA signaling (Wang, 2001; Zhang *et al.*, 2004). In animal and plant cells, the main phospholipids and related compounds involved in intra-cellular communication processes (English, 1996) are phosphatidic acid (PA), lysophosphatidic acid (LPA), diacylglycerol (DAG), sphingosine-1-phosphate (Coursol *et al.*, 2003; Ng *et al.*, 2001), inositol hexakisphosphate (Lemtiri-Chlieh *et al.*, 2000, 2003) and inositol triphosphate. The levels of these molecules are regulated by the activity of several enzymes which have been studied in animal and plant cells. For example, phosphatidyl inositol diphosphate phospholipase C (PIP_2-PLC) hydrolyzes PIP_2 to produce inositol triphosphate and DAG which is phosphorylated by DAG kinase to produce PA. The role of PLC in ABA responses was demonstrated in guard cells (Hunt *et al.*, 2003; Staxen *et al.*, 1999) and seedlings (Sanchez and Chua, 2001). The hydrolysis of phospholipids by phospholipase D (PLD) also produces

PA. This reaction is an important step in ABA signaling because the shunt of PLD hydrolysis activity by primary alcohols counteracts ABA effect in guard cells (Jacob *et al.*, 1999), aleurone cells (Ritchie and Gilroy, 1998) and suspension cells (Hallouin *et al.*, 2002). Moreover, PA is able to mimic ABA induction of stomatal closure, ABA inhibition of stomatal opening (Jacob *et al.*, 1999) and ABA inhibition of α-amylase production triggered by gibberellic acid in barley aleurone cells (Ritchie and Gilroy, 1998). However, when PA accumulates or when PA is externally applied, it remains unclear whether the physiological responses observed are due to PA itself or to PA metabolites. PA can be deacylated by phospholipase A_2 (PLA_2) to produce LPA and free fatty acids, which are signaling compounds in plant responses to auxin (Paul *et al.*, 1998), to wounding (Ryu and Wang, 1998) and to hyperosmotic stress (Meijer *et al.*, 2001). PA can also be dephosphorylated by PA phosphatase to produce DAG. But, in plant cells, in contrast to animal cells, the function of DAG remains unknown and no protein kinase C has been identified (Meijer and Munnik, 2003).

Plant and yeast cells have the ability to phosphorylate PA in diacylglycerol pyrophosphate (DGPP) through a PA kinase activity (Wissing and Behrbohm, 1993a; Wu *et al.*, 1996). DGPP was also found in *Trypanosoma cruzi* (Marchesini *et al.*, 1998) but no PA kinase activity has been detected in mammalian cells. In *Catharanthus roseus*, PA kinase is a plasma membrane protein of 39 kDa (Wissing and Behrbohm, 1993b) but little additional information is available as no PA kinase gene has yet been identified. DGPP phosphatase activity, that hydrolyzes DGPP into PA, belongs to the lipid phosphate phosphatase family and uses DGPP and PA as substrates (Oshiro *et al.*, 2003). Thus PA and DGPP are metabolically related and one can wonder whether DGPP could be, like PA, a signaling phospholipid. In animal cells, pharmacological effects of DGPP were recently reported. DGPP activates mitogen-activating protein kinase activity which phosphorylates a cytosolic PLA_2 responsible for protein kinase C translocation to the membrane (Balboa *et al.*, 1999). DGPP was also shown to antagonize LPA receptors in human platelets (Fischer *et al.*, 2001). In plants, DGPP is accumulated in response to drought (Munnik *et al.*, 2000), hyperosmotic stress (Meijer *et al.*, 2001; Munnik *et al.*, 2000; Pical *et al.*, 1999), pathogenesis attack (van der Luit *et al.*, 2000) and symbiosis relation (den Hartog *et al.*, 2001). Although three genes of DGPP phosphatase have been identified in *Arabidopsis thaliana* (Pierrugues *et al.*, 2001), to our knowledge no experimental data about the role of DGPP in transduction pathways in plants have been reported so far.

We have investigated the role of PA and PA-derived phospholipids in ABA transduction pathways in *A. thaliana* suspension cells. Here we present evidence that supports the involvement of DGPP in ABA signaling and demonstrate that it is a putative second messenger in ABA transduction pathways.

Results

A lipid component synthesized in ABA-treated cells is able to mimic ABA

In Arabidopsis suspension cells we have previously observed that PLD activity was necessary for ABA responses especially for *RAB18*, which is an ABA-specifically inducible gene (Figure 1a; Hallouin *et al.*, 2002). Therefore, we attempted to establish the presence of lipid messengers of ABA transduction pathways in Arabidopsis cells. In a first set of experiments, we applied a sonicated emulsion of the total lipids extracted from control (TL_C) or ABA-treated cells (TL_{ABA}) to a fresh aliquot of cells. In order to prevent any artifact, we added ^{3}H-ABA to measure ABA level in the total lipids extracted from cells. Less than 0.05% of ^{3}H-ABA was retrieved in the total lipid extract. Therefore we considered that the effects recorded with TL_{ABA} (Figures 1 and 2) were not due to ABA contamination. The ability of TL_C and TL_{ABA} to induce the expression of *RAB18* was tested. TL_{ABA} (2 mM) extracted from cells treated by ABA for 1–20 min caused an ABA-like expression of *RAB18* which was not observed with 2 mM TL_C (Figure 1b). The stimulation of *RAB18* expression was higher with TL_{ABA} obtained after 10 min of ABA treatment than with TL_{ABA} obtained after a shorter ABA treatment. TL_{ABA} extracted from ABA-treated cells for 10 min were able to trigger *RAB18* expression on a dose-dependent manner from 0.5 to 2 mM (Figure 1c). These results are specific to TL_{ABA} as addition of total lipids had no effect on the abundance of housekeeping gene transcripts (data not shown).

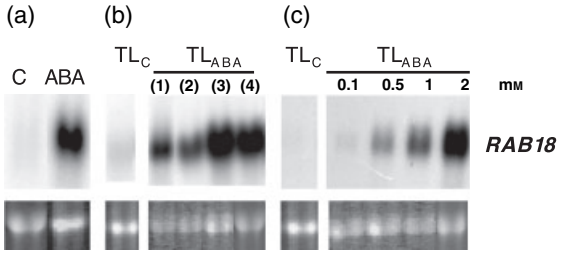

Figure 1. Total lipids from ABA-treated *Arabidopsis thaliana* suspension cells trigger *RAB18* expression.
Northern blot analysis of total RNA (10 μg) from:
(a) Control (C) or 10^{-5} M ABA-treated cells (ABA) for 3 h.
(b) Cells incubated for 3 h with 2 mM total lipids extracted from control (TL_C) or 10^{-5} M ABA-treated cells (TL_{ABA}, 1–4) for 1 min (1), 5 min (2), 10 min (3) or 20 min (4).
(c) Cells incubated for 3 h with 2 mM TL_C and 0.1–2 mM TL_{ABA} extracted from cells treated for 10 min with 10^{-5} M ABA.
Ethidium bromide staining of rRNAs is shown as control. The data presented are representative of four independent experiments.

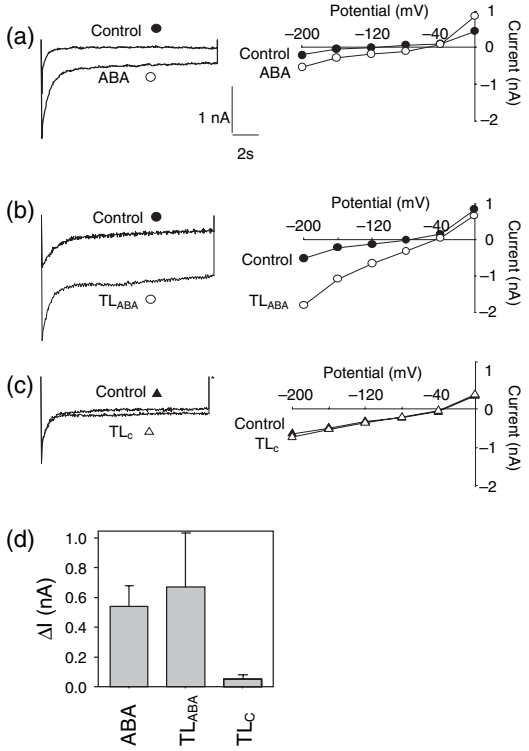

Figure 2. Total lipids from ABA-treated *Arabidopsis thaliana* suspension cells activate whole-cell anion currents.
Whole-cell currents were activated by a depolarizing prepulse (+100 mV for 4.5 sec, not shown). Then, hyperpolarizing pulses from −200 mV to 0 mV in 40 mV steps were applied for 15 sec. Representative current traces at −200 mV and corresponding current–voltage relationship determined for currents recorded after 9-sec deactivation are shown. Holding potential was −40 mV.
Currents were recorded from intact cells before (control) and after application of (a) ABA 10^{-5} M, (b) 1 mM total lipids extracted from cells treated with 10^{-5} M ABA (TL_{ABA}) for 10 min and (c) 1 mM total lipids extracted from control cells (TL_C).
(d) Difference in the anion currents (ΔI) at −200 mV before and after ABA, TL_{ABA} or TL_C application. The results show the mean ± SD, $n = 4$.

In Arabidopsis cells ABA stimulation of plasma membrane anion currents (Figure 2a) is required for *RAB18* expression (Ghelis *et al.*, 2000). Moreover, we demonstrated that ABA activation of anion currents occurred downstream the ABA stimulation of PLD activity (Hallouin *et al.*, 2002). Accordingly, we tested the capacity of total lipids to stimulate plasma membrane anion currents. One millimolar of TL_{ABA} had the same efficiency as 10^{-5} M ABA to activate anion currents (Figure 2b), whereas 1 mM of TL_C was inactive (Figure 2c). The intensity of the currents recorded was 0.67 ± 0.36 nA (at −200 mV) with 1 mM TL_{ABA} and 0.05 ± 0.03 nA (at −200 mV) with 1 mM TL_C (Figure 2d).

These results demonstrate that, within 1 min of ABA application, a lipid component was synthesized in ABA-treated cells and able to mimic ABA action. Furthermore, this lipid component remained present for at least 20 min in ABA-treated cells.

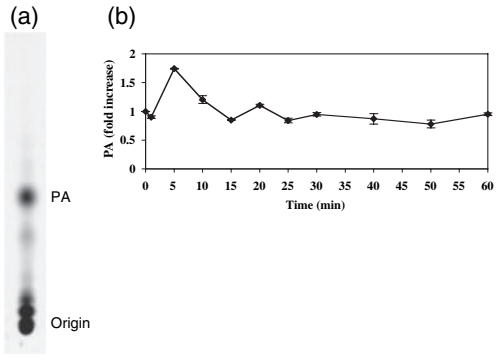

Figure 3. ABA induces a transient increase in PA in *Arabidopsis thaliana* suspension cells.
Cells were labeled for 18 h with $^{33}PO_4^{3-}$ and then incubated with 10^{-5} M ABA for 60 min before lipid extraction.
(a) PhosphorImager picture of TLC plate showing the separation of PA.
(b) Time course of PA content in ABA-treated cells. The results show the mean ± SEM, $n = 5$.

ABA induces accumulation of PA and DGPP

In order to specify the consequences of ABA application on lipid levels, the cells were labeled with ^{33}P for 18 h and then supplemented with 10^{-5} M ABA for 1 h. We first focused on PA, the product of PLD activity. PA was separated from total lipids with an appropriate thin-layer chromatography (TLC) solvent system (Figure 3a; de Vrije and Munnik, 1997) and quantified. The pool of PA represented 3.2% of the total phospholipids. Within 5 min of ABA application, the PA level increased 1.7-fold then, between 5 and 30 min, decreased to the level measured in the control cells and remained unchanged (Figure 3b). The lipid samples were also analyzed in TLC with an alkaline solvent system to allow the separation of the major phospholipid families (Munnik *et al.*, 1996). PC, PE, PI and PG were the most abundant phospholipids in Arabidopsis cells, they represented 39, 32, 16 and 7%, respectively, of the total lipids (Figure 4a). No changes in the size of the pools of these structural phospholipids were recorded under ABA treatment. Standard DGPP and LPA were not well separated within this TLC system. But DGPP was isolated from LPA in a 2-D system (Figure 4b). Other unidentified phospholipids were detected but only DGPP level changed after ABA application. DGPP level was elevated twofold after 10 min of ABA treatment and sevenfold after 20 min (Figure 4c). Between 20 and 30 min, the DGPP level decreased to the level measured in the control cells and remained unchanged. No changes in LPA content were recorded (Figure 4c). From these observations we focused our attention on PA and DGPP as putative messengers in ABA signaling.

DGPP is likely a second messenger in ABA signaling

As ABA activation of anion currents was abolished when PLD activity was shunted by primary alcohols (Hallouin

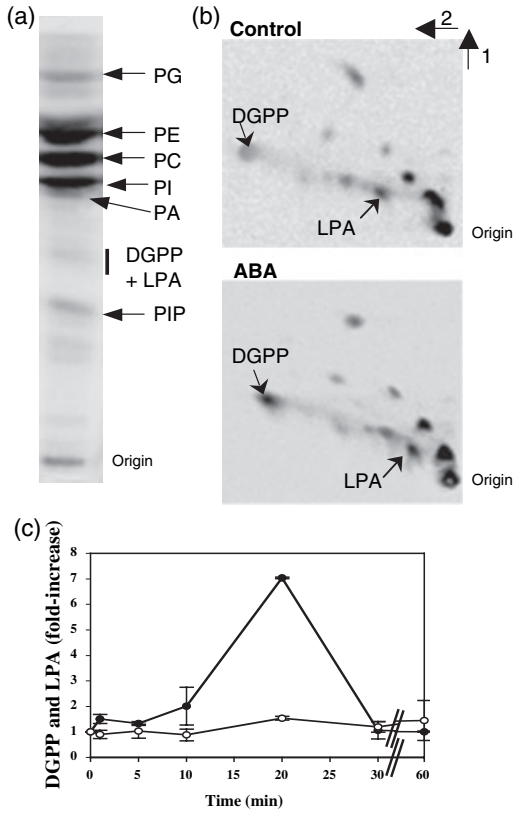

Figure 4. ABA induces an increase in DGPP in *Arabidopsis thaliana* suspension cells.
Cells were labeled for 18 h with $^{33}PO_4^{3-}$ and then incubated with 10^{-5} M ABA for 60 min before lipid extraction.
(a) PhosphorImager picture of 1-D TLC plate showing the separation of phospholipids.
(b) PhosphorImager picture of 2-D TLC plates showing the separation of LPA and DGPP extracted from control cells and 20 min ABA-treated cells. DGPP and LPA were identified with standards.
(c) Time course of LPA (open circle) and DGPP (close circle) contents in ABA-treated cells. The results show the mean ± SEM, $n = 3$.

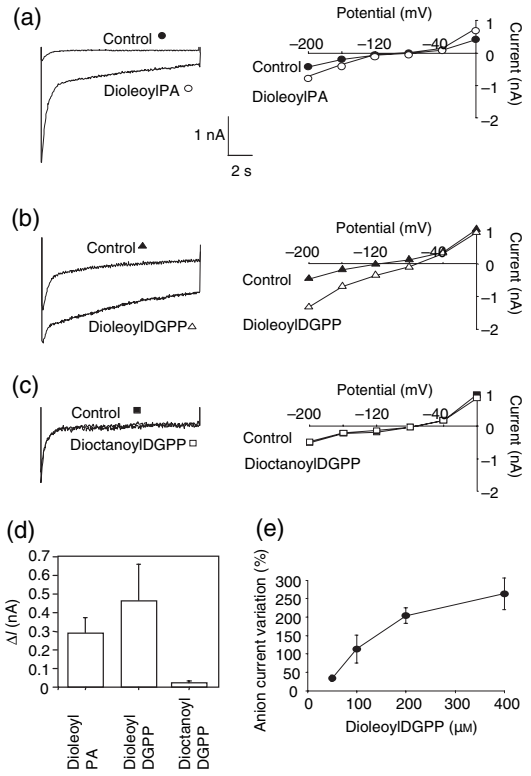

Figure 5. Comparative effect of PA and DGPP on plasma membrane anion currents of *Arabidopsis thaliana* suspension cells.
Whole-cell currents were activated by a depolarizing prepulse (+100 mV for 4.5 sec, not shown). Then, hyperpolarizing pulses from −200 mV to 0 mV in 40 mV steps were applied for 15 sec. Representative current traces at −200 mV and corresponding current–voltage relationship determined for currents recorded after 9-sec deactivation are shown. Holding potential was −40 mV.
Currents were recorded from intact cells before (control) and after application of 100 μM of dioleoylPA (a), dioleoylDGPP (b) or dioctanoylDGPP (c).
(d) Difference in anion currents (ΔI) at −200 mV before and after 100 μM of dioleoylPA ($n = 4$), dioleoylDGPP ($n = 11$) or dioctanoylDGPP ($n = 7$) application. The results show the mean ± SD; *t*-test, $\alpha = 0.05$.
(e) Dose–response at −200 mV of anion current with dioleoylDGPP at 50 μM ($n = 3$), 100 μM ($n = 11$), 200 μM ($n = 3$) or 400 μM ($n = 3$). The results show the mean ± SD.

et al., 2002), we considered that anion currents could be targets of PA or DGPP. Therefore, standard phospholipids were tested for their capacity to stimulate anion currents and to trigger *RAB18* expression. DioleoylPA and dioleoylDGPP mimicked ABA to activate plasma anion currents (Figure 5a–d). The intensities of anion currents at −200 mV were 0.30 ± 0.06 nA and 0.46 ± 0.19 nA, respectively, with dioleoylPA (100 μM) and dioleoylDGPP (100 μM). However, the action of dioleoylPA and dioleoylDGPP were not statistically different (*t*-test, $\alpha = 0.05$). Moreover, the stimulation of anion currents activity was dose-dependent with dioleoylDGPP from 50 to 400 mM (Figure 5e). The short fatty acid chains DGPP and dioctanoylDGPP (up to 400 μM) had no effect on anion currents (0.02 ± 0.01 nA at −200 mV; Figure 5c,d).

In addition, 400 μM of dioleoylPA, diarachidonoylPA or PAmix were not able to trigger *RAB18* expression. By contrast, application of 100–400 μM dioleoylDGPP stimulated *RAB18* expression in a dose-dependent manner. With the short fatty acid chain dioctanoylDGPP, applied up to 400 μM, no effect on *RAB18* expression was observed (Figure 6). Structural phospholipids PS, PC or standard dioleoylphosphatidyl butanol or oleoylLPA were not able to induce *RAB18* expression (data not shown).

Discussion

In Arabidopsis suspension cells, endogenous lipids produced in ABA-treated cells mimic ABA for induction of *RAB18* expression and activation of plasma membrane anion currents (Figures 1 and 2). This demonstrates that a lipid second messenger of ABA signaling is synthesized in ABA-treated cells. As ABA stimulates PLD activity (Hallouin

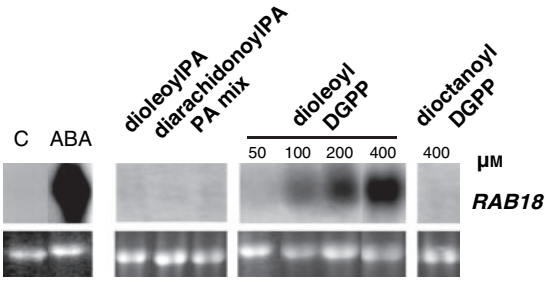

Figure 6. DioleoylDGPP triggers *RAB18* expression in *Arabidopsis thaliana* suspension cells.
Northern blot analysis of total RNA (10 µg) extracted from control (C) or 10^{-5} M ABA-treated cells (ABA) for 3 h or from cells incubated for 3 h with: dioleoylPA (400 µM), diarachidonoylPA (400 µM), PAmix (400 µM), dioleoylDGPP (50–400 µM) or dioctanoylDGPP (400 µM).
Ethidium bromide staining of rRNAs is shown as control. The data presented are representative of five independent experiments.

et al., 2002; Takahashi *et al.*, 2001), we first measured the changes in the pool of PA. A transient 1.7-fold increase in PA level was observed 5 min after 10^{-5} M ABA application (Figure 3). Similar increases in PA, from 1.5- to 2-fold, have been described in response to ABA treatment in guard cells (Jacob *et al.*, 1999) and in aleurone cells (Ritchie and Gilroy, 1998; Villasuso *et al.*, 2003). Thus, a small increase in PA level appears to be typical of ABA responses. In contrast, higher increases in PA level due to both PLD and PLC activities have been reported in various responses which probably do not implicate ABA. For example, in Arabidopsis suspension cells, under cold stress, the threefold increase in PA originated preferentially from PLC activity (Ruelland *et al.*, 2002). In suspension cells of tomato (van der Luit *et al.*, 2000) and alfalfa (den Hartog *et al.*, 2003), several elicitors triggered a 2- to 4-fold increase in PA. In *Chlamydomonas moewusii* submitted to hyperosmotic stress, up to a ninefold increase in PA was recorded (Arisz *et al.*, 2003; Munnik *et al.*, 2000). We also analyzed, by gas chromatography, the fatty acid composition of the PA formed in ABA-treated cells in order to determine the molecular species. We observed (data not shown) that it matched with the one found in PC of cells and differed from that of Arabidopsis PIP_2, the substrate of PIP_2-PLC (Ruelland *et al.*, 2002). Hence, this result confirmed that, after ABA perception, the PA increase might not have originated from PIP_2-PLC but from other PLCs or PLD activities.

In guard cells and aleurone cells, previous reports have shown that PA was able to trigger ABA-specific responses (Jacob *et al.*, 1999; Ritchie and Gilroy, 1998). Thus, we tested the hypothesis that PA could be a second messenger in ABA signaling. In Arabidopsis suspension cells ABA stimulation of PLD activity occurred prior to the ABA activation of plasma membrane anion currents (Hallouin *et al.*, 2002). Furthermore, activation of anion currents was necessary for *RAB18* expression (Ghelis *et al.*, 2000). DioleoylPA was able to activate plasma membrane anion currents (Figure 5) but *RAB18* expression was not stimulated by any PA tested (Figure 6). Therefore, PA is a second messenger of ABA for the activation of anion currents but another signaling lipid, present in TL_{ABA} (Figure 1), is additionally required for *RAB18* expression. In plant and animal cells, few studies have suggested the involvement of PA in the regulation of K^+ ion channels (Fan *et al.*, 2003; Jacob *et al.*, 1999). Here we show that anion channel activity is modulated by PA. Nevertheless, PA produced by PLD activity regulates the chloride efflux in pollen tube (Zonia and Munnik, 2004; Zonia *et al.*, 2002) and in T84 cells (Oprins *et al.*, 2001; Vajanaphanich *et al.*, 1993).

After ABA application, the transient increase in PA suggests that, once formed, PA is metabolized. Simultaneous changes in PA and PA metabolites have already been reported in various physiological processes. For example, increases in PA and LPA levels were recorded in plant defense against wounding (Lee *et al.*, 1997). However, in our experiments, the LPA level remained unchanged indicating that PLA_2 activity was not stimulated in ABA-treated Arabidopsis cells (Figure 4). Furthermore, treatment of cells with standard LPA did not induce *RAB18* expression (data not shown). PA can as well be dephosphorylated by PA phosphatase in DAG (Waggoner *et al.*, 1999). However, previous experiments have shown that DAG was unchanged in cells similarly treated by ABA (Jacob *et al.*, 1999; Ritchie and Gilroy, 1998). Accordingly, it is unlikely that DAG is a second messenger in ABA signaling, although we had not measured it in Arabidopsis cells. In plants, PA can also be metabolized in DGPP (Wissing and Behrbohm, 1993b). Thus, we measured the DGPP level after ABA application. We observed an increase in DGPP (peak at 20 min, Figure 4) after the stimulation of PA production (peak at 5 min, Figure 3). This result is in agreement with the data obtained in barley aleurone cells (Villasuso *et al.*, 2003). ABA treatment evoked changes in PA level after 5 min and changes in PA and LPA/DGPP levels after 30 min. Concomitant increases in PA and DGPP were also observed in plants submitted to hyperosmotic stress (Munnik *et al.*, 2000), rhizobium symbiosis process (den Hartog *et al.*, 2001) or pathogenesis attack (van der Luit *et al.*, 2000). The accumulation of DGPP might result from the stimulation of a PA kinase activity. PA kinase activity has been measured in several plant species (Wissing and Behrbohm, 1993a) and is localized to the plasma membrane (Wissing and Behrbohm, 1993a; Wissing *et al.*, 1994). However, the regulation of PA kinase activity and the physiological role of PA kinase in plants remain unclear.

We have shown that DGPP, in addition to PA, has the capacity to activate anion currents (Figure 5). As anion currents are specifically activated by ABA (Ghelis *et al.*, 2000), this indicates that DGPP, as well as PA (see above), is a second messenger in ABA signaling. Further evidence of

the signaling role of DGPP is comes from the fact that application of dioleoylDGPP triggered the accumulation of *RAB18* messengers (Figure 6). Although the pharmacological effects of DGPP have been described in animal cells (Balboa *et al.*, 1999; Fischer *et al.*, 2001), the mechanism of action of DGPP is unknown. The capacity of both dioleoylPA and dioleoylDGPP to stimulate anion currents suggests that the oleoyl chains, or at least the long acyl chains, are important for the biological activity of DGPP. This interpretation is further reinforced by the absence of an effect recorded with the short fatty acid chains dioctanoylDGPP (Figures 5 and 6). The long fatty acid chains of DGPP may allow proteins to be translocated to the membrane through hydrophobic interactions (van Leeuwen *et al.*, 2004; Munnik *et al.*, 1998). The pyrophosphate group might also be important for DGPP biological activity, especially in electrostatic interaction with protein targets. Whatever the mechanism of DGPP action, our observations show that DGPP plays a role in ABA transduction pathways upstream of the anion currents. Furthermore, it leads us to question whether DGPP is also a lipid messenger in other ABA responses and in other signaling pathways.

Experimental procedures

Plant material

Arabidopsis thaliana L. ec. Columbia cells were obtained by Axelos *et al.* (Axelos *et al.*, 1992). They were cultured at 24°C, under continuous white light (40 μmol m^{-2} sec^{-1}) with an orbital agitation at 130 rpm, in 500 ml Erlenmeyer flasks containing 200 ml Jouanneau and Péaud-Lenoél culture medium (Jouanneau and Péaud-Lenoél, 1967). A 25-ml aliquot of suspension cells was transferred to a fresh medium every week. All the experiments were conducted on 3-day-old cells. The pH of the culture medium was 6.8. ABA was added in dimethylsulfoxide (DMSO) at 0.1% final concentration and we checked that DMSO had no effect on lipid biosynthesis. Moreover, the viability of the cells during the experimental treatment was systematically checked with trypan blue tests (data not shown).

Lipids extraction, separation by TLC and measurement of phospholipid level

Total lipids were extracted from 100 ml of control or ABA-treated suspension cells with 60 ml methanol/chloroform (2:1 vol/vol) for 30 min at 4°C, then 20 ml chloroform and 20 ml 0.9% NaCl were added. After shaking and separation, the organic phase was removed under vacuum and total lipids were dried by N_2.

For measurement of phospholipid content, cells were labeled with $^{33}PO_4^{3-}$ (74 Bq ml^{-1} of suspension) for 18 h. The lipids were then extracted from 1 ml suspension cells as mentioned above. Lipid samples were dissolved in 20 μl chloroform and spotted onto TLC silica plates (Silica Gel 60; Merck, La Jolla, CA, USA). A specific separation of PA was obtained on plates developed consecutively with acetone and the organic upper phase of ethyl acetate/iso-octane/acetic acid/H_2O (13:2:3:10 vol/vol/vol/vol) as described by de Vrije and Munnik (1997). Other phospholipids were separated on K^+ oxalate activated plates with an alkaline solvent chloroform/methanol/ammonia/H_2O (90:70:4:16 vol/vol/vol/vol) as described by Munnik *et al.* (1995). As LPA and DGPP migrated very closely in this latter system and as the amounts of DGPP and LPA were very low, we separated DGPP and LPA in a 2-D system. Lipids from 2.5 g of vacuum-filtrated cells were first separated with the alkaline solvent. Then, the silica strip containing LPA and DGPP, boarded at the bottom with phosphatidylinositol phosphate (PIP) and at the top with PA, was scrapped from TLC plates and extracted in methanol. DGPP and LPA were separated in a 2-D system described by Lepage (1967): first migration in chloroform/methanol/H_2O (130:50:8 vol/vol/vol), second migration in chloroform/acetone/methanol/acetic acid/H_2O (100:40:20:20:10 vol/vol/vol/vol/vol). Radiolabeled lipid spots were visualized with PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA, USA) and analyzed with ImageQuant software (Amersham, Orsay, France). Radioactivity incorporated in a given phospholipid was expressed as percentage of the radioactivity detected in the whole lipid fraction. Standard phospholipids exposed to iodine vapor were used to identify phospholipids. PA was identified by comparison with both dioleoylPA (Sigma-Aldrich, St Louis, MO, USA) and a mixture of PA (PAmix) obtained from the hydrolysis of yolk egg phosphatidylcholine (PC; Sigma). PC, phosphatidylethanol amine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS) were from Sigma. OleoylLPA, dioleoylDGPP, dioctanoylDGPP, diarachidonoylPA and dioleoylphosphatidyl butanol were from Avanti Polar Lipids (Alabaster, AL, USA).

Measurement of ABA in total lipid extract

Tritiated-ABA (3.10^4 Bq; Amersham) was added to the suspension cells before lipid extraction. Then the radioactivity in the total lipids obtained was measured by scintillation counting (Beckman, LS 6000IC; Beckman, Fullerton, CA, USA).

Determination of the phosphorus content of phospholipids

The phosphorus content of phospholipids was used for their quantification (Rouser *et al.*, 1970). An aliquot of phospholipid was dried under N_2 then mineralized in 250 μl 70% perchloric acid at 180°C for 2 h. The inorganic phosphate obtained was supplemented with 400 μl $MoNH_4$ 1.25% (w/vol) and 400 μl sodium ascorbate 5% (w/vol). After 5 min of incubation at 100°C, OD at 797 nm was measured.

Assay of gene expression

Five milliliters of suspension cells was incubated with ABA or lipids for 3 h under the conditions of culture. Lipids were emulsified by sonication for 1 min, four times, at 4°C, in 1 ml of culture medium then added to 4 ml suspension cells. Northern blot analyses were performed according to the protocol previously described (Jeannette *et al.*, 1999). The 684 bp *RAB18* cDNA probe used (GenBank accession number X68042) contained the coding sequence (with the exception of the first 100 bp of 5′ sequence after the ATG codon) and the 3′ non-coding sequence ending with the polyadenylation site of the gene (Lang and Palva, 1992).

Electrophysiology

Anion currents were measured as previously described (Hallouin *et al.*, 2002). Briefly, cells were immobilized by means of a microfunnel and were impaled with a borosilicate capillary glass

microelectrode filled with 600 mM KCl (electrical resistance from 50 to 80 MΩ). ABA and sonicated lipids were introduced via a polyethylene catheter. Whole cell currents from intact cells were measured using the technique of the discontinuous single voltage-clamp microelectrode (Jeannette *et al.*, 1999). The membrane potential being held at −40 mV, anion currents were activated by a depolarizing prepulse (+100 mV for 4.5 sec), then hyperpolarizing pulse from −200 mV to 0 mV in 40 mV steps were applied for 15 sec. We systematically checked that cells were correctly clamped by comparing the protocol voltage values with those actually imposed.

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References

Arisz, S., Valianpour, F., van Gennip, A. and Munnik, T. (2003) Substrate preference of stress-activated phospholipase D in *Chlamydomonas* and its contribution to PA formation. *Plant J.* **34**, 595–604.

Axelos, M., Curie, C., Mazzolini, L., Bardet, C. and Lescure, B. (1992) A protocol for transient gene expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. *Plant Physiol. Biochem.* **30**, 123–128.

Balboa, M., Balsinde, J., Dillon, D., Carman, G. and Dennis, E. (1999) Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *J. Biol. Chem.* **274**, 522–526.

Coursol, S., Fan, L.-M., le Stunff, H., Spiegel, S., Gilroy, S. and Assmann, S. (2003) Sphingolipid signaling in *Arabidopsis* guard cells involves heterotrimeric G proteins. *Nature*, **423**, 651–654.

English, D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signalling. *Cell. Signal.* **8**, 341–347.

Fan, Z., Gao, L. and Wang, W. (2003) Phosphatidic acid stimulates cardiac K_{ATP} channels like phosphatidylinositols, but with novel gating kinetics. *Am. J. Physiol. Cell. Physiol.* **284**, 94–102.

Finkelstein, R., Gampala, S. and Rock, C. (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell*, **14**, S15–S45.

Fischer, D., Nusser, N., Virag, T., Yokoyama, K., Wang, D., Baker, D., Bautista, D., Parrill, A. and Tigyi, G. (2001) Short-chain phosphatidates are subtype-selective antagonists of lysophosphatidic acid receptors. *Mol. Pharmacol.* **60**, 776–784.

Ghelis, T., Dellis, O., Jeannette, E., Bardat, F., Cornel, D., Miginiac, E., Rona, J.-P. and Sotta, B. (2000) Abscisic acid specific expression of *RAB18* involves activation of anion channels in *Arabidopsis thaliana* suspension cells. *FEBS Lett.* **474**, 43–47.

Hallouin, M., Ghelis, T., Brault, M., Bardat, F., Cornel, D., Miginiac, E., Rona, J.-P., Sotta, B. and Jeannette, E. (2002) Plasmalemma ABA perception leads to *RAB18* expression via phospholipase D activation in *Arabidopsis thaliana* suspension cells. *Plant Physiol.* **130**, 265–272.

den Hartog, M., Musgrave, A. and Munnik, T. (2001) Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. *Plant J.* **25**, 55–60.

den Hartog, M., Verhoef, N. and Munnik, T. (2003) Nod factor and elicitors activate different phospholipid signaling pathways in suspension-cultured alfalfa cells. *Plant Physiol.* **132**, 311–317.

Hunt, L., Millis, L., Pical, C., Leckie, C., Aitken, F., Kopka, J., Mueller-Roeber, B., McAinsh, M., Hetherington, A. and Gray, J. (2003) Phospholipase C is required for the control of stomatal aperture by ABA. *Plant J.* **34**, 47–55.

Jacob, T., Ritchie, S., Assmann, S.M. and Gilroy, S. (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proc. Natl Acad. Sci. USA*, **96**, 12192–12197.

Jeannette, E., Rona, J.-P., Bardat, F., Cornel, D., Sotta, B. and Miginiac, E. (1999) Induction of *RAB18* gene expression and activation of K^+ outward-rectifying channels depend on an extracellular perception of ABA in *Arabidopsis thaliana* suspension cells. *Plant J.* **18**, 13–22.

Jouanneau, J.P. and Péaud-Lenoél, C. (1967) Growth and synthesis of proteins in cell suspensions of kinetin-dependent tobacco. *Physiol. Plant.* **20**, 834–850.

Knight, H. and Knight, M. (2001) Abiotic stress signalling pathways: specificity and cross-talk. *Trends Plant Sci.* **6**, 262–267.

Lang, V. and Palva, E.T. (1992) The expression of rab-related gene *RAB18* is induced by abscisic acid during cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **20**, 951–962.

Lee, S., Suh, S., Kim, S., Crain, R., Kwak, J.M., Nam, H.-G. and Lee, Y. (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wound plants. *Plant J.* **12**, 547–556.

van Leeuwen, W., Okrész, L., Bögre, L. and Munnik, T. (2004) Learning the lipid language of plant signalling. *Trends Plant Sci.* **9**, 378–384.

Lemtiri-Chlieh, F., MacRobbie, E.A.C. and Brearley, C.A. (2000) Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells. *Proc. Natl Acad. Sci. USA*, **97**, 8687–8692.

Lemtiri-Chlieh, F., MacRobbie, E.A.C., Webb, A.A.R., Manison, N.F., Brownlee, C., Skepper, J.N., Chen, J., Prestwich, G.D. and Brearley, C.A. (2003) Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proc. Natl Acad. Sci. USA*, **100**, 10091–10095.

Lepage, M. (1967) Identification and composition of turnip root lipids. *Lipids*, **2**, 244–250.

Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 199–222.

van der Luit, A., Piatti, T., van Doorn, A., Musgrave, A., Felix, G., Boller, T. and Munnik, T. (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiol.* **123**, 1507–1515.

Marchesini, N., Santandes, V. and Machado-Domenech, E. (1998) Diacylglycerol pyrophosphate: a novel metabolite in the *Trypanosoma cruzi* phosphatidic acid metabolism. *FEBS Lett.* **436**, 377–381.

Meijer, H.J.G. and Munnik, T. (2003) Phospholipid-based signaling in plants. *Annu. Rev. Plant Biol.* **54**, 265–306.

Meijer, H.J.G., Arisz, S., van Himbergen, J., Musgrave, A. and Munnik, T. (2001) Hyperosmotic stress rapidly generates lysophosphatidic acid in *Chlamydomonas*. *Plant J.* **25**, 541–548.

Munnik, T., Arisz, S.A., de Vrije, T. and Musgrave, A. (1995) G-protein activation stimulates phospholipase D signaling in plants. *Plant Cell*, **7**, 2197–2210.

Munnik, T., de Vrije, T., Irvine, R.F. and Musgrave, A. (1996) Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. *J. Biol. Chem.* **271**, 15708–15715.

Munnik, T., Irvine, R.F. and Musgrave, A. (1998) Phospholipid signalling in plant. *Biochim. Biophys. Acta*, **1389**, 222–272.

Munnik, T., Meijer, H., ter Riet, B., Hirt, H., Frank, W., Bartels, D. and Musgrave, A. (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant J.* **22**, 147–154.

Ng, C.K.-Y., Carr, K., McAinsh, M.R., Powell, B. and Hetherington, A.M. (2001) Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. *Nature*, **410**, 596–599.

Oprins, J., van der Burg, C., Meijer, H., Munnik, T. and Groot, J. (2001) PLD pathway involved in carbachol-induced Cl^- secretion: possible role of TNF-α. *Am. J. Physiol. Cell Physiol.* **280**, C789–C795.

Oshiro, J., Han, G.S. and Carman, G. (2003) Diacylglycerol pyrophosphate phosphatase in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **1635**, 1–9.

Paul, R., Holk, A. and Scherer, G. (1998) Fatty acids and lysophospholipids as potential second messengers in auxin action. Rapid activation of phospholipase A_2 activity by auxin in suspension-cultured parsley and soybean cells. *Plant J.* **16**, 601–611.

Pical, C., Westergren, T., Dove, S., Larsson, C. and Sommarin, M. (1999) Salinity and hyperosmotic stress induce rapid increases in phosphatidyl 4,5-bisphosphate, diacylglycerol pyrophosphate and phosphatidylcholine in *Arabidopsis thaliana* cells. *J. Biol. Chem.* **274**, 38232–38240.

Pierrugues, O., Brutesco, C., Oshiro, J., Gouy, M., Deveaux, Y., Carman, G., Thuriaux, P. and Kazmaier, M. (2001) Lipid phosphate phosphatase in *Arabidopsis*. *J. Biol. Chem.* **276**, 20300–20308.

Ritchie, S. and Gilroy, S. (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. *Proc. Natl Acad. Sci. USA*, **95**, 2697–2702.

Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids*, **5**, 494–496.

Ruelland, E., Cantrel, C., Gawer, M., Kader, J.C. and Zachowski, A. (2002) Activation of phospholipases C and D is an early response to cold exposure in Arabidopsis suspension cell. *Plant Physiol.* **130**, 999–1007.

Ryu, S.B. and Wang, X. (1998) Increase in free linoleic acids associated with phospholipase D-mediated hydrolysis of phospholipids in wounded castor bean leaves. *Biochim. Biophys. Acta*, **1393**, 193–202.

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

Schroeder, J., Allen, G., Hugouvieux, V., Kwak, J. and Waner, D. (2001) Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 627–658.

Staxen, I., Pical, C., Montgomery, L., Gray, J., Hetherington, A. and McAinsh, M. (1999) Abscisic acid induces oscillation in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proc. Natl Acad. Sci. USA*, **94**, 1779–1784.

Takahashi, S., Katagiri, T., Hirayama, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-triphosphate independent of abscisic acid in Arabidopsis cell culture. *Plant Cell Physiol.* **42**, 214–222.

Vajanaphanich, M., Kachintorn, U., Barrett, K., Cohn, J., Dharmsathaphorn, K. and Trayor-Kaplan, A. (1993) Phosphatidic acid modulates Cl^- secretion in T84 cells: varying effects depending on mode of stimulation. *Am. J. Physiol. Cell Physiol.* **264**, C1210–C1218.

Villasuso, A.L., Molas, M.L., Racagni, G., Abdala, G. and Machado-Domenech, E. (2003) Gibberellin signal in barley aleurone: early activation of PLC by G protein mediates amylase secretion. *Plant Growth Regul.* **41**, 197–225.

de Vrije, T. and Munnik, T. (1997) Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petals. *J. Exp. Bot.* **48**, 1631–1637.

Waggoner, D., Xu, J., Singh, I., Jasinska, R., Zhang, Q.-X. and Brindley, D. (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. *Biochim. Biophys. Acta*, **1439**, 299–316.

Wang, X. (2001) Plant phospholipases. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 211–213.

Wissing, J.B. and Behrbohm, H. (1993a) Phosphatidate kinase, a novel enzyme in phospholipid metabolism. Purification, subcellular localization, and occurrence in the plant kingdom. *Plant Physiol.* **102**, 1243–1249.

Wissing, J.B. and Behrbohm, H. (1993b) Diacylglycerol pyrophosphate, a novel phospholipid compound. *FEBS Lett.* **315**, 95–99.

Wissing, J.B., Kornak, B., Funke, A. and Riedel, B. (1994) Phosphatidate kinase, a novel enzyme in phospholipid metabolism. *Plant Physiol.* **105**, 903–909.

Wu, W.I., Liu, Y., Riedel, B., Wissing, J.B., Fischl, A.S. and Carman, G.M. (1996) Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 1868–1876.

Zhang, W., Qin, C., Zhao, J. and Wang, X. (2004) Phospholipase Dα1-derivated phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. *Proc. Natl Acad. Sci. USA*, **101**, 9508–9513.

Zonia, L. and Munnik, T. (2004) Osmotically induced cell swelling versus cell shrinking elicits specific changes in phospholipid signals in tobacco pollen tubes. *Plant Physiol.* **134**, 813–823.

Zonia, L., Codeiro, S., Tupy, J. and Feijo, J. (2002) Oscillatory chloride efflux at the pollen tube apex has a role in growth and osmoregulation and is targeted by inositol 3,4,5,6-tetra*kis*phosphate. *Plant Cell*, **14**, 2233–2249.