Induction of Abscisic Acid-Regulated Gene Expression by Diacylglycerol Pyrophosphate Involves Ca^{2+} and Anion Currents in Arabidopsis Suspension Cells[1][W]

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Diacylglycerol pyrophosphate (DGPP) was recently shown to be a possible intermediate in abscisic acid (ABA) signaling. In this study, reverse transcription-PCR of ABA up-regulated genes was used to evaluate the ability of DGPP to trigger gene expression in Arabidopsis (*Arabidopsis thaliana*) suspension cells. *At5g06760*, *LTI30*, *RD29A*, and *RAB18* were stimulated by ABA and also specifically expressed in DGPP-treated cells. Use of the Ca^{2+} channel blockers fluspirilene and pimozide and the Ca^{2+} chelator EGTA showed that Ca^{2+} was required for ABA induction of DGPP formation. In addition, Ca^{2+} participated in DGPP induction of gene expression via stimulation of anion currents. Hence, a sequence of Ca^{2+}, DGPP, and anion currents, constituting a core of early ABA-signaling events necessary for gene expression, is proposed.

Plants use various strategies to endure water shortage. In vegetative tissues, rapid limitation of water loss occurs through the closure of stomata, whereas long-term adaptation to drought and seed development are accompanied by the expression of a set of genes encoding proteins involved in protective processes. Abscisic acid (ABA) accumulates under hydric stress and is essential, via the control of guard cell movements and gene expression, for the establishment of drought resistance processes. Regulation of many stress-induced genes is mediated through ABA; however, the existence of ABA-independent pathways is also established (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2003; Riera et al., 2005). Among the set of ABA-inducible genes, late-embryogenesis abundant (LEA) protein genes are expressed during seed maturation and in vegetative parts of plants exposed to water limitation (Skriver and Mundy, 1990; Roberts et al., 1993; Bray, 1997). The signaling cascade leading to ABA-regulated genes is currently well understood with respect to DNA transcription. Promoters of ABA-responsive genes have specific ABA cis-acting elements able to bind trans-acting factors (Finkelstein et al., 2002). By contrast, knowledge of the sequence of events linking ABA perception to nuclear events is more fragmentary, although an ABA receptor was recently identified (Razem et al., 2006). Several kinases and phosphatases, G proteins, ion channels, Ca^{2+}, cADP-Rib, and lipid compounds are known to interact in complex signaling networks, but targets and partners of these elements are mostly not identified (Leung and Giraudat, 1998; Finkelstein and Rock, 2002).

Among the second messengers of ABA, Ca^{2+} plays an important role through changes in cytosolic Ca^{2+} concentration. For example, in GA-treated aleurone protoplasts, ABA diminishes cytosolic Ca^{2+} concentration (Ritchie and Gilroy, 1998). In guard cells, ABA provokes a fast rise in cytosolic Ca^{2+} concentration (McAinsh et al., 1990) that regulates anion efflux (Schroeder and Hagiwara, 1989; Hedrich et al., 1990), inhibits inward K^+ currents (Schroeder and Hagiwara, 1989; Grabov and Blatt, 1999), and triggers vacuolar K^+ release (MacRobbie, 2000).

Lipids and phospholipases are involved in most of the signaling pathways in plants (Meijer and Munnik, 2003; Testerink and Munnik, 2005). In ABA-regulated processes, phosphatidic acid (PA) produced by phospholipase D (PLD) activity and/or by phospholipase C coupled to diacylglycerol kinase is a messenger in guard cell movement, leaf senescence, and inhibition

[1] This work was supported in part by the Ministère de l'Education Nationale, France (grants to C.Z. and S.P.).

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[W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.080218.

of α-amylase production in aleurone protoplasts (Fan et al., 1997; Ritchie and Gilroy, 1998; Jacob et al., 1999). Moreover, the importance of PA in ABA responses was recently underlined by the demonstration that PA produced by PLDα was bound to the protein phosphatase 2C ABI1 to regulate its activity (Zhang et al., 2004).

In plants and yeast (*Saccharomyces cerevisiae*), PA can specifically be phosphorylated by PA kinase activity to form diacylglycerol pyrophosphate (DGPP; Wissing and Behrbohm, 1993a; Munnik et al., 1996; Wu et al., 1996). DGPP is a minor phospholipid, which, concomitantly to PA, increases in response to various abiotic and biotic stresses (Pical et al., 1999; Munnik et al., 2000; van der Luit et al., 2000; den Hartog et al., 2001; de Jong et al., 2004). After ABA application, the level of DGPP was also increased in Arabidopsis (*Arabidopsis thaliana*) suspension cells and seeds (Katagiri et al., 2005; Zalejski et al., 2005). Consequently, DGPP was hypothesized to play a role in plant signaling and, especially, in ABA responses (van Schooten et al., 2006). Regulation of the metabolic pathway that leads to ABA-induced DGPP accumulation is beginning to be elucidated. The lipid phosphate phosphatases (LPPs) responsible for the dephosphorylation of DGPP to PA and of PA to diacylgylcerol are encoded by four genes in Arabidopsis (Pierrugues et al., 2001; Katagiri et al., 2005). Expression of *AtLPP1* was shown to be induced by genotoxic stress and to be present in leaves (Pierrugues et al., 2001), whereas *AtLPP2* and *AtLPP3* were found in seeds where AtLPP2 is a negative regulator of ABA signaling during germination (Katagiri et al., 2005). PA kinase activity was found to be associated with plasma membranes in various plant species but, unfortunately, a PA kinase gene has not yet been identified (Wissing and Behrbohm, 1993b).

In Arabidopsis suspension cells, we have demonstrated that ABA provoked *RAB18* expression through stimulation of PLD activity (Jeannette et al., 1999; Hallouin et al., 2002). In this study, we show that DGPP is able to trigger the expression of three ABA up-regulated genes (*At5g06760*, *LTI30*, and *RD29A*) in addition to *RAB18*. Experiments performed to locate DGPP versus Ca^{2+} influx demonstrated that Ca^{2+} occurs both up- and downstream of DGPP formation in the ABA-signaling cascade that leads to gene expression. Our observations support the existence of an early core of common events necessary for ABA-induced expression of several genes.

RESULTS

DGPP Induces Expression of ABA Up-Regulated Genes

In Arabidopsis suspension cells, we previously observed that DGPP content was increased consecutively to ABA treatment and that the application of dioleoylDGPP was able to trigger the expression of *RAB18* (Zalejski et al., 2005). To evaluate the importance of DGPP for the expression of genes induced by ABA, three supplementary genes were chosen. *At5g06760*, *LTI30* (also named *DHNXERO2*, *At3g50970*), and *RD29A* (also named *LTI78* or *COR78*, *At5g52310*) were selected because they were characterized as ABA up-regulated genes (Leonhardt et al., 2004). Reverse transcription (RT)-PCR analysis of *At5g06760*, *LTI30*, and *RD29A* shows that their expression was stimulated by 10 μM ABA in the suspension cells within 3 h (Fig. 1; see also Supplemental Fig. 1). Application of dioleoylDGPP (300 μM, 3 h) also induced expression of these genes (Fig. 1). Expression of *At5g06760* and *LTI30* was neither induced by dioctanoylDGPP nor by dioctanoylPA and dioleoylPA. Weak expression of *RD29A* was recorded with the short fatty acid chains dioctanoylDGPP and dioctanoylPA, whereas dioleoylPA was less efficient than dioleoylDGPP in triggering *RD29A* expression (Fig. 1). From these data, we conclude that dioleoylDGPP was the most efficient phospholipid able to induce expression of several ABA up-regulated genes. The efficiency of dioleoylDGPP may be attributed to the long fatty acid unsaturated chains associated with the pyrophosphate moiety of the molecule.

Role of Ca^{2+} in DGPP Stimulation of Gene Expression

In Arabidopsis suspension cells, we observed that ABA caused a Ca^{2+} influx via fluspirilene- and pimozide-sensitive channels (Ghelis et al., 2000b). This Ca^{2+} entrance was required for *RAB18* expression. Hence, we questioned whether Ca^{2+} was also necessary for ABA- and DGPP-induced *At5g06760*, *LTI30*, and *RD29A* expression. Application of fluspirilene (50 μM) or pimozide (50 μM) decreased expression of *At5g06760*, *LTI30*, and *RD29A* triggered by ABA (Fig. 2). Thus, it appears that the increase in cellular Ca^{2+} concentration was, as well as DGPP, involved in ABA regulation of *At5g06760*, *LTI30*, and *RD29A* expression. To determine whether DGPP and Ca^{2+} acted independently or belonged to the same ABA-signaling cascade, two

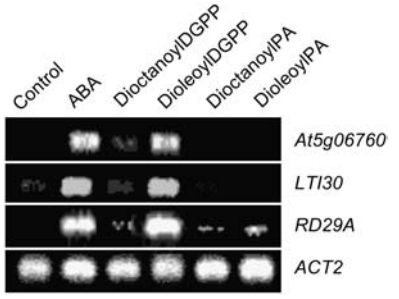

Figure 1. DioleoylDGPP induces expression of ABA up-regulated genes. RT-PCR analysis of *At5g06760*, *LTI30*, and *RD29A* expression in Arabidopsis suspension cells is shown. Total RNA (2.5 μg) was isolated from cells incubated for 3 h with 10 μM ABA or 300 μM dioctanoylPA, dioleoylPA, dioctanoylDGPP, or dioleoylDGPP. PCR products were separated on 2% agarose gel and stained with ethidium bromide. *ACT2* was amplified as a control. The data presented are representative of three independent experiments.

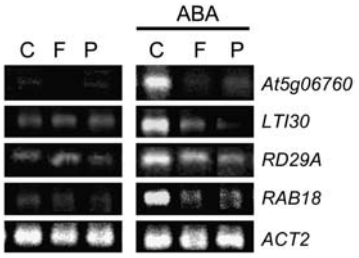

Figure 2. ABA regulation of gene expression requires Ca^{2+}. RT-PCR analysis of *At5g06760, LTI30, RD29A,* and *RAB18* expression in Arabidopsis suspension cells is shown. Total RNA (2.5 μg) was isolated from control cells (C) or cells incubated for 3 h with fluspirilene (F; 50 μM) or pimozide (P; 50 μM) and without or with ABA (10 μM). PCR products were separated on 2% agarose gel and stained with ethidium bromide. *ACT2* was amplified as a control. The data presented are representative of three independent experiments.

experiments were performed. First, we measured the DGPP level when ABA-treated cells were supplemented or not with Ca^{2+} channel inhibitors or a Ca^{2+} chelator. Cells were labeled with ^{33}P for 18 h and incubated for 15 min with fluspirilene (50 μM), pimozide (50 μM), or the Ca^{2+} chelator EGTA (5 mM), and then with or without ABA (10 μM) for 20 min. Lipids were then extracted, DGPP was separated by a two-dimensional (2-D) thin-layer chromatography (TLC) system and quantified. In ABA-treated cells, the amount of DGPP was increased 7-fold (Fig. 3). Fluspirilene and pimozide alone had no effect on DGPP basal level but, when they were added simultaneously with ABA, accumulation of DGPP was inhibited. Addition of fluspirilene inhibited DGPP formation only partially, whereas pimozide caused total inhibition of ABA-induced DGPP accumulation. EGTA alone triggered a slight increase in DGPP level and when added to ABA, provoked weak inhibition of DGPP formation (Fig. 3). Second, expression of *At5g06760*, *LTI30*, *RD29A*, and *RAB18* was recorded in cells simultaneously treated with DGPP and the Ca^{2+} channel inhibitors. Expression of the four genes induced by DGPP was diminished in the presence of fluspirilene and pimozide. Furthermore, pimozide was the most efficient inhibitor of DGPP action on gene expression (Fig. 4).

Taken together, data from Figures 2 to 4 demonstrate that Ca^{2+} acts both up- and downstream of DGPP formation in the ABA pathway, leading to *At5g06760*, *LTI30*, *RD29A*, and *RAB18* expression.

DGPP Induces Gene Expression through Ca^{2+} Stimulation of Anion Currents

To further explore how DGPP and Ca^{2+} act in the ABA-signaling pathway that leads to gene expression, we studied plasmalemma anion currents because they were shown to be stimulated by DGPP and necessary for *RAB18* expression (Ghelis et al., 2000a; Brault et al., 2004; Zalejski et al., 2005). We first examined the effect of the anion channel blocker 9-anthracene carboxylic acid (9-AC) on gene expression. Figure 5 shows that 9-AC (50 μM) partially inhibited ABA-induced expression of *At5g06760*, whereas expression of *LTI30*, *RD29A*, and *RAB18* was totally inhibited. When gene expression was triggered by DGPP, addition of 9-AC also inhibited gene expression. Therefore, DGPP action on gene expression required anion channel activity.

Whole-cell voltage-clamp assays were performed to elucidate the relation between Ca^{2+} and plasma membrane anion currents. DioleoylDGPP stimulated plasma membrane anion currents that induced membrane depolarization (Fig. 6, A, B, and D). DGPP evoked 300% activation in anion current intensity at −200 mV compared to the control (Fig. 6F). Addition of 9-AC to DGPP-treated cells reversed DGPP action (Fig. 6, A and F). When fluspirilene or pimozide were added after DGPP, the increase in anion currents was reversed (from 300% to 200% with fluspirilene and from 300% to 100% with pimozide) and repolarization of the plasma membrane was observed (Fig. 6, B, D, and F). Conversely, when fluspirilene or pimozide were added prior to DGPP, no significant increase in anion currents was recorded and no depolarization of the plasma membrane was observed (Fig. 6, C, E, and F). These data demonstrate that DGPP stimulation of plasmalemma anion currents is mediated by the activity of Ca^{2+} channels. Moreover, this step is necessary for expression of several ABA-regulated genes.

DISCUSSION

In this study, we demonstrated that expression of the ABA-regulated genes *At5g06760*, *LTI30*, and *RD29A* can be triggered by dioleoylDGPP in Arabidopsis cells. This observation extends findings from our previous study that was restricted to the role of dioleoylDGPP

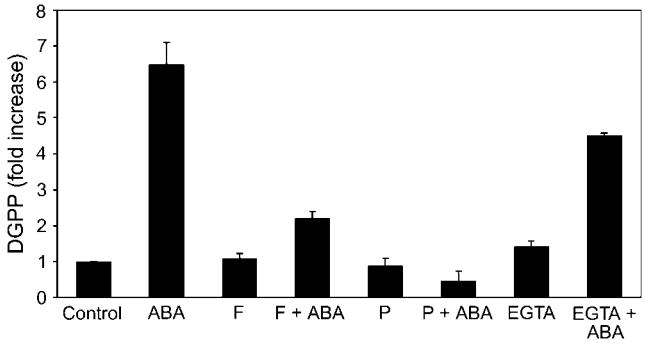

Figure 3. ABA stimulation of DGPP formation is impaired when Ca^{2+} influx is blocked. Cells were labeled for 18 h with $^{33}PO_4^{3-}$, incubated for 15 min with fluspirilene (F; 50 μM), pimozide (P; 50 μM), or EGTA (5 mM), and then for 20 min with ABA (10 μM) before lipid extraction. DGPP was separated with a 2-D TLC system and quantified. The relative DGPP level is expressed with reference to the level in the control (untreated cells). The results show the mean ± SEM; $n = 3$.

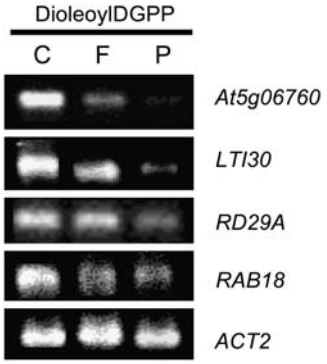

Figure 4. DGPP acts upstream of Ca^{2+} to trigger the expression of ABA up-regulated genes. RT-PCR analysis of *At5g06760, LTI30, RD29A,* and *RAB18* expression in Arabidopsis suspension cells is shown. Total RNA (2.5 μg) was isolated from cells incubated for 3 h with 300 μM dioleoylDGPP and fluspirilene (F; 50 μM) or pimozide (P; 50 μM). PCR products were separated on 2% agarose gel and stained with ethidium bromide. *ACT2* was amplified as a control. For gene expression in the control, F-, or P-treated cells, see Figure 2. The data presented are representative of three independent experiments.

on *RAB18* expression (Zalejski et al., 2005). Because the DGPP level was transiently increased after ABA perception (Zalejski et al., 2005), our results provided further evidence for the role of DGPP as a plant second messenger of ABA. By comparison with dioleoylDGPP, the nonphysiological dioctanoylDGPP was less efficient in triggering *RD29A* expression and unable to trigger *At5g06760* and *LTI30* expression. Thus, the effect recorded with dioctanoylDGPP could be a pharmacological-like effect. Interestingly, our results also showed that *RD29A* was expressed under dioleoylPA treatment. However, the effect of dioleoylPA was not recorded with all three of the chosen genes; for this reason, we assumed that PA's ability to mimic ABA was due to its own specificity and not to its subsequent phosphorylation into DGPP. This hypothesis is in accordance with the observation that PA production played a key role in the induction of *RD29A* in cold-treated Arabidopsis suspension cells (Vergnolle et al., 2005).

At5g06760 encodes a LEA protein of group 1 and *LTI30* and *RAB18* encode LEA proteins of group 2, also called dehydrins (Ramanjulu and Bartels, 2002). Due to their high hydrophilicity, LEA proteins are thought to play a major role in plant, animal, and prokaryote resistance to water deficit (Garay-Arroyo et al., 2000; Browne et al., 2002; Wise and Tunnacliffe, 2004). In plants, the predicted protective function of LEA proteins is further reinforced by many studies that correlate plant tolerance against drought, low temperature, and salinity with LEA protein expression (Lang and Palva, 1992; Lang et al., 1994; Welin et al., 1994; Nylander et al., 2001; Ramanjulu and Bartels, 2002). Moreover, in yeast, introduction of plant LEA confers increased resistance to freezing (Imai, 1996) and osmotic stress (Swire-Clark and Marcotte, 1999). Accordingly, increases in DGPP observed under osmotic stress (Pical et al., 1999; Munnik et al., 2000), salinity (Pical et al., 1999), or biotic stresses (van der Luit et al., 2000; den Hartog et al., 2001; de Jong et al., 2004) may induce LEA expression in vegetative tissues and therefore may be essential for plant survival in stress conditions. In ABA-treated seeds, the increase observed in DGPP level (Katagiri et al., 2005) could also regulate LEA expression and therefore participate in seed resistance against desiccation.

In ABA-treated cells, when Ca^{2+} influx was blocked or Ca^{2+} was chelated in the cell culture medium, the increase in DGPP level was impaired (Fig. 3). This observation demonstrates that the increase in DGPP was dependent on Ca^{2+} entry through diphenylbutylpiperidine-sensitive channels. Thus, the question is to determine how Ca^{2+} could participate in the control of the DGPP level. DGPP is the phosphorylated form of PA produced by PA kinase activity (Wissing and Behrbohm, 1993a, 1993b; Munnik et al., 1996). Conversely, DGPP can be dephosphorylated by the DGPP phosphatase activity of LPP (Pierrugues et al., 2001; Katagiri et al., 2005). Thus, the level of DGPP depends upon these two enzymatic activities and Ca^{2+} may either activate PA kinase or inhibit LPP or regulate both enzyme activities. Unfortunately, control of DGPP metabolism is currently not fully understood and a PA kinase gene has not been identified so far in plants or yeast (van Schooten et al., 2006). Nevertheless, PA kinase activity has been measured in several plant species (Wissing and Behrbohm, 1993b; Wissing et al., 1994). Accordingly, DGPP was also found in many species like Chlamydomonas (Munnik et al., 1996, 2000), *Craterostigma plantagineum* (Munnik et al., 2000), and tomato (*Lycopersicon esculentum*; van der Luit et al., 2000). However, to our knowledge, Ca^{2+} has never been reported as required for PA kinase regulation. Besides that, recent studies have given valuable information on plant LPPs that have added to data on

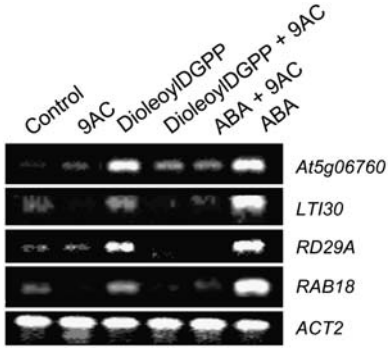

Figure 5. DGPP stimulation of gene expression occurs through anion current activation. RT-PCR analysis of *At5g06760, LTI30, RD29A,* and *RAB18* expression in Arabidopsis suspension cells is shown. Total RNA (2.5 μg) was isolated from cells incubated with dioleoylDGPP (300 μM), 9-AC (50 μM), and/or ABA (10 μM) for 3 h. PCR products were separated on 2% agarose gel and stained with ethidium bromide. *ACT2* was amplified as a control. The data presented are representative of three independent experiments.

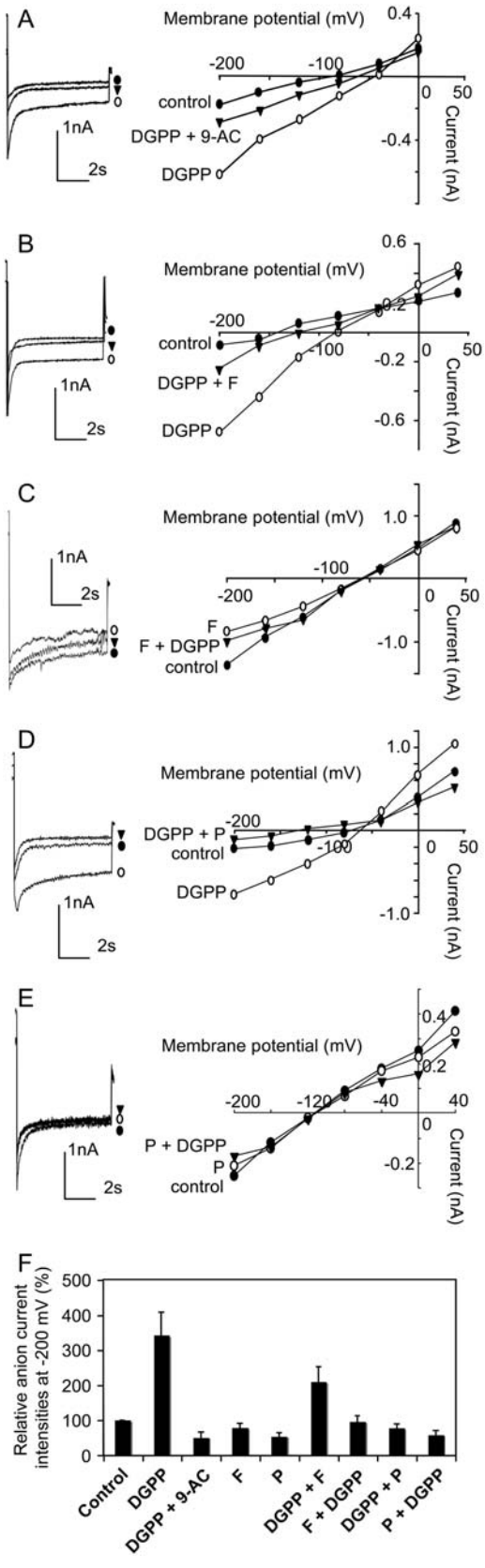

Figure 6. DGPP activation of anion currents requires Ca^{2+} channel activity. Whole-cell currents were activated by a depolarizing prepulse (+100 mV for 3.5 s; data not shown). Then, hyperpolarizing pulses ranging from −200 to +40 mV, in 40-mV steps, were applied for 6.5 s. Representative current traces at −200 mV and the current-voltage relationships determined for currents recorded after 10-s deactivation are shown. Holding potential was −40 mV. Currents were recorded from intact cells before (control) and 1 min after application of dioleoylDGPP (DGPP) or inhibitors. A, 100 μM DGPP for 5 min, then 100 μM anthracene-9-carboxylic acid (9-AC). B, 100 μM DGPP for 5 min, then 50 μM fluspirilene (DGPP + F). C, 50 μM fluspirilene (F) for 5 min, then 100 μM DGPP (F + DGPP). D, 100 μM DGPP for 5 min, then 50 μM pimozide (DGPP + P). E, 50 μM pimozide (P) for 5 min, then 100 μM DGPP (P + DGPP). F, Relative anion current intensities recorded at −200 mV, in percentage. Data represent the means of three replications ± SD.

yeast DGPP phosphatases (Oshiro et al., 2003). In Arabidopsis, *AtLPP1* was isolated by differential displays in UV-radiated cells and three more genes (*AtLPP2*, *AtLPP3*, and *AtLPP4*) were found, based on genomic sequence similarities (Pierrugues et al., 2001; Katagiri et al., 2005). The predicted structure of the four plant LPPs is closely related to yeast LPP with six transmembrane-spanning domains and three domains involved in phosphatase activity (Oshiro et al., 2003). In yeast, inhibition of DGPP phosphatase activity by Ca^{2+} was reported (Wu et al., 1996). Therefore, it is possible that in Arabidopsis suspension cells, Ca^{2+} inhibited DGPP phosphatase activity that participates in the regulation of the DGPP level. In addition, it is likely that Ca^{2+} regulates a signaling intermediate upstream of PA kinase or DGPP phosphatase activities. The existence of a Ca^{2+}-binding domain on PLDs (Wang, 2001) supports the hypothesis that Ca^{2+} may also regulate the DGPP level through PLD-mediated PA synthesis. Finally, posttranslational modifications of the enzyme responsible for DGPP metabolism may also occur.

Electrophysiological experiments have shown that dioleoylDGPP enhanced the activity of 9-AC-sensitive anion channels. Furthermore, RT-PCR analysis showed that dioleoylDGPP stimulation of gene expression occurred via anion current activation. Anion currents are involved in many physiological processes, including plant responses to pathogens (Wendehenne et al., 2002) or oxidative stress (Cazalé et al., 1998), but their precise role in signaling is poorly understood (Barbier-Brygoo et al., 2000). In suspension cells, the mechanism by which outward anion currents are required for gene transcription is unclear. One possibility is that plasmalemma depolarization induced by anion efflux (Brault et al., 2004) could be responsible for the activation of unknown targets. Otherwise, efflux of certain anions could be part of this specific response. In guard cells, ABA-regulated anion efflux plays a key osmotic role because it participates in loss of turgor (Blatt, 2000). In this study, we observed that addition of Ca^{2+} channel inhibitors impaired the activity of anion currents triggered by dioleoylDGPP. Hence, in accordance with several previous observations (Ghelis et al., 2000a, 2000b; Brault et al., 2004), a link between Ca^{2+}

and anion channels is suggested in Arabidopsis suspension cells. In guard cells, the dependence of anion channel activities on the cytosolic Ca^{2+} concentration increase was demonstrated (Schroeder and Hagiwara, 1989; Hedrich et al., 1990; Blatt, 2000), although activation of anion channels may also occur independently of Ca^{2+} (Schwarz and Schroeder, 1998; Levchenko et al., 2005). Thus, ABA regulation of anion currents in suspension cells is similar to that in guard cells.

Altogether, our results support the existence of an early signaling plasma membrane core of events necessary for ABA induction of gene expression. The arguments in favor of plasmalemma localization of DGPP, Ca^{2+} channels, and anion channels can be summarized as follows. First, PA kinase activity was associated with plasmalemma in various species, thus strongly suggesting that metabolism of DGPP occurs in the plasma membrane (Wissing and Behrbohm, 1993a). Moreover, in Arabidopsis suspension cells, mass spectrometry identification of AtLPP3 in the plasmalemma fraction (Marmagne et al., 2004) is consistent with the prediction of transmembrane domains in plant LPP (Pierrugues et al., 2001) and the membrane location of yeast (Han et al., 2001) and Arabidopsis LPP (Katagiri et al., 2005). Although we have no information on DGPP cellular transport, data from suspension cells are in favor of early intervention of DGPP in plasmalemma, where ABA perception occurs (Jeannette et al., 1999). Second, the cellular pool of Ca^{2+} involved in DGPP formation and the one involved in stimulation of anion currents originate, at least partially, from extracellular Ca^{2+}. Indeed, application of pimozide, fluspirilene, or EGTA impaired both the decrease in extracellular Ca^{2+} (Ghelis et al., 2000b) and, conversely, the increase in cytosolic Ca^{2+} triggered by ABA (Brault et al., 2004). Third, DGPP application enhances plasmalemma anion currents. This working model is obviously incomplete, but it provides a framework for the search for missing links, especially the DGPP targets.

Interestingly, a transcriptomics approach that was developed in guard cell protoplasts showed that *At5g06760*, *LTI30*, and *RAB18* genes were ABA induced in guard cells (Leonhardt et al., 2004). In addition, immunolocalization assays have shown that RAB18 was located in guard cells (Nylander et al., 2001). Albeit for one given gene the signaling pathway leading to its expression is likely dependent on the cell type, DGPP could play a role in ABA signaling leading to LEA expression in guard cells. The presence of DGPP has not, so far, been demonstrated in guard cells, but it was reported that PA could mimic ABA in stomatal closure (Jacob et al., 1999). Furthermore, the ABA-signaling components present in suspension cells (i.e. Ca^{2+} and anion currents) also play key roles in guard cells. Consequently, it is possible that, after ABA perception, a common module of plasmalemma components triggers both guard cell shrinkage and LEA gene expression. Hence, a specific role of DGPP could be to relay ABA in the induction of gene expression. Phosphorylation of PA in DGPP would be involved in several cellular responses.

MATERIALS AND METHODS

Arabidopsis Suspension Cells

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia cells were obtained by Axelos et al. (1992) and cultured at 24°C, under continuous white light (40 μE m^{-2} s^{-1}) with an orbital agitation at 130 rpm in 500-mL Erlenmeyer flasks containing 200 mL of culture medium (Jouanneau and Péaud-Lenoël, 1967). A 25-mL aliquot of suspension cells was transferred to fresh medium every week. All experiments were conducted on 3-d-old cells. The pH of the culture medium was 6.8 and viability of cells during the experimental treatment was systematically checked with trypan blue tests (data not shown).

Chemicals

ABA, pimozide, fluspirilene, EGTA, 9-AC, dioleoylPA, and dioctanoylPA were from Sigma. DioleoylDGPP and dioctanoylDGPP were from Avanti Polar Lipids. ABA, pimozide, and fluspirilene were added in dimethylsulfoxide at 0.1% final concentration in suspension cells. EGTA was added in water and 9-AC was diluted in methanol (0.1% final concentration). Lipids were sonicated in 1 mL of culture medium, four times, for 1 min at 4°C, and then added to suspension cells.

Lipid Labeling and Measurement of DGPP Level

Measurement of DGPP content was performed as previously described (Zalejski et al., 2005). Cells were labeled with $^{33}PO_4^{3-}$ (74 Bq/mL of suspension) for 18 h. Lipids from 2.5 g of vacuum-filtrated cells were first separated on K^+ oxalate-activated plates with an alkaline solvent chloroform:methanol:ammonia:water (90:70:4:16; v/v/v/v) as described by Munnik et al. (1995). Then the silica strip containing DGPP boarded down with phosphatidylinositol phosphate and up with PA was scrapped from TLC plates and extracted in methanol. DGPP was separated in a 2-D system described by Lepage (1967): first migration in chloroform:methanol:water (130:50:8; v/v/v), second migration in chloroform:acetone:methanol:acetic acid:water (100:40:20:20:10; v/v/v/v/v). Radiolabeled lipid spots were visualized with PhosphorImager (Storm; Molecular Dynamics) and analyzed with ImageQuant software (Amersham). Endogenous DGPP was identified by reference to the migration of standard DGPP. The relative DGPP level was expressed with reference to the level in untreated cells.

RT-PCR Analyses

Five milliliters of suspension cells were treated with ABA, lipids, and the above-mentioned chemicals for 3 h under the culture conditions. Then, cells were collected by vacuum filtration and total RNA was extracted with LiCl according to the protocol previously described (Verwoerd et al., 1989). For RT-PCR, 2.5 μg of total RNA were treated with 1 μL of DNase I (1 unit/μL; Sigma) added to 1 μL of DNase I buffer in a final volume of 8 μL. After 25 min at room temperature, 1 μL of Stop solution (Sigma-Aldrich) was added. The RT mix was prepared with 1 μL of 70 μM oligo(dT), 1 μL of 10 mM dNTP mix (Finnzymes), 1 μL of Moloney murine leukemia virus RTase (200 units/μL; Finnzymes), 1 μL of Out Ribonuclease Inhibitor (40 units/μL; Invitrogen), and 2 μL of reaction buffer (10×; Finnzymes) in a final volume of 9 μL. This RT mix was added to the RNA treated by Dnase I (final volume 20 μL) and cDNA synthesis was performed at 42°C for 60 min. The RTase was then inactivated at 70°C for 10 min. Two microliters of synthesized cDNA were used for PCR amplification by 0.5 units of Taq DNA polymerase (New England Biolabs) in a total volume of 25 μL under the following conditions: 94°C for 2 min, followed by 27 cycles consisting of 15 s at 94°C then 30 s at 59°C for *At5g06760*, *LTI30*, *RAB18*, or 64°C for *RD29A* and 1 min at 72°C. The procedure was ended at 72°C for 5 min. PCR was performed for 27 cycles within a linear range of amplification of *At5g06760*, *LTI30*, *RD29A*, *RAB18*, and *ACT2* genes. The number and temperature of cycles were optimized for each specific primer pair. We used the following oligonucleotide primers (Eurogentec): *At5g06760*,

forward 5′-GAGGAAAGTGTACGGTTTG-3′ and reverse 5′-CGTCATATC-GCTCGCC-3′; *LTI30*, forward 5′-AAGATTAAAGAGCAACTGCC-3′ and reverse 5′-AACGAAACCAGAAGTAGATATT-3′; *RD29A*, forward 5′-CGA-TGCACCAGGCGTAACAGG-3′ and reverse 5′-CCAGCTCAGCTCCTGACT-CGTC-3′; *RAB18*, forward 5′-TTGGGAGGAATGCTTCACC-3′ and reverse 5′-TTGTTCGAAGCTTAACGGC-3′; and *ACT2*, forward 5′-AACATTGTG-CTCAGTGGTGG-3′ and reverse 5′-TCATCATACTCGGCCTTGG-3′. Nine microliters of the PCR products were loaded and separated on 2% agarose Tris-acetate EDTA gel and visualized by ethidium bromide (2 mg/L) for photography.

Electrophysiology

Voltage-clamp measurements of whole-cell currents from intact cells were carried out at room temperature (20°C–22°C) using the technique of the discontinuous single-voltage-clamp microelectrode (Ghelis et al., 2000a). Cells were immobilized by means of a microfunnel (approximately 30–80 μm o.d.) and controlled by a pneumatic micromanipulator (de Fonbrune). Impalements were carried out with borosilicate capillary glass microelectrodes (0.5-μm diameter) filled with 600 mM KCl (electrical resistance from 50–100 MΩ). In this technique, both current passing and voltage recording used the same microelectrode. Sonicated dioleoylDGPP, 9-AC, pimozide, and fluspirilene were diluted in the bathing medium. The membrane potential was held at −40 mV. Anion currents were activated by a depolarizing prepulse (+100 mV for 3.5 s), then hyperpolarizing pulses ranging from −200 to +40 mV in 40-mV steps for 6.5 s were imposed. We systematically checked that cells were correctly clamped by comparing the protocol voltage values with those really imposed.

ACKNOWLEDGMENTS

We thank Dr. M. Brault for cell cultures and assistance with electrophysiological experiments. We acknowledge Dr. B. Sotta and A. Zachowski for critical reading of the manuscript and helpful discussions.

Received March 16, 2006; revised May 2, 2006; accepted May 23, 2006; published June 9, 2006.

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