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Research article

Differences in phosphatidic acid signalling and metabolism between ABA and GA treatments of barley aleurone cells

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ABSTRACT

Phosphatidic acid (PA) is the common lipid product in abscisic acid (ABA) and gibberellic acid (GA) response. In this work we investigated the lipid metabolism in response to both hormones. We could detect an in vivo phospholipase D activity (PLD, EC 3.1.4.4). This PLD produced [^{32}P]PA (phosphatidic acid) rapidly (minutes) in the presence of ABA, confirming PA involvement in signal transduction, and transiently, indicating rapid PA removal after generation. The presence of PA removal by phosphatidate phosphatase 1 and 2 isoforms (E.C. 3.1.3.4) was verified in isolated aleurone membranes in vitro, the former but not the latter being specifically responsive to the presence of GA or ABA. The in vitro DGPP phosphatase activity was not modified by short time incubation with GA or ABA while the in vitro PA kinase – that allows the production of 18:2-DGPP from 18:2-PA – is stimulated by ABA. The long term effects (24 h) of ABA or GA on lipid and fatty acid composition of aleurone layer cells were then investigated. An increase in PC and, to a lesser extent, in PE levels is the consequence of both hormone treatments. ABA, in aleurone layer cells, specifically activates a PLD whose product, PA, could be the substrate of PAP1 and/or PAK activities. Neither PLD nor PAK activation can be monitored by GA treatment. The increase in PAP1 activity monitored after ABA or GA treatment might participate in the increase in PC level observed after 24 h hormone incubation.

1. Introduction

In plants, phosphatidic acid (PA) plays a key role as a precursor in the biosynthesis of glycerophospholipids (GPL) and triacylglycerols (TAG), and as an important signal-transducing molecule [1,2]. It is also formed as a product during membrane GPL catabolism. Among hormone-responsive cells in cereal grains, the most intensively studied are located in aleurone, a secretory tissue that surrounds the starchy endosperm. In barley aleurone layers, synthesis of hydrolytic enzymes (mainly α-amylase) is induced by gibberellin (GA), thus providing resources for seed germination and early seedling growth, whereas abscisic acid (ABA) inhibits this response [3].

In addition to *de novo* synthesis in the endoplasmic reticulum, PA may be generated from plasma and chloroplast membrane glycerophospholipids (GPL) like phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphoinositides by the hydrolytic actions of phospholipases, such as phospholipase D (PLD), or phospholipase C (PLC) followed by diacylglycerol kinase (DGK) [4,5]. PLD or PLC/DGK-generated PA, has been proposed to have intracellular messenger functions, since it activates a number of physiological events in plants through the activation of specific protein targets [1,5]. Whether the PA molecules engaged in these lipid–protein interactions come from PLD or PLC/DGK remains to be elucidated.

The fact that PA accumulates rapidly and transiently in response to GA or ABA in membranes isolated from aleurone layers [6] is consistent with its role as a second messenger. In addition to enzymes involved in PA generation, those responsible for PA removal shortly after its increase may play a role in terminating PA

Abbreviations: ABA, abscisic acid; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DGPPase, diacylglycerol pyrophosphate phosphatase; FFA, free fatty acids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; GA, gibberellic acid; PAP1 and PAP2, NEM-sensitive and NEM-insensitive forms of phosphatidate phosphohydrolase, respectively; NPC, Non-specific phospholipase C; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; TAG, triacylglycerols; TL, total lipid; TLC, thin layer chromatography.

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signalling events. Reduction of PA levels after PLD activation may be effected by an active PA kinase (PAK) that phosphorylates PA to yield diacylglycerol pyrophosphate (DGPP) or by lipid phosphate phosphatases (LPPs), which dephosphorylate PA to produce DAG.

One of the aims of this study was to determine the ability of aleurone to produce PA through PLD activity, and also to reduce PA levels through the activity of relevant PA phosphatases after exposures to GA or ABA. For this purpose we incubated aleurone layers for several hours in the presence of [^{32}P]Pi to produce endogenous [^{32}P]GPL to be targeted by PLD in the absence or presence of hormones. In these samples, the time-course of [^{32}P]GPL class labelling showed some peculiarities of aleurone layer metabolism, such as an intriguingly high [^{32}P]PA/[^{32}P]PC labelling ratio as time elapsed. For this reason we decided to study the long-term effects of incubation medium on the lipids of non-labelled aleurone samples in the absence and the presence of these hormones. By surveying fatty acid composition and levels of main lipid classes in aleurone layers, we found that PLD appears to be the key enzyme that responds to ABA, the generated 18:2-PA being the lipid messenger whose action is terminated by the subsequent actions of PA-phosphatases to give DAG and PA-kinase to give 18:2-DGPP.

2. Results

2.1. ABA effect on PA metabolism

The rate at which [^{32}P]Pi was incorporated into individual aleurone barley phospholipids is shown in Fig. 1. At early times, PA and DGPP were the most actively labelled GPL (Fig. 1A). In addition to PA, [^{32}P]Pi was mainly taken up in phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (Fig. 1B), followed by phosphatidylinositol (PI) and phosphatidylcholine (PC) during the first hour. Phosphatidylinositol monophosphate (PIP), diacylglycerol pyrophosphate (DGPP) and phosphatidic acid (PA) were labelled relatively quickly, whereas labelled PIP_2 was not observed. The fact that signal lipids reached a maximum much earlier than the other phospholipids obviously reflects that these lipids all possess a mono ester phosphate group which approaches isotopic equilibrium, much more rapidly than the diester phosphate group present in the other phospholipids. The fast labelling of PA and DGPP suggested the presence of active DGK and PAK, acting on their respective endogenous substrates that accounted for low proportions of membrane lipids.

All phospholipid classes reached a steady state of labelling within 180–300 min. Based on this observation, ABA effects on PA levels and PLD activity were studied after labelling samples for longer than 300 min. For convenience, we chose overnight as an adequate pre-labelling time to label aleurone constituent GPL with [^{32}P]Pi.

Aleurone layers thus incubated were then treated with ABA for short periods to study the effects of the hormone on the labelling of PA as a function time (Fig. 2). A significant increase in [^{32}P]PA was observed after adding ABA in a concentration previously shown to inhibit amylase secretion (5 μM) (Fig. 2A and B). The maximal increase (1.5-fold) was reached between 5 and 10 min after adding the hormone. The amount of [^{32}P]PA then decreased (25% with respect to this maximum) between 10 and 40 min, to increase again at 60 min. In contrast, no increase was observed in [^{32}P]PA after GA treatment by 10 min (Fig. 2C). To confirm that ABA-induced increase in [^{32}P]PA had been mainly synthesized via ABA stimulation of PLD, this enzymatic activity was evaluated (Fig. 3). This assay was based on the ability of PLD to use a primary alcohol, in this case butanol, in frank preference to water as an acceptor for the phosphatidyl moiety to form phosphatidyl butanol (PBut) (Fig. 3). Ten minutes incubation in the presence of ABA sufficed to induce a 175 ± 15% increase in the amounts of [^{32}P]PBut and a 112 ± 5% increase in that of [^{32}P]PA, in comparison to controls considered 100% (data not shown). Because in the presence of butanol the activity of PLD almost exclusively produces PBut and ABA treatment did not change inositol phosphates (InsPs) and inositol trisphosphate ($InsP_3$) levels [4], these results show that the PA produced after ABA stimulation came from PLD activation.

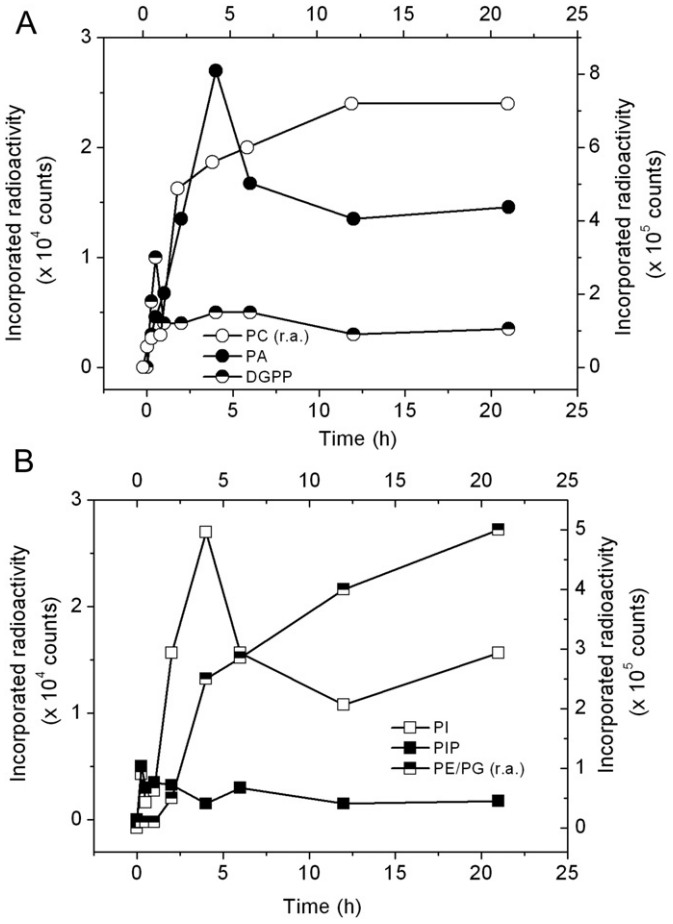
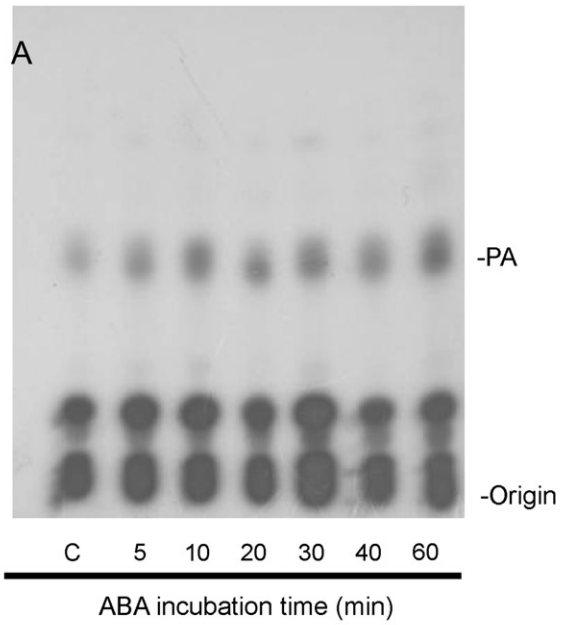


Fig. 1. Labelling of barley aleurone phospholipids as a function of time. Barley aleurones were incubated the presence of [^{32}P]Pi. Lipids were extracted and separated by TLC. Results are expressed as counts incorporated into each lipid class. r.a., right axis scale. (A) [^{32}P]Pi incorporated in PC, PA and DGPP. (B) [^{32}P]Pi incorporated in PG, PE, PI and PIP. Results are representative of three individual experiments.

2.2. Hormone effects on phosphatidate phosphohydrolases and DGPPase activities

In barley, cellular PA levels can be expected to be down-regulated by the activity of PA phospholydrolases (PAP). In this study PAP activities were in vitro detected in the 105,000 × *g* membrane fraction obtained from aleurones stimulated with ABA or GA (24 h) and they were differentiated on the basis of their NEM-sensitivity. This thiol-reactive compound inhibits PAP1 but does not affect PAP2 (currently known as lipin and LPPs, respectively) activity [6].

In the present study, PAP2 in vitro activity in control aleurone samples was nearly 15 times higher than PAP1 activity (58 ± 1.1 nmol/(h × mg of protein)) vs 4 ± 0.1 nmol/(h × mg of protein) (each represented as 100% in Fig. 5A). Incubation with GA decreased significantly (24%) PAP2 activity but ABA did not change it significantly (Fig. 4). In contrast, both hormones stimulated 100% PAP1 activity.



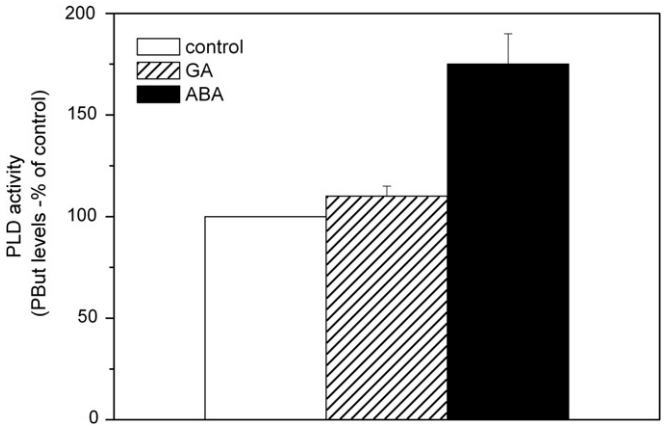


Fig. 3. Barley aleurone in vivo PLD activity in response to ABA. Aleurone layers were incubated in labelling buffer in the presence of [^{32}P]Pi overnight, and then treated with 0.75% (v/v) 1-butanol, in the absence (control) or in the presence or of ABA for 10 min. Lipids were extracted, separated by TLC and detected by autoradiography. Results are expressed as the increase of PBut with respect to control samples, and are mean values ± SE from three independent experiments.

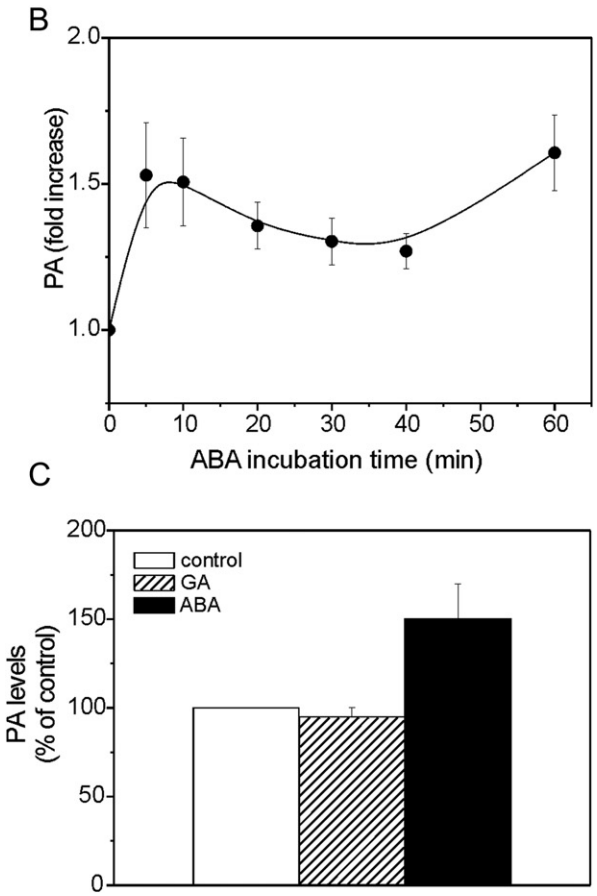


Fig. 2. Effect of ABA on in vivo PA generation in barley aleurone. Barley aleurones were incubated in labelling buffer in the presence of [^{32}P]Pi overnight, then ABA was added and incubations proceeded for the specified minutes. Lipids were extracted and separated by TLC. In (A) a representative TLC is shown, with lipids separated with the solvent containing ethyl acetate [19], quite convenient for the resolution of PA. In (B), PA level is expressed as the number of times its labelling was stimulated by ABA with respect to its label in its absence. In (C) PA level in response to 10 min GA or ABA treatment. Each point represents mean values ± SE from three individual experiments.

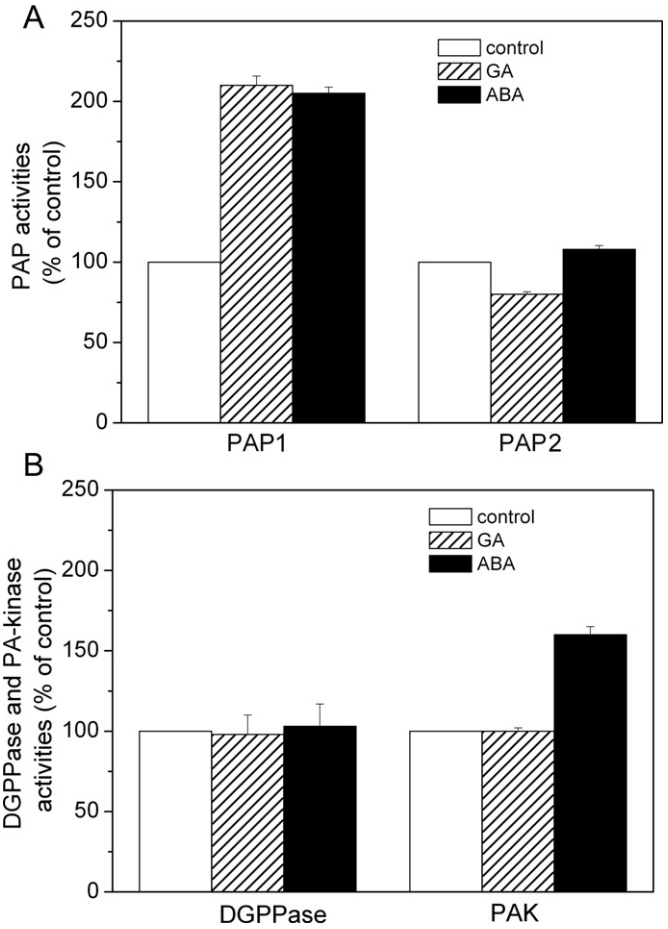


Fig. 4. Phosphatidate phosphohydrolase, diacylglycerol pyrophosphate phosphatase and phosphatidate kinase activities in membranes as measured in vitro. Enzymatic activities were assayed in the 105000 × g membrane fraction obtained from aleurones stimulated with ABA or GA (24 h). PAP1 and PAP2 activities were determined using [2-^{3}H]-PA plus dipalmitoyl phosphatidylcholine or [^{3}H]-PA alone as substrate for PAP1 or PAP2, respectively. DGPP phosphatase activity was measured by the release of water-soluble [^{32}Pi]Pi using chloroform-soluble [β-^{32}P]DGPP. For PAK assay, stimulated aleurone membranes were phosphorylated with [γ-^{32}P]ATP after ABA treatment as showed in Ref. [6]. Values shown are mean SE from three independent experiments.

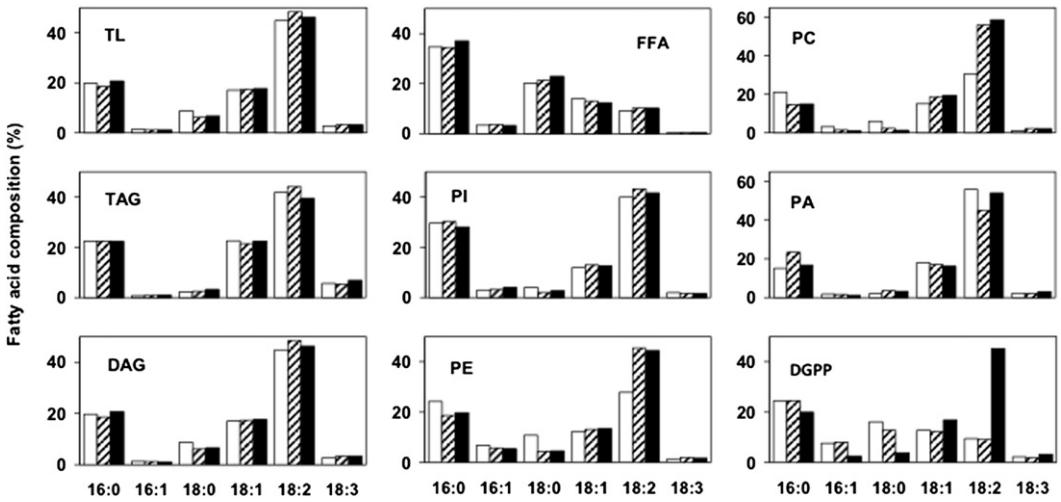


Fig. 5. Effects of 24 h incubation with GA or ABA on the fatty acid composition of lipid classes of barley aleurone layers. Barley aleurone layers were incubated for 24 h in media containing $CaCl_2$ in the absence or in the presence of GA or ABA. Aliquots of total lipid (TL) extracts, as well as lipid classes isolated by TLC, were converted to fatty acid methyl esters and analysed by GC. Figures represent % weight with respect to the total fatty acids in each lipid. Results are from one representative experiment. White, hatched and black bars: untreated controls, GA-treated and ABA-treated samples, respectively.

Levels of DGPP were previously shown to increase significantly after short-term incubations in the presence of GA or ABA [4] revealing the activity of a hormone-responsive PA kinase in aleurone membranes as a regulator of signalling PA levels (Fig. 4B). Another enzyme that could potentially contribute to modify PA levels after GA or ABA could be DGPP phosphatase (DGPPase), the enzyme involved in DGPP hydrolysis. However, our results showed that incubations in the presence of GA or ABA did not modify the in vitro activity of this phosphatase in membranes (Fig. 4B).

2.3. Changes in lipid fatty acid composition and levels associated with hormonal response

In order to survey characteristics of the lipid classes produced after long-term incubations, such as content and fatty acid composition, non-labelled samples of aleurone layers were subjected to long-term incubations in the absence and presence of GA or ABA, followed by lipid class separation and fatty acid analysis. Otherly written, we checked the effects of a long term (24 h) incubation of ABA or GA on lipid composition. After an incubation in control medium, the fatty acid composition of the total lipid (TL) of barley aleurone had linoleic (18:2$n-6$), oleic (18:1$n-9$), palmitic (16:0), linolenic (18:3$n-3$), and palmitoleic (16:1$n-7$) acids, in that order, as the major acyl groups (Fig. 5), with an average unsaturated/saturated fatty acid ratio higher than 4.0. Neither fatty acid composition nor this ratio was significantly modified in TL when these aleurone samples were incubated with ABA for 10, 30, or 60 min (data not shown).

The fatty acid profiles of the neutral lipids (TAG, DAG, FFA) and glycerophospholipids (GPL) classes studied after long-term incubations (24 h) in the absence or presence of GA or ABA are shown in Fig. 5. In controls, glycerolipids (TAG and DAG), as well as all GPL except DGPP, had 18:2$n-6$ as their major acyl group. The fatty acid composition of TAG closely resembled that of TL, a fact that could be expected, since TAG were major lipid components of aleurone layers (70% of the mass of the TL) (Fig. 6). The next more abundant lipid class, DAG, contained relatively more 18:0 and 18:2$n-6$, and less 18:3$n-3$, than TAG. In control samples, saturated fatty acids like 16:0 and 18:0 were major fatty acids only in DGPP and free fatty acids (FFA).

All GPL from samples incubated in the absence of hormones were poorer in 18:2$n-6$ than the corresponding GPL of samples incubated with GA or ABA (Fig. 5), with the remarkable exception of PA. This PA had the highest percentages of 18:2$n-6$ of all lipid classes examined, either neutral or polar.

PC was the lipid class showing the largest percentage of 18:2$n-6$ in the presence of both hormones (55% and 58% of PC fatty acids), and the lowest (30%) in their absence.

A remarkable change in fatty acid composition was undergone by DGPP after incubations with ABA (Fig. 5), but not with GA. This lipid showed one of the largest ABA-dependent increase in percentage of 18:2$n-6$ of all lipids examined.

The *amounts* of each of the lipid classes, as represented by the total content of their fatty acids, are shown in Fig. 6. In control aleurone samples, TAG was the most abundant lipid class, followed by DAG and (the sum of) GPL. Considering individual GPL, PA was the most abundant lipid class in control samples, followed (by far) by PI, PC, and PE, in that order.

After aleurone layer incubation in the presence of GA, the amounts of PI, PE, and even much more actively that of PC, increased significantly, while that of PA decreased (Fig. 6). After incubation with ABA, a similar rising trend as with GA was observed for the mentioned GPL (more with GA than with ABA). DAG levels also increased with both (although more with ABA than with GA). The most specific effect of ABA, not observed with GA, was to increase the amount of DGPP (Fig. 6).

Because it was the major fatty acid, the amount of 18:2$n-6$ in each of the lipids under study virtually replicated the changes in total fatty acids (Fig. 6, lower panels). The amount of 18:2, proportional to that of molecular species of each lipid that contain this fatty acid, showed even more drastically than the total fatty acids the trends of changes induced by hormonal treatments. Thus, after incubations with GA or ABA the amount of 18:2-PA was lower, while that of 18:2-PI, 18:2-PE, and especially of 18:2-PC, were markedly higher, than in controls. The increase of DGPP induced by ABA was mostly due to increases in the amount of molecular species containing 18:2$n-6$.

3. Discussion

In this study, aleurone layers incubated for a number of hours in Ca^{2+}-containing media, manifested a phospholipase D activity clearly apparent by the relatively high proportion of [^{32}P]PA produced after long-term incubations in the presence of [^{32}P]Pi as

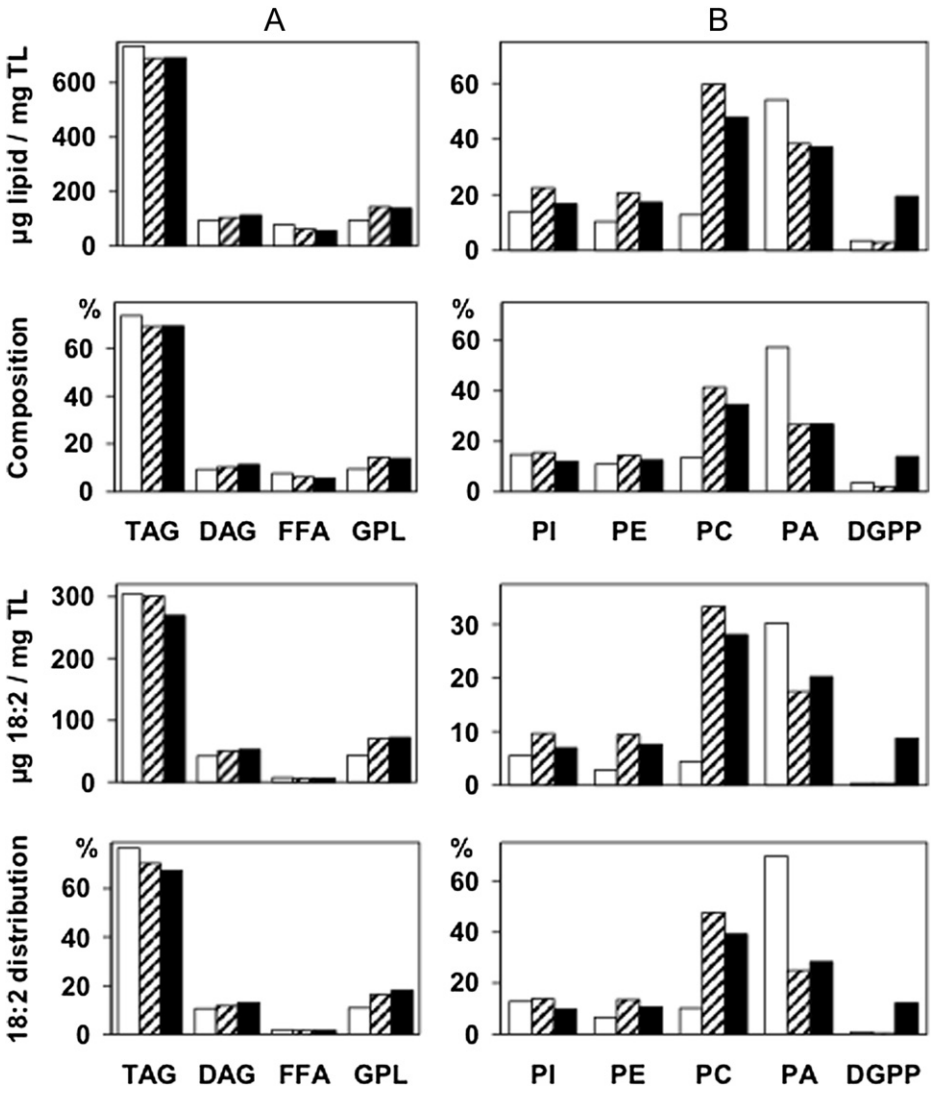


Fig. 6. Effects of 24 h incubation with GA or ABA on aleurone layer content and composition of main lipid classes, and contribution of 18:2$n-6$ to the changes in lipids. Fatty acids from lipids whose composition is shown in Fig. 5 were quantified using an internal standard. The amounts of each lipid are represented by the sum of their total fatty acids, with no further corrections. The amount of 18:2 in each lipid is given separately. Results from 10 aleurone layers in control conditions (white bars) and in the presence of GA or ABA (hatched and black bars, respectively) are compared. The amounts are given as µg of total fatty acids (or µg of 18:2$n-6$) in each lipid, per mg of TL in samples. Note that the vertical axis on the graphics which contain amounts of TAG and (the sum of) GPL (A), is about 10 times higher that those on the right (B), which show amounts of individual GPL classes in the same samples. Instead, the composition (%) graphics represent the contribution of each of the lipid classes there depicted to the corresponding totals, taken as 100%. Thus, panels (B) are proportional to the GPL composition (%) and the distribution (%) of 18:2$n-6$ among GPL classes.

precursor, as well as from the high amounts of PA produced after similar long-term incubations in non-labelled samples. Taken together, these results suggest that a significant part of the endogenous GPL, and part of those synthesized from [^{32}P]Pi during long-term incubations, were hydrolysed by PLD.

3.1. Short-term lipid changes

The short-term (minutes) responses of lipids to hormones are of interest because they point to specific enzymes that modulate cell signalling through controlling the generation of bioactive lipids. The production of PA is mediated by families of multiple enzymes that regulate the timing, location, amount, and molecular species of PA [2]. In aleurone layers whose lipids had been pre-labelled as described with [^{32}P]Pi, the presence of PLD not only was expressed, but was significantly stimulated (nearly 1.5-fold) after short-term (minutes) incubations in the presence of ABA. Evidence of PLD activity, and the fact that it was rapidly stimulated by ABA, was also confirmed using non-labelled samples of aleurone layers incubated in the presence of butanol (data not shown). ABA stimulation of PLD activity has been shown to occur at the plasma membrane and to be mediated by G-protein activity [7].

The fact that ABA-stimulated [^{32}P]PA increase was rapid but transient suggests that part of the produced PA was rapidly metabolized after being generated. One of the lipid products contributing to PA decrease was likely to be [^{32}P]DGPP. PA and DGPP comprise more than 50% of the total phosphorylated lipids of aleurones incubated with [γ-^{32}P]ATP for just 2 min [6]. After short-term incubations (minutes) of aleurone layers in the presence of ABA, increased levels of PA and DGPP are main products [4]. The PA produced by phospholipase D is rapidly phosphorylated by PA kinase to DGPP during the ABA signalling [6].

While PLC is the main player in the signalling of GA in aleurone [4], the increased DAG levels being then moderated by a rapid

stimulation of DAG-kinase and PA-kinase [4], PLD appears to be the key enzyme that responds to ABA, the generated PA being the lipid messenger whose action is terminated by the subsequent actions of PA-phosphatases to give DAG and PA-kinase to give DGPP. In other words, PA is the common lipid product in GA and ABA responses, although formed in membranes by PLC/DGK activities [4] and by PLD activities [6], respectively. This is interesting because PA mediates cellular functions through different modes of action, including modulation of enzymatic activities and/or structural effects on cell membranes [2], and because signalling PA produced by different metabolic routes at the plasma membrane might have different functions in different subcellular compartments of the cell.

3.2. Long-term changes in lipid labelling

After long-term incubations in the continuous presence of [^{32}P]Pi as precursor (as observed in Fig. 1) labelling of PC, PE, PG and PI occurred in aleurone, in this case necessarily involving slower biosynthetic pathways. Two sequential acylation steps need to take place to biosynthesize the PA destined to the biosynthesis of GPL and TAG in endoplasmic reticulum, or galactoglycerolipids in plastids, through the so-called Kennedy's pathway [8]. The generated PA then looses its phosphate through the action of PA phosphatase(s) (PAP) to become the glycerol skeleton of more complex lipids.

In our case [^{32}P]Pi necessarily had to label CMP-PA to result in [^{32}P]PI, and to label ATP and/or CTP to lead to the ^{32}P-labelled CDP-bases that then react with (an unlabelled) DAG to produce the observed [^{32}P]PC and [^{32}P]PE. We previously found that barley has two homologues of PAP [6], which do not contain a transport sequence, for they both play their role in the endoplasmic reticulum. We observed here that GA and ABA both stimulated significantly, and to similar extents, PAP1 activity as measured in vitro. The 9-fold more active PAP2 was decreased marginally with GA and was virtually unchanged with ABA. The possibility that each PAP isoform could produce DAG that are separately involved in GPL and TAG biosynthesis is worth investigating in the future.

3.3. GA- and ABA-induced changes in aleurone lipid content and composition

The lipid composition showed that PA was the major GPL class present after long-term incubation of aleurone layers in the *absence* of hormones, confirming that simultaneously with biosynthesis, indicated by [^{32}P]Pi incorporation, there was an active lipid catabolism that evidently involved the PLD activity we confirmed in this study.

In the absence of hormones, PA not only was several times higher than PC and PE in amount, but was considerably richer in 18:2n − 6 (close to 60% of the fatty acids) than these two lipids, especially than PC.

In the presence of GA or ABA, the amounts of PC and PE were higher, and that of PA was lower, than in their absence. This reversion mainly involved the species that contained 18:2n − 6; the percentage of 18:2n − 6 was lower in PA, and evidently higher in PC (nearly 60% of PC fatty acids) than in control PC (30%).

A GPL class whose amount increased several fold, mostly due to an increase in its species containing 18:2n − 6, was DGPP. This increase *exclusively* occurred in the presence of ABA. Thus, the composition studies would indicate that the molecules of PA and DGPP form part also during the ABA response in barley aleurone [6].

Part of the hormone-dependent reduction in PA levels could have been converted by PAPs into DAG during incubations. The latter were also higher in amount, and richer in 18:2n − 6, in samples incubated with GA and ABA than the DAG from samples incubated with no hormones. This increase could reflect the combined activity of the two PAP isoforms we studied. Thus, PAP2 was basally much more active than PAP1, but the latter was frankly activated by the two hormones. The fact that the amount of DAG increased with both, but less with GA than with ABA, may indicate a more active GA-dependent use of DAG for biosynthetic purposes. It seems to correlate with the presence of GA resulting in higher levels of PC and PE than that of ABA.

The origin of DAG in non-labelled samples is more difficult to ascertain than that of PA. Thus, GPL including PI could contribute to these DAG through PLC activation, and TAG hydrolysis could also lead to increased DAG levels, considering that the amounts of TAG were really massive and that during incubations in the presence of both hormones there was a reduction in TAG levels. It is interesting that FFA were the only lipids to decrease with both hormones, and that there was little free 18:2n − 6 and negligible free 18:3n − 3 in aleurone layers.

In conclusion, the amount of TAG, FFA, and PA decreased while most GPL increased in the presence of GA. In the presence of ABA, TAG, FFA, and PA also decreased, with the difference that part of the produced PA was diverted into DGPP (Fig. 7). Excluding most of the TAG and part of the DAG, most of which are likely to be confined in lipid vacuoles, PA and major GPL coexist in aleurone layer membranes. Changes in their ratios as the ones observed here may be expected to result in large changes in membrane biophysical properties. An important determinant of the biological functions of phosphatidic acid is its anionic headgroup, which has a peculiar

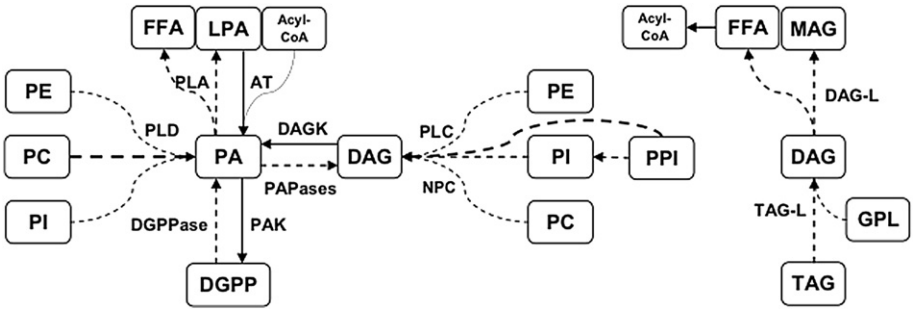


Fig. 7. Diagram showing the lipid classes and enzymes involved in the generation and removal of PA referred to in the text. Broken and continuous lines represent hydrolytic and energy-requiring reactions, respectively. The thicker broken lines from PC and PPI (polyphosphoinositides) point to these two membrane GPLs as putative main sources for the generation of signalling PA and DAG, respectively, at the plasma membrane. TAG stored in lipid-rich vacuoles of aleurone could be hydrolyzed by lipases (L) to eventually release stored fatty acids for use as an energy source or membrane GPL biosynthesis. Stimulation of these lipases could be one of the GA or ABA responses elicited by the generated PA as a lipid messenger. Enzymes using PA and DAG for the biosynthesis complex GPL and TAG, taking place at endoplasmic reticulum membranes, are not included in this chart.

ionization and is crucial in lipid–protein interactions [9]. It can thus be expected that changing PA/PC ratios as the ones shown in this study may result in crucial changes in the physical properties of membranes in the presence versus the absence of hormones.

In coincidence with the present results, freezing at a sublethal temperature induced a decline in PC, and an increase in PA, among minor changes in other membrane lipids, in leaves of wild-type *Arabidopsis* [10]. These changes were attributed to freezing stress, and were significantly reduced in plants deficient in the most abundant form of PLD, PLDα. By a similar token, one could speculate that part of the lipid changes we observed in our aleurone layer controls were associated to sustained PLD activation caused by an as yet inadvertent source of stress. The fact that the negative changes in lipids were in part successfully neutralized by incubation in the presence of GA and ABA agree with the notion that these hormones, in addition to the rapid generation of bioactive lipids, work in favour of membrane lipid integrity and plant tissue preservation.

4. Materials and methods

4.1. Plant material

Barley grains (*Hordeum vulgare* L, cv. Himalaya) were de-embryonated, surface sterilized, and allowed to imbibe in sterile water in the dark for 4 days at room temperature. Aleurone layers were isolated by gently scraping away the starchy endosperm with metal spatulas. Ten layers were incubated for different periods at 25 °C in the dark, with gentle shaking, in a medium containing 20 mM $CaCl_2$ in the absence (controls) or in the presence of GA or ABA (5 μM) [11].

4.2. [^{32}P]Pi phospholipid labelling, extraction and analysis

Aleurones (10 layers) were incubated in 1 mL label medium (20 mM $CaCl_2$ and 20 mM sodium-succinate, pH 6.5) containing 50 μCi carrier-free [^{32}P]orthophosphate, abbreviated as [^{32}P]Pi. Treatments were stopped at specified times by adding 250 μL of 25% v/v perchloric acid, vortexing for 5 min, and maintaining sample-containing tubes for 30 min at room temperature. After discarding perchloric acid, aleurone lipids were extracted by adding 400 μL chloroform/methanol/hydrochloric acid (50/100/1, v/v/v) and freezing and thawing the mixture by means of liquid nitrogen. After 5 min of vigorous mixing, lipid extracts were transferred to clean tubes and 400 μL chloroform and 214 μL 0.9% (w/v) NaCl were added to produce a two-phase system [12]. After vortexing (1 min) and centrifugation (1000 × *g*, 5 min), the upper phase was removed and the lower phase washed with 500 μL chloroform/methanol/1 M hydrochloric acid (3/48/47, v/v/v). Lipid extracts were dried by vacuum centrifugation, dissolved in a suitable volume of chloroform, and immediately subjected to TLC analysis. An alkaline solvent system, chloroform/methanol/25% ammonium hydroxide/water (45/35/2/8, v/v/v/v), was used to separate labelled GPL as described by Den Hartog et al. [12].

4.3. Phospholipase D activity

PLD activity was measured by the production of phosphatidyl butanol (PBut), essentially as described by Den Hartog et al. [12]. After labelling lipids with [^{32}P]Pi as described, aleurone layers, untreated or treated with hormones, were incubated in the presence of 0.75% v/v n-butanol for 30 min at 24 °C. Reactions were stopped and lipids were extracted for separation of PA and PBut from other phospholipids, which was achieved by using the organic upper phase of the biphasic system, ethyl acetate/isooctane/formic acid/H_2O (13/2/3/10, v/v/v/v) as the TLC solvent. Radiolabelled lipids were located by autoradiography on Kodak film. Spots were scraped off the plates, and fractions were counted in a liquid scintillation counter.

4.4. Phosphatidate phosphohydrolase (PAP) activities

Lipid phosphatases were measured in samples of a membrane fraction prepared from aleurone layers as previously described [6]. Briefly, aleurone layers prepared as above were incubated in a shaking bath for 15 min at 25 °C, in 20 mM succinate buffer (pH 6.5) containing 20 mM $CaCl_2$, then stimulated with ABA or GA (final concentration 5 μM) for 24 h, washed three times with cold buffer, and suspended in 10 volumes of 50 mM HEPES (pH 7.4) containing 0.25 M sucrose, 5 mM KCl, 1 mM EDTA, and protease inhibitors (1 μg mL^{-1} Leupeptin; 1 mM PMSF, 1 μg mL^{-1} Aprotinin). This suspension was frozen in liquid N_2 and thawed (three times), homogenized, and centrifuged at 1000 × *g* for 15 min to remove unbroken cells and cell debris. The supernatant was centrifuged at 105,000 × *g* for 60 min to obtain membrane fraction. The membranes were washed, resuspended with 50 mM HEPES, pH 7.4, and used as a source of phosphatase activities.

The radiolabelled PA used as substrate was prepared from [2-^{3}H]-PC, as described by Pasquaré et al. [13]. Radioactivity and phosphorus content [14] were then measured to determine the specific radioactivity of [2-^{3}H]PA, 0.1–0.2 μCi/μmol. Briefly, PAP activities were differentiated according to NEM-sensitivity [15,16]. Membrane proteins were incubated with the substrate 0.6 mM [2-^{3}H]-PA (0.1–0.2 μCi/μmol); substrate was prepared as showed elsewhere [6] and resuspend in a buffer in presence or absence of PC [15,16]. All assays for determination of PAP1 or PAP2 activities were conducted for 30 min at 37 °C. Enzyme assays were stopped by addition of chloroform/methanol (2/1, v/v). Blanks were prepared in the same way, except that the membranes were boiled for 5 min before use. The 1,2-diacyl[^{3}H]glycerols and [^{3}H]monoacylglycerol produced from PAP activity were isolated by TLC and counted. PAP activity was expressed as the sum of nmol of ([^{3}H]diacylglycerol and [^{3}H]monoacylglycerol) × (h × mg protein)$^{-1}$.

Lipids were extracted from membranes with chloroform/methanol (2/1, v/v) and the extracts were washed with 0.2 volumes $CaCl_2$ (0.05%) [17]. Lipids were resolved by gradient-thickness TLC on silica gel G [18] using hexane/diethyl ether/acetic acid (35/65/1, v/v/v). After development, phospholipids including [2-^{3}H]-PA remained at the origin of the TLC plates. Lipids were located by exposing the plates to iodine vapours and scraped off into vials for liquid scintillation counting after adding 0.4 mL water and 10 mL 5% Omnifluor in toluene/Triton X-100 (4/1, v/v).

4.5. DGPP phosphatase activity

DGPP phosphatase activity was assayed as described by Han and Carman [19], based on release of water-soluble [^{32}P]Pi from chloroform-soluble [β-^{32}P]DGPP (2000 cpm/pmol). The reaction mixture contained 50 mM citrate buffer (pH 5.0), 0.1 mM DGPP, 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein, in a total volume of 0.1 mL. DGPP was synthesized from PA using enriched-membrane fraction and *H. vulgare* PA kinase [20]. Enzyme assays were conducted for 20 min at 30 °C in duplicate.

4.6. Separation of lipids for fatty acid analysis

For lipid and fatty acid analysis, 10 aleurone layers per sample were incubated in the presence or absence of GA or ABA for 24 h as described above and lipids were extracted using chloroform/methanol mixtures [21]. Aliquots were taken from lipid extracts for the determination of total lipid (TL) fatty acid composition.

Phospholipids were subjected to two-dimensional TLC [10] to resolve them into classes, recovering also free fatty acids (FFA) and neutral lipids (NL). The latter were resolved into TAG and DAG by means of TLC using hexane/ether (80/20, v/v). After TLC, lipids were located with dichlorofluorescein and eluted from the silica support by thoroughly mixing it with water/methanol/chloroform (1/5/5, v/v/v), followed by centrifugation (three times) and partition of eluates with 4.5 volumes of water [21]. After adding an appropriate internal standard, dried samples of the separated lipid classes in screw-capped tubes were dissolved in (N_2-saturated) anhydrous methanol containing 0.5 N H_2SO_4 and warmed at 45 °C overnight under N_2 to obtain the corresponding fatty acid methyl esters (FAME). After methanolysis, TLC using pre-cleaned silica gel G plates and hexane/ether (95/5, v/v) as solvent was used to purify FAME. The latter were recovered from the silica support by mixing it with water and methanol, followed by hexane (1/1/1, v/v/v). The hexane layer was recovered after centrifugation, repeating the hexane extraction twice more.

Fatty acid analysis was performed using a Varian 3700 gas chromatograph equipped with two (2 m × 2 mm) glass columns packed with 15% SP 2330 on Chromosorb WAW 100/120 (Supelco Inc., Bellefonte, PA) and two flame ionization detectors. The carrier gas was N_2 (30 mL/min). The column oven temperature was programmed from 155 °C to 230 °C at a rate of 5 °C/min. Injector and detector temperatures were 220 °C and 230 °C, respectively.

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