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Diacylglycerol pyrophosphate inhibits the α-amylase secretion stimulated by gibberellic acid in barley aleurone

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ABA plays an important regulatory role in seed germination because it inhibits the response to GA in aleurone, a secretory tissue surrounding the endosperm. Phosphatidic acid (PA) is a well-known intermediary in ABA signaling, but the role of diacylglycerol pyrophosphate (DGPP) in germination processes is not clearly established. In this study, we show that PA produced by phospholipase D (E.C. 3.1.4.4) during the antagonist effect of ABA in GA signaling is rapidly phosphorylated by phosphatidate kinase (PAK) to DGPP. This is a crucial fact for aleurone function because exogenously added dioleoyl-DGPP inhibits secretion of α-amylase (E.C. 3.2.1.1). Aleurone treatment with ABA and 1-butanol results in normal secretory activity, and this effect is reversed by addition of dioleoyl-DGPP. We also found that ABA decreased the activity of an Mg^{2+}-independent, N-ethylmaleimide-insensitive form of phosphatidate phosphohydrolase (PAP2) (E.C. 3.1.3.4), leading to reduction of PA dephosphorylation and increased PAK activity. Sequence analysis using *Arabidopsis thaliana* lipid phosphate phosphatase (LPP) sequences as queries identified two putative molecular homologues, termed *HvLPP1* and *HvLPP2*, encoding putative Lpps with the presence of well-conserved structural Lpp domains. Our results are consistent with a role of DGPP as a regulator of ABA antagonist effect in GA signaling and provide evidence about regulation of PA level by a PAP2 during ABA response in aleurone.

Introduction

The aleurone is a secretory tissue that surrounds the starchy endosperm of the cereal grain. GA induces synthesis of hydrolytic enzymes (principally α-amylase) in aleurone, providing resources for seed germination and early seedling growth (Lovegrove and Hooley 2000, Ritchie et al. 2002). ABA inhibits the response of aleurone to GA, evoking changes in cytoplasm pH (Heimovaara-Dijkstra et al. 1994a, van der Veen et al. 1992), membrane potential (Heimovaara-Dijkstra et al. 1994b) and in cytosolic calcium levels (Gilroy 1996, Ritchie and Gilroy 1998). The phospholipid signaling pathway that inhibits α-amylase secretion remains poorly understood. Phospholipids are a major component of all biological membranes and play a key role in signal transduction processes (Meijer and Munnik 2003, Testerink and Munnik 2005). In ABA-regulated processes, phosphatidic acid (PA) produced by phospholipase D (PLD) is an important messenger responsible for inhibition of α-amylase production in aleurone (Ritchie and Gilroy 1998). Enzymes that metabolize PA also play an important role in switching the PA signal on/off. The two principal routes leading to PA production are PLD-mediated hydrolysis of structural phospholipids and the combined action of

Abbreviations – DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DPPC, dipalmitoyl phosphatidylcholine; FFA, free fatty acid; LPA, lyso-phosphatidic acid; LPP, lipid phosphate phosphatase; NEM, N-ethylmaleimide; PA, phosphatidic acid; PAK, phosphatidate kinase; PAP, phosphatidate phosphohydrolase; PLC, phospholipase C; PLD, phospholipase D.

phospholipase C (PLC) and diacylglycerol kinase (DGK) (Munnik et al. 1998). Removal of PA is caused by several enzymes (1) phosphatidate kinase (PAK) that phosphorylates PA to yield diacylglycerol pyrophosphate (DGPP) (Carman and Zeimetz 1996, Wissing et al. 1994, Wu et al. 1996); (2) lipid phosphate phosphatases (Lpps) that dephosphorylate PA to produce diacylglycerol (DAG) (Pierrugues et al. 2001); and (3) PA-selective A type phospholipase that deacylates PA to produce lysophosphatidic acid (LPA) and free fatty acids (FFAs). Besides reducing PA function, these enzymatic activities can also generate new lipid messengers, such as DGPP, DAG and FFAs (Wang et al. 2006).

PA and DGPP are accumulated transiently in response to various abiotic and biotic stresses (den Hartog et al. 2001, de Jong et al. 2004, Meijer and Munnik 2003, Munnik et al. 2000, Pedranzani et al. 2003, Pical et al. 1999, van der Luit et al. 2000), or after ABA treatment (Katagiri et al. 2005, Zalejski et al. 2005). It has therefore been suggested that DGPP may function as a signaling molecule itself (Balboa et al. 1999, Munnik et al. 1996, Santander et al. 2002), particularly in response to ABA (van Schooten et al. 2006, Villasuso et al. 2003). Consistent with this idea, DGPP was shown to trigger expression of four ABA upregulated genes (Zalejski et al. 2005, 2006).

Little is known about regulation of the metabolic pathway that leads to ABA-induced DGPP accumulation. DGPP is produced rapidly together with PA, but high levels of DGPP are rapidly eliminated. The plant DGPP phosphatase activity described by Riedel et al. (1997), termed LPP, preferentially not only dephosphorylates DGPP to PA but also dephosphorylates PA to DAG, allowing sequential conversion of DGPP to PA to DAG (Pierrugues et al. 2001). Phosphatidate phosphohydrolases type 2 (PAP2) are Mg^{2+}-independent and N-ethylmaleimide (NEM)-insensitive forms (Carman 1997, Waggoner et al. 1999) and were renamed lipid phosphate phosphohydrolases (LPPs) (Brindley and Waggoner 1998). LPPs from *Saccharomyces cerevisiae* and mammalian cells exhibit activity toward a variety of substrates such as LPA, S1P and C1P, besides PA (Toke et al. 1998a, 1998b, Waggoner et al. 1999).

In barley aleurone, we showed previously that PA and DGPP are metabolically related in response to ABA, suggesting that synthesis and metabolism of both may be coupled to the activity of PLD/LPP/PAK and proposed a role for DGPP in α-amylase secretion (Villasuso and Machado-Domenech 2004, Villasuso et al. 2003). We now provide evidence supporting the hypothesis that DGPP is a key element in the antagonist effect of ABA on GA signaling. PA produced was rapidly phosphorylated to DGPP by PAK and inhibited GA-stimulated α-amylase secretion, mimicking ABA stimulation. ABA decreased the activity of PAP2, consistent with increased PAK activity. Effect of 1-butanol on ABA-stimulated PLD activity was overcome by addition of dioleoyl-DGPP. These observations demonstrate a link between PAK and PAP2 activities. The transient increase of DGPP may activate pathways leading to regulation of aleurone secretory activity.

Materials and methods

Plant material and α-amylase assay

Barley grains (*Hordeum vulgare* L, cv. Himalaya) were deembryonated, 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. Layers (10) were incubated for 24 h at 25°C in the dark, with gentle shaking, in the presence of 20 m*M* $CaCl_2$ with 5 μ*M* ABA, 5 μ*M* GA or both. α-Amylase activity in the incubation medium was measured by the starch I/KI method (Jones and Varner 1967). When α-amylase activity was determined in the presence of dioleoyl-PA, dioleoyl-DGPP or dioctanoyl-DGPP, these compounds (purchased from Avanti Polar Lipids, Alabaster, AL) were added from 1000-fold concentrated stock solutions in chloroform. Prior to use, they were dried under N_2 stream and emulsified by sonication for 30 s, six times, in 50 m*M* HEPES.

Preparation of aleurone membrane

Aleurone layers prepared as above were incubated in a shaking bath for 15 min at 25°C in 20 m*M* succinate buffer (pH 6.5) containing 20 m*M* $CaCl_2$, then stimulated with ABA (final concentration 5 μ*M*) at the indicated times, washed three times with cold buffer and suspended in 10 volumes of 50 m*M* HEPES (pH 7.4) containing 0.25 *M* sucrose, 5 m*M* KCl, 1 m*M* EDTA and protease inhibitors (1 μg ml^{-1} leupeptin, 1 m*M* phenyl methane sulfonil fluoride (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 m*M* HEPES, pH 7.4, and used as a source of lipid kinase activities.

Lipid kinase activity determination

The membrane fraction isolated as above (60 μg protein) was added to thermally equilibrated (30°C) 50 m*M* HEPES buffer, pH 7.4, 0.1 m*M* EDTA, 0.5 m*M* DTE, 10 m*M* $MgCl_2$, 0.1 m*M* sodium orthovanadate, 1 m*M*

Mg^{2+}-ATP and [γ-32P]ATP (370 MBq). Lipid kinase activities were assayed simultaneously using endogenous lipids as substrates, unless otherwise stated. Lipid phosphorylation was allowed to proceed for 2 min at 30°C in a final volume of 100 μl. The incubation mixture was then quenched with 1.5 ml chloroform/methanol (1:2, v/v) (Racagni-Di Palma et al. 2002). Protein content of membrane samples was determined by the Bradford (1976) method with bovine albumin as standard. Effects of DGK inhibitors, R59949 (3-{2-[4-(bis-(4-fluorophenyl) methylene]-1-piperidinyl)ethyl}-2,3-dihydro-2-thioxo-4(1H) quinazolinone), R59022 (6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5H-thiazolo(3,) pyrimidine-5-one) and D609, were tested by preincubating the aleurone layer for 30 min with the inhibitors.

Phospholipid extraction and separation

Lipids were extracted from membranes as described previously (Racagni-Di Palma et al. 2002), and phospholipids were separated by TLC. Samples were spotted on silica gel plates coated with potassium oxalate solution and heated at 110°C for 60 min just before use. Plates were developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v) for the first dimension, and chloroform/pyridine/formic acid (35:30:7, v/v) for the second dimension. Positions of radiolabeled lipids were determined by autoradiography on Kodak film. Spots were scraped off the plates, and fractions counted in a liquid scintillation counter.

Determination of PAP activities

PAP activities were differentiated according to NEM sensitivity (Hooks et al. 1998, Jamal et al. 1991). For determination of NEM-insensitive PAP activity (PAP2), the assay mixture consisted of 50 m*M* Tris–maleate buffer, pH 6.5, 1 m*M* DTT, 1 m*M* EDTA plus 1 m*M* EGTA, 4.2 m*M* NEM and 100 μg of the membrane protein in a volume of 0.1 ml. The reaction was started by adding 0.6 m*M* [2-^{3}H]-phosphatidate.

NEM-sensitive PAP activity (PAP1) was determined in an assay mixture containing 50 m*M* Tris–maleate buffer, pH 6.5, 1 m*M* DTT, 1 m*M* EDTA and 1 m*M* EGTA, 0.2 m*M* Mg^{2+} and 100 μg of the membrane protein in a volume of 0.1 ml. The reaction was started by adding 0.6 m*M* [2-^{3}H]-phosphatidate plus 0.4 m*M* phosphatidylcholine. Parallel incubations were carried out after preincubating the membrane with 4.2 m*M* NEM for 10 min. The difference between the two types of activity was labeled as PAP1. 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 identically, except that the membrane was boiled for 5 min before use. PAP activity products 1,2-diacyl[^{3}H]glycerol was isolated and measured as described below. PAP activity was expressed as the sum of nmol of [^{3}H]-DAG and [^{3}H]monoacylglycerol mg^{-1} protein h^{-1}.

Preparation of radiolabeled 1,2-diacyl-sn-glycerol-3-phosphate

Radiolabeled PA was obtained from [2-^{3}H]-phosphatidylcholine, which was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (7.4 GBq $mmol^{-1}$) as described (Pasquaré de García and Giusto 1986). Lipids were extracted from tissues as described by Folch et al. (1957). [2-^{3}H]phosphatidylcholine was isolated by one-dimensional TLC and eluted therefrom (Arvidson 1968). [2-^{3}H]-PC was then hydrolyzed with phospholipase D (Kates and Sastry 1969) and PA, the hydrolysis product, was purified by one-dimensional TLC on silica gel H, developed with chloroform/methanol/acetic acid/acetone/water (9:3:3:12:1.5, v/v). The substrate was eluted from silica gel with neutral solvents to avoid formation of LPA and then converted to free acid by washing twice using an upper phase containing 0.1 *M* sulfuric acid and then an upper phase containing water. Radioactivity and phosphorus content (Rouser et al. 1970) were measured to determine specific radioactivity. [2-^{3}H]PA had a specific radioactivity of 4–7 kBq μmol^{-1}. To determine NEM-sensitive PAP activity, the substrate was prepared by sonicating 3.33 m*M* [2-^{3}H]-phosphatidate (4–7 kBq μmol^{-1}) and 2.22 m*M* dipalmitoyl phosphatidylcholine (DPPC) in 5.56 m*M* EGTA and 5.56 m*M* EDTA. For determination of NEM-insensitive PAP activity, an emulsion was prepared as described above except that phosphatidylcholine (PC) was omitted (Hooks et al. 1998, Jamal et al. 1991).

Lipids were extracted with chloroform/methanol (2:1, v/v) and washed with 0.2 volumes $CaCl_2$ (0.05%) (Folch et al. 1957). Neutral lipids were separated by gradient-thickness TLC on silica gel G (Giusto and Bazán 1979) and developed with hexane/diethyl ether/acetic acid (35:65:1, v/v). When the chromatogram was developed, [2-^{3}H]-PA and phospholipids were retained at the spotting site. Lipids were visualized by exposing the chromatogram to iodine vapor and scraped off for liquid scintillation counting following addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene/Triton X-100 (4:1, v/v).

Sequence alignment and phylogenetic analysis

Sequence analysis was performed using tools provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/), ExPASy Molecular Biology

Server (http://us.expasy.org) and Arabidopsis Information Resource (http://www.arabidopsis.org). Amino acid sequences of open reading frames were initially aligned using ClustalW (Thompson et al. 1994) with BioEdit Sequence Alignment Editor 4.8.8 (Hall 1999), and the alignment was then visually refined. Phylogenetic analysis was performed using the neighbor-joining method with PAM distances computed on 55 reliably aligned sites (Saitou and Nei 1997).

Results

DGPP inhibits α-amylase secretion in GA-treated aleurone layers

In plants, the increase in PA formation in response to a variety of physiological stresses is often accompanied by increase of a more polar lipid known as DGPP. The conversion of PA into DGPP by PAK suggests that DGPP functions to attenuate the PA signal and that this phospholipid is just an inactivated form of PA. However, in view of its initial low concentration and its rapid and transient accumulation in response to stimuli, DGPP should be considered a signaling molecule (van Schooten et al. 2006). ABA inhibits α-amylase secretion in aleurone, preventing seed germination. To test the possible role of DGPP as a signaling molecule in the ABA antagonist effect on GA signal, aleurone layers were pretreated with 50 μ*M* dioleoyl-DGPP, stimulated with 5 μ*M* GA, and α-amylase secretion was recorded in the incubation medium after 24 h. Addition of 50 μ*M* dioleoyl-DGPP caused approximately 50% inhibition in secreted α-amylase relative to maximal secretion obtained with GA (GA, 16 ± 0.6 U ml^{-1}, n = 10; GA + dioleoyl-DGPP, 7.4 ± 1 U ml^{-1}, n = 5, $P < 0.05$, *t*-test) (Fig. 1). The response in presence of ABA and GA was similar to the response in the presence of ABA alone (GA + ABA, 5 ± 0.8 U ml^{-1}; ABA, 4 ± 0.8 U ml^{-1}, n = 6, $P > 0.05$, *t*-test). Moreover, the inhibition of amylase activity was dose-dependent with dioleoyl-DGPP from 10 μ*M* to 200 μ*M*. Other phospholipids were also tested to determine the specificity of the DGPP effect on α-amylase secretion. When layers were pretreated with 50 μ*M* dioleoyl-PA, α-amylase activity was reduced approximately 50% (8.2 ± 1 U ml^{-1}; n = 5, $P < 0.05$, *t*-test), similar to DGPP effect. PA breakdown products such as DAG or LPA and phosphatidylinositol phosphate (PIP) were also tested but had no detectable effect on α-amylase secretion (Fig. S1). We also analyzed the fatty acid composition of the PA and DGPP formed in ABA-treated cells to determine the molecular species. Analysis of fatty acid composition by gas chromatography showed that there was an increase in the content of the main unsaturated fatty acids, 18:1 and 18:2 in

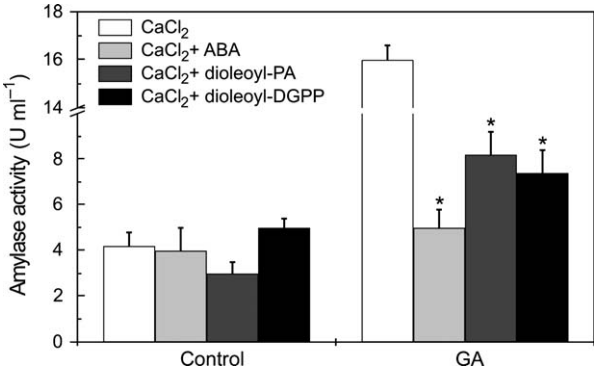

Fig. 1. Effect of dioleoyl-PA and dioleoyl-DGPP on α-amylase secretion. Aleurone layers were pretreated with 20 m*M* $CaCl_2$ and 50 μ*M* dioleoyl-PA or dioleoyl-DGPP for 3 h, then incubated 24 h with 5 μ*M* GA or 5 μ*M* ABA and assayed for α-amylase activity. Values shown are mean ± SEM, n > 5, **P* < 0.05, *t*-test.

aleurone treated with ABA during 30 and 60 min with respect to control (Villasuso AL, Racagni G, Aveldaño M, Machado E, UNRC, unpublished results). We therefore decided to test a physiological form, dioleoyl-PA, as an alternative to the non-physiological chain-type arachidonoyl/stearoyl-PA previously assayed (Ritchie and Gilroy 1998). Forms of PA and DGPP with shorter acyl chains, e.g. dioctanoyl-PA and dioctanoyl-DGPP, showed no effect on α-amylase secretion (Fig. S1). Dioleoyl-DGPP was used in subsequent experiments.

ABA induces an increase in PAK activity that is dependent on PA level

The above results suggested a role of DGPP in ABA antagonist response of aleurone layers. We therefore examined possible changes in endogenous levels of DGPP in response to ABA. Phosphorylation assay of endogenous PA was carried out with the 105 000 *g* membranes fraction in the presence of radiolabeled ATP. The pattern of radiolabeled phospholipid extracted from barley aleurone membrane and separated by two-dimensional TLC is shown in Fig. 2A, B. The concentration of DGPP in resting cells is less than 20% that of other phospholipids in barley aleurone. DGPP concentration increases when signaling is triggered by ABA (Fig. 2B). The phosphorylation of endogenous PA under standard conditions was linear for 5 min with 60 μg protein, 13 pmol min^{-1} mg^{-1} protein. DGPP formation was dependent on pH and protein concentration. PAK showed an optimal pH between 7 and 7.5, with a rapid decrease in activity below 6.6. When the DGPP/LPA fraction was separated by a two-dimensional solvent system, DGPP and LPA were found to comprise 90 and 10% of the fraction,

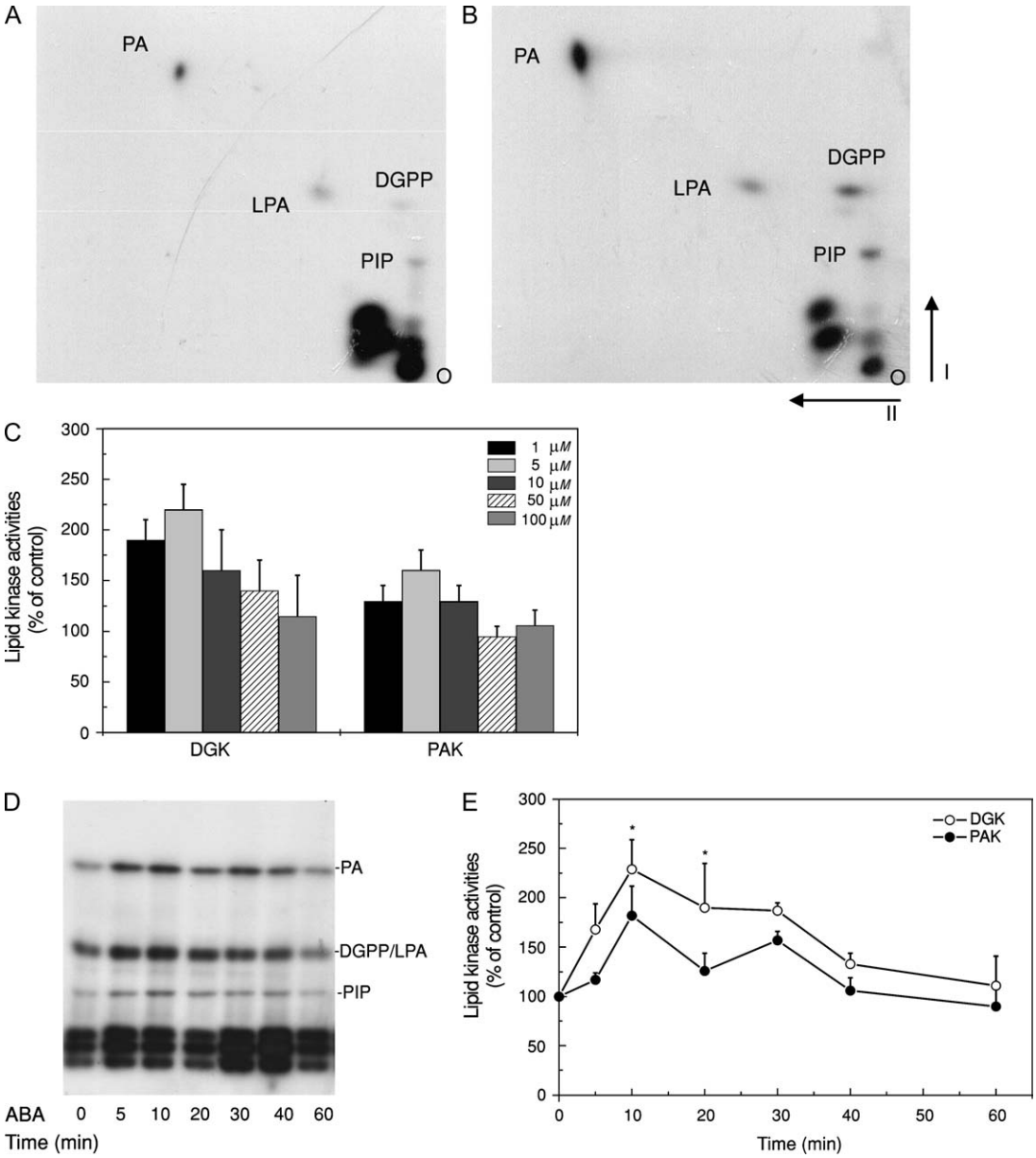

Fig. 2. PAK and DGK activities. Stimulated aleurone membranes were phosphorylated with [γ-^{32}P]ATP for 2 min at 30°C after ABA treatment. Lipids were extracted and separated by one-dimensional TLC with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v) and by two-dimensional TLC with chloroform/pyridine/formic acid (35:30:7, v/v) for the second dimension. (A and B) Autoradiography of two-dimensional TLC showing separation of LPA and DGPP extracted from control cells and ABA-treated (30 min) cells. (C) Dose-dependent ABA effect. (D) Autoradiography of one-dimensional TLC showing the time-course of ABA effect. (E) Time-course of ABA effect on DGK and PAK activities. Results are expressed as percent of non-stimulated control (defined as 100%) $\pm$SEM, n = 6, $^*P < 0.05$, *t*-test.

respectively (Fig. 2A, B). Treatment of aleurone layers with a range of ABA concentrations (1–100 μM) for 30 min showed that DGPP accumulation was dose-dependent, reaching a maximum at 5 μM ABA (Fig. 2C). Formation of PA remain increased at ABA concentrations >10 μM, while DGPP formation did not increase at the higher end of this range, suggesting that DGPP synthesis may have been limited by PA accumulation.

Results of a time-course experiment using 5 μM ABA is shown in Fig. 2D, E. PA and DGPP formation occurred within 1 min, but DGPP accumulation was observed only after 5 min, reaching maximal values at 10 and 30 min:

182 ± 30% and 157 ± 9% (relative to control, defined as 100%), respectively. PAK activity increased 80% after 10 min ABA treatment and remained high for at least 20 min. Complete time-course analyses of ABA stimulation effect on DGK and PAK showed similar kinetics (Fig. 2E; $P > 0.05$, *t*-test). The response to ABA was also observed as a PLD rapid and transient activation producing PA time-dependent accumulation. This accumulation was maximal, approximately 1.5-fold with respect to the control value, within the 20 min after stimulation (data not shown). Therefore, this PLD activity determined in the membrane fraction used for kinases determination coincided with the timing of PLD activation in response to ABA previously reported by Ritchie and Gilroy (1998).

Therefore, the formation of DGPP during ABA treatment could be a consequence of PA produced by PLD action, or of PA produced from DAG by DGK. Using DGK inhibitors type I (R59022) and type II (R59949), an inhibitory effect on DGK activity was recorded (Fig. 3). The observed increase in DGPP by ABA was abolished. Because PC-PLC could also have increased DAG levels, we tested the effect of PC-PLC inhibitor D609 (Fig. 3) and found that it reduced DGK and PAK activities. The possibility that DGK inhibition may block the inhibitory effect of ABA on α-amylase secretion was tested by pretreating aleurone layers with the above compounds and assessing α-amylase activity at 24 h. These treatments had no significant effect on ABA inhibitory activity (GA + ABA, 5 ± 0.8 U ml^{-1}; GA + ABA + DGK inhibitors type I, type II or D609, 4.7 ± 0.5 U ml^{-1}, n = 3, $P > 0.05$, *t*-test).

We were also able to increase DGPP level by modifying PAK activity through exogenous addition of the substrate, dioleoyl-PA, to the enzymatic assay. Addition of 50 μ*M* dioleoyl-PA to membranes obtained from layers treated with ABA increased DGPP formation approximately 40% (white bar) relative to the control value without dioleoyl-PA addition (light grey bar) (Fig. 4). The amount of DGPP formed appeared to be related to the amount of PA formed during ABA stimulation. This PAK activity increase following exogenous addition of dioleoyl-PA suggested that DGPP was a metabolic product of PA. In contrast, exogenous addition of 50 μ*M* dioleoyl-DGPP had an inhibitory effect on PAK and DGK activities. This indicates end-product inhibition of PAK activity and possibly a low DGPP phosphatase activity. No change in DGK or PAK activities was observed in control membranes in the presence of exogenous substrate (black and dark grey bars). The inhibition of α-amylase secretion by dioleoyl-PA may occur through its phosphorylation, yielding DGPP, rather than by a dephosphorylation reaction (shown in Fig. 1).

DGPP overcomes PLD inhibition by 1-butanol

Primary alcohols such as 1-butanol inhibit PA production because PLD can catalyze the transfer of a phosphatidyl group onto an alcohol molecule, producing a phosphatidylalcohol instead of PA. This transphosphatidylation reaction is unique to PLD and competitively inhibits PA production. In view of the above results, we performed experiments to determine whether DGPP is able to reverse the 1-butanol effect in aleurone layers treated with GA plus ABA. Application of dioleoyl-DGPP plus 1-butanol at 0 min after ABA addition reduced α-amylase activity, suggesting that DGPP blocked the 1-butanol

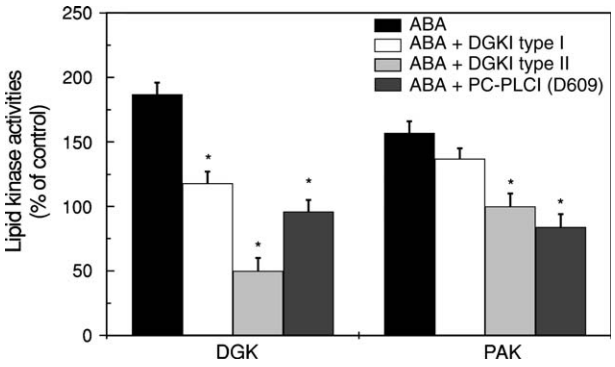

Fig. 3. Effect of DGK inhibitors (R59022 and R59949) and PC-PLC inhibitor (D609) on DGK and PAK. Aleurone layers were preincubated with 100 μ*M* R59022, 150 μ*M* R59949 and 150 μ*M* PC-PLC D609 for 20 min, then stimulated with 5 μ*M* ABA for 30 min and homogenized. Phosphorylation assay was performed as described in Materials and methods. Results are expressed as percent of non-stimulated control (defined as 100%) ±SEM, n = 3, $*P < 0.05$, *t*-test.

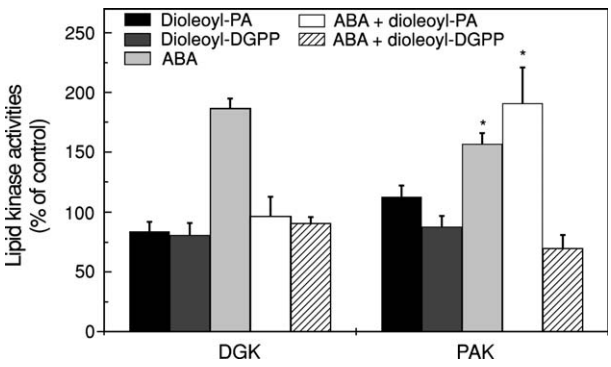

Fig. 4. Effect of dioleoyl-PA and dioleoyl-DGPP on DGK and PAK activities. Membrane fractions obtained from aleurone controls, or from aleurone treated with ABA for 30 min, were incubated with 50 μ*M* dioleoyl-PA or dioleoyl-DGPP for 20 min at room temperature. Enzymatic activities were measured as described in the Fig. 2. Results are expressed as percentage of control value (without hormone). Values shown are mean ± SEM, n = 6, $*P < 0.05$, *t*-test.

effect (Fig. 5, GA + ABA + 1-butanol, 8 ± 0.4 U ml^{-1}; GA + ABA + 1-butanol + dioleoyl-DGPP, 4 ± 0.7 U ml^{-1}, n = 3, $P < 0.05$, *t*-test). The competitive inhibition of PLD activity by 1-butanol was overcome by exogenous application of DGPP, similar to PA (GA + ABA + 1-butanol + dioleoyl-PA, 3.5 ± 0.5 U ml^{-1}, n = 3, $P < 0.05$, *t*-test). This finding suggested again that PA produced by PLD in vivo is phosphorylated by PAK. However, the effect of exogenous DGPP could have been attenuated by phosphatase activity, yielding PA and DAG. To test this possibility, PAP activity was measured in the 105 000 *g* membrane fraction obtained from ABA-treated aleurone.

ABA inhibits PAP activity

DGPP degradation occurs by a two-step dephosphorylation reaction catalyzed by DGPP phosphohydrolases. The enzyme removes the β-phosphate to form PA, then removes the phosphate from PA to form DAG. The reactions catalyzed by DGPP phosphohydrolase are Mg^{2+}-independent and NEM-insensitive (Carman 1997). We differentiated PAP activities on the basis of NEM sensitivity; this thiol-reactive compound inhibits PAP1 activity but does not affect PAP2 activity. PAP1 was assayed using [^{3}H]PA plus DPPC. PAP2 activity is coupled mainly to PLD activity because the main product of hydrolytic PLD activity, PA, is the substrate for PAP2 to yield DAG. PAP2 was determined in the presence of NEM using [^{3}H]PA as substrate.

In aleurone, PAP1 activity was modified when assayed under hormone action because neither activity was detected in the 105 000 *g* membrane fraction stimulated with ABA (30 min) or GA (5 min). Fig. 6 also shows that ABA treatment decreased PAP2 activity (24%) (light grey bar). In contrast, GA signal modified PAP2 activity to a lesser extent (13%) than ABA, indicating the presence of a different mechanism for metabolizing PA. These data suggest that PAP2 plays a role in regulation of PA and DGPP levels, in the ABA antagonist effect.

Identification of barley LPP genes

To better understand barley LPPs, we identified the predicted *LPP* genes in the barley database. The Institute for Genomic Research website (http://www.tigr.org) was searched for homologues of *LPP* genes. This search identified two predicted genes, which were noted at TIGR as TC134328 and TC148186 and assigned as phosphatidate phosphatase. BLAST searches showed that the sequences of barley have high similarity or identity to those of *Arabidopsis* LPPs (Table 1). They were therefore called *HvLPP1* and *HvLPP2* (*H. vulgare* lipid phosphate phosphatase), and their products called HvLpp1p and HvLpp2p.

There was 73% or more amino acid sequence similarity (55% identity) between *Arabidopsis* and barley LPPs. The sizes of HvLPP1 and HvLPP2 are 1165 and 1626 bp, and they code for putative proteins of 319 and 312 amino acids, respectively. Alignment analysis between HvLpp1p and HvLpp2p showed that both sequences share a high amino acid identity (70%); however, HvLpp2p possess residues in the N- and C-terminal (60 and 296 amino acids in length, respectively) that do not align with HvLpp1p

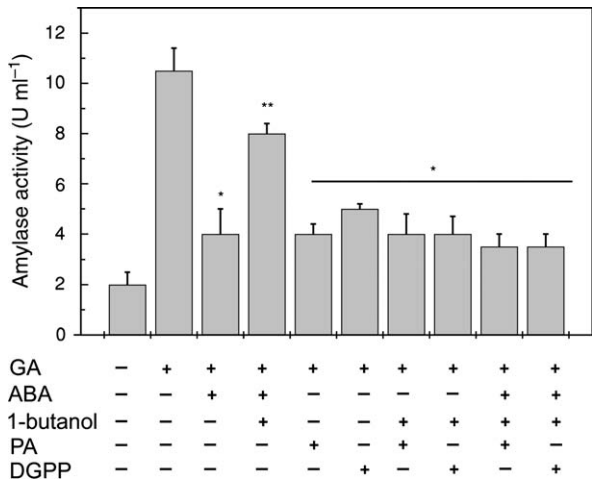

Fig. 5. Effect of 1-butanol on α-amylase activity. Aleurone layers were treated with 5 μ*M* GA or ABA, 0.1% 1-butanol and 50 μ*M* dioleoyl-PA or dioleoyl-DGPP. Amylase activity was assayed after 24-h treatment. Values shown are mean ± SEM, n = 3, **P* < 0.05, ***P* > 0.05, *t*-test.

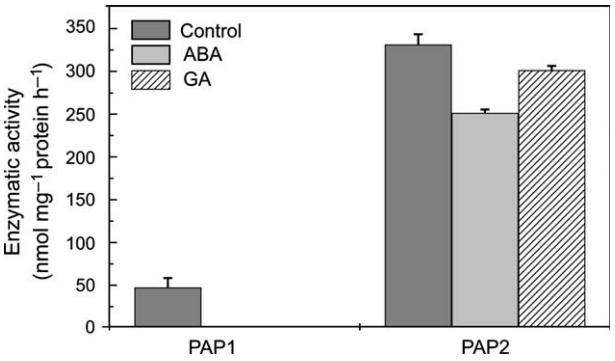

Fig. 6. PAP activity. PAP1 and PAP2 activities were determined using [^{3}H]-PA (0.6 m*M*) plus DPPC (0.4 m*M*) or [^{3}H]-PA (0.6 m*M*) as substrate for PAP1 or PAP2, respectively. Other assay conditions are described in Materials and methods. PAP activities are expressed as nmol [^{3}H]-DAG plus [^{3}H]-MAG mg^{-1} protein h^{-1}. Values shown are mean ± SEM, n = 3. MAG, monoacylglycerol.

Table 1. Amino acid similarity and identity of barley and *Arabidopsis*. For each pairwise comparison, similarity values are followed by identity values in parentheses.

	HvLpp1p (TC134328)		HvLpp2p (TC148186)	
	I/P	Expect values	I/P	Expect values
AtLpp1p (Q9ZU49)	55 (73)	2×10^{-80}	55 (73)	1×10^{-97}
AtLpp2p (Q9XI60)	70 (80)	5×10^{-76}	69 (81)	2×10^{-102}
AtLpp3p (AAF32467)	67 (85)	5×10^{-75}	63 (80)	1×10^{-99}

sequence. The open reading frame of 1165 nucleotides on the cDNA codes for a putative 35.7-kDa integral membrane protein, containing six highly hydrophobic regions of sufficient length to be membrane-spanning, designated as TM1-6 (Fig. 7). The putative protein contains a three-domain phosphatase sequence motif that is conserved in several LPPs in *S. cerevisiae*, *A. thaliana*, and in mammalian cells (Stukey and Carman 1997). The protein also showed the presence of conserved amino acid residues that are important for phosphate phosphatase activity (Toke et al. 1999, Zhang et al. 2000). Similar results were obtained in analysis of HvLpp2, which codes for a 34.8-kDa putative protein. A phylogenetic tree was constructed for members of the LPP protein family, including HvLpp1p and HvLpp2p. The *H. vulgare* proteins were grouped with those of *S. cerevisiae* and *A. thaliana* (Fig. 8). The predicted amino acid sequences of these HvLpps candidates showed that any of them had putative transit peptides, which allow them target to chloroplasts.

Discussion

In this study, regulation of DGPP level is shown to be an important mechanism involved in the antagonistic effect of ABA on GA-stimulated α-amylase secretion in aleurone. We demonstrated previously that ABA increases PA level approximately 1.5-fold within a few minutes after stimulation and suggested that synthesis and metabolism of both PA and DGPP are coupled to PLD/LPP activity (Villasuso et al. 2003).

In barley aleurone, the principal route leading to PA production is PLD activity, which is sensitive to ABA (Ritchie and Gilroy 1998). In the present study, increase of PAK activity in membrane fraction from ABA-stimulated aleurone was transient, similar to stimulation of PLD activity by ABA. Our results suggest that the ABA receptor, other related components, and PAK activity are all closely associated within the plasma membrane, consistent with the current model of ABA effect (Gilroy and Jones 1994, Razem et al. 2006, Ritchie and Gilroy 1998).

The transient increase in PA observed following application of ABA suggests that PA is metabolized immediately after being formed. Simultaneous changes in PA and PA breakdown products have been reported previously in various physiological processes. However, LPA level in our experiments remained unchanged, indicating that phospholipase A_2 (PLA_2) activity was not stimulated in ABA-treated barley aleurone. Treatment of aleurone with LPA did not inhibit α-amylase secretion (Fig. S1).

PA can also be dephosphorylated by PAP in DAG (Waggoner et al. 1999), and our experiments showed that this effect is inhibited by ABA (Fig. 6, light grey bar). It is unlikely that this DAG behaves as a second messenger in ABA signaling because we observed that ABA increased DGK activity, whereas treatment of aleurone with DAG had no effect on α-amylase secretion (Fig. S1). These differential biological activities can be attributed to different shapes and biophysical properties of DAG and PA. Rate of DAG uptake, and its stability, may also modulate its effect. We found that addition of DGPP to the kinase assay blocked the increase of DGK activity by ABA, suggesting an inhibitory effect on DGK. Nevertheless, the role of DGK in barley germination remains largely unknown.

In membrane lipid metabolism, PA is synthesized by two sequential acylation steps in the 'Kennedy pathway' (Kennedy 1957), which is common to three different classes of glycerolipids: phospholipids (phosphatidylcholine and phosphatidylethanolamine), galactolipids and triacylglycerol. As the last step of the Kennedy pathway is catalyzed by PAP, this enzyme may be involved in lipid metabolism of both plastids and ER. We found that barley has two homologues of PAP, which do not contain predictable transit peptides. Therefore, they are unlikely to be candidates for plastids (Nakamura et al. 2007).

PA can also be metabolized to DGPP (Wissing et al. 1994). Following ABA application, we measured an increased level of DGPP (Fig. 2), which is consistent with results in *Arabidopsis*. Zalejski et al. (2005) reported that ABA application produced a transient 1.7-fold increase in PA within 5 min, followed by a seven-fold increase in DGPP at 20 min. These findings suggest that DGPP formation in aleurone is a consequence of increased PAK activity and inhibition of PAP activity, as reported by Wu et al. (1996).

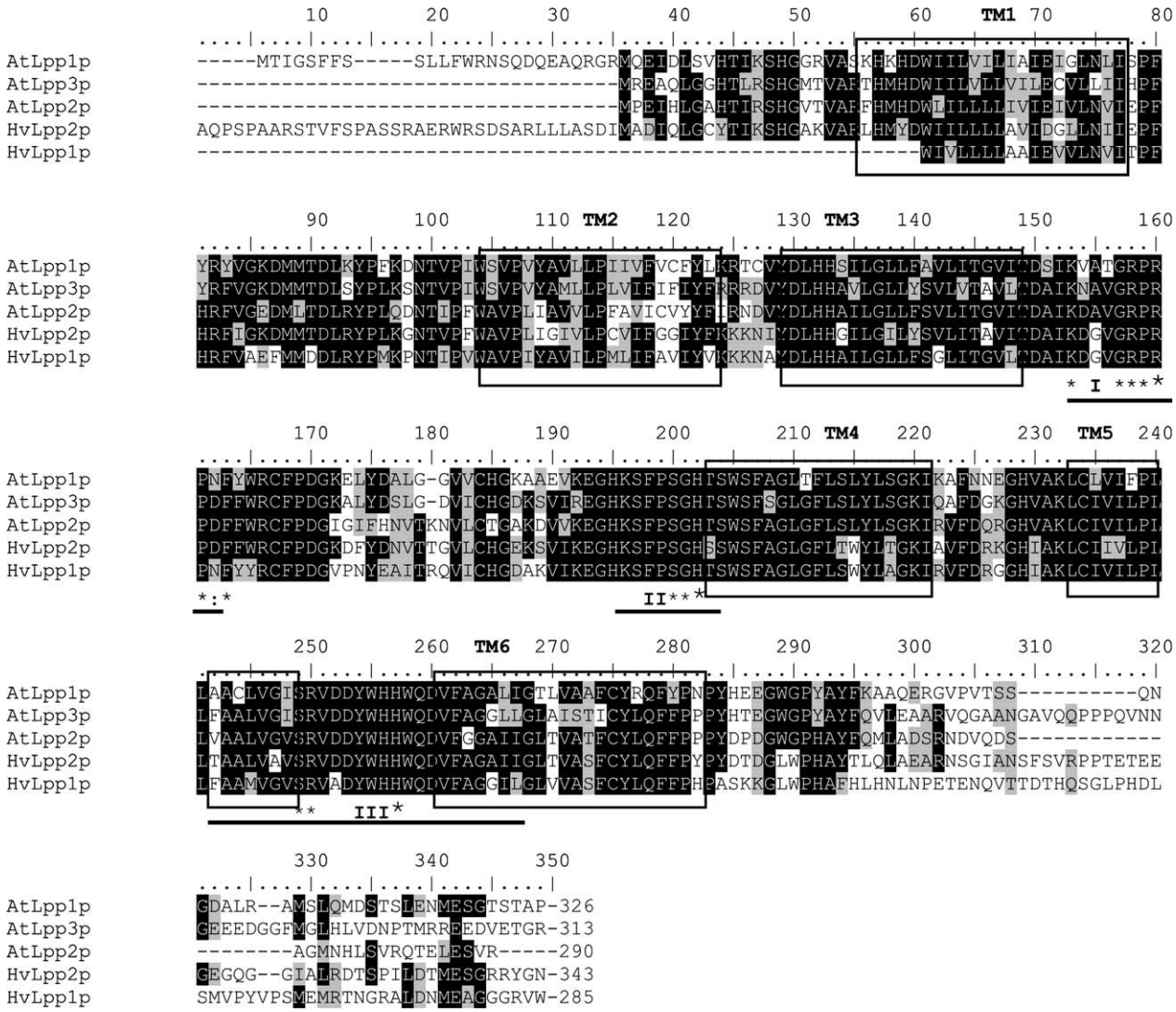

Fig. 7. Amino acid sequence comparison between the deduced HvLpp1p and HvLpp2p, and *Arabidopsis thaliana* lipid phosphate phosphatases (Lpp). In the aligned amino acid sequences, invariant amino acids are boxed in black and conserved amino acids are boxed in grey. Transmembrane regions are indicated as open boxes. Black bars indicate three domains of the phosphatase sequence motif. Asterisks indicate conserved amino acid residues of all Lpp proteins.

We also observed that exogenously added DGPP inhibits GA response and that exogenous addition of dioleoyl-PA increases DGPP accumulation. These findings suggest that DGPP is a metabolic product of PA, that dioleoyl-DGPP (in addition to dioleoyl-PA) is able to inhibit α-amylase secretion and that DGPP acts as a second messenger in the antagonistic effect of ABA signaling. This idea is consistent with the observed accumulation of RAB18 messenger following application of dioleoyl-DGPP (Zalejski et al. 2005). The ability of dioleoyl-DGPP to inhibit α-amylase secretion in aleurone suggests that long fatty acid chains are essential for the biological activity of DGPP. Dioctanoyl-DGPP, which has short fatty acid chains, did not inhibit the secretion. Similar results were obtained by Zalejski et al. (2005). Dioctanoyl-DGPP, but not dioleoyl-DGPP, was shown to be an antagonist for a LPA receptor (LPA3) (Fischer et al. 2001). The long fatty acid chains of DGPP may allow translocation of proteins to the membrane through hydrophobic interactions (Munnik et al. 1998, van Leeuwen et al. 2004). Zhang et al. (2004) showed that dioleoyl-PA binds to ABI, a protein phosphatase 2C, more efficiently than other PA species. The pyrophosphate group may also be important for biological activity of DGPP, particularly in electrostatic interaction with protein targets. A combination of electrostatic forces and hydrogen bond

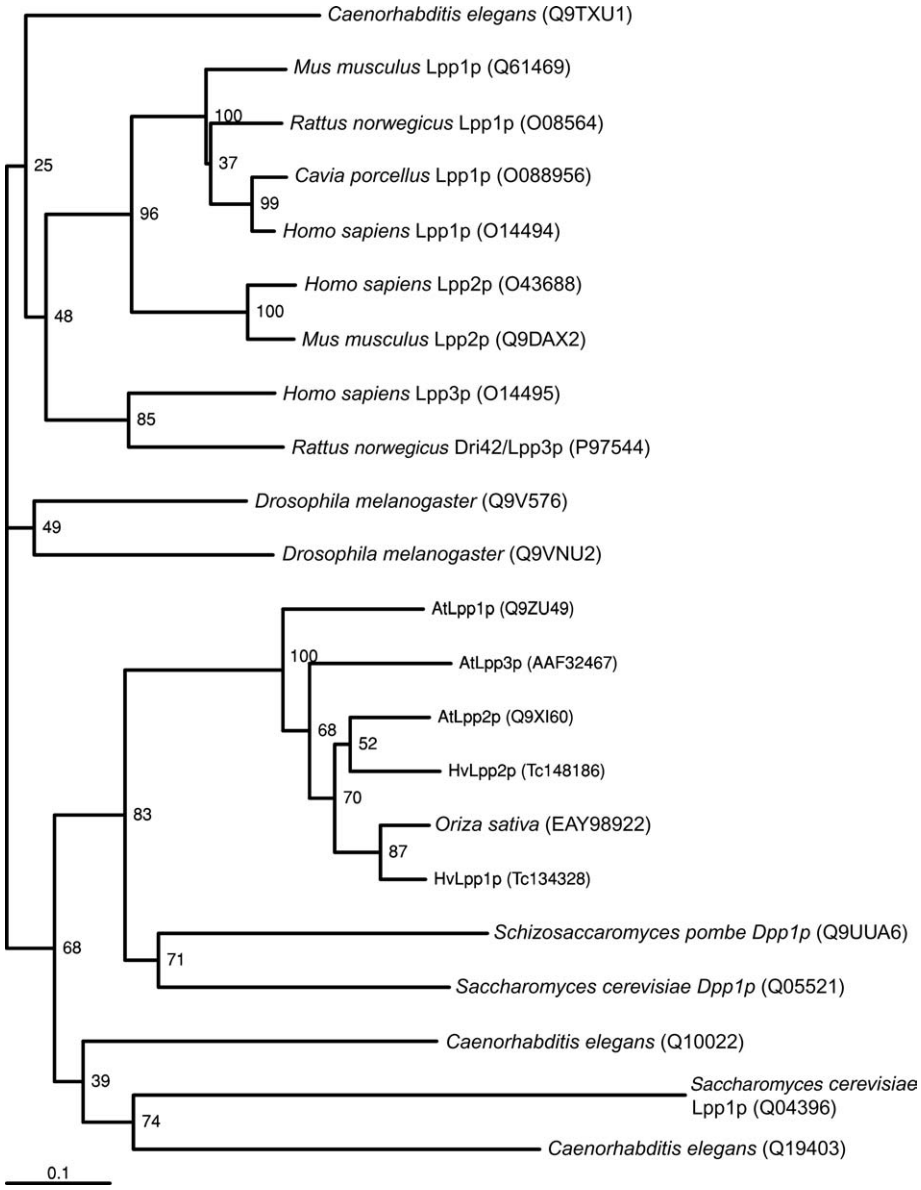

Fig. 8. Phylogenetic analysis of the lipid phosphate phosphatase protein (Lpp) family. The *Hordeum vulgare* HvLpp1p, HvLpp2p proteins, the *Arabidopsis* AtLpp1p, AtLpp2p, AtLpp3p proteins, and the *Saccharomyces cerevisiae* Dpp1p, Lpp1p proteins were analyzed. All available mammalian Lpp proteins, the *Drosophila* Wunen protein, as well as the uncharacterized putative Lpp-like proteins identified in the *Oriza sativa*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Schizosaccaromyces pombe* genomes were used for comparison. The tree was built applying the neighbor-joining method to PAM distances computed on 55 reliably aligned sites. SwissProt accession numbers (in brackets) designate all protein sequences. The length of horizontal branches is such that the evolutionary distance between two proteins is proportional to the total length of the horizontal branches that connect them. Bootstrap values are shown at the nodes.

interactions with basic amino acids, similar to that seen in PA binding to proteins, may occur between DGPP and its target proteins (Kooijman et al. 2007).

The fact that DGPP and PA formation was rapid, and that levels of the two phospholipids remained high and similar to each other up to 30 min, suggest the presence of enzyme activities that utilize DGPP and PA as substrates and attenuate the signal. This finding is consistent with the coupling of PLD and LPP activities (Villasuso et al. 2003). To confirm the association, we determined the activities of two PAP isoforms, PAP1 and PAP2, which are sensitive to ABA. We demonstrate here the presence of both PAP isoforms, PAP1 and PAP2, based on their enzymatic properties with respect to Mg^{2+} and NEM

sensitivity. The two PAP isoforms displayed different behaviors in response to ABA, indicating distinct functions. The inhibitory effect of ABA suggests that the activities of these enzymes control cellular aleurone levels of messengers such as DGPP, PA, and DAG. To determine whether Lpp genes are present in the *H. vulgare* genome, we searched public databases for putative Lpp-coding sequences. Bioinformatic search of the *H. vulgare* database revealed the presence of sequences highly homologous to *Arabidopsis* proteins, with structural similarities to PAPs of yeast and mammalian cells, including the presence of a three-domain phosphatase sequence motif. Phylogenetic analysis groups the candidate protein into the main branch with *Arabidopsis* protein. Our findings support a role of DGPP in ABA transduction pathways upstream of the α-amylase secretion, involving PAP, in the antagonistic effect of ABA in aleurone.

In summary, results presented in this study help elucidate the mechanism of phosphorylation/dephosphorylation involved in PA and DGPP synthesis in barley aleurone. Ongoing studies by our group focus on DGPP targets involved in secretory aleurone function to further clarify the role of DGPP in GA signaling.

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References

Arvidson GAE (1968) Structural and metabolic heterogeneity of rat liver glycerophosphatides. Eur J Biochem 4: 478–486

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

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brindley RR, Waggoner W (1998) Mammalian lipid phosphate phosphohydrolases. J Biol Chem 273: 24281–24284

Carman G (1997) Phosphatidate phosphatases and diacylglycerol pyrophosphate phosphatases in *Saccharomyces cerevisiae* and *Escherichia coli*. Biochim Biophys Acta 1348: 45–55

Carman G, Zeimetz G (1996) Regulation of phospholipid biosynthesis in the yeast *Saccharomyces cerevisiae*. J Biol Chem 271: 13293–13296

den Hartog M, Musgrave A, 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

de Jong CF, Laxalt AM, Bargmann BO, de Wit PJ, Joosten MH, Munnik T (2004) Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. Plant J 39: 1–12

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

Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226: 497–509

Gilroy S (1996) Signal transduction in barley aleurone protoplasts is calcium dependent and independent. Plant Cell 8: 2193–2209

Gilroy S, Jones RL (1994) Perception of gibberellin and abscisic acid at the external face of the plasma membrane of barley (Hordeum vulgare L.) aleurone protoplasts. Plant Physiol 104: 1185–1192

Giusto NM, Bazán NG (1979) Phospholipids and acylglycerols biosynthesis and ^{14}C production from [^{14}C]glycerol in the bovine retina: the effects of incubation time oxygen and glucose. Exp Eye Res 29: 155–168

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95–98

Heimovaara-Dijkstra S, Heistek JC, Wang M (1994a) Counteractive effects of ABA and GA_3 on extracellular and intracellular pH and malate in barley aleurone. Plant Physiol 106: 359–750

Heimovaara-Dijkstra S, Vanduijn B, Libbenga KR, Heidekamp F, Wang M (1994b) Abscisic Acid-induced membrane potential changes in barley aleurone protoplasts: a possible relevance for the regulation of rab gene expression. Plant Cell Physiol 35: 743–750

Hooks SB, Ragan SP, Lynch KR (1998) Identification of a novel human phosphatidic acid phosphatase type 2 isoform. FEBS Lett 427: 188–193

Jamal Z, Martin A, Gomez-Muñoz A, Brindley DN (1991) Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. J Biol Chem 266: 2988–2996

Jones RL, Varner JE (1967) The bioassay of gibberellins. Planta 72: 53–59

Katagiri T, Ishiyama T, Kato S, Tabata M, Kobayashi K, Shinosaki K (2005) An important role of phosphatididic acid in ABA signalling during germination in *Arabidopsis thaliana*. Plant J 43: 107–117

Kates M, Sastry PS (1969) Phospholipase D in Methods Enzymol. Academic Press, London, pp 197–203

Kennedy EP (1957) Biosynthesis of phospholipids. Fed Proc Am Soc Exp Biol 16: 847–853
Kooijman EE, Tieleman DP, Testerink C, Munnik T, Rijkers DTS, Burger KN, de Kruijff B (2007) An electrostatic/Hydrogen bond switch as the basis for the specific interaction of phosphatidic acid with proteins. J Biol Chem 282: 11356–11364
Lovegrove A, Hooley R (2000) Gibberellin and abscisic acid signalling in aleurone. Trends Plant Sci 5: 102–110
Meijer HJG, Munnik T (2003) Phospholipid-based signalling in plants. Annu Rev Plant Biol 54: 265–306
Munnik T, de Vrije T, Irvine E, Musgrave A (1996) Identification of diacyl-glycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. J Biol Chem 271: 15708–15715
Munnik T, Irvine RF, Musgrave A (1998) Phospholipid signalling in plants. Biochim Biophys Acta 1389: 222–272
Munnik T, Meijer H, ter Riet B, Hirt H, Frank W, Bartels D, Musgrave A (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. Plant J 22: 147–154
Nakamura Y, Tsuchiya M, Ohta H (2007) Plastidic phosphatidic acid phosphatases identified in a distinct subfamily of lipid phosphate phosphatases with prokaryotic origin. J Biol Chem 282: 29013–29021
Pasquaré de García SJ, Giusto NM (1986) Phosphatidate phosphatase activity in isolated rod outer segment from bovine retina. Biochim Biophys Acta 875: 195–202
Pedranzani H, Racagni G, Alemano S, Miersch O, Ramírez I, Peña Cortez H, Taleisnik E, Machado-Domenech EE, Abdala G (2003) Salt tolerance tomato plants show increased levels of jasmonic acid. Plant Growth Regul 41: 149–158
Pical C, Westergren T, Dove S, Larsson C, Sommarin M (1999) Salinity and hyperosmotic stress induce rapid increases in phosphatidyl 45-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, Kasmaier M (2001) Lipid phosphate phosphatases in Arabidopsis. J Biol Chem 276: 20300–20308
Racagni-Di Palma G, Brito-Argáez L, Hernández-Sotomayor SMT (2002) Phosphorylation of signalling phospholipids in *Coffea arabica* L cells. Plant Physiol Biochem 40: 899–906
Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. Nature 439: 290–294
Riedel B, Morr M, Wu W, Carman GM, Wissing JB (1997) Metabolism of diacylglycerol pyrophosphate by suspension cultured *Catharanthus roseus* cells: Identification and characterization of diacylglycerol pyrophosphate phosphatase in plants. Plant Sci 128: 1–10
Ritchie S, Gilroy S (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. Proc Nat Acad Sci USA 95: 2697–2702
Ritchie SM, Swanson SJ, Gilroy S (2002) From common signalling components to cell specific response: insights from the cereal aleurone. Physiol Plant 115: 342–351
Rouser G, Fleischer S, Yamamoto A (1970) Two dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots lipids. Lipids 5: 494–496
Saitou N, Nei M (1997) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
Santander V, Bollo M, Machado-Domenech E (2002) Lipid kinases and Ca^{2+} signalling in *Trypanosoma cruzi* stimulated by a synthetic peptide. Biochem Biophys Res Commun 293: 314–320
Stukey J, Carman GM (1997) Identification of a novel phosphatase sequence motif. Protein Sci 6: 469–472
Testerink C, Munnik T (2005) Phosphatidic acid: a multifunctional stress signalling lipid in plants. Trends Plant Sci 10: 368–375
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680
Toke DA, Bennett WL, Oshiro J, Wu WI, Voelker DR, Carman GM (1998a) Isolation and characterization of the Saccharomyces cerevisiae LPP1 gene encoding a Mg2+-independent phosphatidate phosphatase. J Biol Chem 273: 14331–14338
Toke DA, Bennett WL, Dillon DA, Wu W, Chen X, Ostrander DB, Oshiro J, Cremesti A, Voelker DR, Fishl AS, Carman GM (1998b) Isolation and characterization of the Saccharomyces cerevisiae DPP1 gene encoding diacylglycerol pyrophosphate phosphatase. J Biol Chem 273: 3278–3284
Toke DA, McClintick ML, Carman GM (1999) Mutagenesis of the phosphatase sequence motif in diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. Biochemistry 38: 14606–14613
van der Luit AH, Piatti T, van Doorn A, Musgrave A, Felix G, Boller T, Munnik T (2000) Elicitation of suspension-culture tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. Plant Physiol 123: 1507–1516
van der Veen R, Heimovaara-Dijkstra S, Wang M (1992) Cytosolic alkalinization mediated by abscisic acid is necessary but not sufficient for abscisic acid-induced gene expression in barley aleurone protoplasts. Plant Physiol 100: 699–705
van Leeuwen W, Okresz L, Bogre L, Munnik T (2004) Learning the lipid language of plant signalling. Trends Plant Sci 9: 378–384

van Schooten B, Testerink C, Munnik T (2006) Signalling diacylglycerol pyrophosphate a new phosphatidic acid metabolite. Biochim Biophys Acta 1761: 151–159
Villasuso AL, Machado-Domenech EE (2004) Gibberellin signalling pathway: role of phospholipids in barley aleurone. Plant Growth Regul 43: 101–108
Villasuso AL, Molas ML, Racagni G, Abdala G, Machado-Domenech EE (2003) Gibberellin signal in barley aleurone: early activation of PLC by G protein mediates amylase secretion. Plant Growth Regul 41: 197–205
Waggoner D, Xu J, Singh I, Jasinska R, Zhang Q-X, Brindley D (1999) Structural organization of mammalian lipid phosphate phosphatases: implications for signal transduction. Biochim Biophys Acta 1439: 299–316
Wang X, Devaiah SP, Zhang W, Welti R (2006) Signalling functions of phosphatidic acid. Prog Lipid Res 45: 250–278
Wissing JB, Kornak B, Funke A, Riedel B (1994) Phosphatidate kinase a novel enzyme in phospholipid metabolism. Plant Physiol 105: 903–909
Wu WI, Liu Y, Riedel B, Wissing JB, Fischl AS, Carman GM (1996) Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. J Biol Chem 271: 1868–1876
Zalejski C, Zhang Z, Quettier AL, Maldiney R, Bonnet M, Brault M, Demandre C, Miginiac E, Rona JP, Sotta B, Jeannette E (2005) Diacylglycerol pyrophosphate is a second messenger of abscisic acid signalling in *Arabidopsis thaliana* suspension cells. Plant J 42: 145–152
Zalejski C, Paradis S, Maldiney R, Habricot Y, Miginiac E, Rona JP, Jeannette E (2006) Induction of abscisic acid-regulated gene expression by diacylglycerol pyrophosphate involves Ca^{2+} and anion currents in *Arabidopsis* suspension cells. Plant Physiol 141: 1555–1562
Zhang QX, Pilquil CS, Dewald J, Berthiaume LG, Brindley DN (2000) Identification of structurally important domains of lipid phosphate phosphatase-1: implications for its sites of action. Biochem J 345: 181–184
Zhang W, Quin C, Zhao J, Wang X (2004) Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulateds abscisisc signalling. Proc Natl Acad Sci USA 101: 9508–9513

Supplementary material

The following supplementary material is available for this article:

Fig. S1. Effect of phospholipids and diacylglycerol on α-amylase secretion.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1399-3054.2008.01148.x

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