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Short communication

Arabidopsis thaliana lipid phosphate phosphatase 2 is involved in abscisic acid signalling in leaves

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ABSTRACT

Lipid phosphate phosphatases (LPPs, E.C. 3.1.3.4) catalyse the dephosphorylation of diacylglycerol pyrophosphate (DGPP) and phosphatidic acid (PA), which are secondary messengers in abscisic acid (ABA) signalling. In this study, we investigated the effect of ABA on the expression of *AtLPP* genes as they encode putative ABA-signalling partners. We observed that *AtLPP2* expression was down-regulated by ABA and we performed experiments on *Atlpp2-2*, an *AtLPP2* knockout mutant, to determine whether *AtLPP2* was involved in ABA signalling. We observed that *Atlpp2-2* plantlets contained about twice as much PA as the wild-type Col-0 and exhibited higher PA kinase (PAK) activity than Col-0 plants. In addition, we showed that ABA stimulated diacylglycerol kinase (DGK) activity independently of AtLPP2 activity but that the ABA-stimulation of PAK activity recorded in Col-0 was dependent on *AtLPP2*. In order to evaluate the involvement of AtLPP2 activity in guard cell function, we measured the ABA sensitivity of *Atlpp2-2* stomata. The inhibition of stomatal opening was less sensitive to ABA in *Atlpp2-2* than in Col-0. Watered and water-stressed plants of the two genotypes accumulated ABA to the same extent, thus leading us to consider *Atlpp2-2* an ABA-signalling mutant. Taken together our observations show that *AtLPP2* is a part of ABA signalling and participate to the regulation of stomatal movements.

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1. Introduction

The phytohormone abscisic acid (ABA) participates in the whole plant life cycle from seed development to flowering [1]. In addition, under unfavourable environmental conditions, ABA accumulation induces stomatal closure in the leaf epidermis and modifies the expression of numerous genes in different tissues [2]; [3]. ABA-signalling pathways are complex. ABA, perceived by several receptors, induces the phosphorylation and dephosphorylation of many target proteins, relaying the signal via multiple second messengers [1,4–7]. Among these ABA response messengers are lipid molecules including phosphatidic acid (PA) and diacylglycerol pyrophosphate (DGPP). For example, ABA treatment induces a transient increase in PA and DGPP in guard cells, cells in suspension, aleurone layer cells and seeds [8–11]. In *Arabidopsis thaliana* cells in suspension, addition of DGPP triggers the expression of ABA-specific genes [12], and application of PA or DGPP counteracts the GA-stimulated α–amylase secretion in barley seeds [11]. In Arabidopsis guard cells, PA can bind to ABI1 thus inhibiting its protein phosphatase activity [13]. In addition, genetic studies have strengthened the tenet that PA is involved in ABA signalling. Antisense phospholipase Dα (*PLD*α) and knockout *PLD*α*1* constructs were used to show that the activity of PLDα participates in ABA-promoted stomatal movements [13–15]. In barley seeds, recent data have indicated that after ABA application the rise in PA and DGPP levels depends on diacylglycerol kinase (DGK) and phosphatidic acid kinase (PAK) activities, respectively [11]. Besides enzymes that synthesise PA and DGPP, enzymes that degrade them are also important because they can potentially attenuate the biological effects of these lipid mediators on signalling pathways. Phosphatidate phosphatases (PAPs, E.C. 3.1.3.4) dephosphorylate PA producing DAG and inorganic

Abbreviations: ABA, abscisic acid; DAG, diacylglycerol; DGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DMSO, dimethyl sulfoxyde; P_i, inorganic phosphate; LPP, lipid phosphate phosphatase; MS, Murashige and Skoog; PA, phosphatidic acid; PAK, phosphatidic acid kinase; PAP, phosphatidate phosphatase; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

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phosphate (P_i). Two types of PAP enzymes are distinguished according to the Mg^{2+} requirement for catalytic activity. Conventional PAPs, named PAP1, are Mg^{2+} dependent whereas PAP2, also generally named lipid phosphate phosphatases (LPPs), are Mg^{2+} independent [16]. PAP2/LPPs regulate not only the level of PA, but also the level of DGPP because they can remove the β phosphate from DGPP to form PA and P_i [17,18]. In *A. thaliana*, five PAP1 were identified from cyanobacterial orthologs, among them, three were shown to be located in the chloroplast where they may be essential for galactolipid synthesis [19]. Based on the deduced protein sequence similarities with yeast *LPPs*, four genes encoding PAP2/LPPs were identified in *A. thaliana* [10,20]. AtLPP2 was shown to act in ABA inhibition of seed germination thus confirming the role of PA and DGPP in ABA signalling [10]. However, little is known about the role of AtLPP2 in phospholipids formation under ABA treatment.

In this study, we observed that the expression of *AtLPP2* is down-regulated by ABA. We also show that *Atlpp2-2* mutant, which is deficient in *AtLPP2* mRNA, has a higher level of PA and a higher level in PAK activity than the wild-type Col-0. Stomata of *Atlpp2-2* mutant are less sensitive to the ABA-induced inhibition of their opening than Col-0. Consequently, we suggest that AtLPP2 is part of the ABA-signalling network regulating some aspects of leaf physiology.

2. Results

2.1. Regulation of AtLPP gene expression by ABA

Several data indicate that among ABA-signalling proteins, some of them are encoded by genes that are transcriptionally regulated by ABA [1]. Therefore, we investigated the effect of ABA on the expression of *AtLPP* genes as they encode putative ABA-signalling partners. Semi-quantitative analysis of *AtLPP* genes showed that their level of expression in leaves of wild-type plants treated for 3 h with ABA was differentially modified according to the gene considered. Indeed, the level of expression of *AtLPP1* was not modified in Col-0, but the expression of *AtLPP2* decreased and the expression of *AtLPP3* increased in leaves (Fig. 1). *AtLPP4* mRNA was not detected in leaves (Fig. 1). These results suggest that AtLPP2 and AtLPP3 could play a role in ABA response. Here, we choose to study the involvement of AtLPP2 by the means of the mutant *Atlpp2-2* that contains a T-DNA insertion in the *AtLPP2* gene.

2.2. Atlpp2-2 accumulates more PA than Col-0 and has a higher PAK activity

First, we verified by RT-PCR that the mutant *Atlpp2-2* did not express *AtLPP2* mRNA so it can be considered as an RNA-null or knockout (Supplementary Fig. S1). Then, we measured the endogenous level of PA in *Atlpp2-2* in comparison to Col-0 plants. Leaves of *Atlpp2-2* mutant had twice more PA (8.6 ± 1.4 nmol g^{-1} FW) than Col-0 (4.7 ± 1.1 nmol g^{-1} FW; Fig. 2A). Thus, the disruption of the *AtLPP2* gene modifies the level of PA accumulation in leaf. In the mutant lacking PAP2 activity, the level of PA is the result of the equilibrium between PLD and DGK activity that produces it and the PAK activity that uses it. Here, we focus our study on the measurement of the activity of these lipid kinases. In microsomal extracts, DGK and PAK activities were almost twice higher in *Atlpp2-2* than in Col-0 plants (Fig. 2B). The activities of DGK and PAK were 15 ± 9.5 pmol min^{-1} mg^{-1} of protein and 18 ± 0.3 pmol min^{-1} mg^{-1} of protein in Col-0 and 33 ± 7.5 pmol min^{-1} mg^{-1} of protein and 45 ± 9.3 pmol min^{-1} mg^{-1} of protein in *Atlpp2-2*, respectively. This suggests that PAP2 activity originating from *AtLPP2* participates to the regulation of the activity of DGK and PAK.

2.3. Effect of ABA on DGK and PAK activities

We measured the microsomal lipid kinase activities in Col-0 and *Atlpp2-2* plants submitted to a 5–60 min ABA treatment. In

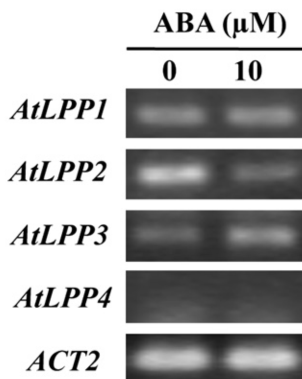

Fig. 1. The expression of *AtLPP2* and *AtLPP3* is regulated by ABA. Semi-quantitative RT-PCR analysis of *AtLPP1*, *AtLPP2* and *AtLPP3* expression in Col-0 leaves treated for 3 h with 10 µM ABA. *ACT2* was used as a control. Data are representative of 3 independent experiments.

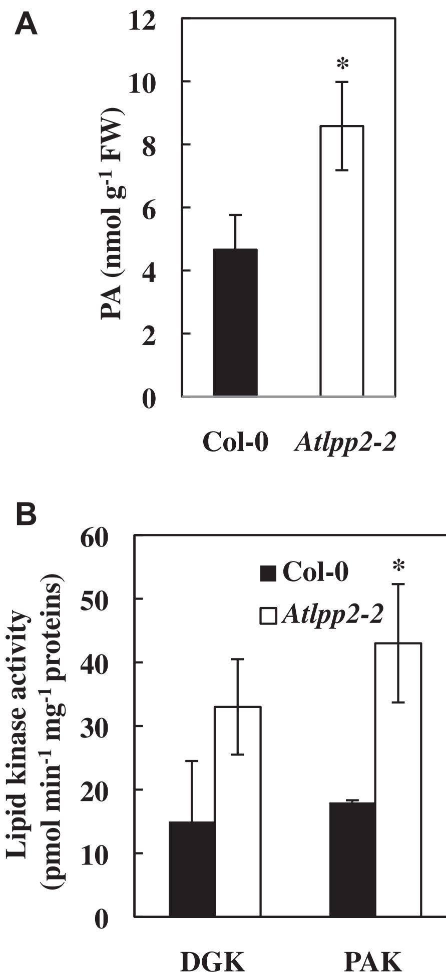

Fig. 2. PA content and lipid kinase activities in *Atlpp2-2*. (A) PA content in Col-0 and *Atlpp2-2* leaves. (B) DGK and PAK activities in Col-0 and *Atlpp2-2* plants. Lipid kinase activities were determined from membrane fractions extracted from 15-d-old plants. Membrane aliquots were incubated for 4 min at 30 °C with 370 MBq ^{32}P-ATP, then lipids were extracted, separated by TLC, scraped off from the plates and quantified by scintillation counting. In (A) and (B): black bars, Col-0; white bars, *Atlpp2-2*. Data are means ± SD, $n = 3$ and were compared using Student's *t test*. Asterisks indicate that the mean value is significantly different from that of the control ($* = P < 0.05$).

Atlpp2-2, as in Col-0, DGK activity was stimulated by ABA between 2 and 3 fold in 20 min (Fig. 3A). A 2.2 and a 2.8 fold increase in DGK activity were recorded in both genotypes at 10 and 30 min, respectively. By contrast, in response to ABA, the microsomal PAK activity was affected in *Atlpp2-2*. In Col-0, a rapid and transient increase in the PAK activity, reaching a maximal value after 30 min of ABA treatment, was observed. In *Atlpp2-2* plants, PAK activity was also stimulated by ABA but to a lesser extent. For example, after 10 min, a 3 fold increase in PAK activity was recorded in Col-0, when a 1.8 fold increase was observed in *Atlpp2-2*. Consequently, the mutant lacking AtLPP2 activity was also impaired in ABA-stimulation of PAK activity. This suggests that, within the time of ABA treatment, the AtLPP2 activity is necessary for the stimulation of PAK activity.

2.4. Stomatal response to ABA is modified in Atlpp2-2 plants

Freshly harvested epidermal peels were used to measure the stomatal aperture of *Atlpp2-2 in planta*. One hour after the start of the photoperiod, the stomatal apertures of *Atlpp2-2* leaves were smaller (2.1 ± 0.1 μm) than those of Col-0 leaves (2.5 ± 0.1 μm; Fig. 4A). In other words *Atlpp2-2* stomata were *in planta* less open than Col-0 stomata. To compare the stomatal sensitivity of each genotype to ABA, epidermal peels were treated with ABA for 3 h and the stomatal aperture measured. As shown in Fig. 4B, 0.1 μM ABA provoked the stomatal closure in the wild-type as well as in *Atlpp2-2* mutant. However, 1 and 10 μM ABA caused less closure of stomata in *Atlpp2-2* than in Col-0 suggesting that *Atlpp2-2* is slightly less sensitive to ABA than Col-0. In parallel, we compared the effectiveness of ABA to inhibit stomatal opening of Col-0 and *Atlpp2-2* plants. Therefore, epidermal peels were harvested 1 h before the end of the dark period, when the stomata were closed. At this time, the stomata apertures were 1.36 ± 0. 09 μm ($n = 56$) and 1.25 ± 0.08 ($n = 33$) for Col-0 and Atlpp2-2, respectively. Fig. 4C shows that the inhibition of stomatal opening induced by ABA in Col-0 was affected in *Atlpp2-2*. For example, 1 μM ABA inhibited 33% of the stomatal opening in Col-0 and 14% of the stomatal opening in *Atlpp2-2*. Therefore, *Atlpp2-2* stomata are less sensitive to ABA than Col-0 stomata with regard to the inhibition of stomatal opening.

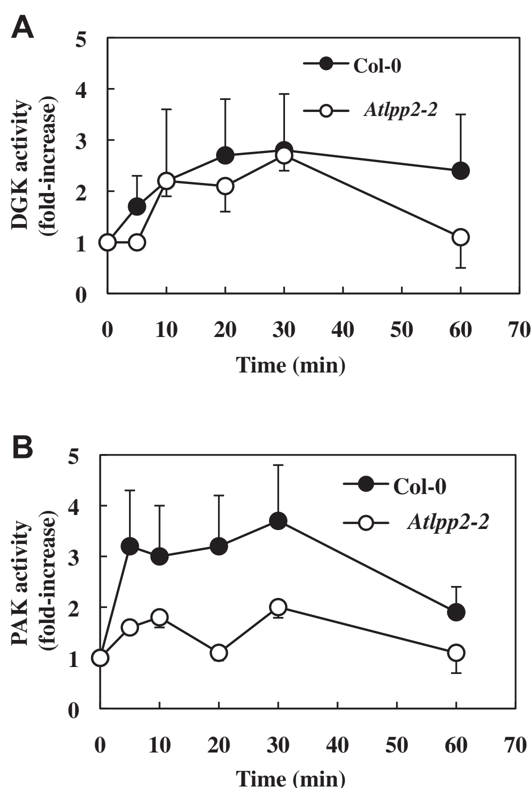

Fig. 3. ABA effect on DGK and PAK activities in *Atlpp2-2*. (A) Time course of DGK activity in Col-0 and *Atlpp2-2* plantlets treated with 10 μM ABA. (B) Time course of PAK activity in Col-0 and *Atlpp2-2* plantlets treated with 10 μM ABA. Lipid kinase activities were determined from membrane fractions extracted from 10 μM ABA-treated 15-d-old plants. Membrane aliquots were incubated for 4 min at 30 °C with 370 MBq ^{32}P-ATP, then lipids were extracted, separated by TLC, scraped off from the plates and quantified by scintillation counting. In (A) and (B): closed circles, Col-0; open circles, *Atlpp2-2*. Data are means ± SD, $n = 3$.

2.5. ABA content is unaltered in Atlpp2-2 leaves

The altered stomatal responses to ABA recorded in *Atlpp2-2* could be due to a difference between the two genotypes in their sensitivity to ABA or reflect a difference in their ability to produce and/or accumulate this hormone [21]. In order to understand the origin of *Atlpp2-2* phenotype, we measured the ABA content of rosette leaves. In 5-week-old plants, Col-0 and *Atlpp2-2* have similar ABA levels (about 800 pmol g^{-1} DW; Fig. 5). In plants that were not watered for 10 d, the level of ABA was higher (about 3500 pmol g^{-1} DW) than in watered plants but, again, no differences were found between the two genotypes (Fig. 5). Moreover, no phenotypic differences between *Atlpp2-2* and the wild-type were observed when plants were not watered and no differences in water losses were recorded (Supplementary Fig. S2).

3. Discussion

In this study, we have considered the role of *AtLPP2* in leaves in response to ABA by means of *Atlpp2-2*, a T-DNA insertional knockout mutant. We characterised *Atlpp2-2* mutant and found that it contained a 2-fold higher level of PA compared to Col-0. This demonstrates the functionality of AtLPP2 in leaves and is consistent with in vitro data showing that PA is a substrate of AtLPP2 enzyme [20]. However, the difference in PA content between *Atlpp2-2 and* Col-0 was smaller in leaves (about 2-fold) than in seedlings which have 4-fold more PA than Col-0 seedlings [10]. This difference could be explained by the fact that only two LPP genes, *AtLPP2* and *AtLPP3*, are expressed in seeds during germination [10], whereas three genes, *AtLPP1*, *AtLPP2* [10, 20] and *AtLPP3* (our observations), are expressed in leaves. Consequently, the effect of *AtLPP2* disruption might be stronger in seeds than in leaves.

In order to elucidate the impact of AtLPP2 on PA metabolism, DGK and PAK activities were evaluated in *Atlpp2-2*. It is important to point out that these enzymatic assays were performed using the endogenous substrates of the microsomal fraction. Hence, the measures recorded likely reflect the in vivo kinase activities. We observed that DGK activity was not significantly different between Col-0 and *Atlpp2-2*. Furthermore, time-course analyses of ABA effect on DGK in Col-0 and *Atlpp2-2* showed similar kinetics. This suggests that ABA stimulated DGK activity independently of AtLPP2. Interestingly, the involvement of the nonspecific phospholipase C, NPC4, coupled to DGK activity was recently demonstrated in ABA responses [22]. *AtLPP2* gene disruption modified PAK activity as *Atlpp2-2* plants showed 2-fold higher PAK activity than Col-0 suggesting that the amount of protein and/or its activity were regulated by either the substrates, or the products, of AtLPP2 activity. We favour the possibility of a metabolic regulation because PA level was also increased in *Atlpp2-2*. Under ABA treatment, the stimulation of PAK observed in Col-0 was attenuated in *Atlpp2-2*. However, it is important to note that in untreated *Atlpp2-2* plants, the PAK activity was already higher than in ABA-treated Col-

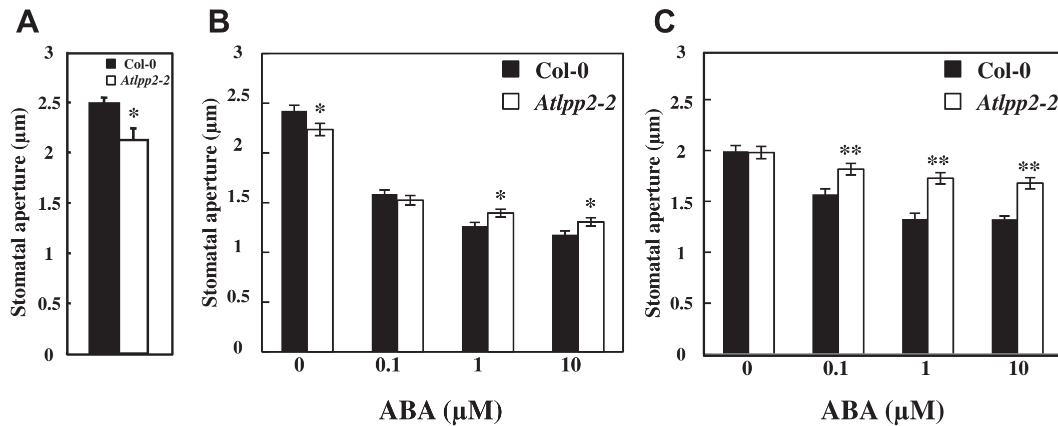

Fig. 4. Sensitivity of *Atlpp2-2* stomata in response to ABA. (A) Stomatal aperture *in planta*. Leaf epidermal peels were harvested 1 h after the start of the light period and stomata were immediately observed. Data are means ± SEM, $n = 108$ for Col-0 and $n = 87$ for *Atlpp2-2*. (B) Stomatal closure induced by ABA. Leaf epidermal peels harvested before the end of the dark period were incubated for 4 h in the light. ABA was then added, or not, as indicated and peels were incubated in the light for 3 h. Values represent the means ± SEM, $300 \geq n \geq 380$. (C) Inhibition of stomatal opening induced by ABA. Leaf epidermal peels were harvested before the end of the dark period and incubated with, or without, ABA in the light for 4 h. Values are the means ± SEM, $146 \geq n \geq 489$. In (A), (B) and (C): black bars, Col-0; white bars, *Atlpp2-2*. Data were compared using Student's *t test*. Asterisks in (A), (B) and (C) indicate that the mean value is significantly different from that of the control (* = $P < 0.05$ and ** = $P < 0.01$, respectively).

0 plants. Consequently, it could be possible that, in the mutant, PAK activity was not stimulated by ABA.

The role of PA in the regulation of stomatal closure by ABA is now well established [14,15]. The stimulation of PLDα1 activity by ABA, produces PA that binds to ABI1, hence promoting the closure of stomata [13]. In addition, PA produced by PLDα1 activity binds to the NADPH oxydase RbohD thus resulting in ROS production that triggers stomatal closure [14]. In their study, Mishra et al. [23] have proposed that, under ABA treatment, the binding of PA produced by PLDα1, to GPA1 promotes the inhibition of stomatal opening. Hence, PA is also involved in the inhibition of stomatal opening. Here we have shown that the stomata of *Atlpp2-2* were, *in planta*, less opened compared to wild-type plants. It is possible to explain this observation taking into account the increased level of PA recorded in the mutant. This latter modulates the activity of PA targets, *i.e.* ABI1 and/or RbohD, and, thus promotes, *in planta*, a larger stomatal closure in *Atlpp2-2* than in Col-0. In parallel, we have shown that in *Atlpp2-2* the stomata were less sensitive to ABA than in Col-0 suggesting that AtLPP2 positively regulates stomatal movements. In *Atlpp2-2*, we recorded at 1 μM and 10 μM ABA, a slight inhibition of

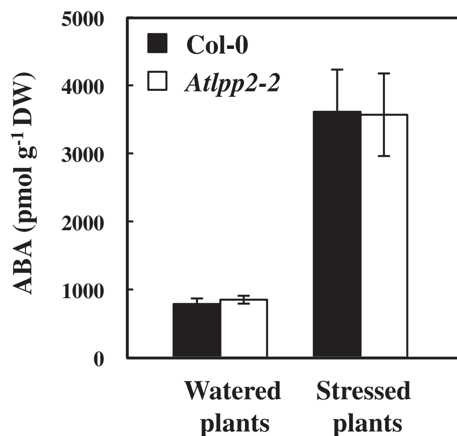

Fig. 5. ABA content is not modified in *Atlpp2-2*. ABA was extracted and quantified by ELISA in leaves of 5-week-old watered plants and in leaves of plants subjected to water stress (see Materials and Methods). Data are means ± SD, $n = 3$. Black bars, Col-0; white bars, *Atlpp2-2*.

the promotion of stomatal closure that indicated that AtLPP2 participated to this process. The effect of *Atlpp2-2* mutation on ABA inhibition of stomatal opening was significant from 0.1 μM to 10 μM ABA. It is difficult to interpret these results taken into account the established function of PA. According to the current knowledge, we expected that the lacking of *AtLPP2* resulting in an increase in PA level should favour hypersensitivity to ABA in *Atlpp2-2*. But, we cannot exclude the existence of other pathways that participate to the promotion of stomatal closure and inhibition of stomatal opening. Especially, the fatty acid composition of PA could be altered in *Atlpp2-2*, therefore the mutant exhibited an insensitivity to ABA. Indeed, Zhang et al. have shown the composition of PA species is essential for its binding capacity [14].

Our observations show that the expression of *AtLPP2* is inhibited by ABA in leaves. In seeds, ABA also inhibits *AtLPP2* expression (data not shown), an observation consistent with the data of Katagiri et al. [10] showing that AtLPP2 is a negative regulator in ABA-induced inhibition of germination. We have observed an antagonistic regulation of *AtLPP2* and *AtLPP3* expression by ABA in leaves. Considering that AtLPP2 and AtLPP3 proteins are located in the plasma membrane, this opposite regulation could play a role in ABA signalling [10,24]. However, it is noteworthy to notice that, if compensation exists between *AtLPP2* and *AtLPP3* expression, it does not hide the *Atlpp2-2* stomatal phenotype. We observed no change in the level of *AtLPP1* expression in ABA-treated leaves, which suggests that the stimulation of *AtLPP1* expression recorded in seedlings challenged with drought is ABA-independent [10].

Finally, as the alteration of ABA sensitivity observed in *Atlpp2-2* could be due either to an alteration in the ABA content or to a defect in signalling, we measured the hormone content. Whatever the water status of the plants, no differences in leaf ABA content between *Atlpp2-2* and Col-0 were found, thus suggesting that AtLPP2 activity was not involved in leaf ABA metabolism. As *Atlpp2-2* and Col-0 have the same ability to accumulate ABA in their leaves we propose that the *Atlpp2-2* was a mutant in ABA signalling. By contrast, in stratified seeds, there was less ABA in *Atlpp2-1* than in Col-0, leading to the idea that ABA synthesis was regulated by AtLPP2 in seeds [10]. This discrepancy is likely due to different modes of regulation of ABA metabolism in leaves and seeds [25].

In summary, the present results demonstrate the participation of AtLPP2 in response to ABA. Our observations suggest that AtLPP2 plays a positive role in the regulation of stomatal movement by

ABA. To better understand the role of AtLPP2 in stomata, the challenge will be to determine PA and DGPP level and the regulation of lipid kinases and phosphatases in guard cells during ABA response.

4. Materials and methods

4.1. Chemicals

ABA and PA were purchased from Sigma Aldrich, USA. ABA was diluted in dimethylsulfoxide (DMSO) and added at 0.1% (v/v) final DMSO concentration. All other chemicals used were of the highest reagent grade.

4.2. Plant material and growth conditions

Seeds of the *A. thaliana* (ecotype Columbia-0) T-DNA insertion mutant SALK_005663, which has a T-DNA insertion in *AtLPP2* (*Atlpp2-2*), were obtained from the European Arabidopsis Stock Centre (Nottingham, UK). Plants used for stomatal assays and for measuring ABA and PA contents were grown on soil/vermiculite (3:1) in a growth chamber at 21–22 °C, 75% relative humidity under an 8-h photoperiod (170 μE m^{-2} s^{-1}) to favour the vegetative development. Plants were watered when necessary. ABA content was also measured in plants that were not watered for 10 d before the analysis. Plants used for determining PAK and DGK activities were cultured in vitro on 0.8% agar in 0.5 Murashige and Skoog (MS) medium at 21 °C under a long day cycle (16-h day, 8-h night) with 170 μE m^{-2} s^{-1}.

4.3. Measurement of stomatal aperture

Stomatal apertures were measured on paradermal sections of abaxial epidermis taken from rosette leaves of 6-week-old plants. To measure stomatal aperture *in planta* (or so-called), epidermal peels were harvested 1 h after the start of the light period and observed immediately. To measure stomatal closure after ABA treatment, leaves were harvested before the end of the dark period. Paradermal sections of abaxial epidermis were incubated at 21 °C for 4 h in the light in opening medium 20 mM KCl, 10 mM iminodiacetic acid, 10 mM MES–KOH (pH 6.25). Then 0.1% (v/v) DMSO (control) or ABA at 0.1, 1 or 10 μM (in 0.1%, v/v, final DMSO concentration) was added to the opening medium for 3 h in the same conditions. To measure ABA inhibition of light-induced stomatal opening, epidermal peels taken before the end of the dark period, i.e. when stomata were closed, were incubated in the light for 4 h in opening medium with ABA. Stomatal apertures were measured by viewing with a light microscope (Microphot-FXA, Nikon, Tokyo, Japan) fitted with a camera (Digital Sight DS-L1, Nikon, Tokyo, Japan).

4.4. Determination of PA levels

Leaves of 6-week-old plants were allowed to settle for 5 min in boiling distilled water then ground in a blender in 12 mL of methanol/chloroform/acetic acid (100/50/1.5, v/v/v) with addition of 8 mL of 0.9% (w/v) NaCl, 4 mL of chloroform and 20 mL of boiling water. After shaking to mix and separation of the phases, the organic phase containing the total lipids was removed and dried under nitrogen. Lipid samples were dissolved in 20 μL of chloroform and spotted onto thin layer chromatography (TLC) silica plates (Silica Gel 60, Merck, La Jolla, CA, USA). The specific separation of PA was obtained on plates developed consecutively with acetone and the organic upper phase of ethyl acetate/isooctane/acetic acid/H_2O (13/2/3/10, v/v/v/v) as described by Munnik et al. [26]. Phospholipids were visualized with iodine vapour. PA scraped from TLC plates was mineralised at 180 °C overnight in prechloric acid and the phosphorus content was determined according to the method described by Rouser et al. [27].

4.5. In vitro lipid kinase assays

Membrane fractions were prepared from 2-week-old plants cultured in vitro. Plants were incubated in 0.5 MS medium for 15 min in a shaking bath at 21 °C then treated with ABA (final concentration 10 μM), washed 3 times with cold 0.5 MS medium, and suspended in 10 volumes of 50 mM HEPES (pH 7.4) containing 0.25 mM sucrose, 5 mM KCl, 1 mM EDTA and protease inhibitors (1 μg mL^{-1} leupeptin, 1 mM phenylmethanesulfonyl fluoride, 1 μg mL^{-1} aprotinin). This suspension was frozen in liquid nitrogen and thawed (3 times), homogenised, and centrifuged at 1000 × *g* for 15 min. The supernatant was centrifuged at 105,000 × *g* for 60 min to obtain the membrane fraction, which was washed, resuspended in 50 mM HEPES (pH 7.4) and the lipid kinase activities measured. The amount of protein in membrane samples was determined by the Bradford method [28] with BSA as the standard. Lipid kinase activity was determined in 60 μg aliquots of membrane proteins (105,000 × *g* membrane fraction) added to 50 mM HEPES buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM dithioerythritol, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM Mg^{2+}–ATP, and [γ-^{32}P]ATP (370 MBq), pre-warmed at 30 °C. Lipid kinase activities were assayed using endogenous lipids as substrates. Lipid phosphorylation was allowed to proceed for 4 min at 30 °C in a final volume of 100 μL. The reaction was then quenched by adding 1.5 mL of chloroform/methanol (1/2, v/v) to the mixture, lipids were extracted from the membranes as described previously ([11]) and phospholipids were separated by TLC. Samples were spotted on silica gel plates coated with 1% potassium oxalate solution and heated at 110 °C for 60 min just before use. Plates were developed with chloroform/methanol/acetone/acetic acid/H_2O (40/14/15/12/7, v/v/v/v/v). Positions of radiolabelled lipids were determined by autoradiography on Kodak film. Spots were scraped off plates and fractions were counted in a liquid scintillation counter. Lipid activities were expressed as pmol min^{-1} mg^{-1} of protein.

4.6. Measurement of ABA content

Leaves from 5-week-old plants were harvested, frozen in liquid nitrogen and lyophilised. ABA was extracted and quantified using the ELISA method described in Quettier et al. [29] with slight modifications. In brief, 20 mg of powdered samples were extracted with 10 mL of 80% methanol supplemented with butylhydroxytoluol (40 mg L^{-1}) for 16 h at 4 °C in darkness. ^{3}H-ABA (GE Healthcare, USA) was added as a standard. The extracts were then filtered through 0.2 μm-pore Minisart filters (Sartorius, Germany), passed through C18 Sep-Pak cartridges (Millipore, USA), concentrated under vacuum and taken up in 0.2 mL of 0.1% trifluoroacetic acid (TFA) in 10% acetonitrile. Samples were injected onto a 4.6 × 250 mm C18 Luna Phenomenex (USA) high performance liquid chromatography (HPLC) column. Elution was performed using a 0.1% TFA/acetonitrile gradient at a flow rate of 1 mL min^{-1}. Aliquots of eluted fractions were dried under vacuum (SpeedVac, Savant, USA), methylated with ethereal diazomethane, evaporated and taken up with 1 mL Milli Q water (Millipore, USA). Aliquots were processed for scintillation counting to determine ABA recovery or for ELISA [29].

4.7. Semi-quantitative RT-PCR analysis

Fully developed leaves of 5-week-old plants were immersed *in planta* for 1 min in 0.05% Silwet L-77® (GE Healthcare, USA) solution added, or not, with 10 μM ABA. After 3 h of incubation in the former

growth conditions, leaves were harvested. About 100 μg of leaf were frozen in liquid nitrogen and ground using a mixer mill. The powder was added to 1 mL TRIZOL (Invitrogen, France). After centrifugation, total RNA was precipitated with 70% ethanol and resuspended in RNase free water.

Reverse transcriptase-PCR (RT-PCR) was performed as described in Quettier et al. ([29]). Gene primers (Eurogentec, France) were 5′-GCCTTGATTTTGCCAGAGAG-3′ and 5′-ACACAAGCACGATTGGATCA-3′ for *AtLPP1* (*At2g01180*); 5′-CCGTTTCCCGCTTTAATACA-3′ and 5′-GGAAACAACGCCAAAAGAAA-3′ for *AtLPP2* (*At1g15080*); 5′-CACAGGATGAGAGAGCCACA-3′ and 5′-AAGACCATGACGTGTGTCCA-3′ for *AtLPP3* (*At3g02600*); 5′-ATCACGCCATTCTCGGTATC-3′ and 5′-CCGTTTTCGTCGTAAGGGTA-3′ for *AtLPP4* (*At3g18220*); 5′- AACATTGTGCTCAGTGGTGG-3′ and 5′-TCATCATACTCGGCCTTGG-3′ for *ACT2* (*At3g18780*). Twenty-seven amplification cycles were programmed with annealing temperatures of 59 °C for *AtLPP1*, *AtLPP2*, *AtLPP3*, *ACT2* and 64 °C for *AtLPP4*. The PCR was ended at 72 °C for 5 min. Nine μL of PCR products were loaded and separated on 2% agarose Tris-acetate EDTA gel and visualized with ethidium bromide (2 mg L^{-1}) and UV for photography.

4.8. Statistical analysis

Results are shown as the means ± SD for at least three independent experiments. Student's t-Tests were performed using the program provided by the College of Saint Benedict and Saint John's University (http://www.physics.csbsju.edu/stats/t-test.html).

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Appendix. Supplementary material

Supplementary material related to this article can be found at doi:10.1016/j.plaphy.2011.01.010.

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