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Roles of *Arabidopsis* Patatin-Related Phospholipases A in Root Development Are Related to Auxin Responses and Phosphate Deficiency

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ABSTRACT **Phospholipase A enzymes cleave phospho- and galactolipids to generate free fatty acids and lysolipids that function in animal and plant hormone signaling. Here, we describe three *Arabidopsis patatin-related phospholipase A* (*pPLA*) genes *AtPLAIVA*, *AtPLAIVB*, and *AtPLAIVC* and their corresponding proteins. Loss-of-function mutants reveal roles for these pPLAs in roots during normal development and under phosphate deprivation. *AtPLAIVA* is expressed strongly and exclusively in roots and *AtplaIVA*-null mutants have reduced lateral root development, characteristic of an impaired auxin response. By contrast, *AtPLAIVB* is expressed weakly in roots, cotyledons, and leaves but is transcriptionally induced by auxin, although *AtplaIVB* mutants develop normally. *AtPLAIVC* is expressed in the floral gynaecium and is induced by abscisic acid (ABA) or phosphate deficiency in roots. While an *AtplaIVC-1* loss-of-function mutant displays ABA responsiveness, it exhibits an impaired response to phosphate deficiency during root development. Recombinant AtPLA proteins hydrolyze preferentially galactolipids and, less efficiently, phospholipids, although these enzymes are not localized in chloroplasts. We find that AtPLAIVA and AtPLAIVB are phosphorylated by calcium-dependent protein kinases *in vitro* and this enhances their activities on phosphatidylcholine but not on phosphatidylglycerol. Taken together, the data reveal novel functions of pPLAs in root development with individual roles at the interface between phosphate deficiency and auxin signaling.**

Key words: **Abiotic/environmental stress; hormonal regulation; protein phosphorylation/dephosphorylation; signal transduction; gene regulation; *Arabidopsis*.**

INTRODUCTION

In animals and plants, phospholipase A_2 (PLA) enzymes participate in numerous developmental and pathological processes through the production of free fatty acid (FFA) and lysophospholipid (LPL) signaling molecules (Scherer, 2002; Meijer and Munnik, 2003; Shimizu et al., 2006). Based on sequence homologies, three major groups of phospholipase A_2 genes are known: calcium-dependent cytosolic phospholipases A_2 ($cPLA_2$) (Dessen, 2000), two related groups of animal calcium-independent (iPLA) (Balsinde and Balbao, 2005) and plant patatin-related phospholipases A (pPLA) (Holk et al., 2002), and secreted phospholipases A_2 ($sPLA_2$) (Ståhl and Stymme, 1998). Two of these major groups are found in the *Arabidopsis* genome: a family of four genes encoding $sPLA_2$s and a family of 10 genes encoding *patatin-related phospholipases A* (*AtPLAs*; Ryu, 2004). A further group of PLA enzymes comprising 12 genes (PLA_1) has also been described in *Arabidopsis*. This group includes DEFECTIVE ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL), which were shown to have phospholipase A_1 (PLA_1) activity and liberate linolenic acid as a precursor for

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jasmonic acid (JA) biosynthesis (Ishiguro et al., 2001; Hyun et al., 2008). Sequences related to animal cPLA$_2$s are not found in plants (Holk et al., 2002; Ryu, 2004).

The protein family of patatin-related phospholipases A, also referred to as PLP (La Camera et al., 2005), was named after the first sequenced homolog, patatin (Racusen, 1984; Andrews et al., 1988; Mignery et al., 1988). The AtPLA family can be classified further into three sub-groups according to amino acid sequence homologies and intron/exon structures (Holk et al., 2002). The location of each of the 10 *AtPLA* genes on the five *Arabidopsis* chromosomes is indicated by a Roman number followed by a capital letter when more than one *pPLA* gene exists on one chromosome (e.g. *AtPLAIIA* and *AtPLAIIB*). All of the pPLA proteins tested so far were shown to possess phospholipase A$_2$ activity. The catalytic rates of AtPLAs were higher when galactolipids were used as substrates compared to phospholipids (Matos et al., 2001; La Camera et al., 2005; Yang et al., 2007). Although related to patatins, which are major storage proteins in potato tubers, AtPLAs lack an N-terminal signal peptide and are localized to the cytosol (Holk et al., 2002).

Plant PLA activity is rapidly induced by different external signals and the PLA reaction products (released fatty acids and lysolipids) function as second messengers that regulate distinct proteins or downstream processes (Scherer, 1996; Meijer and Munnik, 2003). Thus, AtPLAs are thought to be important for early signal transduction events (Scherer, 2002; Ryu, 2004). In contrast to the wealth of information on auxin-induced transcription and auxin transport (Paponov et al., 2008; Teale et al., 2008), little is known about signaling steps prior to transcription. Lysophospholipid levels increased after 1–2 min and fatty acid levels within 5 min of auxin application to parsley and soybean cell cultures (Scherer and André, 1989, 1993; Paul et al., 1998). Such rapid profiles of auxin-induced PLA activity does not allow for biosynthesis of new proteins (Calderon-Villalobos et al., 2006), suggesting that PLA family proteins are post-translationally activated by auxin *in planta*. However, the identity of respective PLA enzymes and mechanisms by which the proteins are regulated has not yet been identified. Inhibitors of animal PLA$_2$ enzymes also inhibited auxin-induced rapid release of FFA and LPL in cell culture cells, as well as elongation of zucchini hypocotyl segments, etiolation of *Arabidopsis* hypocotyls, and auxin-induced transcription (Scherer and Arnold, 1997; Paul et al., 1998; Holk et al., 2002; Scherer et al., 2007). The same compounds inhibited enzyme activities of two recombinant AtPLAs tested so far *in vitro* (Holk et al., 2002; Rietz et al., 2004). Therefore, sequence homology and biochemical characteristics of AtPLAs suggest them as likely candidates to signal auxin, and possibly other effector-mediated responses in the cytosol.

Rapid activation of PLA activity has also been demonstrated in plant defense-related processes. Defense elicitors induced the production of FFA and LPL within minutes of application in tomato leaves (Narváez-Vásquez et al., 1999) and in *Eschscholtzia californica* (Viehweger et al., 2002, 2006) or *Petroselinum crispum* (Scherer et al., 2000, 2002) cultured cells. Viehweger et al. (2006) were the first to show that an elicitor induces a transient rise of lysophosphatidylcholine (LPC) followed by activation of a Na^+/H^+ exchange transporter that acidifies the cytosol. Cytosol acidification could also be induced by exogenously applied LPC and was both necessary and sufficient to induce biosynthesis of secondary products for defense (Viehweger et al., 2006). Function of LPC as a signal molecule was further demonstrated during mycorrhiza initiation in potato (Drissner et al., 2007). Besides rapid activation of PLA activity by elicitors, more prolonged accumulation of *pPLA* transcript and protein levels was observed after pathogen infection in *Arabidopsis* (La Camera et al., 2005) and tobacco (Dhondt et al., 2000, 2002). In the latter case, this was accompanied by increased PLA activity. Analysis of promoter-GUS plants revealed transcriptional activation of *AtPLAIIA* within 24 h of treatment with salicylic acid, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, wounding or iron and phosphate deficiency, pointing to various functions of PLAs in the plant (Rietz et al., 2004). More recently, a family of 14 *pPLA*-related genes was described in *Dictyostelium* of which one is involved in chemotaxis signaling (Chen et al., 2007). As in plants, *Dictyostelium* does not possess cPLA$_2$ genes.

Previously, we cloned members of the *Arabidopsis AtPLA* gene family as potential key components of early signal relay during plant development (Holk et al., 2002). Here, we characterize the mode of expression of three *patatin-related PLA* genes, *AtPLAIVA*, *AtPLAIVB*, and *AtPLAIVC* and, by analysis of corresponding loss-of-function mutants, identify functions in root and seedling development related to auxin response and phosphate deficiency. We further present evidence for post-translational regulation of AtPLAs via phosphorylation by calcium-dependent protein kinases. Our results provide a first functional link between members of AtPLA proteins and root growth regulation in *Arabidopsis*.

RESULTS

Arabidopsis AtPLAIVA, AtPLAIVB, and AtPLAIVC Are Conserved Acyl Hydrolases

AtPLAIVA, *AtPLAIVB*, and *AtPLAIVC* are located in tandem close to the telomeric region on the long arm of chromosome 4 (Figure 1A). The corresponding proteins have 52–78% amino acid identity and contain a conserved 'GXSXG' lipase motif as part of a predicted catalytic Ser/Asp dyad (Figure 1B) and, as a third highly conserved amino acid, an arginine in the 'DGGGXR' motif of plant pPLAs. This latter sequence corresponds to the 'SGGGXR' motif of animal cPLAs (Dessen, 2000; Holk et al., 2002; Rydel et al., 2003; Gosh et al., 2006).

Previously, we demonstrated enzymatic phospholipase A activity of recombinant AtPLAIVA *in vitro* on fluorescently labeled substrates (Holk et al., 2002). In the present study, we measured release of free fatty acids from various natural lipids to test for substrate preferences. Recombinant AtPLAIVA,

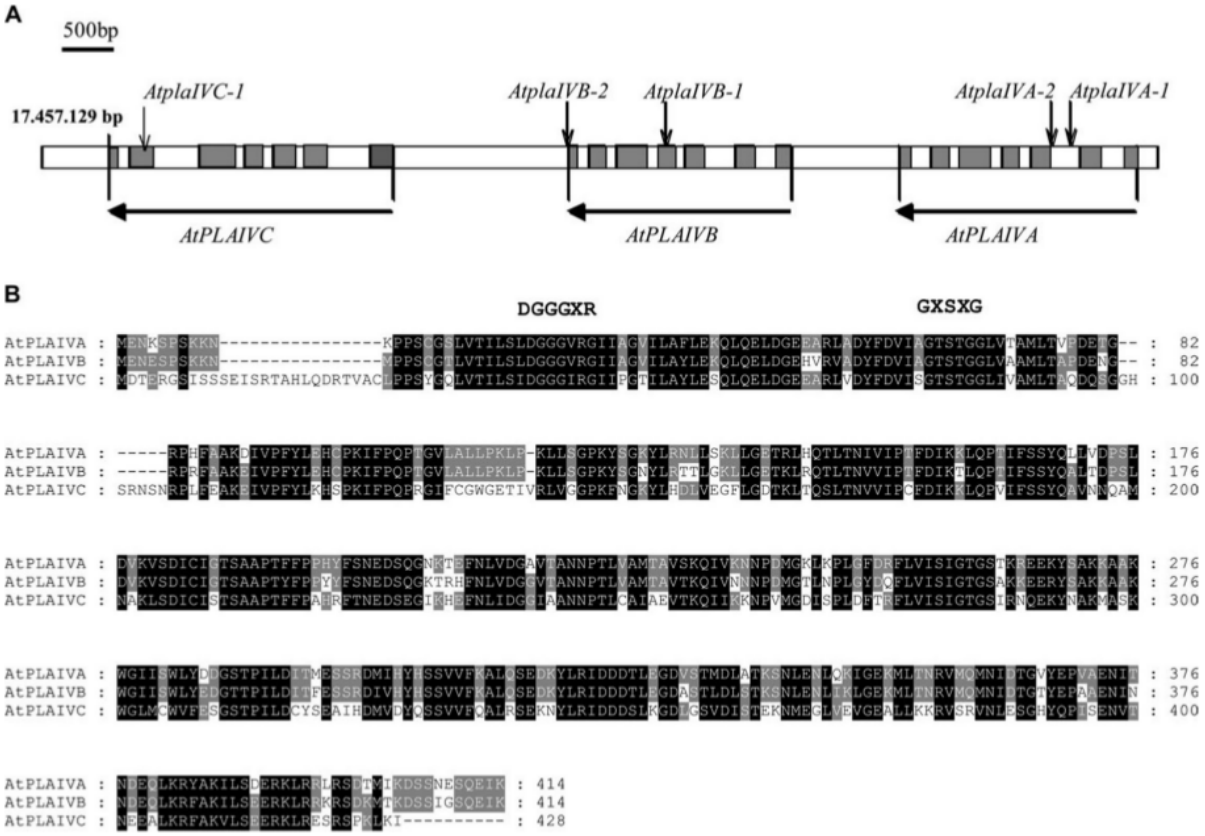

Figure 1. Tandem Gene Structure and Protein Sequence Alignment of *AtPLAIVC* (At4g37050), *AtPLAIVB* (At4g37060), and *AtPLAIVA* (At4g37070).

(A) Genomic structure of the genes. The exon/intron structure is indicated by dark gray exons into the scheme and arrows indicate positions of insertions in the mutants (*AtplaAIVA-1 AtplaIVA-2*, *AtplaIVB-1*, *and AtplaIVB-2 all in Col-O; AtplaIVC-1 in Ws*). Arrows below the sequences indicate the direction of transcription.

(B) Sequence alignments of AtPLAIVA, AtPLAIVB, and AtPLAIVC proteins. Amino acid identity is indicated in dark, similarity in gray. The phosphate binding element DGGGXR and the catalytic serine (GXSXG)/aspartate dyad are indicated above the sequences.

AtPLAIVB, and AtPLAIVC expressed in *Escherichia coli* were able to cleave the neutral lipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol (PG), and the polar lipids phosphatidylcholine (PC) and phosphatidylinositol (PI), but not the storage lipid, triacylglycerol (TAG) (Figure 2A). Their specific activities ranged from 10 to 50 nmol FA mg^{-1} min^{-1} for AtPLAIVC to 150–400 nmol FA mg^{-1} min^{-1} for AtPLAIVA and AtPLAIVB. AtPLAIVC differed from AtPLAIVA and AtPLAIVB in its turnover rate by a factor of ~10. Whereas linolenic acid was the major product released from galactolipids and phosphatidylglycerol, linoleic acid was the dominant product released from phosphatidylcholine and phosphatidylinositol (Figure 2B and 2C). Also, palmitic acid was found with phosphatidylinositol as substrate. Notably, AtPLAIVC differed from AtPLAIVA and AtPLAIVB in that it did not hydrolyze palmitic acid from phosphatidylinositol and required a reducing environment for activity. These data point to different kinetic requirements of the three AtPLA enzymes that may influence their *in vivo* activities.

AtPLAIVA Is Expressed in Roots and Knockout Lines Have Decreased Lateral Root Numbers

To gain further insights into the role of each *AtPLA* gene in development and/or sensing changes in the environment, independent *Arabidopsis* stable transformants of promoter::GUS (*uidA*: *β-glucuronidase*) plants (giving, respectively, PIVA:*uidA*, PIVB:*uidA*, and PIVC:*uidA*) were selected and examined throughout development and in response to hormones and abiotic stresses. Also, insertion mutants of all *AtPLAIV* genes were isolated (Figure 1) and found to be transcript-null lines (Supplemental Figure 1). For *AtPLAIVA* and *AtPLAIVB*, two lines were obtained and investigated and the single line *AtplaIVC-1* contained only one insertion, as shown by Southern blot (Supplemental Figure 2). Under normal growth

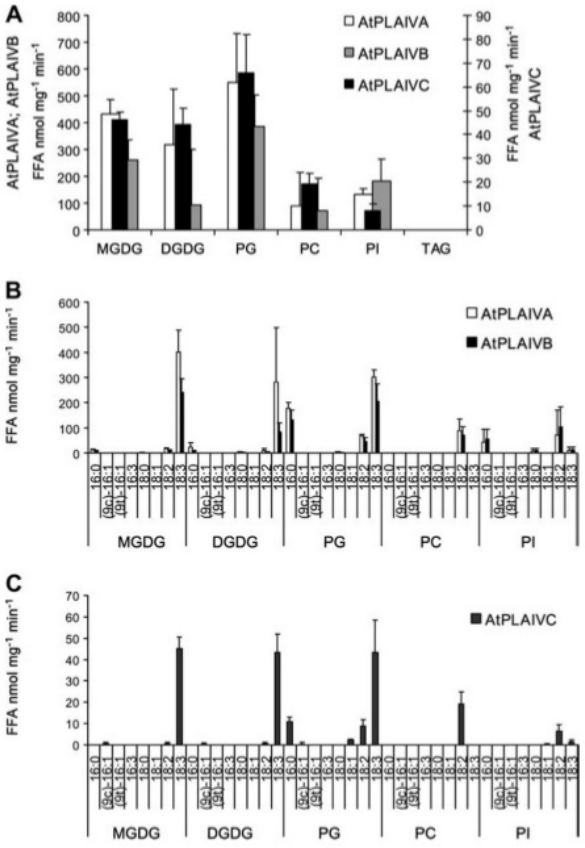

Figure 2. Substrate Specificity of Recombinant AtPLAIVA, AtPLAIVB, and AtPLAIVC Enzymes.

(A) Total fatty acid release from galactolipids and phospholipids. MGDG, monogalactoglycerolipid; DGDG, digalactoglycerolipid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; TAG, triacylglycerol. Note that AtPLAIVC specific activity is about 10 times lower (n = 3; ± SD). White bars: AtPLAIVA; gray bars: AtPLAIVB; black bars: AtPLAIVC.

(B) Release of individual fatty acids from galactolipids and phospholipids by recombinant AtPLAIVA and AtPLAIVB. MGDG, monogalactoglycerolipid; DGDG, digalactoglycerolipid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; TAG, triacylglycerol (n = 3; ± SD).

(C) Release of individual fatty acids from galactolipids and phospholipids by recombinant AtPLAIVC. Legend see (B).

conditions in soil, none of the mutants exhibited obvious morphological alterations compared to the corresponding wild-type accessions.

We found that PIVA:*uidA* expression was restricted to the root and root hairs but was absent from the root tip (Figure 3A–3E). Stem, leaves, and flowers did not show GUS activity and the pattern of expression was not altered by treatment with any of the tested hormones or conditions. We therefore focused on developmental differences in the roots, related to the promoter:*uidA* expression analysis. When grown on

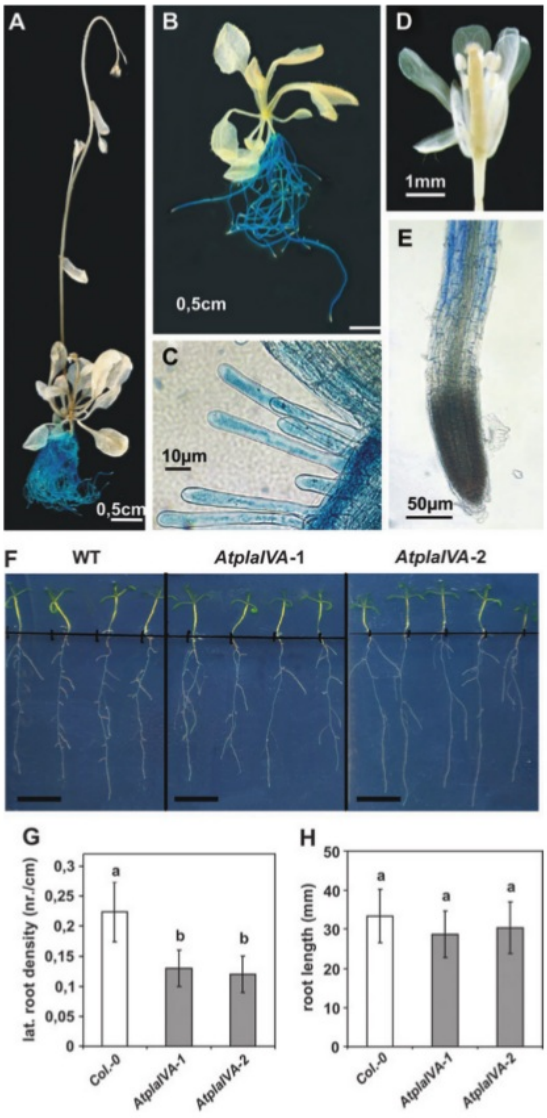

Figure 3. GUS Activity in Transgenic *Arabidopsis* Plants of PIVA:*uidA* Fusions and Growth Analysis of *AtplaIVA-1* and *AtplaIVA-2*.

Representative expression patterns observed in at least 10 independent transgenic lines are shown (20 min staining time each).

(A) 35-day-old plant.

(B) 22-day-old plant.

(C) Root detail with root hairs.

(D) Flower.

(E) Root tip.

(F) Ten-day-old seedlings grown on B5 (1:50) minimal diluted medium of wild-type Columbia-0, *AtplaIVA-1*, and *AtplaIVA-2* (bar = 1 cm).

(G) Lateral root densities of plants grown as in (F) (n = 39 for wt, n = 22–28 for mutants; ± SD). Different letters indicate statistical different values (ANOVA; $P < 0.01$).

(H) Lateral root lengths of plants grown as in (F) (n = 39 for wt, n = 22–28 for mutants; ± SD). Different letters indicate statistical different values (ANOVA; $P < 0.01$).

vertical agar plates, *AtplaIVA-1* and *AtplaIVA-2* seedlings developed only half the number of lateral roots compared to Columbia wild-type (Figure 3F and 3G), while the primary root length was unchanged (Figure 3H). This defect suggested an impaired root response to auxin (Casimiro et al., 2003). However, when treated with auxin, the roots of *AtplaIVA-1* and *AtplaIVA-2* responded to the same extent as wild-type roots with respect to decreased root length (Supplemental Figure 3) and lateral root formation (Supplemental Figure 4). Thus, general responsiveness to auxins seems not to be impaired in the *AtplaIVA* mutants. This genotype is reminiscent of other auxin signaling mutations such as in IAA proteins having regulatory functions in lateral root formation (Casimiro et al., 2003).

AtPLAIVB Is Expressed at Low Levels in Roots, Cotyledons, and Leaves

GUS expression in PIVB:*uidA* plants exhibited a pattern similar to PIVA:*uidA* in the root but was also observed in cotyledons and leaves (Figure 4A–4C). Upon auxin treatment, PIVB:*uidA* and native *AtPLAIVB* RNA became up-regulated (Figure 4F and 4G), but the corresponding *AtplaIVB* mutants exhibited only slightly reduced lateral root numbers and no clear apparent root or shoot morphology compared to wild-type in the vertical plate assay (Figure 4D and 4E). Again, root growth of *AtplaIVB-1* and *AtplaIVB-2* responded to exogenous auxin in the same way as wild-type plants (Supplemental Figures 3 and 4). We reasoned that loss of the *AtPLAIVB* expression is likely compensated for by other members of the *AtPLA* family under most growth conditions, especially because it is expressed at a lower level than the other two genes investigated here (Figure 4H).

AtPLAIVC Expression Is Specific to the Gynaecium and Is Stimulated by ABA

By contrast, GUS expression driven by PIVC:*uidA* was absent in seedlings and adult root and leaf tissues (Figure 5A) but was strong in the flower, specifically the stigma, ovary, and funiculus of the ovary (Figure 5B–5D). Notably, treatment with ABA (but no other hormone) induced GUS expression in root tissues in a concentration-dependent manner between 1 and 10 μM ABA (Figure 5E). Increased expression of endogenous *AtPLAIVC* mRNA by ABA was confirmed in wild-type plants (Figure 5F). Since ABA is a major hormone controlling the plant response to drought stress (Seki et al., 2007), we tested conditions of low humidity in the absence of exogenous ABA on PIVC:*uidA* expression and here it was also increased (Figure 5G). However, *AtplaIVC-1* mutants displayed wild-type responses to imposed drought stress.

Previously, we showed that GUS expression under control of the *AtPLAIIA* promoter was induced in roots upon phosphate deprivation (Rietz et al., 2004). To test whether the three AtPLA family members presented here are involved in responses to low phosphate supply, *AtPLA* transcripts and morphology were investigated. When wild-type seedlings were grown on 1:50 diluted B5 medium (Zhang and Forde, 1998; Signora et al., 2001) in limited phosphate supply, shortening of the primary roots and increased density of lateral roots were observed, as is typical for phosphate deficiency (López-Bucio et al., 2002). However, *AtplaIVC-1* mutant plants continued primary root growth to almost twice the length of the wild-type and developed only 60% of wild-type lateral root density (Figure 6C, 6G, and 6H). When 1 mM $KHPO_4$ was supplied, both wild-type and mutant showed very similar root architecture (Figure 6A, 6E, and 6F). RNA levels of *AtPLAIVC* did not respond to phosphate limitation (not shown). The response to 0.6 μM ABA of wild-type and *AtplaIVC-1* suppression of lateral roots and shortening of main roots were similar both in medium supplied with 1 mM $KHPO_4$ or without it (Figure 6B and 6D–6H). Under phosphate limiting conditions, both wild-type and *AtplaIVC-1* plants responded more strongly to ABA by decreasing root length but displayed higher root densities (Figure 6C, 6D, 6G, and 6H).

Altogether, the responses of *AtplaIVC-1* seedlings to ABA were similar to wild-type and suggested only a weak insensitivity phenotype when compared to the wild-type seedlings, which may explain why a drought phenotype could not be found. This implies that *AtPLAIVC* is necessary to perceive or mediate phosphate deficiency rather than being an important part of the ABA signaling pathway, although complementation by other *AtPLA* members in ABA responses can not be excluded.

We also noted that *AtplaIVC-1* mutants had longer hypocotyls than wild-type plants (Figure 6I). Since the response to phosphate deficiency is not known to influence hypocotyl length, this finding points towards a further role of *AtPLAIVC* in plant development.

AtPLAIVA and AtPLAIVB Phospholipases Are Targets of Calcium-Dependent Protein Kinases

Components of signal transduction cascades are often regulated by phosphorylation. Analysis of the AtPLAIVA, AtPLAIVB, and AtPLAIVC primary sequences revealed potential sites for phosphorylation by CDPKs in their C-terminal portions: a serine residue preceded by basic amino acids, notably at positions –1 and –3 (Cheng et al., 2002). We therefore tested whether CDPKs could target these PLAs for phosphorylation. To narrow down the number of potential kinases, we first correlated *AtPLA* and *CDPK* expression patterns based on the analysis of AtPLA expression presented here and published microarray data (www.genevestigator.ethz.ch/gv/index.jsp; Zimmermann et al., 2004) and also considered their sub-cellular localization (Dammann et al., 2003; Benetka et al., 2008). Coincidence of cytosolic localization and co-expression in root tissue with AtPLAs led us to predict that CPK3, CPK4, CPK6, and CPK29 could target these AtPLAs for phosphorylation. These CDPKs were expressed as recombinant proteins and tested in *in vitro* kinase assays with recombinant AtPLAIVA protein as substrate. Among the tested kinases, CPK3 and CPK4 phosphorylated AtPLAIVA whereas CPK6 and CPK29 exhibited negligible activity (Figure 7A). Conversely, only AtPLAIVA and, to a lesser

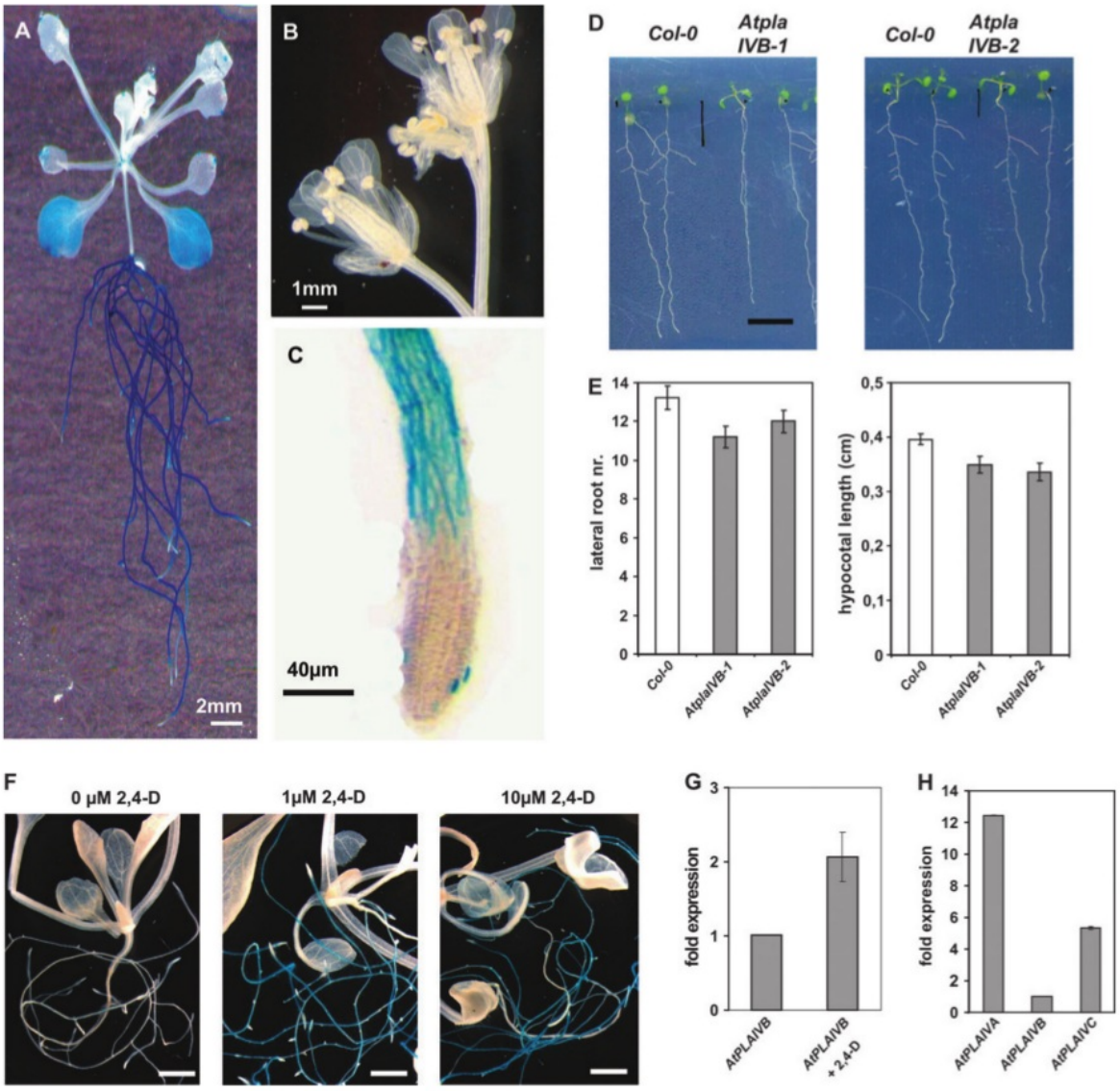

Figure 4. GUS Activity in Transgenic *Arabidopsis* Plants of PIVB:*uidA* Fusions and Growth Analysis of *AtplaIVB-1* and *AtplaIVB-2*.

Representative expression patterns observed in at least 10 independent transgenic lines are shown (24 h GUS staining).

(A) 15-day-old plant.

(B) Flowers.

(C) Root tip.

(D) Phenotypes of *AtplaIVB-1* and *AtplaIVB-2*. Seedlings were grown on B5 (1:50) minimal diluted medium. In each panel, two Col-0 wild-type seedlings are to be seen on the left side and two mutant seedlings on the right side (bar = 5 mm).

(E) Lateral root number and hypocotyl length of plants grown as in (D) (n = 12–16; ± SD).

(F) PIVB:*uidA* plants after 24-h treatments with 0 µM 2,4-D, 1 µM 2,4-D, and 10 µM 2,4-D (bars = 2 mm). GUS staining after auxin induction was performed for 2 h.

(G) Fold expression of *AtPLAIVB* after 24-h 10 µM 2,4-D relative to no 2,4-D by qPCR.

(H) Fold expression of *AtPLAIVA* and *AtPLAIVC* relative to *AtPLAIVB* in seedlings by qPCR.

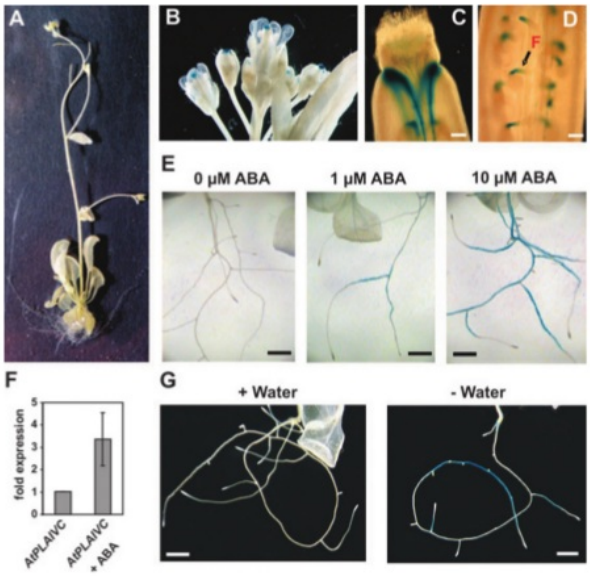

Figure 5. GUS Activity in Transgenic *Arabidopsis* Plants of PIVC:*uidA* Fusions (18 h GUS Staining).

(A) 35-day-old plants.
(B) Flowers.
(C) Gynoecium with stigma and style (bar = 100 µm).
(D) Detail of style with funiculi (F) (bar = 100 µm).
(E) PIVC:*uidA* plants treated with ABA; seedlings after 24 h with 0 µM, 1 µM, or 10 µM ABA (bar = 2 mm).
(F) Fold expression of *AtPLAIVC* in seedlings grown on medium without or with 10 µM ABA for 14 d by qPCR.
(G) PIVC:*uidA* plants kept for 24 h on Whatman membrane plus water (control) and minus water to induce drought stress (bar = 2 mm; treatment: see Methods).

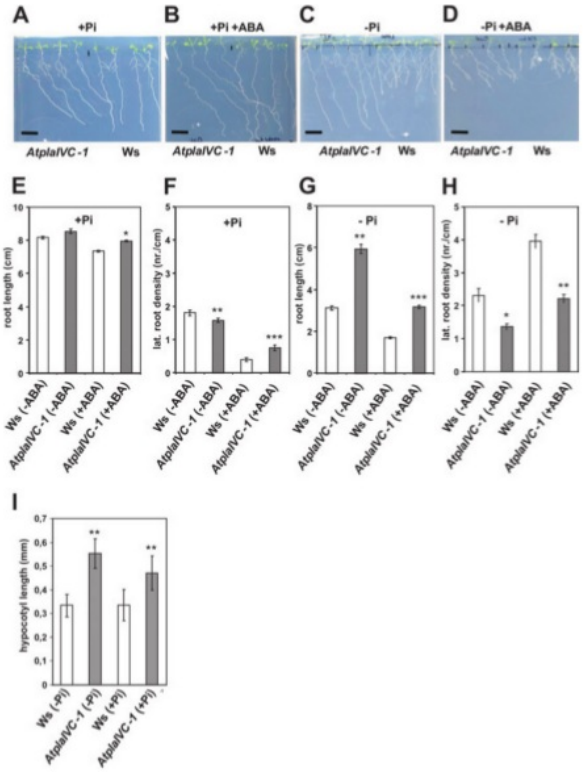

Figure 6. Growth Analysis of *AtplaIVC-1*.

(A–D) Seedlings grown on B5 (1:50) minimal diluted medium (bar = 1 cm). Stars above columns indicate significant differences between *AtplaIVC-1* and the corresponding Ws treatments at $P < 0.05$ (*), $P < 0.001$ (**), and $P < 0.0001$ (***) level following Student's *t*-test. (A) Medium supplemented with 1 mM $KHPO_4$. (B) Medium supplemented with 1 mM $KHPO_4$ + 0.6 µM ABA. (C) No addition. (D) Medium supplemented with 0.6 µM ABA only.
(E) Primary root lengths quantified from (A) and (B).
(F) Lateral root density quantified from (A) and (B).
(G) Primary root lengths quantified from (C) and (D).
(H) Lateral root density quantified from (C) and (D).
(I) Hypocotyl length quantified from (A) and (C) (n = 15–28; $P < 0.001$ for each relevant pair).

extent, AtPLAIVB but not AtPLAIVC were phosphorylated by CPK3 (Figure 7B). Since the predicted CDPK phosphorylation sites are located at the very C-terminus of the AtPLAs, we expressed C-terminally truncated versions of AtPLAIVA and AtPLAIVB in which the predicted CDPK recognition site within the last 16 amino acids was deleted (Figure 7C and 7D). Alternatively, the predicted target serine residues at position 399 in AtPLAIVA and AtPLAIVB were exchanged to alanines in the full-length proteins. The AtPLAIVA and AtPLAIVB C-terminal deletions were not accepted as substrates for CPK3. However, Ser399 to Ala exchange mutant proteins were phosphorylated, although less efficiently than the corresponding wild-type proteins (Figure 7C). These data show that the phosphorylation sites for CPK3 reside in the C-termini of the PLAs and appear to include but are not limited to Ser399.

To test the functional consequence of CPK3-mediated phosphorylation on phospholipase A activity, we measured the specific activities of phosphorylated and non-phosphorylated AtPLAIVA and AtPLAIVB *in vitro*. Purified recombinant AtPLAs were incubated with CPK3 for 15 min under kinase reaction conditions before addition of the lipid substrate. Since phosphorylation could influence PLA substrate selectivity, both phosphatidylglycerol (PG) as an uncharged lipid and phosphatidylcholine (PC) possessing a positively charged head group were used in the assays. Hydrolysis of PG was marginally repressed while PC was cleaved three times more efficiently by phosphorylated AtPLAIVA and two times higher by phosphorylated AtPLAIVB than by the corresponding non-phosphorylated proteins (Figure 8).

DISCUSSION

In plants and animals, free fatty acids and lysolipids are released from lipids in response to various stimuli and act as mediators of developmental processes and during defense

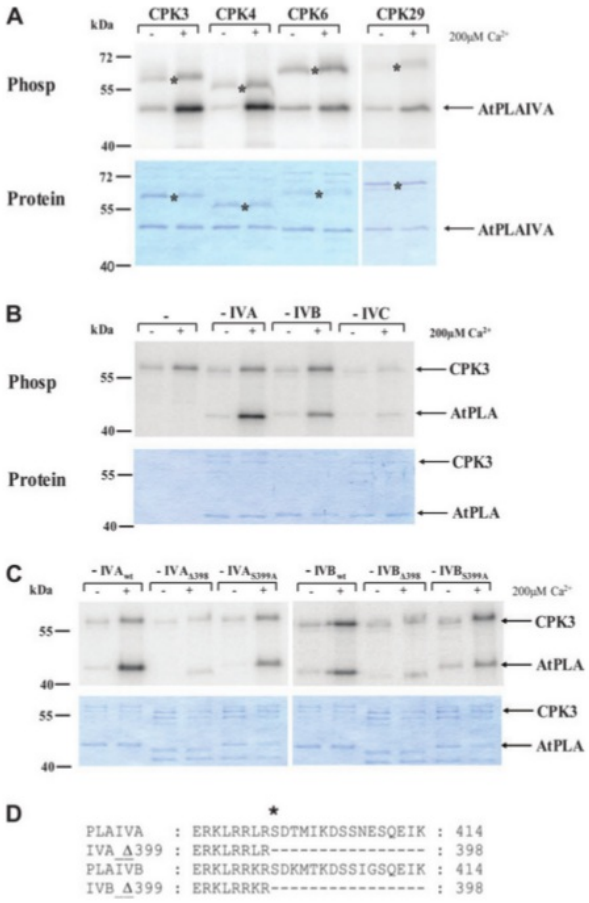

Figure 7. *In Vitro* Phosphorylation of Recombinant AtPLAIVA, AtPLAIVB, and AtPLAIVC Proteins by Recombinant Calcium-Dependent Protein Kinases.

(A) Phosphorylation of recombinant AtPLAIVA by CPK3, CPK4, CPK6, and CPK29. Asterisks denote the positions of the auto-phosphorylated CPKs (upper part) and in the protein gel (lower part). Position of phosphorylated AtPLAIVA protein is indicated by arrow and was calcium-dependent.

(B) Calcium-dependent phosphorylation of AtPLAIVA, AtPLAIVB, and AtPLAIVC by CPK3. Positions of auto-phosphorylated CPK3 and the PLAs are indicated by arrows.

(C) Phosphorylation of wild-type AtPLAIVA and AtPLAIVB proteins, mutated proteins (ser399 → ala399), and proteins truncated at position 398.

(D) Sequence comparison of the C-termini of the phosphorylated AtPLA proteins. Phosphorylation assays were three times repeated, always resulting in the same pattern.

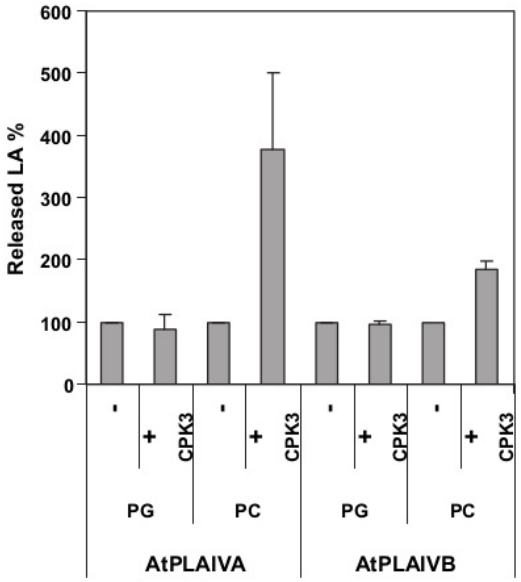

Figure 8. Phospholipase A Assay of Non-Phosphorylated and CPK3 Phosphorylated Recombinant AtPLAIVA and AtPLAIVB with Phosphatidylglycerol (PG) and Phosphatidylcholine (PC).

LA, linoleic acid (n = 3; ± SD).

against pathogens (Scherer, 2002; Meijer and Munnik, 2003; Shimizu et al., 2006). Increases of phospholipase A activity in plants were observed in response to auxin (Scherer and André, 1989, 1993; Paul et al., 1998), elicitors (Narváez-Vásquez et al., 1999; Scherer et al., 2000, 2002; Viehweger et al., 2002) and upon pathogen infection (Dhondt et al., 2000; Yang et al., 2007). While in animals, the superfamily of phospholipase A_2 enzymes has been described to cleave fatty acids from phospholipids, identification of corresponding proteins and their functions in plants remain unclear. The active centre of animal $iPLA_2$s and patatin-related phospholipases A (pPLA) is composed of a general esterase catalytic site motif GXSXG including the active site serine (Ghosh et al., 2006) (Figure 1B). Preceding the active centre, a catalytically essential arginine as part of the consensus sequence DGGGXR is involved in phosphate-binding of the substrate. Structural analysis of a patatin protein revealed similar spatial orientation of both motifs compared to $cPLA_2$ but otherwise pPLAs and $cPLA_2$ are not homologous (Dessen, 2000; Holk et al., 2002; Rydel et al., 2003). Whereas the general esterase motif is present in PLA_1 enzymes, the phosphate-binding motif is absent (Aoki et al., 2007). Since $cPLA_2$ (Dessen, 2000) and $iPLA_2$ (Balsinde and Balboa, 2005) enzymes in animals participate in various cellular signaling events, similar functions might be expected for plant pPLAs.

We previously identified 10 genes of the *patatin-related phospholipase A* family in the *Arabidopsis* genome (*AtPLA*) encoding for putative enzymes that could produce free fatty acids and lysolipids during auxin and elicitor signal transductions (Holk et al., 2002). Three proteins of the *Arabidopsis* AtPLA family, AtPLAI, AtPLAIVA, and AtPLAIIA, were shown to possess phospholipase A activity and *AtPLAI* and *AtPLAIIA* genes became activated upon pathogen infection, wounding or salicylate treatment (Rietz et al., 2004; La Camera et al., 2005; Yang et al., 2007). *AtPLA* genes involved in auxin signal transduction were not identified in auxin-response genetic

screens, possibly due to functional redundancy among this enzyme class. By confirming enzymatic activity and investigating potential functions in hormone and stress-related processes, we provide a first insight into the activities and roles of AtPLAIVA, AtPLAIVB, and AtPLAIVC in plants. The three genes *AtPLAIVA*, *AtPLAIVB*, and *AtPLAIVC* form a tandem arrangement on chromosome 4 (Figure 1A) suggesting the occurrence of gene duplications from one ancestral gene. This notion is supported by the high degree of homology between AtPLAIVA and AtPLAIVB (Figure 1B).

AtPLAIVA, AtPLAIVB, and AtPLAIVC Proteins Cleave Glyco- and Phospholipids

Patatin-related PLA enzymes expressed in *E. coli* or baculovirus systems appear to have a substrate preference for galactolipids that are most abundant in chloroplastic membranes (this work, Matos et al., 2001; La Camera et al., 2005; Yang et al., 2007). AtPLAIIA, AtPLAIVA, and AtPLAIVC are not, however, localized in plastids and AtPLAI shows only partial plastid localization (Holk et al., 2002). Under normal physiological conditions, galactolipids are absent in non-plastid membranes and may only be replaced by galactolipids from endomembranes and plasma membrane phospholipids under phosphate starvation (Andersson et al., 2005; Kobayashi et al., 2006). PC and PE were tested as substrates in *in vivo* auxin or elicitor stimulation of PLA activities (Scherer and André, 1989; Paul et al., 1998; Scherer et al., 2000, 2002; Viehweger et al., 2002, 2006; Heinze et al., 2007). Our *in vitro* assays showed that mainly linoleic acid and palmitic acid were released from PC and PI, but not the jasmonic acid precursor linolenic acid. It remains to be shown whether AtPLA enzymes can localize to chloroplasts under certain conditions and thus be linked then to JA biosynthesis, as has been suggested (La Camera et al., 2005; Yang et al., 2007), or whether galactolipid-hydrolyzing PLA_1 enzymes adopt this function (Ishiguro et al., 2001; Hyun et al., 2008).

Functions of *AtPLAIVA* and *AtPLAIVB* Related to Auxin Response in Roots

AtPLAIVA was strongly expressed throughout the root except in root tips and expression was not induced or diminished by hormones such as auxin. However, reduced lateral root formation in *AtplaIVA*-null mutants was an obvious defect. Auxin is a major promotive signal of lateral root formation (Casimiro et al., 2003). Since induction of lateral roots by auxin and primary root growth inhibition was similar in *AtplaIVA* mutants and wild-type seedlings, we discounted a general defect in auxin responses in these mutants. Partially reduced lateral root formation suggests that other genes in the *AtPLA* family may compensate for loss of *AtPLAIVA*. Auxin sensitivity of root growth was not changed in any of the knockout lines used here, which also suggests functional redundancy between *AtPLA* genes (Supplemental Figures 3 and 4). A change in auxin transport influences lateral root formation (Casimiro et al., 2001) and we cannot exclude this happening in *AtplaIVA* seedlings. Alterations in auxin biosynthesis were not investigated, since root length, which is also regulated by auxin, was unaffected in these mutants. Hence, *AtPLAIVA* seems to be involved in formation of lateral roots, as do many regulatory proteins, particularly Aux/IAAs (Liscum and Reed, 2002). Previously, we showed that pPLA inhibitors inhibit transcription of several *Aux/IAA* genes as well as the engineered auxin-activated *DR5* promoter (Scherer et al., 2007) and suppressed elongation in etiolated seedlings and hypocotyl segments (Scherer and Arnold, 1997; Holk et al., 2002). Those results and the data presented here point to a role of *AtPLA* genes in lateral root formation, which is probably linked to auxin signaling during root development.

Of all hormones tested, only auxins up-regulated *AtPLAIVB* expression (Figure 4F and 4G). However, no obvious auxin-induced root phenotype was observed in *AtplaIVB*-null mutants (Supplemental Figures 3 and 4). Also, other members of the AtPLA family (not investigated here) may be redundant with *AtPLAIVB* in mediating auxin-related processes. Analyzing publicly available DNA microarrays using the Genevestigator database revealed *AtPLAIIA* (At2g26560) and *AtPLAIIIA* (At3g54950) as encoding such potential AtPLA compensatory activities, since their transcripts also increase in response to IAA.

Loss of *AtPLAIVC* Prevents Adjustment of Root Architecture to Phosphate Starvation

Major changes in root architecture in response to phosphate deficiency are suppression of primary root length and increased numbers and longer lateral roots (López-Bucio et al., 2002; Nacry et al., 2005; Jain et al., 2007; Pérez-Torres et al., 2008). Both responses were repressed in the *AtplaIVC-1* mutant (Figure 6). Changes in root architecture due to phosphate deficiency were reported to involve cytokinin (Franco-Zorrilla et al., 2005; Kobayashi et al., 2006; Wang et al., 2006), gibberellin (Devaiah et al., 2009), auxin (López-Bucio et al., 2002, 2005; Nacry et al., 2005; Jain et al., 2007; Pérez-Torres et al., 2008), and ABA (Signora et al., 2001; de Smet et al., 2006; Ribot et al., 2008; Wasilewska et al., 2008). However, a connection between *AtPLAIVC* and cytokinin or gibberellin signaling seems unlikely from our data. Results rather suggest a role for auxin in PLA activation and for ABA and drought in transcriptional activation of *AtPLAIVC* (Figure 5E–5G). Growing *AtplaIVC-1* under drought conditions did not cause obvious differences in growth between mutant and wild-type and provision of ABA to *AtplaIVC-1* seedlings only partially reduced sensitivity in lateral root formation (Figure 6F). ABA represses lateral root formation and decreases root length (de Smet et al., 2006) and the *AtplaIVC* mutant retained both ABA responses. Strikingly, the ABA response was partially suppressed by phosphate, which may be a similar phenomenon to nitrate suppression of ABA response to inhibition of lateral root formation (Signora et al., 2001). Possibly, regulation of this gene by ABA affects functions in the gynoecium, where the gene is expressed strongly and exclusively

under normal conditions (Figure 5B–5D), although an obvious flower phenotype was not detectable. Seed maturation is accompanied by ABA synthesis and young pods have the highest ABA content (Müller et al., 2002). The observed induction of *AtPLAIVC* by ABA correlated with this pattern of expression.

A link between phosphate deficiency and auxin signaling is generally accepted, although the precise mechanisms are unclear. Recent publications suggest changes in both auxin concentration of lateral roots and auxin transport (Nacry et al., 2005; Jain et al., 2007) and revealed up-regulation of the auxin-responsive *DR5* and the *TIR1* auxin receptor promoters in response to phosphate deficiency. The authors interpreted this as an increase in auxin sensitivity presumed to modulate the structural changes associated with phosphate starvation (Pérez-Torres et al., 2008) because application of auxin to seedlings evokes reduced primary root growth and an increase in lateral root formation, reminiscent of root architectures from phosphate-starved plants (López-Bucio et al., 2002). Nacry et al. (2005) interpret their finding of increased free IAA content due to phosphate starvation in the root as a potential cause of root architecture changes, especially because auxin pathway mutants were compromised in adaptation to low phosphate. Since primary root growth of the *AtplaIVC-1* mutant continued during phosphate deficiency and the lateral root density did not increase (Figure 6C, 6G, and 6H), AtPLAIVC protein is necessary for root adjustment and thus may participate in auxin-directed root growth regulation. To test this hypothesis, further analysis of auxin distribution in *AtplaIVC-1* roots and epistatic analysis of auxin pathway mutants will be undertaken.

Longer hypocotyls of the *AtplaIVC-1* mutant were observed under conditions of low and normal phosphate, with or without ABA. Hence, neither phosphate deficiency nor ABA appears to be linked to this phenotype. Inhibition of hypocotyl extension by light is well known (Chen et al., 2004) so that a partial defect in de-etiolation may provide an explanation for the longer hypocotyls observed in *AtplaIVC-1*, but do not exclude auxin-related phenotypes.

Calcium-Dependent Protein Kinases Regulate AtPLAIVA and AtPLAIVB Activities

Rapid PLA activation upon auxin application (<5 min) (Scherer and André, 1989, 1993; Paul et al., 1998) or in response to elicitors and pathogens (<2 min) (Scherer et al., 2000, 2002; Viehweger et al., 2002, 2006) suggest post-translational control, since new proteins in plants are not synthesized earlier than 30–45 min after stimulus application (Calderon-Villalobos et al., 2006). We tested direct phosphorylation of AtPLAs by suitable candidate kinases that were selected based on co-expression in the same tissue, co-localization in the same cellular compartment, and the presence of potential phosphorylation sites in the AtPLAs. Several potential phosphorylation sites were predicted in the C-terminal part of the AtPLAs investigated here and most of those were consensus sequences for CDPK phosphorylation (Cheng et al., 2002). The observed specificity of CPK3 for AtPLAIVA and AtPLAIVB (Figure 8B) is in agreement with the known substrate specificities of CDPKs (Cheng et al., 2002), favoring a basic residue at position –3 to the phosphorylated Ser or Thr. This is the case for AtPLAIVA and AtPLAIVB but not for AtPLAIVC, which has an acidic residue in this position (aa 420 in Figure 1B). Specific phosphorylation of at least AtPLAIVA and AtPLAIVB by CPK3 and CPK4 but not by CPK6 or CPK29 may therefore serve as a key point for regulation of specific PLA enzymes (Figure 7A). In line with the observed localization of these CDPKs (Dammann et al., 2003; Benetka et al., 2008; Mehlmer and Teige, unpublished), a cytosolic localization of the AtPLA substrates has been shown (Holk et al., 2002). Our prediction of C-terminally located phosphorylation sites, particularly at amino acid 399 in AtPLAIVA and AtPLAIVB, was experimentally verified and loss of the single serine or truncation of the C-terminus strongly reduced phosphorylation by CPK3 (Figure 7C). Furthermore, when we tested the phosphorylated proteins for changes in catalytic activity and substrate specificity, we observed a two to three-fold increase in the cleavage of PC, but not of PG. So far, nothing is known about phospholipase A phosphorylation in plants. In animals, $cPLA_2$ enzymes release arachidonic acid and LPC from PC, both mediators in physiological and pathological responses (Shimizu et al., 2006). *In vivo* and *in vitro* experiments demonstrated that serines in $cPLA_2$ are phosphorylated by a MAP kinase, increasing the activity to a similar extent as observed for AtPLAs (Lin et al., 1993; Tian et al., 2008). The molecular mechanism of increased $cPLA_2$ activity by phosphorylation is not well understood. Das et al. (2003) propose enhanced PC binding through a conformational change of the protein facilitating hydrophobic interaction while Tian et al. (2008) found phosphorylation to interfere with PLA_2-associated proteins that inhibit lipid cleavage in the bound state *in vivo*. In the case of AtPLAIVA and AtPLAIVB, PG hydrolysis is not changed upon phosphorylation, but the hydrolysis of PC is. This offers the possibility of increased ionic attraction of the positively charged choline group of PC by the negatively charged phosphate modification. The observed selective enhancement of PLA activity with PC as substrate is consistent with a previous analysis demonstrating that PC is the source of rapid auxin-induced FFA release (Paul et al., 1998). To our knowledge, this mechanism of substrate-specific enzyme activation has not been described before for phospholipases A. Intriguingly, AtPLAIVC was only weakly accepted as substrate by the tested kinases but needs a reducing environment *in vitro* to become active, suggesting that a redox-related mechanism may regulate its activity (Buchanan and Balmer, 2005). PLA complex formation of one (or several) PLAs with Gα after elicitor application was suggested as another form of regulation (Viehweger et al., 2006; Heinze et al., 2007). Our data and those of previous studies clearly favor a model of rapid AtPLA activation by different stimuli *in vivo* before transcription changes.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana T-DNA insertion lines of *AtPLAIVA* (SALK_036785.54.90.x = *AtplaIVA-1* and SALK_027625.39.00.x = *AtplaIVA-2*), *AtPLAIVB* (SALK_073180.18.00.x = *AtplaIVB-1* and SALK_090933.54.20.x = *AtplaIVB-2*) were obtained from the Salk collection (Columbia ecotype) (Alonso et al., 2003). The T-DNA insertion line for *AtPLAIVC* (*AtplaIVC-1*) was isolated from the Wisconsin collection (Wassilewskija ecotype) (Sussman et al., 2000) and the insertion was localized from sequencing the PCR product of the genomic DNA at bp 17457546. Single insertion was verified by Southern blot (Supplemental Figure 2). Plants homozygous for the T-DNA insertion were identified by PCR-based genotyping. *Arabidopsis* seedlings were grown in long-day (16 h light, 8 h dark, 1100 lx m^{-2}). Seedlings were pre-grown for 3 d on MS/2 agar supplemented with 2% glucose and then transferred. Standard growth medium for phenotype experiments contained 1/50 B5 salts (Gamborg et al., 1968) minus vitamins, supplemented with 1 mM $CaCl_2$ and 70 μM $NaBO_4$, 0.5% saccharose, 1.2% agar, containing 21.8 μ phosphate. When indicated, the medium was supplemented with 1 mM NaH_2PO_4 or hormones. pH was buffered to pH 5.7 with 23 mM MES. To induce phosphate deficiency, no phosphate was in the medium. To treat promoter:*uidA* plants with hormones, seedlings were grown on MS/2-agar + 2% glucose for 12 d, then transferred into liquid MS/2 medium + 2% glucose including indicated concentrations. Tested hormones comprise 2,4-dichlorphenoxyacetic acid (0.1–10 μM), indole-3-acetic acid (0.1–10 μM), gibberellic acid (1–100 μM), abscisic acid (1–100 μM), 1-aminocyclopropane-1-carboxylate (5–500 μM), 6-benzylaminopurine (1–10 μM), jasmonic acid (5–500 μM), and salicylic acid (10–100 μM). *In vitro* drought stress was generated by transferring 12-day-old seedlings into a Petri dish (Ø 3.5 cm) containing 50 μl H_2O soaked by Whatman paper, wrapped with Parafilm and incubated for 24 h. Control plants were kept in 2 ml H_2O. Growth experiments were repeated two to three times or more.

Plasmid Constructs and Plant Transformation

To investigate gene transcription of *AtPLAIVA*, *AtPLAIVB*, and *AtPLAIVC*, the corresponding 5′ regulatory DNA sequences (= promoter PIVA – PIVC) were isolated by PCR from genomic wild-type DNA and cloned in front of the *β-glucuronidase* (*uidA*) open reading frame. The oligonucleotides used to amplify respective genomic fragments were: IVa-f ((*Xho*I) 5′-TGA CTC GAG CGA CAT TTA AAA TTA GAA CG-3′), IVa-r ((*Bam*HI) 5′-AGT TGG ATC CGA ATA GTT GAT CGA TCT TC-3′) for PIVA; IVb-f (5′-CAC CCT TTG TGT GAA ATT G-3′), IVb-r (5′-AGT TGA TCA ATC TTC TTT TGA AC-3′) for PIVB; IVc-f ((*Xho*I) 5′-CCC TCG AGA CCG AGG AAA GGA AAT TAA GG-3′), IVc-r (*Bam*HI) 5′-CGG GAT CCG TCT TCT CCT TTA CAA TCT C-3′). The underlined nucleotides depict additional restriction sites at both ends of PIVA and PIVC to allow cloning into binary pGPTV containing the *uidA* gene for stable plant transformation (Becker et al., 1992). PIVB was cloned in the binary pKGWFS7 vector (Karimi et al., 2002) using the Gateway system (Stratagene) that also allows promoter GUS studies. Both binary vectors were transformed into *Agrobacterium tumefaciens* GV3101 using either triparental mating (van Haute et al., 1983) or following a protocol from Weigel and Glazebrook (2001). The transformation of *Arabidopsis thaliana* Columbia-0 was performed according to Bechtold et al. (1993) supported by vacuum infiltration of the *Agrobacterium*.

GUS Assays

Arabidopsis stable transformants of PIVA:*uidA*, PIVB:*uidA*, and PIVC:*uidA* were selected for each construct and expression patterns were examined throughout development in plants grown *in vitro* or in soil and in response to treatments. Representative expression patterns observed in at least 10 independent transgenic lines are shown. The measurement of GUS activity was performed according to Jefferson et al. (1987). For histochemical analysis, samples were incubated in 50 mM $NaPO_4$ pH 7.0, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 0.5 mM K-ferrocyanide, 0.5 mM K-ferrycyanide, and 0.5% Triton X-100 (v/v) for 20 min to 16 h at 37°C. Endogenous pigments were extracted with 70% ethanol. For chemical treatments, seedlings of transformed PLA promoter-*uidA* plants were grown on 1/2MS-agar + 2% glucose for 12 d and transferred into 24-well plates filled with liquid 1/2MS + 2% glucose with or without different concentrations of 2,4-dichlorphenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), gibberellic acid, abscisic acid (ABA), 1-aminocyclopropancarboxylate, 6-benzylaminopurine, jasmonic acid, or salicylic acid (see Methods). Drought stress was produced by transferring 12-day-old seedlings on moistened Whatman paper compared to water as control.

Nucleic Acid Analysis

For quantitative RT–PCR experiments, 4 μg of total RNA was prepared with the NucleoSpin® RNA Plant kit according to the manufacturer's instructions (Macherey and Nagel) and converted with RevertAid™ H Minus First Strand cDNA Synthesis kit to cDNA (Fermentas). Primers were selected with the help of Primer 3-Web 0.4.0 software (http://frodo.wi.mit.edu/primer3/) and Netprimer software (www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html) and checked for primer efficiency and against primer dimers. Primers were: IVA-F: 5′-GGA AAT CAT TCG TGC TTT TGT GTG AA-3′; IVA-R: 5′-ACG TCC ATT ACT TTA TAT GCT GTG AG-3′; IVB-F: 5′-CCG AGG AAA GGA AAT TAA GGA GAA-3′; IVB-R: 5′-TAC AGC CGG AAA ATC ACT CTC G-3′; IVC-F: 5′-CCT TGA TTG AGA TTG TAA AGG AGA AGA G-3′; IVC-R: 5′-AGA TAG AAC AGT TGC TTG TCT TCC-3′; 18S rRNA-for 5′-GGC TCG AAG ACG ATCA GAT ACC-3′; 18S rRNA-rev 5′-TCG GCA TCG TTT ATG GTT-3′. Quantitative PCR reactions were performed with 1 μL of six-fold diluted cDNA, 200 nM primers and 0.2 Power SYBR® Green PCR master mix from Invitrogen in a StepOnePlus™

(Applied Biosystems) machine. For each pair of primers, a threshold value and PCR efficiency value were determined using a cDNA diluted 10-fold each in five dilution steps. For all primer pairs, PCR efficiency was >99% including the internal standard gene, 18S rRNA. The specificity of PCR amplification was examined by monitoring the presence of single peak in the melting curves of qPCR. Amplicons were checked for fragment length on 4% agarose gels. For each determination, two biological repeats and three technical repeats each were performed in the subsequent PCR reaction. Relative expression was calculated according to the ΔΔCt method using the equation: relative expression = $2^{-[\Delta Ctsample - \Delta Ctcontrol]}$, with $\Delta Ct = Ct_{sample\ gene} - Ct_{reference\ gene}$, where Ct refers to the threshold cycle determined for each gene in the early exponential amplification phase (Livak and Schmittgen, 2001). The control treatment was set as one-fold expression level. Statistical analysis was performed with the REST 2008 software (Pfaffl et al., 2002).

To test the T-DNA insertion lines for transcript abundance, corresponding cDNAs were amplified by PCR using following oligonucleotides: IVa-forw: 5′-TTA CTT CGA CGT GAT AGC TGG A-3′, IVa-rev: 5′-GGT ATG CAA AAA TTC TCT CGG-3′ for *AtPLAIVA* (At4g37070); IVb-forw: 5′-AGG AGA TGC AAG CAC TCT GG-3′, IVb-rev: 5′-CCT TTC CTC GGA GAG GAT TT-3′ for *AtPLAIVB* (At4g37060), and IVc-forw: 5′-ATT CTT CTC GGA ACG AAG AGC-3′, IVc-rev: 5′-GTT TGA GGC CAA GAA ATC GT-3′ for *AtPLAIVC* (At4g37050). The number of T-DNA insertion sites was analyzed by Southern. Genomic DNA (10 μg) of *AtplaIVC-1* was digested with either *Xba*I or *Eco*RI and hybridized with a α-^{32}P dCTP probe using a Random Primed DNA labeling Kit (Roche). The probe was amplified by PCR using S-for 5′-TGCTGTCGGCTTTAACCTCT-3′ and S-rev 5′-GGCACAGCACATCAAAGAGA-3′.

Protein Expression and Purification

The vector construct for recombinant expression of *AtPLAIVA* was described (Holk et al., 2002). In analogy, the coding sequences of *AtPLAIVB* and *AtPLAIVC* were amplified from respective cDNA clones using IVb-for1 ((*Sac*I) 5′-CGA GCT CGA GAA CGA ATC GCC C-3′), IVb-rev1 ((*Pst*I) 5′-AAC TGC AGT TTT ACT TGA TCT CTT GTG A-3′), IVc-for1 ((*Bam*HI) 5′-CGG GAT CCG ATA CAG AGA GAG GAT CAA-3′), and IVc-rev1 ((*Kpn*I) 5′-TAG GTA CCT CAT ATC TTG AGT TTA GGA-3′) and transferred in the pQE-30 expression vector (Qiagen) through the indicated restriction enzymes, containing an N-terminal 6xHis-tag, to facilitate protein purification. The expression vectors were transformed into XL-1 Blue MRF′ cells (Stratagene). For expression, 200 ml LB-medium plus 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} tetracydin was inoculated with 8 ml overnight culture and grown until OD600 0.9 at 37°C. After the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), growth continued for 2 h at 23°C. Cells were spun down and frozen at –80°C until further processing. The recombinant protein was purified via affinity binding to nickel-nitrilotriacetic acid (Ni-NTA) following the manufacturer's instructions (Qiagen). Protein concentration was calculated from a Coomassie stained SDS–PAGE by the program Scion Image (www.scioncorp.com) with bovine serum albumin as a standard.

The coding regions of *AtCPK3* (At4g23650), *AtCPK4* (At4g09570), *AtCPK6* (At2g17290), and *AtCPK29* (At1g76040) were amplified by PCR introducing an N-terminal *Nco*I site and a C-Terminal *Pst*I site and subsequently cloned as *Nco*I–*Pst*I fragments into the pTWIN1 expression vector (New England Biolabs, Ipswich, MA, USA). Recombinant proteins were purified from the BL21 *E. coli* strain according to the manufacturers' protocol.

Protein Kinase Assays

In vitro kinase assays using recombinant CPKs and AtPLAIVs were performed as essentially described in Teige et al. (2004). The reaction mix (20 μl) contained each 10 μg of substrate and 4 μg of recombinant kinase and was performed for 20 min at room temperature in kinase buffer (20 mM HEPES pH 7.5; 25 mM $MgCl_2$; 0.2 mM DTT; 0.2 mM ATP; 6 μCi ^{32}P-ATP) either in the presence of 0.2 mM Ca^{2+} (+Ca), or in the presence of 1 mM EGTA (–Ca). The reaction was stopped by adding 1/5 volume of five times SDS-loading buffer (Laemmli, 1970) and separated on 12% SDS–PAGE and analyzed by autoradiography. Kinase assays were performed three times and a representative single experiment is shown.

Lipase/Phospholipase A Assay

Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphaticylglycerol (PG), and L-alpha-phosphatidylinositol (PI), all from plant and fatty acid standards *cis*-9-hexadecenoic acid, *trans*-9-hexadecenoic acid, *cis*-7,10,13-hexadecatrienoic acid, octadecanoic acid, *cis*-9-octadecenoic acid, and *cis*-9,12,15-octadecatrienoic acid were purchased from Larodan/CPS Chemie GmbH (Düren, Germany). Other chemicals were purchased from Sigma/Fluka. A 200-μl reaction mix was prepared by drying lipid substrates under a stream of nitrogen gas and re-suspending in reaction buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM $CaCl_2$, 2 mM DTT (only AtPLAIVC), 0.01% hexadecyltrimethylammonium bromide, 0.4 μg μl^{-1} lipid). Solubilization of the lipids was supported by short sonicator bursts. To start the reaction, 20 μl of recombinant protein (~2 μg) was added and incubated at 28°C for 30–60 min. In cases of protein phosphorylation prior to phospholipase A assay, AtPLAs were incubated for 15 min at RT with CPK3 as described for the kinase assay before adding the lipid substrate. Subsequently, reaction mix was extracted with 2 vol. of chloroform/methanol (1:2) plus 1 vol. of 0.1 M KCl. After phase separation, the chloroform phase was dried under N_2 gas. The detection of free fatty acids was based on a pre-column derivatization with 2-nitrophenylhydrazine (2-NPH) according to Miwa et al. (1985). Dried lipids were re-suspended in 50 μl of 40 mM 2-NPH in 40 mM HCl/ethanol (1:1) plus 50 μl N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride in ethanol/3% pyridine in ethanol (1:1) and

heated to 80°C for 5 min. After adding 30 μl of 15% KOH in CH_3OH/H_2O (4:1), the reaction was heated for another 5 min at 80°C and cooled to RT. Analysis of the derivatives was performed by HPLC (Agilent 1100 series) equipped with a ZORBAX SB-Aq (3.0 × 150 mm, 3.5 μm) column and a diode array detector. A sample volume of 20 μl was injected and eluted with 100% H_2O, 0.1% trifluoroacitic acid (buffer A), and 98% acetonitrile, 0.1% trifluoroacitic acid (buffer B) running a gradient of 5 min 65%, 10 min 65–85%, and 5 min 85–100% buffer B. The eluted, derivatized fatty acids were measured by light absorption at 400 nm. Heptadecanoic acid served as internal standard to determine fatty acid amounts from the integrated peak area calculated by the software Chemstation (Agilent). Fatty acids were identified by commercial standards. Results shown are averages from three experiments.

Accession Numbers

AtPLAIVA: At4g37070, *AtPLAIVB*: At4g37060, *AtPLAIVC*: At4g37050, *AtCPK3*: At4g23650, *AtCPK4*: At4g09570, *AtCPK6*: At2g17290, *AtCPK29*: At1g76040

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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