Short title: pPLAIIIα affects germination rate

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Phospholipase pPLAIIIα increases germination rate and resistance to turnip crinkle virus when overexpressed

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One-sentence Summary
Overexpression of a patatin-related phospholipase in Arabidopsis increases seed germination rate and confers turnip crinkle virus resistance.

**Author Contributions:**

O.R.L. conceived the project and designed the experiments. J.H.J., N.Q.N., and Y.J.K., performed the experiments, except for the lipidomics experiment, which was performed by B. L., and hormone analysis, which was performed by H.J.S. O.R.L., F.B., and J.H.J. analyzed the data and wrote the manuscript.

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**ABSTRACT**

Patatin-related phospholipase As (pPLAs) are major hydrolases acting on acyl-lipids and play important roles in various plant developmental processes. pPLAIII group members, which lack a canonical catalytic serine motif, have been less studied than other pPLAs. We report here the characterization of \( pPLAIII \alpha \) in Arabidopsis (\textit{Arabidopsis thaliana}) based on the biochemical and physiological characterization of \( pPLAIII \alpha \) knockouts, complementants and overexpressors, as well as heterologous expression of the protein. In vitro activity assays on the purified recombinant protein showed that despite lack of canonical phospholipase motifs, \( pPLAIII \alpha \) had a phospholipase A activity on a wide variety of phospholipids. Overexpression of \( pPLAIII \alpha \) in Arabidopsis resulted in a decrease in many lipid molecular species, but the composition in major lipid classes was not affected. Fluorescence tagging indicated that \( pPLAIII \alpha \) localizes to the plasma membrane. While Arabidopsis \( pplaIII \alpha \) knockout mutants showed some phenotypes comparable to other \( pPLAIIIs \), such as reduced trichome length and increased hypocotyl length, control of seed size and germination were identified as distinctive \( pPLAIII \alpha\)-mediated functions. Expression of some \( PLD \) genes was strongly reduced in the \( pplaIII \alpha \) mutants. Overexpression of \( pPLAIII \alpha \) caused increased resistance to turnip crinkle virus, which associated with a two-fold higher salicylic acid:jasmonic acid ratio and an increased expression of the defense gene pathogenesis-related protein 1(\textit{PR1}). These results therefore show that \( pPLAIII \alpha \) has functions that overlap with those of other \( pPLAIIIs \) but also distinctive functions, such as the control of seed germination. This study also provides new insights into the pathways downstream of \( pPLAIII \alpha \).
INTRODUCTION

Lipases are a diverse group of hydrolases that break down acyl-lipids. Most of them hydrolyze the carboxyl ester bond between a fatty acid and the glycerol backbone, but lipase sequences diverge widely, and even the loose GXSXG esterase consensus for the catalytic serine is not always present. Based on their preferred substrate, in vitro lipases are usually classified into triacylglycerol lipases, phospholipases, galactolipases, and others. However, many lipases often act in vitro on a variety of lipid classes, and the determination of their physiological role is not trivial. Genome analysis of Arabidopsis (Arabidopsis thaliana) has indicated that there are as many genes annotated as potentially involved in lipid breakdown as there are involved in lipid biosynthesis (Beisson et al., 2013). Elucidation of the cellular function of the many putative plant lipases is thus a major challenge.

By hydrolyzing membrane phosphoglycerolipids, phospholipases participate in many aspects of plant cellular biology, such as signal transduction, cell growth regulation, membrane remodeling in response to environmental stresses and lipid metabolism (Wang X, 2001; Meijer and Munnik, 2003; Ryu, 2004; Matos and Pham-Thi, 2009; Scherer et al., 2010). Phospholipases of A-type (PLA) hydrolyze the carboxyl ester bond specifically at the sn-1 or sn-2 position of glycerophospholipids or in some cases at both positions. Plant PLA₂ families are classified into two groups: low-molecular-weight PLA₂s (PLA₂α, β, γ, and δ) and patatin-related PLAs (pPLAs), the latter of which are homologous to the potato (Solanum tuberosum) tuber storage protein patatin. The pPLAs act on glycerogalactolipids as well as glycerophospholipids to release free fatty acids and the corresponding lysolipids (Lee et al., 2010; Scherer et al., 2010).
In Arabidopsis, the 10 members of the pPLA family have been classified into three groups based on gene structure and amino acid sequence similarity: pPLAI, pPLAII (α, β, γ, δ, ε) and pPLAIII (α, β, γ, δ) (Holk et al., 2002; Scherer et al., 2010). Both pPLAI and pPLAIIIs are involved in plant responses to pathogens, auxin signaling, and phosphate deficiency. Although the recently characterized pPLAIIIs lack the canonical catalytic serine-containing motif GXSXG (Holk et al., 2002; Scherer et al., 2010), some pPLAIId and pPLAIIIβ possess a lipase activity with broad substrate specificity (Li et al., 2011; Lin et al., 2011; Li et al., 2013). In Arabidopsis, pPLAIId is involved in plant response to auxin (Labusch et al., 2013). Moreover, in rice (Oryza sativa), OspPLAIIα overexpression and knockout (KO) have an opposite effect on the expression of the growth repressor SLENDER1 in the gibberellin signaling pathway (Liu et al. 2015). Overexpressors (OE) of pPLAIIIs display similar stunted growth patterns with additional functions, such as reduced cellulose content in pPLAIIIβ-OE (Li et al., 2011) or lignin in PgpPLAIIIβ-OE and pPLAIIId-OE (Jang et al., 2019; Jang and Lee et al., 2020a; Jang and Lee., 2020b), and increased seed oil in pPLAIId-OE (Li et al., 2013; Li et al., 2015). Activation tagging of pPLAIId (STURDY, Huang et al., 2001) also results in decreased longitudinal cell elongation and stunted growth, as observed in pPLAIId-OE. The recessive rice mutant dep3 with OspPLAIId deficiency displays a dense and erect phenotype with short, wide epidermal cells (Qiao et al., 2011). A comparative proteomic analysis of pPLAIId-OE and WT has shown that one protein significantly differs between the OE and WT line, and it was identified as MICROTUBULE-ASSOCIATED PROTEIN (MAP)18 (Zheng et al., 2014). Many physiological functions have been thus associated with pPLAIIs in Arabidopsis or other plants, but the molecular pathways involved mostly remain to be elucidated.
To further shed light on the function of pPLAIIIα, we studied the activity of the recombinant protein \textit{in vitro}, characterized overexpression and \textit{KO} mutants in Arabidopsis at physiological and molecular levels, and performed lipidomic analyses.
RESULTS

Despite Lacking Canonical Motifs, pPLAIIIα has Retained Lipase Activity

Arabidopsis pPLAIIIα (At2g39220) was represented by a single gene encoding a protein of 499 amino acids with a predicted pI of 6.24 and a molecular mass of 54.5 kDa. Like all Arabidopsis pPLAIII proteins, pPLAIIIα lacked the serine (S) of the putative serine (S)-aspartate (D) catalytic dyad because S present in the canonical GxSxG motif was replaced with G (Fig. 1A). However, the second residue of the putative catalytic S-D dyad, aspartate (D), was present in the DGG motif. In addition, it can be noted that the phosphate or anion binding element DSGGXXG was not completely conserved in the pPLAIIIα protein because the second glycine (G) was replaced with serine (S).

Arabidopsis pPLAIIIα thus lacked the canonical phospholipase motif for the catalytic serine found in other characterized pPLAIIIs and one could question whether this protein really possessed phospholipase activity. The recombinant pPLAIIIα protein His-tagged at the C-terminal end was therefore expressed in *Escherichia coli* and purified (Fig. S1). *In vitro* enzymatic assays showed that the His-tagged pPLAIIIα had an acyl-ester hydrolase activity on each of the four major Arabidopsis phospholipid classes, with a slightly higher activity on phosphatidic acid (PA) than on phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) (Fig. 1C).

Taken together, these results show that pPLAIIIα is a noncanonical phospholipase A that hydrolyzes various phospholipids *in vitro.*

Spatial and Temporal Expression Patterns of pPLAIIIα

To determine the expression pattern of pPLAIIIα in Arabidopsis, we generated
PropPLAIIIα::GUS transformants using 2,087 bp upstream from ATG with 15 bp coding sequence (total 2,105 bp). The GUS reporter gene was expressed in most organs including inflorescences, flowers, siliques, stems, and leaves (Fig. 2A-J) but displayed further distinct spatial or temporal expression patterns compared to other pPLAIIIs.
promoters (Dong et al., 2014). *pPLAIIIa* was highly expressed in roots, with greater restriction in the vasculature and meristematic zones of the lateral roots (Fig. 2A and C-F). In germinating seeds, GUS activity was observed in embryo cotyledons and vasculature of roots during testa rupture and radical emergence (Fig. 2C and D). Vasculature expression in sepals and petals was also observed in whole flower organs.
(Fig. 2B). In cotyledons and true leaves, stomata expression was restricted in the inner wall of the guard cell region (Fig. 2G). *PropPLAIIIα::GUS* expressed highly in hypocotyls elongating in the darkness compared with those of grown in the light (Fig. 2H). Cross-sectional images of stems indicate strong expression of *pPLAIIIα* in the xylem and phloem (Fig. 2I). Strong expression was also observed in the hydathodes of young leaves and trichomes (Fig. 2J). Overall, GUS expression was observed in all organs with more restriction in the vasculature.

**Knockout, Overexpression and Subcellular Localization of Arabidopsis *pPLAIIIα***

To study the function of *pPLAIIIα*, we performed gain- and loss-of-function experiments in Arabidopsis. We first isolated homozygous T-DNA-insertion mutants for *pPLAIIIα* (Fig. 3A). In addition, the full-length genomic DNA sequence of Arabidopsis *pPLAIIIα* was overexpressed in Arabidopsis under the control of the 35S promoter with yellow fluorescence protein (YFP)- or monomeric red fluorescent protein (mRFP)-tagging at the C-terminal end. Immunoblotting using mRFP antibody detected expected size bands only visible in *OE* lines but not in knockouts (Fig. 3B). Both C-terminal YFP and mRFP tagging showed that *pPLAIIIα* localized to plasma membranes (PM) in root cells (Fig. 3C and D). The fluorescence signal of PM was separated from the cell wall, indicating its signal is not wall associated (Fig. 3D). Quantification of *pPLAIIIα* transcript levels in four independent homozygous *OE* lines (Fig. 3E) showed that *pPLAIIIα* was overexpressed very strongly in line 6 (440-fold), line 7 (420-fold), and line 13 (800-fold), and moderately in line 8 (5-fold). No transcripts were detected for two T-DNA insertion lines in *pPLAIIIα*, indicating that they were *KO* mutants (Fig. 3E). Transcript expression levels corresponded to protein levels (Fig. 3B). Complementation
Figure 3. Knockout, overexpression, and complementation lines for pPLAIIIa in Arabidopsis. (A) The T-DNA insertion sites in two Arabidopsis pplailla knockout (KO) mutants and a diagram showing the overexpression construct of pPLAIIIa (pPLAIIIa-OE) under the control of the 35S promoter with YFP or mRFP fusion at the C-terminus. (B) Immunoblotting of mRFP-tagged pPLAIIIa from Col-0, vector control, KO and OE plants. After SDS-PAGE separation, protein was immunoblotted with anti-mRFP antibody. (C) Subcellular localization of pPLAIIIa-YFP in the plasma membrane merged with FM4-64. Scale bar = 10 µm. (D) Plasmolysis of root epidermal cells of the pPLAIIIa-mRFP with 0.2M NaCl for 1 min. Scale bar = 10 µm. (E) Transcript levels of pPLAIIIa genes in the controls, KO, OE, and complementation (COM) lines as measured by RT-qPCR. n=3. (F) Phenotypes of 4-day-old seedlings. Scale bar = 1 cm. (G) The hypocotyl length and (H) root length of the controls, KO, OE, and COM lines. n=19–43. (E, G and H) Data represent the mean ± standard error (SE) of independent replicates. Stars denote statistical significance (t-test) at P < 0.05 (*) and P < 0.01 (**) compared with the controls. Col-0 and an empty vector line were used as control for mutant and OE lines, respectively.

(pPLAIIIa-COM) generated by crossing each OE line in the KO#1 background.
(SAIL830G12) still displayed overexpression of \textit{pPLAIII\textalpha} but compromised where line 7 overexpressed 87-fold and line 13 94-fold. This complementation result could be caused by overexpressing using the 35S promoter. Interestingly, transcripts for the closely related \textit{pPLAIII\textbeta} (72\% identity with \textit{pPLAIII\textalpha}), \textit{pPLAIII\textgamma} (56\%) and \textit{pPLAIII\textdelta} (34\%) (Fig. S2) were slightly modulated by the expression level of \textit{pPLAIII\textalpha} (Fig. S3).

In seedling stages (Fig. 3F), the hypocotyl length of the \textit{pplaIII\textalpha} mutants was longer than that of controls, but \textit{OE} lines were shorter than controls (Fig. 3G). The root lengths were only shorter in two of the \textit{OE} lines and unaltered in the \textit{KO} mutant lines (Fig. 3H). Both \textit{OE} 6 and 7 could be considered as ectopic lines with similar levels of expression, and \textit{OE} line 13 was the most highly expressing line. However, \textit{OE} line 8 was the most moderate line that was perfectly complemented in the \textit{KO#1} (Fig. 3G and H). From the seedling stages, all \textit{OE} lines displayed stunted and dwarf phenotypes with thicker cotyledons (Fig. 3F), which were also confirmed in the subsequent growth stages.

\textbf{Lipidomics Reveal a Wide Range of Acyl-Lipids are Decreased in \textit{pPLAIII\textalpha-OE} on a seedling weight basis}

To determine which lipid molecular species may be affected in the knockout or overexpressor plants, total lipids were extracted from seedlings of a \textit{pplaIII\textalpha} mutant and two strong \textit{pPLAIII\textalpha-OE} lines, and 115 molecular species of acyl-lipids, including phospholipids, galactolipids, and sulfolipids, were analyzed by LC-MS/MS (Fig. 4, Fig. S4 and 5).

In the \textit{pplaIII\textalpha KO} mutant, analysis of the lipid molecular species did not reveal significant differences, including in the phospholipid species potentially localized to the plasma membrane (Fig. 4C and Fig. S4). In \textit{OE} lines, total acyl-lipids were reduced.
about 25% on a seedling fresh weight basis in both lines compared to the vector control.
(Fig. 4A). Significant decreases were observed in many lipid classes (Fig. 4B). This included major phospholipid classes, such as the mostly extraplastidial PC and PE and the plastidial/mitochondrial PG, the minor phospholipid class PA, and some purely plastidial lipid classes such as the galactolipid monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and the sulfolipid class sulfoquinovosyldiacylglycerol (SQDG). For plastidial lipids, both ‘prokaryotic’ species (C18/C16, i.e. C34) and ‘eukaryotic’ species (C18/C18, i.e. C36) were affected (Fig. 4C). The reduction was almost evenly distributed among all glycerolipid classes, and the lipid class composition was thus not significantly affected in the two OE lines (Fig. S5). In the OE lines, a reduction was seen in most of the major molecular species (Fig. 4C) and the minor ones also (Fig. S4). The PA class appeared to be particularly impacted with almost all molecular species of PA reduced by 30-50% (Fig. 4C). Lysolipids were not significantly changed, except for the lysoMGDG and lysoPG species, which were significantly reduced in both OE lines. No accumulation of a particular free fatty acid (FFA) species (Fig. 4C) or of total FFAs (Fig. 4B) was measured in the OE lines.

In conclusion, lipidomic analyses show that total lipid amount per fresh weight and the composition in glycerolipid molecular species remained unchanged in the pplaIIIα KO mutant. In OE lines, FFAs did not accumulate and composition in glycerolipid classes was not significantly changed in both lines.

**pPLAIIIα Expression Level Affects the Size and Shape of many Cells and Organs**

Transversely expanded cell morphology with altered longitudinal cell elongation has been previously reported following the overexpression of pPLAIIIβ and pPLAIIIδ
Overexpression of pPLAIIIa alters the size and shape of many cells and organs. (A, C-E) Statistical analysis of plant height (A), leaf surface area (C), leaf thickness (D), and leaf water content (E) in 4-week-old plants. The data represent the mean ± SE of three independent replicates at P < 0.05 (∗) and P < 0.01 (∗∗) compared with the controls. (B) The aerial part of each 4-week-old plant with all individual leaves. The leaves are arranged from cotyledons (left) to the youngest leaves (right). Scale bar = 1 cm. (F) Floral organs and siliques in the pPLAIIIa-OE line (No. 13) and Col-0. Scale bars = 0.2 mm (top), 2 mm (middle), and 5 mm (lower). (G) Flower in the Col-0 and OE lines. Scale bar = 500 μm. (H) Stems in the Col-0 and OE lines. Scale bars = 500 μm (upper) and 100 μm (lower). (I) The area (mm²) of cross-sectioned stems and (J) cell number of each sectioned stems. Data represent the mean ± standard deviation of ten independent replicates. Stars denote statistical significance (t-test) at P < 0.01 (∗∗) compared with the controls. (K) Pollen structures in Col-0, KO and OE lines. Scale bar =10 μm. All surface images were captured using low vacuum scanning electron microscopy (JSM-IT300, JEOL Korea) at a 10.8 mm working distance and 20.0 kV.

(Huang et al., 2001; Li et al., 2011; Li et al., 2013; Dong et al., 2014). We thus
investigated the possible role of \textit{pPLAIII} in Arabidopsis growth and development by performing a detailed phenotypic analysis of several organs in \textit{OE} and \textit{KO} lines for \textit{pPLAIII}. Overall, \textit{OE} plants were, on average, 9 cm shorter in strongly expressing lines compared with the controls (Fig. 5A). The size of the rosette leaves decreased, and the number of leaves decreased with increasing \textit{pPLAIII} expression (Fig. 5B and C). In three strong \textit{pPLAIII-OE} lines (No, 6, 7, and 13), leaves were 1.7-fold thicker and contained more water (3%) on average (Fig. 5D and E). A reduction in organ size in \textit{pPLAIII-OE} lines was also clearly seen in other organs such as flowers and siliques, (Fig. 5F and G), hypocotyls (Fig. 3G), roots (Fig. 3H), and petioles (Fig. S6).

At the cellular level, when \textit{pPLAIII} was overexpressed, longitudinal elongation patterns from cells were reduced as observed in stems (Fig. 5H) and flowers (Fig. 5F and S7A); conversely, stem cells expanded transversely (Fig. 5I). However, the number of cells per stem cross-section was similar in WT, \textit{KO}, \textit{OE}, and \textit{COM} lines (Fig. 5J). Here, \textit{OE} line 8 was again solely complemented in the \textit{KO} background, and \textit{OE} line 13 displayed partial complementation in stem thickness (Fig. 5I). The effect of \textit{pPLAIII} overexpression on cell size and shape was also observed on trichomes (Fig. S7B and C) and pollen grains (Fig. 5K). Trichomes are predominantly three-branched on the adaxial surfaces of the rosette and cauline leaves of WT (Higginson et al., 2003). The overall trichome size was strongly reduced in \textit{pPLAIII-OE} (Fig. S7B and C), and a greater number of two-branched trichomes were observed compared with that in control (Fig. S7B). While overexpression of \textit{pPLAIII} had a strong effect on the sizes of many organs, silencing of the gene did not produce drastic effects in Arabidopsis plants. However, some differences occurred in \textit{KO} mutants compared with Col-0, including an increase in length of hypocotyls (Fig. 3F and G) and petioles (Fig. S6). This observation suggests
Transcript Levels of Ethylene and GA Biosynthesis Genes Increased during Growth Physiological Studies

The possible involvement of pPLAIIIα in hormonal growth control by auxin, ethylene and gibberellin that are essential for hypocotyl and petiole elongation (Wit et al., 2016; Yang and Li, 2017) in WT plants. pPLAIIIα-OE is involved in the control of seed size and germination. An exception to the reduction in organ size in OE lines occurred in seeds, which increased in width without alteration in length (Fig. 6A and B). Consistently, seed weight increased 23% in pPLAIIIα-OE and decreased 10% in KO mutants (Fig. 6C), thus showing that pPLAIIIα is involved in seed development in WT plants. In highly overexpressing OE lines, it was clear that the increased seed weights were due to the increased seed number per silique (Fig. 6D). The fact that seed weights decreased in KO mutants prompted us to check seed germination rates (Zhong et al., 2016) and kinetics. After 20h in the light, KO mutants showed 19% lower germination rates than the control, and the OE line showed a 10% greater germination rate on average (Fig. 6E). However, all seeds germinated by 30h, which indicated that the germination rate was not affected, but that germination was delayed compared to that in WT. Treatment with gibberellic acid (GA3) did not alter the germination ratio on average (Fig. 6F). Since antagonism between abscisic acid (ABA) and gibberellic acid (GA3) plays a key role in controlling seed germination (Koornneef et al., 2002), we quantified ABA in dry seeds. ABA content increased in the mutant compared to the WT (Fig. 6G). Altogether, these data suggest that the KO of pPLAIIIα delays the initial germination rate by changing endogenous GA and ABA biosynthesis, and that the GA and ABA circadian rhythms are altered by pPLAIIIα.
The antagonistic effects of ethylene and ABA in the regulation of seed germination have also been extensively studied (review, Corbineau et al., 2014). Increased ethylene...
production is associated with an accumulation of ACC oxidase (ACO) transcripts (review, Corbineau et al., 2014). ACO1 and ACO2 are the major ACOs involved in ethylene synthesis. ACO1, ACO2, and ACO4, which exhibits ACC oxidase activity (Gómez-Lim et al., 1993; Linkies et al., 2009; Park et al., 2018), were all upregulated in OE lines, whereas ACO1 and ACO4 expression decreased in KO lines (Fig. 7A).

Numerous data also suggest that ethylene stimulates seed germination by affecting GA biosynthesis (review, Corbineau et al., 2014). To uncover a possible role of bioactive GA biosynthesis, we analyzed expression of four GA oxidase genes. GA2ox and GA3ox encode enzymes that catalyze bioactive gibberellin biosynthesis, whereas
GA2ox1 and GA2ox2 are involved in the conversion of bioactive gibberellins into an inactive form. Two GA2oxs were upregulated 1.4-fold in KO lines, whereas GA20ox1 was upregulated 1.3-fold and GA2ox2 was downregulated 0.6-fold in the OE lines (Fig. 7B). These data explain that the faster germination in OE lines might be due to more active forms of GA and increased levels of ethylene.

**Overexpression of pPLAIIIα Delayed Senescence by Reducing ROS**

Suppression of the PA-generating PLDa1 leads to reduced superoxide synthesis, and addition of exogenous PA to leaves promotes reactive oxygen species (ROS) production (Sang et al., 2001). Besides, in several plant systems, minor lipid PA content has been studied in relation to stress responses (Wang et al., 2006) through the modulation of ROS (Hong et al., 2009). Therefore, we decided to monitor hydrogen peroxide (H2O2) levels in 8-week-old mutant and OE lines (Fig. 8A) using 3,3-diaminobenzidine tetrahydrochloride (DAB) staining (Fig. 8B). DAB is oxidized by hydrogen peroxide in the presence of heme-containing proteins to generate a dark-brown precipitate (Thordal-Christensin et al., 1997). In OE lines, leaves were greener in eight-week-old plants, when the leaves of WT plants had already become chlorotic (Fig. 8A). All plant leaves were green up to 28 days, but the levels of H2O2 were lower when pPLAIIIα was highly expressed, as observed in the OE lines compared with the WT (Fig. 8B) following DAB staining. To confirm that the delayed senescence was caused by pPLAIIIα, we analyzed the expression of a representative downstream gene, **SENESCENCE-ASSOCIATED GENE 12 (SAG12)**, which encodes a cysteine protease (Lohman et al., 1994) upregulated during senescence. Expression of SAG12 was 10-fold lower in four strong OE lines (Fig. 8C), which shows that high expression of pPLAIIIα retards senescence.
By contrast, expression of SAG13, which may be induced by stress or cell death, was only slightly increased in two of the four OE lines analyzed (Fig. 8D).

**Overexpression of pPLAIIIa Confers Turnip Crinkle Virus Resistance by Altering Salicylic Acid (SA) and Jasmonic Acid (JA) Contents**

Overexpression of phospholipase activity by SOBER1 reduces PA levels and suppresses plant immunity to the bacterial effector AvrBsT (Kirik and Mudgett, 2009). However, a plant immunity study focusing on virus relative to the level of PA has not been previously reported. Considering that plant viruses are pathogens associated with major threats, resistance to turnip crinkle virus (TCV) in Arabidopsis, which is one of the few manipulative plant-virus systems, was tested in mutant and OE lines (Fig. 9). Formation of the hypersensitive response (HR) was visible in TCV-inoculated plant leaves 3-days post-inoculation (DPI) (Fig. 9A and B) and in inflorescences at 12 DPI.
(Fig. S8A), with the most severe effects in the KO mutant. Only highly expressing pPLAIIIα-OE (No. 13) displayed an intact inflorescence (Fig. 9B). These phenotypes...
were consistent with the corresponding patterns of TCV gene expression, whereby the relative gene expression dramatically decreased in OE No. 13 and increased in the KO mutant (Fig. 9C and S8B). Following TCV infection, several defense genes, such as pathogenesis-related protein 1 (PR1), PR2, and PR5, are expressed, and SA accumulates (Kachroo et al., 2000). To determine whether this mechanism could be involved in higher virus resistance of pPLAIIIa-OE, the gene expression patterns of two representative SA and JA pathway genes were analyzed using leaves at 3 DPI (Fig. 9D and E). PR1 was upregulated in both OE lines, whereas PDF1.2 expression significantly decreased in OE line No. 13. The two-fold increase in PR1 gene expression in the KO mutant and OE lines may be explained by two endogenous antagonistic plant hormones. SA content was higher in the KO mutant and the OE line 13 compared with the controls (Fig. 9F). This finding indicates that both SA level and PR1 gene expression are modulated by the threshold level of relative pPLAIIIa expression or activity in response to developmental and environmental cues. However, the endogenous JA level was two times higher in the mutant and 25% lower in OE lines compared with that in the controls (Fig. 9G). Thus, differential cellular levels of SA and JA seem to be coordinately involved in TCV resistance due to the function of pPLAIIIa.
While the molecular and biochemical functions of \( pPLAIII\beta \) and \( pPLAIII\delta \) have been characterized in Arabidopsis (Li et al., 2011; Li et al., 2013), \( pPLAIII\alpha \) has only been studied in rice (Liu et al., 2015). Here, we characterized \( pPLAIII\alpha \) KO and OE lines in Arabidopsis at the biochemical, morphological and physiological levels. Phenotypes that confirm and extend those reported in rice were observed, such as reduced longitudinal growth and enlarged seed width and a strong relative decrease in the contents of PA as well as most other polar glycerolipid classes. But we also provide evidence for the association of \( pPLAIII\alpha \) with new processes, such as seed germination and virus resistance, and we give new molecular insights into \( pPLAIII\alpha \) signaling via regulation of expression of \( PLD \) genes.

**Lipid Substrates of pPLAIII\( \alpha \) in vivo**

Overexpression of Arabidopsis \( pPLAIII\beta \) increases all lipid species analyzed; phospholipids, including PE, PC, PI, PS, PA, and PG; and galactolipids, including MGDG and DGDG (Li et al., 2011). Similarly, overexpression of Arabidopsis \( pPLAIII\delta \) also tends to increase the levels of lipid species, including seed lipid reserves (Dong et al., 2014; Li et al., 2015). These counterintuitive results showing increased lipid content in plants overexpressing lipid-degrading enzymes may be explained by the fact that \( pPLAIII\beta \) may normally act in acyl editing mechanisms involved in lipid biosynthesis. Acyl editing mechanisms involve the removal by phospholipases of specific fatty acids from membrane phospholipids (for example PC) to yield lysophospholipids and a pool of acyl-CoAs enriched in specific fatty acids that are used for synthesis of other lipids such as storage lipids (Bates et al. 2012). Increasing the pool of acyl editing-involved
lysophospholipids through overexpression of specific phospholipases may create an
imbalance in membrane lipid composition. This imbalance may in turn trigger an
increased flux of \textit{de novo} fatty acid and membrane lipid synthesis and eventually result
in an overall accumulation of lipids.

By contrast, Arabidopsis \textit{pPLAIIIa-OE} displayed reduced levels of many
molecular lipid species compared to WT (Fig. 4 and Fig. S4). Thus, it seems that
\textit{pPLAIIIa} overexpression does not act on the same mechanisms that are altered by
\textit{pPLAIII\textbeta} and \textit{pPLAIII\textdelta} overexpression (possibly acyl editing cycles as discussed
before). This idea is consistent with the fact that lysophospholipids, which are key
players in acyl editing mechanisms, accumulate in \textit{pPLAIII\textbeta-OE} and \textit{pPLAIII\textdelta-OE} but
not in \textit{pPLAIIIa-OE}. Interestingly, despite the decrease in many lipid species in the \textit{OE}
lines, FFAs did not accumulate in these lines compared to WT, which showed that the
FA breakdown machinery was not overwhelmed by the overexpression of \textit{pPLAIIIa}.

Therefore, the phenotypes observed in the \textit{OE} lines are not likely caused by an excess of
FFAs. Some of these phenotypes may result from the decrease in one or several of the
many molecular lipid species significantly affected in both \textit{OE} lines (Fig. 4 and S4).

The fact that the Arabidopsis protein localizes to the plasma membrane suggests that
\textit{pPLAIIIa} acts directly on phospholipid molecular species and indirectly on plastidial
lipid species such as galactolipids, sulfolipids or PG. Concerning the major plastidial
lipids MGDG and DGDG, it should be noted that in \textit{OE} lines the decrease in their
molecular species impacts similarly both prokaryotic and eukaryotic species (Fig. 4C,
Fig. S4) and does not affect the mol\% fraction (Fig. S5) of these classes. It is thus likely
that the \textit{pPLAIIIa} activity in extraplastidial membranes of \textit{OE}s has an indirect effect on
the overall content in chloroplast membranes, but does not compromise lipid
homeostasis in these organelles.

*In vitro* assays on the partially purified pPLAIIIα showed that the protein hydrolyzed various phospholipids, with a slightly higher activity on PA (Fig. 1B). This seems to be consistent with a decrease in all phospholipid classes with a stronger reduction in PA molecular species in OE lines (Fig. 4C). Measurement of total phospholipase activities in plant extracts did not yield further insight into pPLAIIIα *in vivo* activity as no difference could be detected between WT and OE lines using four different phospholipid substrates (Fig. S9). This result was possibly due to highly active phospholipases other than pPLAIIIα, or to the presence of inhibitors of pPLAIIIα activity in the extract.

*In vitro* activity assays and lipidomic analyses show that pPLAIIIα protein is therefore probably a non-specific phospholipase A acting on a variety of phospholipids in the plasma membrane, with a possible slight preference for PA.

**PA levels and PLD expression**

PA accumulates at significant levels at the plasma membrane in Arabidopsis (Platre et al. 2018). The possibility that PA is one of the phospholipid classes targeted by pPLAIIIα in WT plants is supported by the slight preference of pPLAIIIα for PA in *in vitro* assays (Fig. 1C) and the strong reduction of almost all PA lipid species observed in Arabidopsis *pPLAIIIα-OE* (Fig. 4). These results on Arabidopsis and the previous observation in rice that a preferential decrease in PA occurs when *OspPLAIIIα* is overexpressed (Liu et al., 2015) thus support the view that PA is one of the possible substrates of pPLAIIIα in WT plants of both organisms. However, it should be stressed that hydrolysis of other phospholipid classes by Arabidopsis pPLAIIIα *in vivo* cannot be
ruled out because lipid species from other phospholipid classes are also reduced in OE lines (Fig. 4).

Intriguingly, no increases in PA molecular species were detected in pplaIIIα mutants in rice (Liu et al., 2015) or Arabidopsis (Fig. 4). Since PA is mostly generated by the activation of PLD (Sang et al., 2001), we checked the expression of several PLD isoforms in the Arabidopsis pplaIIIα KO mutants. Interestingly, the expression of the major PLD isoforms PLDa1, PLDζ1, and PLDζ2 decreased significantly compared to the controls in the mutant seedlings (Fig. S10). Taken together these data thus suggest that pPLAIIIα hydrolyzes PA in Arabidopsis WT plants and that absence of this activity in the pplaIIIα KO results in decreased expression of several PLD genes to possibly maintain PA levels in the plasma membrane. Other PA-producing pathways, such as DAG phosphorylation, could also be involved and may warrant further investigation. However, in OEs the situation is clearly more complex than a simple counterbalance of PA levels by modulation of PLD expression. Indeed, in OEs there was an increase in expression of PLD genes only in the stronger OE line, while other ones showed even a decrease in PLD expression. Complexity of the response observed in OEs may be enhanced by the fact that pPLAIIIα is also active on other plasma membrane phospholipids than PA, which may activate membrane homeostasis mechanisms and interfere with lipid signaling.

Role of pPLAIIIα in Cell Elongation and Plant Growth

Overexpression of pPLAIIIβ results in shorter leaves, petioles, hypocotyls, primary roots, and root hairs compared with the WT (Li et al., 2011), which is partly reminiscent of the phenotypic characteristics of pPLAIIIα-OE in Arabidopsis (Fig. 3F-H, 5, and
Conversely, the recessive rice mutant dep3 defective in OspPLAIIIδ displays a taller plant stature (Qiao et al., 2011). Generally, short and stunted morphology is observed in pPLAIIIδ-OE in camelina (Camelina sativa) (Li et al., 2015), pPLAIIIδ-OE in Arabidopsis and Brassica napus (Dong et al., 2014), pPLAIIIB-OE in Arabidopsis (Li et al., 2011), OSAG78-OE (OnpPLAIIIδ-OE) in Arabidopsis (Lin et al., 2011), OspPLAIII-OE in rice (Liu et al., 2015), and PgpPLAIIIB in Arabidopsis and poplar (Populus alba × Populus glandulosa) (Jang et al., 2019; Jang and Lee et al., 2020a).

Since pPLAIIIB-OE and pPLAIIIδ-OE show accumulation of lysolipids and free fatty acids, which have deleterious effects on cell membranes, it is tempting to think that the stunted phenotypes of pPLAIII-OEs are caused by the accumulation of pPLAIII products, which are deleterious to cellular activities. However, our data show clearly that pPLAIIIA-OE have stunted phenotypes in the absence of accumulation of FFAs or lysolipids. These results thus suggest that pPLAIIIs, at least pPLAIIIA, are involved in plant growth and development through the modulation of minor membrane lipid species (such as PA for pPLAIIIA) rather than via large changes in FFAs. Modified PA levels by pPLAIIIA may indirectly regulate cell elongation and plant growth, for example by affecting subcellular localization of regulatory proteins (Yao et al., 2013).

Trichomes are the outermost epidermal cells, which develop on almost all aerial structures of Arabidopsis. The pPLAIIIA-induced changes in trichome branching (Fig. S7B) and pollen structure (Fig. 5K) might also be caused by altered patterns of epidermal cell elongation. Endogenous JA content is involved in trichome patterning (Yoshida et al., 2009). Overexpression of pPLAIIIA reduced the level of JA content (Fig. 8G). Thus, it will be interesting to further investigate how pPLAIIIA-mediated lowered JA affects the trichome branching patterning.
Role of pPLAIIIα in Seed Germination

Overexpression of rice pPLAIIIα in rice (Liu et al., 2015) and Arabidopsis pPLAIIIδ in camelina (Li et al., 2015) thickened seed widths, but the length of seeds was reduced or not changed significantly. The recessive rice mutant dep3, where part of the pPLAIIIδ gene is deleted, shows smaller and rounder seeds but greater grain yields than control (Qiao et al., 2011). Seed sizes and weights can affect germination, but no study has yet been reported from pPLA gene families. Here, we show that alteration of seed morphology and weight can delay germination among pPLAs (Fig. 6). The importance of the plant hormone GA in promoting seed germination is well known, and ABA can act antagonistically. Ethylene also regulates germination and dormancy of many species via complex hormonal signaling networks (Corbineau et al., 2014). The knockout pplaIIIα mutant contained more ABA and increased gene expression of GA2ox1, which is involved in GA inactivation (Fig. 7B). The overexpression of pPLAIIIα displayed more transcripts of GA oxidases, which catalyze bioactive GA (Fig. 7B). Ethylene stimulates seed germination by affecting GA biosynthesis or signaling. Thus, the alternation of pPLAIIIα delays initial germination speed, possibly by modulating active and non-active forms of GA, and increasing ethylene biosynthesis.

Reduced PA and JA Levels May Explain the Low Level of ROS in pPLAIIIα-OE

Characteristic features of leaf senescence are the ordered disassembly of the altered photosynthetic apparatus and the loss of chlorophyll (Yoshida 2003). Expression of a well-studied senescence-response gene, SAG12, markedly decreased in all pPLAIIIα-OE lines (Fig. 8C); the higher the expression of pPLAIIIα, the greater the reduction in
SAG12 transcripts. Suppression of PLDa leads to reduced superoxide synthesis via reduction in the minor phospholipid class PA (Sang et al., 2001). In addition, the formation of PA leads to the production of other lipid messengers, such as JA (Wang et al., 2000). Thus, reduced content of PA (Fig. 4C) and JA (Fig. 9G) due to the overexpression of pPLAIIIα could be one possible reason for the reduction in H₂O₂. Mostly reduced transcript levels of PLD genes in pPLAIIIαOE lines (Fig. S10) should support this notion. Although PLDa1 was slightly upregulated, other PLDs were all downregulated. Alternation of lipid species by the reduction of lysolipids and FFAs (Fig. 4) and hormonal signaling, such as auxin, ethylene, and GAs (Fig. 6 and 7), could also affect the level of ROS (Corbineau et al., 2014).

A higher SA to JA Ratio May Increase the Resistance of pPLAIIIα-OE to Turnip Crinkle Virus

Some evidence suggests that at least one member of the pPLAI and pPLAII subclasses is involved in plant defenses. pplaI mutants are more sensitive to Botrytis cinerea infection without altering JA levels (Yang et al., 2007) whereas, pPLAIIα-deficient mutants are more resistant to Botrytis or avirulent Pseudomonas syringae infection (La Camera et al., 2005). Among three isoforms, pPLAIIIα, β, and δ, only pPLAIIIβ is upregulated upon Botrytis and Pseudomonas infection (La Camera et al., 2005). However, there is no clear evidence of the involvement of pPLAIII genes in plant innate immunity. Here we found that higher expression of pPLAIIIα confers TCV resistance via the regulation of SA and JA (Fig. 9). Increased levels of SA with decreased levels of JA may play crucial roles. In Col-0 and the vector control, the ratio of SA:JA was around 0.9 and 1, respectively. In knockout mutants, this ratio reduced to
0.8 whereas it increased 1.4- to 2-fold in two *p*PLAIIIα-OE lines, No. 8 and 13, respectively (Fig. 9F and G), which indicates a 2-fold higher SA level compared to the JA level in TCV resistance. During TCV infection, the HR is reportedly mediated by increased expression of defense genes, such as pathogenesis-related genes, and the accumulation of SA, phytoalexin, and camalexin (Dempsey et al., 1993; Kachroo et al., 2000). The 2.5-fold increase in *PR1* expression and approximately 50% reduction in *PDF1.2* expression mediated by SA might have increased TCV virus resistance.
MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; Col-0) was used as the wild-type (WT) plant. The *pplaIIIα* knockout mutants (KO#1: SAIL830G12 and KO#2: SALK_040363) were purchased from the stock center (http://www.arabidopsis.org/). Seeds were maintained as reported previously (Jang and Lee et al., 2020a and 2020b).

Transgene Constructs and Arabidopsis Transformation

The modified pCAMBIA1390 vector containing the CaMV 35S promoter, *YFP*, and/or *mRFP* was used (Lee et al., 2010) to express *pPLAIIIα*. The full *pPLAIIIα* genomic fragment was amplified using primers containing *SalI* and *AvrII* site (Table S1). Enzyme-digested PCR products were cloned into the vector (*Pro35s: pPLAIIIα-YFP/mRFP*). The promoter::GUS fusion construct was generated based on the obtained upstream intergenic region of *pPLAIIIα*. The promoter region was amplified using primers as follows: 5′-TC CTG CAG ATC ATC AAT GTA GTC GAA-3′ (forward) and 5′-TC GTC GAC TTG CAT CGT AGT TAA CAT-3′ (reverse). The amplified PCR product was subsequently cloned into a pCAM1390 vector containing a gus* A* reporter gene. All transgene-confirmed constructs were transformed into *Arabidopsis* using *Agrobacterium tumefaciens* C58C1 (pMP90) (Bechtold and Pelletier, 1998). Col-0 and the empty vector line were used as controls for the *pplaIIIα* mutant and *pPLAIIIα-OE*, respectively.

GUS Histochemical Analysis

Histochemical GUS staining was performed by incubating *PropPLAIIIα::GUS*
transformants in staining buffer following a previous report (Kim et al., 2014). Seedlings were photographed under a microscope (ZEISS Axio Observer D1, Germany).

### Observation of Reporter Gene Expression
Fluorescence was observed by confocal laser scanning microscopy (TCS SP5 AOBS/Tandem, Leica Germany). YFP and mRFP were detected using 514/>530, and 543/560—615 nm excitation/emission filter sets, respectively. The images were acquired at the Korea Basic Science Institute, Gwangju, Korea.

### Gene Expression Analysis by Reverse Transcription Quantitative PCR (RT-qPCR)
Total RNA extraction, quantification of total RNA, synthesis of complementary DNA, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed following previous reports (Jang and Lee et al., 2020a and 2020b). Three independent experiments were performed for each primer set (Table S1).

### Lipid Extraction and Lipidomic Analysis
Total lipids were extracted using the following hot isopropanol method. Briefly, 3-week-old Arabidopsis rosettes were cut, immediately weighed and placed in 2 ml boiling isopropanol (85°C) containing 0.01% (w/v) butylated hydroxytoluene. After 10 min heating, samples were cooled down to room temperature, and 1 µg of each internal standard was added (PE 17:0/17:0, MGDG 18:0/18:0, TAG 17:0/17:0/17:0). Samples were then ground for 1 min using an Ultra-turrax T25 apparatus (IKA Labortechnik), and 3 ml of methyl tert-butyl ether (MTBE) was added. After vortexing for 30 s, 1 ml of water was added and the mixture was shaken vigorously for 30 min and allowed to
phase separate by centrifugation at 3,000 g for 2 min. The uppermost (organic) phase was collected, and 1 ml of MTBE was added to the remaining lower phase. The mixture was shaken for 30 s and allowed to phase-separate by centrifugation. The upper phase was then combined with the previous organic phase, and the solvent of the total lipid extract was evaporated to dryness under a gentle stream of nitrogen gas. The total lipid extract was resuspended in 200 µl acetonitrile/isopropanol/ammonium formate (65:30:5, v/v/v, final concentration of ammonium formate 10 mM) and kept at -20°C until use. Quality control (QC) samples were prepared for data quantification by pooling aliquots of the lipid extract samples from Col-0 plants to make a QC stock solution. QC samples were evaporated to dryness under a gentle stream of nitrogen gas and resuspended in 200 µl of the same acetonitrile/isopropanol/ammonium formate mixture used for samples. A QC sample contained, in addition to the three internal standards coming from samples, the following quantification standards added in the molar proportions: PG (17:0/17:0), 4; PA (17:0/17:0), 1; PE (17:0/17:0) 2; PC (17:0/17:0), 4; PI (17:0/14:1), 1; MGDG (18:0/16:0), 28; DGDG (18:0/16:0), 12; SQDG (16:0/18:3), 2; LPG (17:1), 1; LPE (17:1), 1; LPC (17:0), 1; FFA (17:0), 1; (purity of standards was determined before use by LC-MS/MS analysis). Samples and QC samples were subjected to UPLC-MS/MS analyses using an ultimate RS 3000 UPLC system (Dionex) connected to a quadrupole-time-of-flight (QTOF) 5600 mass spectrometer (AB Sciex). Samples were run in negative mode. Lipids were separated using a C8 2.1 × 150 mm 2.6 µm column (Kinetex) and a binary gradient of solution A (60v:40v water/acetonitrile) and solution B (90v:10v isopropanol/acetonitrile). Elution was achieved through a gradient of solution B from 27 to 97% as compared to solvent A within 20 min at a speed of 0.3 ml min⁻¹, and then at 97% for 5 min. Solution B was
then decreased to a 27% enrichment during 7 min for column reequilibration. Relative quantification of lipid molecular species in samples was achieved with MULTIQUANT software (AB Sciex) using intensity values obtained by extracting masses of the different lipids previously identified, and by normalizing based on rosette fresh weight (FW) and the internal standard (to control for lipid extraction). In samples, absolute amounts of lipid molecular species (pmol per mg FW) in each lipid class were estimated using intensities of the corresponding quantification standards of the QC samples (normalized by the internal standard). For lysoMGDGs and lysoDGDGs, estimates were based on one of the lysolipid standards (LPE). The estimated absolute amounts of lipid molecular species were used to calculate total amounts in each lipid class, total lipid amounts and lipid class compositions in each line.

**Protein Purification/Extraction, and Immunoblotting**

The full-length cDNA of pPLAIIIα was cloned into the pET28a vector with the 6xHis. The BL21 (DE3) bacteria expressing pPLAIIIα-His fusion protein were induced with 1 mM isopropyl 1-thio-b-D-galactopyranoside (IPTG), and the fusion protein was purified. The bacterial pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole 1%(v/v) triton X-100 adjust pH to 8.0) containing 1 mg/mL lysozyme and 1 mM phenylmethanesulfonyl fluoride (PMSF). The suspension was sonicated. After centrifugation at 10,000g for 20 min, the supernatant was mixed with Ni-NTA agarose resin (10%, w/v) (Qiagen). The fusion proteins bound to agarose beads were washed with washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole adjust pH to 8.0), and pPLAIIIα proteins were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, adjust pH to 8.0). The proteins from
7-day-old seedlings were extracted using extraction buffer (120 mM Tris-HCl, pH 7.5, 100 mM EDTA, 5% glycerol, 2% SDS, 1% Triton X-100, 300 mM NaCl, 1 mM DTT, 1 mM PMSF) following sonication and centrifugation in the same condition as for the above protein purification. The amount of purified protein was measured with the Bradford method (Bradford, 1976). The immunoblotted membranes were pre-blotted with 5% skim milk for 30 min and incubated with 6x-His tag antibody, HRP (MA1-135, Invitrogen, USA) for 14 h at 4 °C. Anti-RFP polyclonal antibody and goat anti-Rabbit IgG (H+L) HRP were used as the primary and secondary antibody, respectively, for pPLAIIIα-mRFP detection. Bands were visualized by exposing to the HR-A X-ray film after ECL substrate (Thermo Scientific, USA) treatment.

In vitro Enzymatic Assays

Phospholipids (each 16:0-18:1 PA, PC, PE, PG) were purchased from Avanti Polar Lipids. Acyl hydrolyzing activities were assayed in a reaction mixture (25 mM HEPES, pH 7.5, 10 mM CaCl₂, and 10 mM MgCl₂). Each 0.5 mM of lipids was used as substrate and 0.1 μg of purified protein was added to the mixture in a final volume of 100 μl. The reaction samples were incubated at 30°C for 60 min. The released NEFA products were measured with a NEFA-HR colorimetric kit (Wako Pure Chemicals, http://www.wako-chem.co.jp/english/) using an Epoch microplate spectrophotometer (BioTek) at 546 nm.

DAB Staining

For the in situ detection of H₂O₂, leaves were detached and stained with 3,3’-diaminobenzidine (DAB) solution for 4–5 h. DAB solution was generated by dissolving 1 mg/ml DAB in sterile H₂O and adjusting to pH 3.0 with 0.2 M HCl. Additionally, 25
μl of Tween 20 (0.05% v/v) and 2.5 ml of 200 mM Na$_2$HPO$_4$ were added. This process generated 10 mM Na$_2$HPO$_4$ DAB staining solution and increased the pH.

**Turnip Crinkle Virus Infection and HR Response Analysis**

Transcripts synthesized *in vitro* from a cloned cDNA of the TCV genome using T7 RNA polymerase were used for viral infections as described (Dempsey *et al*., 1993). Resistance and susceptibility were confirmed by RT-qPCR.

**Phytohormone Analysis**

Samples were extracted twice with ethyl acetate. The extracts were combined, evaporated, and dissolved in 70% methanol. Isotope-labeled standards ($^2$H$_4$-SA and $^2$H$_6$-JA) were used as internal standards. Ultra-performance liquid chromatography (Waters Corp., MA, USA) coupled with a QTOF instrument (Waters Corp.) was used for the analysis. The chromatographic separation was carried out on an C18 column. The mobile phases consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile). MS analysis was conducted in the negative ion mode with electrospray ionization.

**Accession Numbers**

Arabidopsis Genome Initiative locus identifiers (http://www.arabidopsis.org) for the genes described in this study are depicted in Table S1.

**Supplemental Data**

**Supplemental Figure S1.** SDS-polyacrylamide gel electrophoresis of recombinant pPLAIIIα protein.
**Supplemental Figure S2.** Phylogenetic tree of PLA family proteins in Arabidopsis.

**Supplemental Figure S3.** Transcript levels of other $pPLAIII$ genes are modulated by the activity of $pPLAIIIa$.

**Supplemental Figure S4.** Abundance of minor lipid molecular species in MGDG, DGDG, SQDG, PG, PC and PE lipid classes.

**Supplemental Figure S5.** Lipid class composition based on absolute amounts of lipid molecular species estimated by LC-MS/MS.

**Supplemental Figure S6.** $pPLAIIIa$ expression levels affect petiole length.

**Supplemental Figure S7.** Overexpression of $pPLAIIIa$ affects the direction of cell elongation in filament and style and trichome length.

**Supplemental Figure S8.** Phenotypic differences in $KO$ mutant and $OE$ lines against TCV infection.

**Supplemental Figure S9.** Lipase activity assay using total protein from Col-0, $KO$ and $OE$ plants.

**Supplemental Figure S10.** Relative gene expression patterns of $PLD\alpha 1$, $PLD\zeta 1$, and $PLD\zeta 2$ in $KO$ mutant and $OE$ lines.

**Supplemental Table S1** Primers used in confirmation of DNA insertion and PCR

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**Figure legends**

**Figure 1.** Distinctive conserved motifs of $pPLAIIIa$ and its lipase activity. (A)
Alignment of pPLAIIα with other pPLAIIIs and two pPLAIIs from Arabidopsis. Amino acid sequences were analyzed using the pairwise sequence alignment program. Multiple sequence alignment was performed using the BioEdit program (version 7.1.9). (B) Immunoblot analysis of recombinant pPLAIIα protein using anti-6X His tag antibody on E. coli purified recombinant proteins. (C) In vitro enzymatic assay of recombinant pPLAIIα protein using a non-esterified fatty acid assay kit. pPLAIIα fusion protein and 0.5 mM of each substrate (16:0-18:1 PA, PC, PE, PG) were incubated at 30°C for 60 min. Data represent the mean ± SE of four independent biological replicates. Data were analyzed by one-way ANOVA. Means with different letters represent significantly different (p < 0.05) according to Scheffé's test.

Figure 2. Spatial and temporal gene expression patterns of pPLAIIα in Arabidopsis.
Histochemical analysis of GUS expression harboring PropPLAIIα::GUS at different developmental stages. (A) GUS expression in a fully grown plant, (B) a floral organ, (C) a 1-day-old seedling, (D) 2-day-old seedlings, (E) 4-day-old seedlings, (F) the vasculature of the roots and the meristematic zone of the lateral root, (G) inner cell wall of a guard cell, (H) light- and dark-dependent expression in 6-day-old seedlings, (I) the vasculature of the apical and basal stem, and (J) the mature cauline leaf. All images were brightness adjusted. All scale bars = 100 µm or as specified (A).

Figure 3. Knockout, overexpression, and complementation lines for pPLAIIα in Arabidopsis. (A) The T-DNA insertion sites in two Arabidopsis pplallla knockout (KO) mutants and a diagram showing the overexpression construct of pPLAIIα (pPLAIIα-
OE) under the control of the 35S promoter with YFP or mRFP fusion at the C-terminus. (B) Immunoblotting of mRFP-tagged pPLAIIIα from Col-0, vector control, KO and OE plants. After SDS-PAGE separation, protein was immunoblotted with anti-mRFP antibody. (C) Subcellular localization of pPLAIIIα-YFP in the plasma membrane merged with FM4-64. Scale bar = 10 µm. (D) Plasmolysis of root epidermal cells of the pPLAIIIα-mRFP with 0.2M NaCl for 1 min. Scale bar = 10 µm. (E) Transcript levels of pPLAIIIα genes in the controls, KO, OE, and complementation (COM) lines as measured by RT-qPCR. n=3. (F) Phenotypes of 4-day-old seedlings. Scale bar = 1 cm. (G) The hypocotyl length and (H) root length of the controls, KO, OE, and COM lines. n=19–43. (E, G and H) Data represent the mean ± standard error (SE) of independent replicates. Stars denote statistical significance (t-test) at P < 0.05 (*) and P < 0.01 (**) compared with the controls. Col-0 and an empty vector line were used as control for mutant and OE lines, respectively.

**Figure 4. Total lipid content and abundance of lipid molecular species in OE and KO lines.** (A) Total lipid content, (B) total content of each lipid class, (C) major lipid molecular species content in OE and KO lines. Each molecular species of acyl-lipid was identified and quantified using UPLC-MS/MS in the KO mutant and OE lines compared with the controls. Values were normalized by fresh weight of rosette leaves analyzed. Data represent the mean ± SE of three (controls) to six (KO and OEs) independent biological replicates. Stars denote significant differences at P < 0.05 (*) and P < 0.01 (**) compared with the controls. Molecular species are indicated as the total number of carbon atoms in acyl chains: the total number of double bonds. Dashes (e.g. 18:3-1 and 18:3-2) indicate two species with the same mass and fragmentation pattern but with
different retention times. For MGMG, DGDG, SQDG, PG, PC, PE classes, only the 3 or 4 major species are shown. Minor species for these lipid classes are shown in Figure S4.

**Figure 5. Overexpression of pPLAIIIα alters the size and shape of many cells and organs.** (A, C-E) Statistical analysis of plant height (A), leaf surface area (C), leaf thickness (D), and leaf water content (E) in 4-week-old plants. The data represent the mean ± SE of three independent replicates at P < 0.05 (†) and P < 0.01 (**) compared with the controls. (B) The aerial part of each 4-week-old plant with all individual leaves. The leaves are arranged from cotyledons (left) to the youngest leaves (right). Scale bar = 1 cm. (F) Floral organs and siliques in the pPLAIIIα-OE line (No. 13) and Col-0. Scale bars = 0.2 mm (top), 2 mm (middle), and 5 mm (lower). (G) Flower in the Col-0 and OE lines. Scale bar = 500 µm. (H) Stems in the Col-0 and OE lines. Scale bars = 500 µm (upper) and 100 µm (lower). (I) The area (mm²) of cross-sectioned stems and (J) cell number of each sectioned stems. Data represent the mean ± standard deviation of ten independent replicates. Stars denote statistical significance (t-test) at P < 0.01 (**) compared with the controls. (K) Pollen structures in Col-0, KO, and OE lines. Scale bar =10 µm. All surface images were captured using low vacuum scanning electron microscopy (JSM-IT300, JEOL Korea) at a 10.8 mm working distance and 20.0 kV.

**Figure 6. pPLAIIIα expression associates with increased germination rate and seed size.** (A and B) Mature seeds (A) and size (B) of control, KO, OE, and COM lines. Scale bar = 1 mm. Average n= 50 for seed size. (C) The hundred seed weight of control, KO, and OE lines. Average n = 10. (D) Seed number per silique (n = 10). (E-F) Germination rates of control, KO, and OE lines after 20 h germination under light
conditions. n = 120. (F) The germination rate following treatment with 1 μM of GA$_3$.

(G) ABA content was analyzed using ultra-performance liquid chromatography (UPLC).

Data represent the mean ± standard error (SE) of three independent replicates at P < 0.05 (*) and P < 0.01 (**).

Figure 7. The mRNA levels of ethylene and GA biosynthesis genes in pPLAIIIa-OE lines. (A) Gene expression patterns of ACC oxidases (ACO) and of (B) gibberellin oxidases (GA2ox1, GA2ox2, GA3ox1, and GA20ox1) from control, KO, and OE seeds imbibed for 20 h. Data represent the mean ± standard error (SE) of three independent replicates at P < 0.05 (*) and P < 0.01 (**).

Figure 8. pPLAIIIα-OE lines remain green longer with reduced H$_2$O$_2$. (A) Eight-week-old plants of the control, KO, and pPLAIIIa-OE lines. Scale bar = 5 cm. (B) The sixth or seventh leaves were detached from 4-week-old plants and used for DAB staining. Scale bars = 1 cm. (C and D) Relative gene expression patterns of two senescence marker genes, SAG12 (C) and SAG13 (D). Data represent the mean ± SE of three independent replicates at P < 0.05 (*) and P < 0.01 (**).

Figure 9. Increased SA in pPLAIIIα-OE enhances turnip crinkle virus (TCV) resistance by changing transcript levels of PRI and PDF1.2. (A) Inflorescences of each line 12 days post-inoculation (DPI) of mock and TCV. (B) Magnified images of TCV infected plants from each line. Scale bars (A and B) = 2 cm. (C–E) The relative gene expression of TCV, PRI, and PDF1.2 in the inoculated leaves of TCV- and mock-infected plants. The inoculated leaves were monitored for the presence or absence of the
HR, and RNA was extracted from inoculated leaves on 0 (1 h), 1, 2, and 3 DPI. Expression of \textit{PRI} and \textit{PDF1.2} was analyzed from the leaves at 1 DPI only. (F and G) Hormone contents of SA and JA as measured by UPLC in rosette leaves of 5-week-old plants in control, \textit{KO}, and \textit{OE} lines. (C–G) Four-week-old seedling were used for the analysis. Data represent the mean ± SE of three independent replicates at P < 0.05 (*) and P < 0.01 (**).
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