

This is the author's final, peer-reviewed manuscript as accepted for publication. The publisher-formatted version may be available through the publisher's web site or your institution's library.

The patatin-containing phospholipase A pPLAII α modulates oxylipin formation and water loss in *Arabidopsis thaliana*

Wenyu Yang, Yong Zheng, Sung Chul Bahn, Xiangqing Pan, Maoyin Li, Hieu Sy Vu, Mary R. Roth, Brad Scheu, Ruth Welti, and Xuemin Wang

How to cite this manuscript

If you make reference to this version of the manuscript, use the following information:

Yang, W., Zheng, Y., Bahn, S. C., Pan, X., Li, M., Vu, H. S., . . . Wang, X. (2012). The patatin-containing phospholipase A pPLAII α modulates oxylipin formation and water loss in *Arabidopsis thaliana*. Retrieved from <http://krex.ksu.edu>

Published Version Information

Citation: Yang, W., Zheng, Y., Bahn, S. C., Pan, X., Li, M., Vu, H. S., . . . Wang, X. (2012). The patatin-containing phospholipase A pPLAII α modulates oxylipin formation and water loss in *Arabidopsis thaliana*. *Molecular Plant*, 5(2), 452-460.

Copyright: © The Author 2012. Published by the Molecular Plant Shanghai Editorial Office in association with Oxford University Press on behalf of CSPB and IPPE, SIBS, CAS.

Digital Object Identifier (DOI): doi:10.1093/mp/ssr118

Publisher's Link: <http://mplant.oxfordjournals.org/content/5/2/452.full>

This item was retrieved from the K-State Research Exchange (K-REx), the institutional repository of Kansas State University. K-REx is available at <http://krex.ksu.edu>

The patatin-containing phospholipase A pPLAII α modulates oxylipin formation and water loss in *Arabidopsis thaliana*

Wenyu Yang¹, Yong Zheng¹, Sung Chul Bahn¹, Xiangqing Pan¹, Maoyin Li¹, Hieu Sy Vu², Mary R. Roth², Brad Scheu², Ruth Welti², and Xuemin Wang^{1,3}

¹*Donald Danforth Plant Science Center, St. Louis, Missouri 63132, Department of Biology, University of Missouri, St. Louis, Missouri 63121, USA*

²*Kansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, Kansas 66506*

³*To whom correspondence should be addressed. E-mail: swang@danforthcenter.org; fax 314-587-1519.*

ABSTRACT

The patatin-related phospholipase A (pPLA) hydrolyzes membrane glycerolipids to produce monoacyl compounds and free fatty acids. Phospholipids are cleaved by pPLAII α at the *sn*-1 and *sn*-2 positions, and galactolipids, including those containing oxophytodienoic acids, can also serve as substrates. Ablation of pPLAII α decreased lysophosphatidylcholine and lysophosphatidylethanolamine levels, but increased free linolenic acid. pPLAII α -deficient plants displayed a higher level of jasmonic acid and methyl jasmonate, as well as the oxylipin-biosynthetic intermediates 13-hydroperoxylinolenic acid and 12-oxophytodienoic acid than wild-type plants. The expression of genes involved in oxylipin production was also higher in the pPLAII α -deficient mutant than in wild-type plants. The mutant plants lost water faster than wild type plants did. The stomata of wild type and mutant plants responded similarly to abscisic acid. In response to desiccation, the mutant and wild type leaves produced abscisic acid at the same rate, but after 4 h of desiccation, the jasmonic acid level was much higher in mutant than wild-type leaves. These results indicate that pPLAII α negatively regulates oxylipin production and suggest a role in the removal of oxidatively modified fatty acids from membranes.

INTRODUCTION

The release of fatty acids from cellular membrane glycerolipids has been implicated in various cellular processes including the production of signaling messengers, membrane remodeling, membrane deterioration during senescence, and stress damage (Wang, 2004; Scherer et al., 2010). The patatin-related phospholipase A (pPLA) is a major family of enzymes that hydrolyze membrane glycerolipids to produce lysolipids and free fatty acids. Patatin refers to a group of storage glycoproteins that were originally discovered in potato tubers (Galliard, 1971); patatin-related proteins were later found in other plant species and tissues, as well as in animal cells (Holk *et al.*, 2002). The well-characterized

mammalian cytosolic PLA₂s contain a patatin domain that serves as catalytic site (Schrag and Cygler, 1997; Hirschberg *et al.*, 2001). The Arabidopsis pPLA family consists of 10 genes that are grouped into three subfamilies, pPLAI, pPLAII (α , β , γ , δ , ϵ), and pPLAIII (α , β , γ , δ), based on gene structures and deduced protein sequences (Scherer *et al.*, 2010). pPLAI contains 1257 amino acid residues whereas the size of pPLAII α s and III α s ranges from 382 to 526 amino acid residues. pPLAs are involved in various processes, including fungal and bacterial pathogen infection, phosphate deprivation, auxin response, cellulose deposition, cell elongation, and grain yield (La Camera *et al.*, 2005; Yang *et al.*, 2007; Rietz *et al.*, 2010; Li *et al.*, 2011). However, the metabolic and cellular mechanism by which pPLAs affect plant growth and stress response remain elusive.

One of potential mechanisms of action for pPLAs starts with the hydrolysis of membrane lipids, releasing polyunsaturated fatty acids (PUFAs), such as linolenic acid that serves as a substrate for the synthesis of jasmonic acid (JA) in response to stress. JA and other cyclopentenone derivatives are synthesized via the octadecanoic pathway from linolenic acid in plants. These oxylipins modulate plant response to various stresses, including pathogen infection, insect attack, and drought (Staswick, 1992; Wasternack and Parthier, 1997; Reymond and Farmer, 1998; Farmer, 2001). One PLA₁ was identified as being involved in JA production, pollen maturation, and anther dehiscence in Arabidopsis (Ishiguro *et al.*, 2001), but the identity of the enzyme(s) involved in stress-induced production of PUFAs for oxylipin production it is still unclear. pPLAI has been implicated in the production of basal levels of JA, but not pathogen or wounding-induced JA production (Yang *et al.*, 2007). pPLAI plays a positive role in Arabidopsis resistance to *Botrytis cinerea* (Yang *et al.*, 2007), whereas the suppression of pPLAII α was reported to render Arabidopsis plants more resistant to fungal and bacterial infection (La Camera *et al.*, 2005). This negative effect of pPLAII α on plant-pathogen interactions appears to argue against a role of pPLAII α in promoting JA production. The expression of pPLAII α is induced in response to various biotic and abiotic stresses, including pathogens, low temperature, high salinity, abscisic acid, salicylic acid, methyl jasmonate, ethylene, iron, and phosphate deficiency (Narusaka *et al.*, 2003; Rietz *et al.*, 2004). These observations raise intriguing questions regarding the role of pPLAII α in JA production and plant stress responses.

Recent studies have shown that complex membrane lipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phospholipids, are oxidatively modified (Andersson *et al.*, 2006; Buseman *et al.*, 2006; Seltmann *et al.*, 2010). Oxidized (ox-) MGDG and DGDG containing 12-oxophytodienoic acid (OPDA) and dinor-oxophytodienoic acid (dnOPDA), including Arabidopside A (OPDA/dnOPDA MGDG), B (OPDA/OPDA MGDG), C (OPDA/dnOPDA DGDG), and D (OPDA/OPDA DGDG), are produced in response to wounding, bacterial and fungal infection, dark, aging, and osmotic stress (Andersson *et al.*, 2006; Buseman *et al.*, 2006; Glauser *et al.*, 2008; Kourtchenko *et al.*, 2007; Maeda *et al.*, 2008; Stelmach *et al.*, 2001; Xiao *et al.*, 2010). pPLAI has been shown to use arabidopsides as substrates, and to prefer them as substrates to unoxidized, normal MGDG and DGDG (Yang *et al.*, 2007). In this study, we analyzed pPLAII α 's lipid-hydrolyzing activity and effect on JA formation. The results suggest that this enzyme

negatively regulates oxylipin production, possibly via participating in membrane repair that includes removal of oxidatively modified lipids.

RESULTS

pPLAII α Hydrolyzes Glycerolipids at the *sn*-1 and *sn*-2 Positions.

The full-length cDNA of *pPLAII α* gene encodes a protein of 407 amino acids with a predicted pI of 5.57 and molecular weight of 44239. *pPLAII α* contains the conserved serine hydrolase motif GX SXG at residues 64-68, and a conserved Asp residue at 215 within the patatin domain (residues 21-228). The serine and aspartic acid residues constitute a catalytic dyad for its acyl hydrolase activity (Hirschberg *et al.*, 2001; Rydel *et al.*, 2003). The *pPLAII α* cDNA was expressed in *E. coli* and a protein of 44 kDa corresponding to the predicted size of *pPLAII α* was purified to apparent homogeneity for determination the enzymatic properties of *pPLAII α* (Fig. 1A).

Purified *pPLAII α* was assayed for its ability to hydrolyze various phospholipids, using fluorescently [12-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-dodecanoyl (NBD)]-labeled phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylserine (PS), as substrates. The *pPLAII α* reaction produced both NBD-labeled free fatty acids and NBD-lysophospholipids (Fig. 1B). Because the fluorescent label was at the *sn*-2 position, the detection of both the NBD-lysophospholipids and NBD-free fatty acid indicates that the enzyme releases fatty acids at both the *sn*-1 and *sn*-2 positions. The relative amount of NBD-fatty acids vs NBD-lysophospholipids formed indicates the relative hydrolysis at the *sn*-2 vs *sn*-1 position by the enzyme. *pPLAII α* preferred releasing the fatty acid at the *sn*-1 to *sn*-2 position when PC or PE was the substrate whereas it favors the *sn*-2 position when PG, PA, or PS was the substrate (Fig. 1B and C). The enzyme was less active towards PS and PA in comparison to the other phospholipids used in the assay (Fig. 1B and C). Radioactive 1-stearoyl-2-(1-[¹⁴C]arachidonyl)-phosphatidylinositol (PI) was used to determine the hydrolytic activity toward PI. Consistent with results from the fluorescent lipid assays, both radioactive free fatty acid and lysoPI were generated (Fig. 1C). The amount of [¹⁴C]-fatty acid formed was about five-fold higher than that of [¹⁴C]-lysoPI, suggesting that the enzyme prefers hydrolysis of PI at the *sn*-2 to *sn*-1 position.

pPLAII α Act on Oxidized and Unoxidized Glycerolipids.

To determine the substrate selectivity, total lipids extracted from leaves of a wounded plant were used as substrates for the purified *pPLAII α* . As a control, the same amount of leaf lipids was incubated with empty-vector bacterial proteins that were subjected to the same purification process as *pPLAII α* . After reaction, phospholipid and galactolipid species in the *pPLAII α* and control reactions were measured. Addition of *pPLAII α* decreased PC, PE, PI but little PS and PA species (Fig. 2A and B). Lyso-phospholipids levels rose (LPC and LPE determined) (Fig. 2C). *pPLAII α* also acted on nearly every species of MGDG and DGDG (Fig. 3A), including those containing OPDA (Fig. 3B), resulting in the production of corresponding lysogalactolipids monogalactosyl monoacylglyceride and digalactosyl monoacylglyceride (Fig. 3C). Taken together, the

data show that the enzyme is capable of acting on nearly every membrane lipid substrate determined in wounded *Arabidopsis* leaves.

JA Production Is Upregulated in *pPLAII α* -Deficient Leaves.

A T-DNA insertion knockout (KO) mutant was isolated and the mutant lost the expression of *pPLAII α* , as indicated by real-time quantitative PCR (Fig. 4A). *pPLAII α* KO leaves contained approximately 30% and 15% less LPC and LPE, respectively, but the level of LPG was the same in the KO and wild-type (WT) leaves (Fig. 4B). The decrease occurred with LPC species with 16:0, 18:2, or 18:0, but not with 18:3 whereas only 16:0-LPE was significantly decreased in the KO leaves (Fig. 4C).

In contrast to the change in lysophospholipids, the level of free linolenic acid in *pPLAII α* -KO leaves was significantly higher than that of WT (5A). The KO leaves produced approximately twice the amount of methyl jasmonate (MeJA) and JA of WT plants (Fig. 5A). Measurements of the potential intermediates in the oxylipin biosynthetic pathway showed that the levels of 13-hydroperoxylinolenic acid (13-HPOT) and OPDA in *pPLAII α* -KO leaves were significantly higher than those of WT plants (Fig. 5A).

The expression of the genes for the enzymes in the oxylipin synthesis and responsive processes was monitored by quantitative real-time PCR (Fig. 5B). *pPLAII α* -KO plants displayed higher levels of lipoxygenase 1, 3 (*LOX1*, *LOX3*), allene oxide synthase (*AOS*), allene oxide cyclase 1 and 2 (*AOC1* and *AOC2*), and carboxyl methyltransferase (*MJT*). The fold increases were 2.4, 65, 1.6, 2, 5, and 2 for *LOX1*, *LOX3*, *AOS*, *AOC1*, *AOC2*, *MJT*, respectively. The levels of expression for *LOX2*, hydrogen peroxide lyase (*HPL*), OPDA reductase 3 (*OPR3*), vegetative storage protein 2 (*VSP2*) were comparable between KO and WT plants (Fig. 5B). These results indicate that an oxylipin pathway in *pPLAII α* -KO plants is upregulated at the level of both gene expression and the accumulation of the metabolic products.

Knockout of *pPLAII α* Increases Water Loss and Drought Sensitivity

During growth and handling of transgenic plants, it was noted that the *pPLAII α* -KO plants became dehydrated faster than WT plants. When fresh weights of detached leaves were measured, *pPLAII α* -KO leaves lost 25% more water than WT leaves plants 2 h after detachment (Fig. 6A). When plants were left unwatered, the mutant plants became desiccated and died faster than WT plants (Fig. 6B). When the mutant was complemented with the genomic gene of *pPLAII α* , the rate of water loss and the sensitivity to drought were restored to those of WT (Fig. 6), indicating that the accelerated desiccation is caused by the loss of the *pPLAII α* function.

Water loss in terrestrial plants results mostly from transpiration via stomata, and the plant hormone abscisic acid (ABA) promotes stomatal closure and reduces water loss. Thus, the stomatal response to ABA was compared between *pPLAII α* -KO and WT leaves. ABA in different concentrations caused the similar closure in stomata in WT and *pPLAII α* -KO leaves (Fig. 7). This result suggests that the loss of *pPLAII α* does not alter stomatal response to ABA. To determine how the levels of ABA and JA change during dehydration in *pPLAII α* -KO and WT plants, the hormone levels were measured at

different time intervals in detached leaves. ABA level increased continuously in both wild type and mutant plants during the 4-h period tested, but no significant difference was found between the two genotypes (Fig. 8A). The JA levels increased in the first hr after detachment in both WT and KO leaves. The JA levels displayed no further increase at the 2nd and 3rd h in both genotypes. At 4 h of treatment, JA level in the mutant became almost 3-fold higher than that in WT (Fig. 8B).

DISCUSSION

The present study shows that pPLAII α hydrolyzes various membrane glycerolipids. Comparison of pPLAII α activity with pPLAI and III reveals differences in substrate usage. pPLAI hydrolyzes MGDG four times greater than PG (Yang *et al.*, 2007), whereas pPLAIII β uses PG four times greater than MGDG (Li *et al.*, 2011). pPLAII α has similar activities toward MGDG and PG (Rietz *et al.*, 2010). The specific activity of pPLAII α is much higher than that of pPLAIII β (Li *et al.*, 2011). These differences suggest that activation of individual pPLAs in the cell may selectively hydrolyze different classes of membrane lipids.

The *pPLAII α* -deficient plants displayed increased basal levels of JA and methyl JA. The result indicates that pPLAII α is not involved in providing free fatty acids for JA biosynthesis. On the contrary, pPLAII α acts more like as a repressor of the JA production. The enhanced JA production in the *pPLAII α* -deficient plants is further supported by the increase in several major intermediates, linolenic acids, 13-HPOT, OPDA, and increased expression of genes involved in the JA biosynthesis. The effect of *pPLAII α* -KO on the increased basal JA production is opposite to that of pPLAI-KO that shows a decreased basal level of JA (Yang *et al.*, 2007). These results indicate that individual pPLAs have distinctly different functions. In addition, the increase in LOX1, a 9-LOX that is not directly involved in the JA production, suggests that the production of other oxylipins besides JA is increased in *pPLAII α* -knockout plants. pPLAII α might be involved in the removal of oxidatively modified fatty acids from membranes in membrane remodeling and repair, and the loss of pPLAII α might impair the membrane repair, resulting in increases in oxylipin production.

The increased water loss in *pPLAII α* -deficient plants suggest that pPLAII α plays a role in modulating leaf water status. ABA is a key water stress hormone that triggers stomatal closure and reduces transpirational water loss (Kim *et al.*, 2010). However, ablation of *pPLAII α* does not impact stomatal reponse to ABA or ABA production in response to water deficits. This result indicates that pPLAII α modulates plant response to water deficits in an ABA-independent manner. JA may be involved in the altered response as it was increased significantly more in KO than WT leaves four hrs after desiccation. On the other hand, JA has been reported to induce stomatal closure and its effect depends on the cytosolic Ca²⁺ concentration involving a calcium-dependent protein kinase in guard cells (Munemasa *et al.*, 2011). How JA participates in pPLAII α modulation of plant response to water deficits without affecting ABA is not known. Further study is needed to determine whether and how the increased oxylipin level may play a role in increasing water loss in the *pPLAII α* -deficient plants.

METHODS

cDNA Cloning and Expression of Recombinant pPLAII α Protein in *E. coli*

The full length cDNA of *pPLAII α* was obtained with PCR using a cDNA clone from the *Arabidopsis* Biological Resource Center at Columbus, Ohio, using primers of forward 5' TCCGGATCCATGCAAATGGACAGCCCCA 3' and reverse 5' ATCGTTCGACGA TCCTAATTGGAGCTTTTGCATG 3'. The coding sequence of the cDNA (1221 bp) was cloned into the *Bam*HI and *Sal*I sites of pET28a(+) (Novagen) and both strands were sequenced. The construct was introduced into *E. coli* strain BL-21(DE3). The cells were grown to an OD₆₀₀ of 0.7 and induced with 0.4 mM IPTG for 12 h at 22°C. The purification of the protein was performed as described previously (Pappan *et al.*, 2004) and the protein concentration was determined with the Bradford method (Bradford, 1976). The purity of the protein was analyzed by 8% SDS-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining.

Isolation of *pPLAII α* -KO Mutant and Genetic Complementation

Arabidopsis thaliana plants (ecotype Columbia) were grown in growth chambers under a day/night regimen of 23/18°C and 12/12 h photoperiod under white light of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A T-DNA insertion mutant at the *pPLAII α* locus *At2g26560* was isolated by a screening T-DNA insertion line Salk_059119. The T-DNA was inserted at the last intron and the site of T-DNA insertion was confirmed by sequencing. The loss of *pPLAII α* expression was verified by quantitative real-time PCR. The mutant gene co-segregated with kanamycin resistance in a 3:1 ratio and the homozygous knockout plants were isolated and used in the study. A 3.34-kb genomic fragment of DNA containing the coding region of *pPLAII α* and 1.10 kb 5' and 0.45 kb 3' untranslated regions was amplified by PCR. The PCR product was cloned into the *Asc*I site of pEC291 binary vector. The resulting vector was introduced into the C58C1 strain of *Agrobacterium tumefaciens*, and the *pPLAII α* mutant plants were transformed as described (Clough and Bent, 1998).

Quantitative Real-Time PCR

RNA was isolated from liquid nitrogen-frozen plant leaves using a cetyltrimethylammonium bromide extraction method as described previously (Fan *et al.*, 1997). Ten micrograms of RNA were reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad) containing a blend of oligo (dT) and random hexamer primers. Quantitative RT-PCR was performed on 50 ng cDNA with the iQTM SYBR Green Supermix (Bio-Rad). Amplification of *Arabidopsis* polyubiquitin gene (*UBQ10*) transcripts derived from gene *At4g05320* was used as reference. The gene specific primer pairs used for the PCR are (from 5' to 3'): *pPLAII α* (*At2g26560*): forward CTTGCCAAGACAGGAGATGAACTAC, reverse GCATGAGGTGAACGAATGTCTCGGAT; lipoxygenase1 (*LOX1*, *At1g55020*): forward ACCATCACA GCTCAGCTTCAGACA, reverse TCCAAACTTCTCGAACGCCTCCAA; lipoxygenase2 (*LOX2*, *At3g45140*): forward AGAGCTGGA GCTGGTGTGTTAAG, reverse GAACACCCA TTCCGGTAACACCAT; lipoxygenase 3 (*LOX3*, *At1g17420*):

forward TATGGATTTGCGGCA GAGATCGGA, reverse AGGCTCAGAACT CGGAACCAACAA; allene oxide synthase (*AOS*, *At5g42650*): forward TGGTGGCGAGGTTGTTTGTGATTG, reverse ATTAACGGAGCTTCCTAACGGC GA; allene oxide cyclase 1 (*AOC1*, *At3g25760*): forward CTTAAGCCCAGTGGA GTTGTAAGC, reverse TATACAGGACACGAG AAAGATAAGACT; allene oxide cyclase 2 (*AOC2*, *At3g25780*): forward GCACTGGAG CCTAGCGGAGTTATAAGT, reverse TATATCATTACACAGCGATACGAGAAAC; OPDA reductase (*OPR*, *At2g06050*): forward CGGATTTGGTTTCGCGGTTCAAGA, reverse TCCGTGTAGCCAACAACCTGGATCT; jasmonic acid carboxyl methyltransferase (*MJT*, *At1g19640*): forward TATGTAAGCTCGCCACGATACGCT, reverse AACACGATCAACCGGCTCTAACGA; vegetative storage protein 2 (*VSP2*, *At5g24780*): forward GCAAGTGGTGTACAAGTCAAAGGT, reverse ATCCTCAACCAAATCAGCCCATTG; polyubiquitin (*UBQ10*, *At4g05320*): forward CACTCCACTTGGTCTTGCGT, reverse TGGTCTTTCGGTGAGAGTCTTCA. The PCR amplification was performed with a MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions.

Assaying pPLAII α Activity Using Individual Phospholipids as Substrates

NBD-PC, NBD-PE, NBD-PG, and NBD-PA were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Individual classes of NBD-lipids in chloroform were dried under a nitrogen stream and emulsified in distilled water containing 0.05% Triton X-100 by sonication. Enzymatic activities were assayed in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, and 0.05% Triton X-100. Sixty μ M of NBD-lipids was used as substrate and 3 μ g of purified protein was added to the mixture in a final volume of 200 μ l. The reaction was incubated at 30°C for 60 min and was stopped by adding 700 μ l chloroform/methanol (2:1, v/v) and 200 μ l of 2 M KCl. After vortexing and centrifugation, the aqueous phase was removed and remaining chloroform was dried under a stream of nitrogen. Lipid was dissolved in 10 μ l chloroform and spotted on a thin layer chromatography (TLC) plate (silica gel 60, Merck, Darmstadt, Germany). The TLC plate was developed in chloroform/methanol/ammonium hydroxide/water (65:39:4:4, v/v/v/v). Lipids on the plate were visualized under ultraviolet light. The spots corresponding to free fatty acids and lysophospholipids were scraped into vials. The lipid in the vials was extracted with methanol and its fluorescence was measured at 460 nm (excitation) and 534 nm (emission). For activity assays using radioactive PI as a substrate, 0.6 μ Ci 1-stearoyl-2-[1-¹⁴C arachidonyl]-PI (New England Nuclear) and 3 μ mol of unlabeled soy PI (Sigma, St Louis, MO) were mixed in chloroform. The solvent was evaporated under a nitrogen stream, and the lipid was emulsified in 0.5 ml H₂O containing 0.05% Triton X-100 by sonication. The enzyme assay was performed as described above, using 20 μ l of the radioactive substrate. After separation on a TLC plate, the lipids on the plate were visualized by exposure to iodine vapor. The spots corresponding to the lipid standards were scraped into vials, and their radioactivity was determined by liquid scintillation counting.

Assaying pPLAII α Enzymatic Activity Using Mixed Plant Lipids as Substrates

To use plant lipids as substrates, fully expanded *Arabidopsis* leaves were wounded mechanically with a forceps and total lipids were extracted 45 min after wounding as

described (Zien *et al.*, 2001). Solvents of the lipid extracts were evaporated under a stream of nitrogen, emulsified in distilled water containing 0.05% Triton X-100 by sonication, and used as substrates. Thirty μg of the lipids (1 $\mu\text{g}/\mu\text{l}$) and 3 μg purified pPLAII α were added to each reaction in a final volume of 200 μl . Control reactions contained the same amount of lipids and empty-vector bacterial proteins and were subjected to the same purification process as those reactions containing pPLAII α . The other components in the assay and the reaction condition were the same as described above. After the reaction at 30°C for 60 min, the resulting lipids were extracted and the organic phase was analyzed by the triple quadrupole mass spectrometry.

Lipid Analysis by Mass Spectrometry

Lipid extraction, lipid analysis, and lipid quantification of all lipids except MGMT and DGMT were performed as described by Xiao *et al.*, (2010), except that negative-ion precursor scan mass spectral data for OPDA-containing lipids, were normalized to amounts of 34:6 MGDG in the samples. This was done by multiplying the negative ion [Pre 291.2, i.e. OPDA] signals times the amount (nmol) of 34:6 MGDG determined by Neutral Loss scanning for the MGDG head group [NL 179.1] in positive ion mode and dividing by the signal for 34:6 MGDG in negative ion mode [Pre 277.2, i.e. 18:3]. MGMT and DGMT were quantified similarly to MGDG and DGDG as $[\text{M} + \text{NH}_4]^+$ in positive ion mode with NL179.1 and NL 341.1, except that the internal standards were 18:0 MGMT and 18:0 DGMT. Five replicates of each treatment for each phenotype were processed and analyzed. Paired values were subjected to the Student *t*-test to determine the statistical significance.

Measurements of Jasmonates and ABA

The JA and ABA analysis was performed as described previously (Pan *et al.*, 2008). Briefly, approximately 50 to 100 mg of fresh tissue was sealed in 1.5 mL snap-cap vials. After being frozen in liquid nitrogen, the leaves were ground to powder, and 500 μL of 1-propanol/ H_2O /concentrated HCl (2:1:0.002, v/v/v) with internal standards were added, followed by agitation for 30 min at 4°C. Dichloromethane (1 mL) was added, followed by agitation for another 30 min and then centrifugation at 13,000 $\times g$ for 1 min. The bottom layer was used for jasmonate and ABA analysis. Dihydrojasmonic acid (H_2JA) and heptadecanoic acid (C17:0) were used as internal standards for JA and linolenic acid quantification, respectively. Plant extracts were firstly separated by HPLC, equipped with a reversed-phase column (C18 Gemini 5 μ , 150 \times 2.00 mm, Phenomenex, CA, USA), using a binary solvent system composed of water with 0.1% formic acid and methanol with 0.1% formic acid as a mobile phase at a flow rate of 0.3 mL/min and a gradient of linearly increasing methanol content from 30% to 100% at 30 min. A hybrid triple quadrupole/linear ion trap mass spectrometer (ABI 4000 Q-Trap, Applied Biosystems, Foster City, CA) outfitted with an electrospray ion source, was used in multiple reaction monitoring (MRM) mode. JA, H_2JA , linolenic acid, 13-hydroperoxyoctadeca-9,11,15-trienoic acid (13-HPOT) and oxophytodienoic acid (OPDA) were measured according to the procedure described (Pan *et al.*, 2010).

Measurements of Water Loss and Stomatal Aperture

Arabidopsis plants were grown in growth chambers under a day/night regimen of 23/18°C and 12/12 h photoperiod under white light of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Leaves from 4-week plants were detached, and placed on bench at room temperature and under white light of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The weight of the leaf was recorded at different time points after detachment, and the percentage over initial weight was calculated for the water loss rate of the leaf. Stomatal responses to ABA and JA were analyzed in 4 week-old plants based on the procedure described by Kuhn *et al.* (2006). Briefly, epidermal layers of leaves from 4 week-old plants grown in growth chamber were peeled and floated in stomata opening buffer. After incubation at room temperature under light of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 2.5 h, ABA or JA was added to the solution at various concentrations and the peels were incubated under light for another 2.5 h before the stomata were photographed under a microscope and the stomatal aperture was measured. For measurement of JA and ABA in response to desiccation, leaves from 4 week-old plants were detached and placed on a bench at room temperature under white light of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. At different time points after desiccation, the leaves were placed in liquid nitrogen for hormone preparation and measurement (Pan *et al.*, 2008).

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Foundation (MCB-0922879; IOS-0818740) and the US Department of Energy (DE-SC0001295). The Kansas Lipidomics Research Center's research was supported by grants from the National Science Foundation (MCB-0920663, DBI-0521587, and Kansas Experimental Program to Stimulate Competitive Research Award EPS-0236913), with support from the State of Kansas through the Kansas Technology Enterprise Corporation and Kansas State University, as well from U.S. Public Health Service Grant P20 RR-016475 from the IDeA Network of Biomedical Research Excellence program of the National Center for Research Resources.

REFERENCES

- Andersson, M.X., Kourtchenko, O., Dangl, J.L., Mackey, D., and Ellerström, M.** (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. *Plant J.* **47**, 947-959.
- Bradford, M.M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Buseman, C. M., Tamura, P., Sparks, A. A., Baughman, E. J., Maatta, S., Zhao, J., Roth, M. R., Esch, S. W., Shah, J., Williams, T. D., and Welti, R.** (2006) Wounding stimulates the accumulation of glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoic acid in *Arabidopsis* leaves. *Plant Physiol.* **142**, 28–39.
- Clough, S.J., and Bent, A.** (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

- Devaiah, S. P., Roth, M. R., Baughman, E., Li, M., Tamura, P., Jeannotte, R., Welti, R., and Wang, X.** (2006) Quantitative profiling of polar glycerolipid species from organs of wild-type *Arabidopsis* and a phospholipase D α 1 knockout mutant. *Phytochemistry* **67**, 1907–1924.
- Fan, L., Zheng, S. and Wang, X.** (1997) Antisense suppression of phospholipase D α 1 retards abscisic acid- and ethylene-promoted senescence of postharvest *Arabidopsis* leaves. *Plant Cell* **9**, 2183–2196.
- Farmer, E.E.** (2001). Surface-to-air signals. *Nature* **411**, 854–856.
- Galliard, T.** (1971) Enzymic deacylation of lipids in plants. The effects of free fatty acids on the hydrolysis of phospholipids by the lipolytic acyl hydrolase of potato tubers. *Eur. J. Biochem.* **21**, 90–98.
- Glauser, G., Grata, E., Dubugnon, L., Rudaz, S., Farmer, E.E., and Wolfender, J.L.** (2008) Spatial and temporal dynamics of jasmonate synthesis and accumulation in *Arabidopsis* in response to wounding. *J. Biol. Chem.* **283**, 16400–16407.
- Hirschberg, H. J. H. B., Simons, J.-W. F. A., Dekkar, N. and Egmond, M. R.** (2001) Cloning, expression, purification and characterization of patatin, a novel phospholipase A. *Eur. J. Biochem.* **268**, 5037–5044.
- Holk, A., Rietz, S., Zahn, M., Quader, H. and Scherer, G.F.** (2002) Molecular identification of cytosolic, patatin-related phospholipases A from *Arabidopsis* with potential functions in plant signal transduction. *Plant Physiol.* **130**, 90–101.
- Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I. and Okada K.** (2001) The DEFECTIVE IN ANTHOR DEHISCENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. *Plant Cell* **13**, 2191–2209.
- Kim, T.H., Böhmer, M., Hu, H., Nishimura, N., and Schroeder, J.I.** (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO₂, and Ca²⁺ signaling. *Annu. Rev. Plant Biol.* **61**, 561–591.
- Kourtchenko, O., Andersson, M.X., Hamberg, M., Brunnström, A., Göbel, C., McPhail, K.L., Gerwick, W.H., Feussner, I., and Ellerström, M.** (2007) Oxo-phytodienoic acid-containing galactolipids in *Arabidopsis*: jasmonate signaling dependence. *Plant Physiol.* **145**, 1658–1669.
- Kuhn, J.M., Boisson-Dernier, A., Dizon, M.B., Maktabi, M.H., Schroeder, J.I.** (2006) The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in *Arabidopsis*, and effects of *abh1* on AtPP2CA mRNA. *Plant Physiol.* **140**, 127–139.
- La Camera, S., Geoffroy, P., Samaha, H., Ndiaye, A., Rahim, G., Legrand, M. and Heitz, T.** (2005) A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in *Arabidopsis*. *Plant J.* **44**, 810–825.
- Li, M., Bahn, S.C., Guo, L., Musgrave, W., Berg, H., Welti, R., and Wang, X.** (2011) Patatin-related phospholipase pPLAIII β -induced changes in lipid metabolism alter cellulose content and cell elongation in *Arabidopsis*. *Plant Cell* **23**, 1107–1123.
- Pan, X., Welti, R., and Wang, X.** (2008). Simultaneous Quantification of Plant Hormones by High-performance Liquid-chromatography Electrospray Tandem Mass Spectrometry. *Phytochemistry* **69**: 1773–1781.

- Pan, X., Welti, R., and Wang, X.** (2010) Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. *Nature Protocols* 5: 986-992.
- Pappan, K., Zheng, L., Krishnamoorthi, R. and Wang, X.** (2004) Evidence for and characterization of Ca²⁺ binding to the catalytic region of *Arabidopsis thaliana* phospholipase D β . *J. Biol. Chem.* **279**, 47833–47839.
- Maeda, H., Sage, T.L., Isaac, G., Welti, R., and Dellapenna, D.** (2008) Tocopherols modulate extraplastidic polyunsaturated fatty acid metabolism in *Arabidopsis* at low temperature. *Plant Cell* **20**, 452-470.
- Munemasa, S., Hossain, M.A., Nakamura, Y., Mori, I.C., and Murata, Y.** (2011) The *Arabidopsis* calcium-dependent protein kinase, CPK6, functions as a positive regulator of methyl jasmonate signaling in guard cells. *Plant Physiol.* **155**, 553-561.
- Narusaka, Y., Narusaka, M., Seki, M., Fujita, M., Ishida, J., Nakashima, M., Enju, A., Sakurai, T., Satou, M., Kamiya, A., Park, P., Kobayashi, M., and Shinozaki, K.** (2003) Expression profiles of *Arabidopsis* phospholipase A IIA gene in response to biotic and abiotic stresses. *Plant Cell Physiol.* **44**, 1246-1252.
- Reymond, P., and Farmer, E.E.** (1998). Jasmonate and salicylate as global signals for defense gene expression. *Curr. Opin. Plant Biol.* **1**, 404–411.
- Rietz, S., Holk, A. and Scherer, G.F.** (2004) Expression of the patatin-related phospholipase A gene AtPLA IIA in *Arabidopsis thaliana* is up-regulated by salicylic acid, wounding, ethylene, and iron and phosphate deficiency. *Planta* **219**, 743–753.
- Rietz, S., Dermendjiev, G., Oppermann, E., Tafesse, F.G., Effendi, Y., Holk, A., Parker, J.E., Teige, M., and Scherer, G.F.** (2010) Roles of *Arabidopsis* patatin-related phospholipases a in root development are related to auxin responses and phosphate deficiency. *Mol. Plant.* **3**, 524-538.
- Rydel, T.J., Williams, J.M., Krieger, E., Moshiri, F., Stallings, W.C., Brown, S.M., Pershing, J.C., Purcell, J.P. and Alibhai, M.F.** (2003) The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. *Biochemistry* **42**, 6696–6708.
- Scherer, G.F., Ryu, S.B., Wang, X., Matos, A.R., and Heitz, T.** (2010). Patatin-related phospholipase A: Nomenclature, subfamilies and functions in plants. *Trends Plant Sci.* **15**, 693–700.
- Schrag, J.D., and Cygler, M.** (1997) Lipases and alpha/beta hydrolase fold. *Methods Enzymol.* **284**, 85-107.
- Staswick, P.E.** (1992). Jasmonate, genes, and fragrant signals. *Plant Physiol.* **99**, 804–807.
- Wang, X.** (2004). Lipid signaling. *Curr. Opin Plant Biol.* **7**, 329-336.
- Wasternack, C., and Parthier, B.** (1997). Jasmonate signalled plant gene expression. *Trends Plant Sci.* **2**, 302–307.
- Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H.-E., Rajashekar, C. B., Williams, T. D., and Wang, X.** (2002) Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in *Arabidopsis*. *J. Biol. Chem.* **277**, 31994–32002.
- Xiao S, Gao W, Chen QF, Chan SW, Zheng SX, Ma J, Wang M, Welti R, and Chye ML.** (2010) Overexpression of *Arabidopsis* acyl-CoA binding protein ACBP3

promotes starvation-induced and age-dependent leaf senescence. *Plant Cell* **22**, 1463-1482.

Yang, W., Shivakumar P. D., Pan, X., Giorgis, I., Welti, R., and Wang, X. (2007)

AtPLAI is an acyl hydrolase involved in basal jasmonic acid production and *Arabidopsis* resistance to *Botrytis cinerea*. *J. Biol. Chem.* **282**, 18116–18128.

Zien, C.A., Wang, C., Wang, X. and Welti, R. (2001) In vivo substrates and the contribution of the common phospholipase D, PLD α , to wound-induced metabolism of lipids in *Arabidopsis*. *Biochim. Biophys. Acta* **1530**, 236–248.

FIGURE LEGENDS

Figure 1. pPLAI α activity on labeled phospholipid substrates. *A*, Coomassie blue staining of an 8% SDS-PAGE gel loaded with affinity purified pPLAI α from *E. coli*. M, protein marker; P, purified protein. *B*, fluorescent fatty acid and lysophospholipid released by pPLAI α when NBD-phospholipids were used as substrates. *C*, Production of radioactive fatty acid and lysoPI by pPLAI α when 1-stearoyl-2-[1-¹⁴C]arachidonyl-PI was used as substrate. Values are mean \pm S.D. ($n=3$).

Figure 2. Hydrolysis of phospholipids by pPLAI α using total lipids from mature wounded *Arabidopsis* leaves. Equal amounts of total *Arabidopsis* lipids were incubated with purified pPLAI α (+ pPLAI, open bars) or protein from empty-vector control (- pPLAI, gray bars) at 30°C for 1 h. *A*. Amounts of PC and PE molecular species present in the + pPLAI and - pPLAI mixture after reactions. *B*. Amounts of PI, PS, and PA molecular species present in the + pPLAI and - pPLAI mixture after reactions. *C*. Lysophospholipid species formed in the + pPLAI and - pPLAI mixtures after reactions. Values are means \pm SD ($n = 5$). Asterisks mark differences between the control and +pPLAI α reactions at $p < 0.05$ according to Student's *t* test.

Figure 3. Hydrolysis of galactolipids by pPLAI α using total lipids from mature wounded *Arabidopsis* leaves. Equal amounts of total *Arabidopsis* lipids were incubated with purified pPLAI α (+ pPLAI, open bars) or protein from empty-vector control (- pPLAI, gray bars) at 30°C for 1 h. *A*. Amounts of normal-chain DGDG, MGDG, and PG molecular species present in the + pPLAI and - pPLAI mixture after reactions. *B*. Amounts of OPDA-containing DGDG, MGDG, and PG molecular species in the + pPLAI and - pPLAI mixture after reactions. *C*. MGMG and DGMG species formed in the + pPLAI and - pPLAI mixtures after reactions. Values are means \pm SD ($n = 5$). Asterisks mark differences between the control and +pPLAI α reactions at $P < 0.05$ according to Student's *t* test. One unit is the amount of signal produced by one nmol of internal standard (panels A and C) or one unit of 34:6 MGDG signal (panel B) as described in Materials and Methods. Asterisks mark differences between the control and +pPLAI α reactions at $p < 0.05$ according to Student's *t* test.

Figure 4. Lysophospholipid level in pPLAII α -KO and WT leaves. *A.* Relative level of *pPLAII α* gene expression in wild type and mutant plants was checked with real time quantitative PCR. *B.* Profiles of total LPC, LPE, and LPG in WT and mutants. *C.* Profiles of LPC and LPE species in WT and mutant plants. Values are means \pm SD (n = 5). Statistically significant differences between the wild type and mutant line are marked with asterisks ($p < 0.05$, according to Student's *t* test).

Figure 5. Contents of intermediate compounds and the expression levels of several genes in the oxylipin pathway in WT and KO plants. *A.* The amount of jasmonates in the oxylipin pathway in WT and mutants. 18:3, linolenic acid; 13-HPOT, 13-hydroperoxylinolenic acid; OPDA, 12-oxo-phytodienoic acid, JA, jasmonic acid; MeJA, methyl jasmonic acid. Values are means \pm SD (n = 5). *B.* Expression of several genes in the oxylipin pathway. *PLAIIA*, *pPLAII α* ; *LOX1*, lipoxygenase 1; *LOX2*, lipoxygenase 2, *LOX3*, lipoxygenase 3; *AOS*, allene oxide synthase; *HPL*, hydroperoxide lyase; *AOC1*, allene oxide cyclase 1; *AOC2*, allene oxide cyclase 2; *MJT*, jasmonic acid carboxyl methyltransferase; *OPR*, 12-oxo-phytodienoic acid (OPDA) reductase 3; *VSP2*, vegetative storage protein 2.

Figure 6. Water loss and phenotype of pPLAII α -KO and WT plants. *A.* Water loss as measured by decrease in fresh weight in detached leaves from WT, *pPLAII α -KO* and the complemented plants (Com). Values are means \pm SD (n = 5). *B.* Phenotype of drought sensitivity in WT, *pPLAII α -KO*, and complemented plants. 5 week old plants grown in growth chamber were left without watering for 10 days under 12 h/12 h light/dark period, 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 30% humidity in growth chamber.

Figure 7. Response of stomatal aperture to ABA and JA. Leaf epidermal peels were incubated with different levels of ABA under light for 2.5 h before stomatal aperture was measured. Values are the means \pm SD (n >30).

Figure 8. Changes in ABA and JA levels in detached leaves of WT and pPLAII α -KO mutants. *A.* ABA change in leaves of WT and mutant plants upon desiccation treatment. *B.* JA change in leaves of WT and mutant plants upon desiccation treatment. Values are means \pm SD (n = 5). Statistically significant differences between the wild type and mutant line are marked with asterisks ($p < 0.05$, according to Student's *t* test).

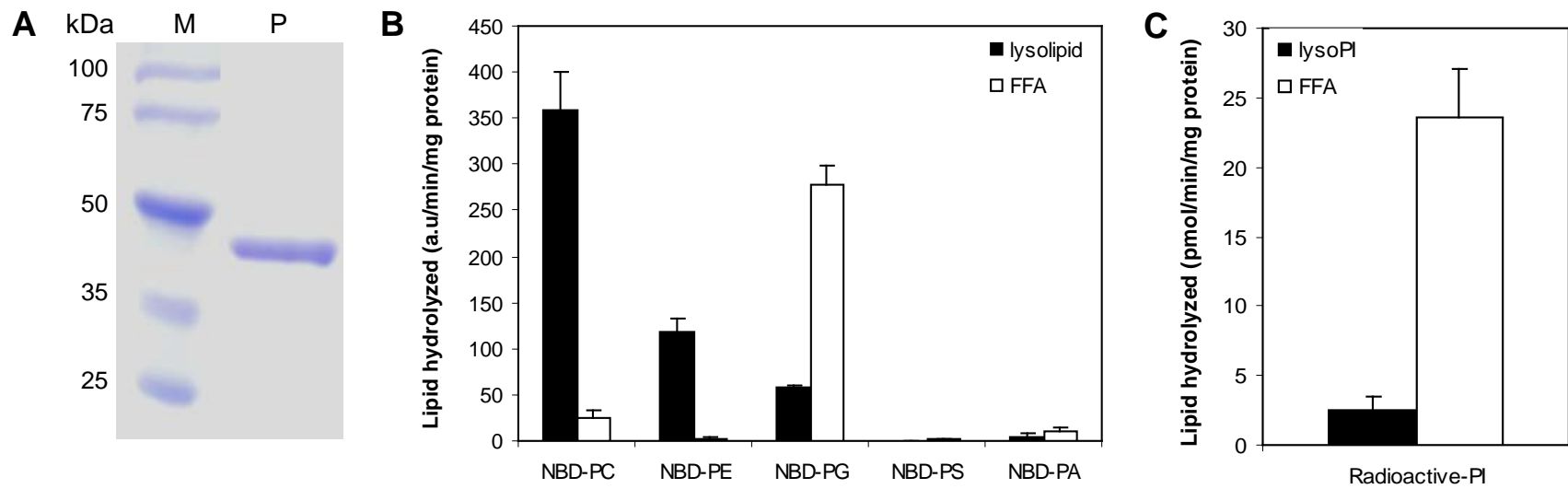


Fig. 1

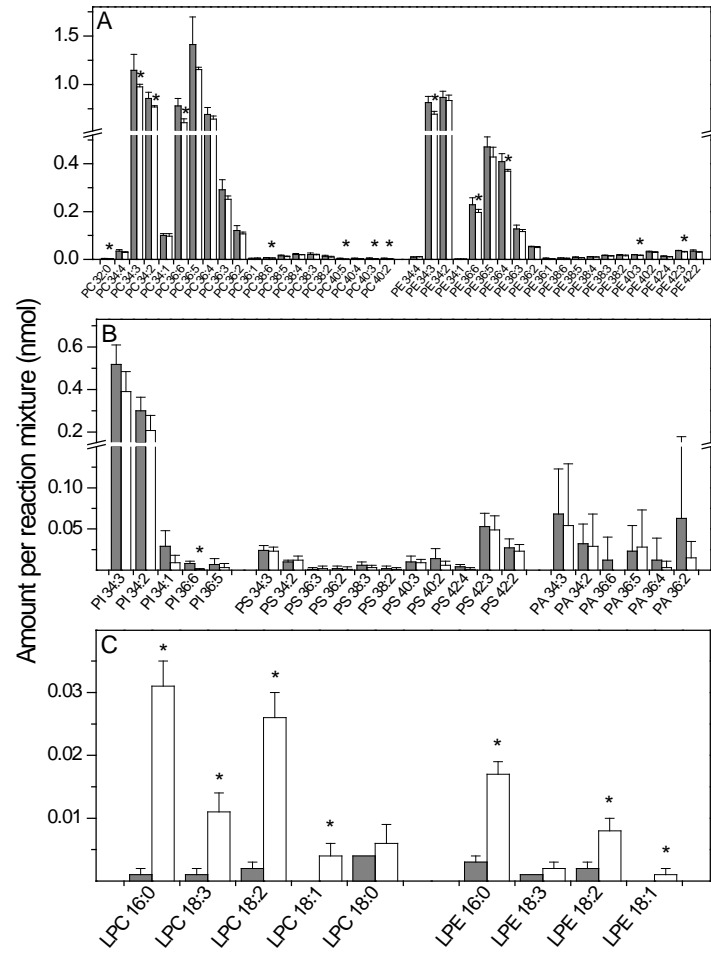


Figure 2

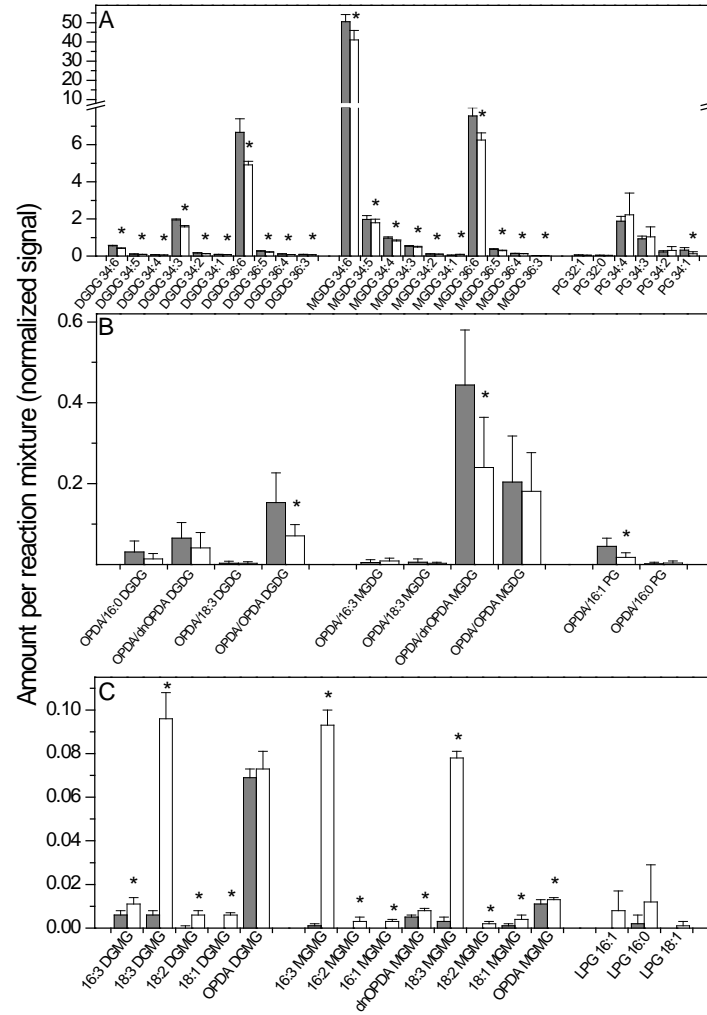


Figure 3

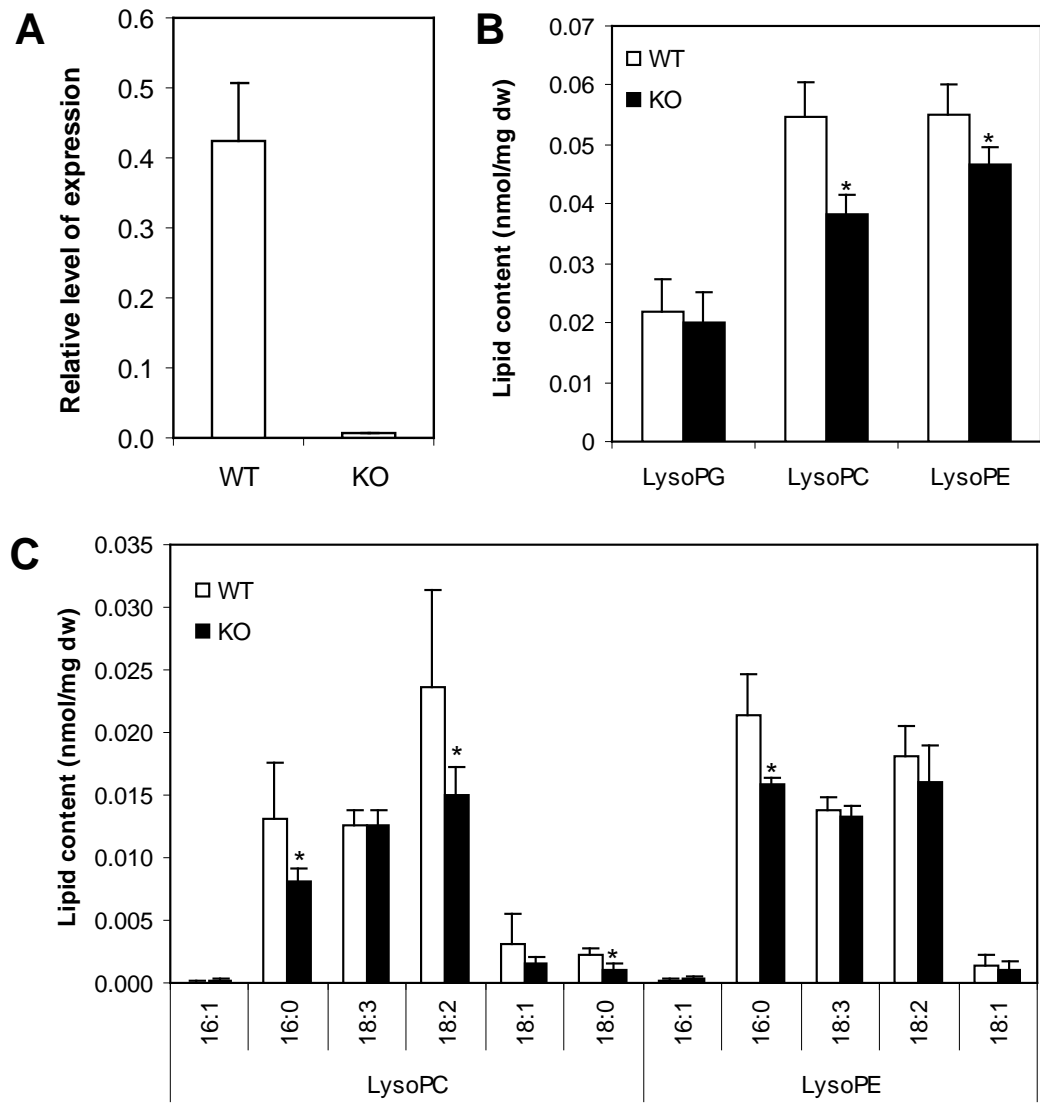


Fig. 4

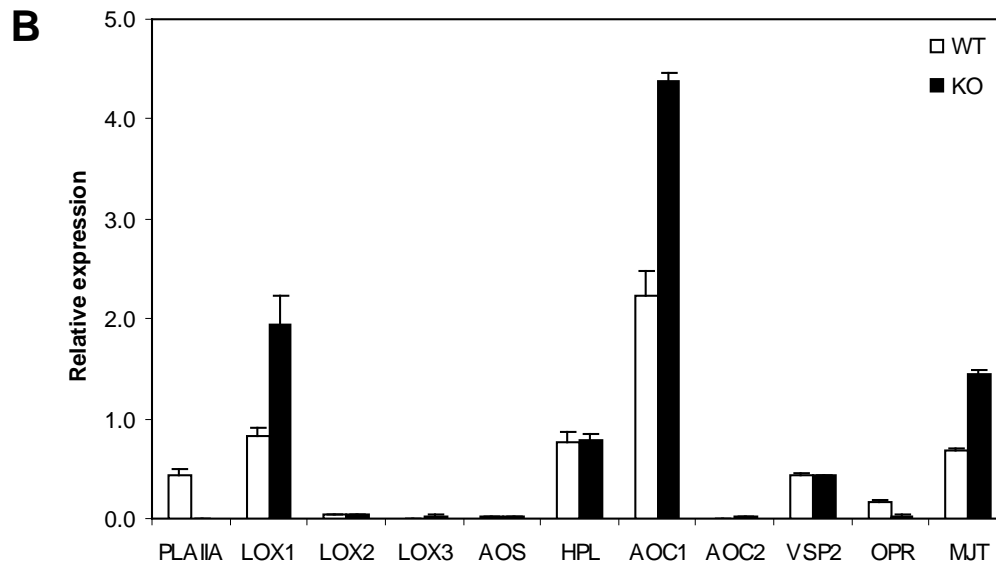
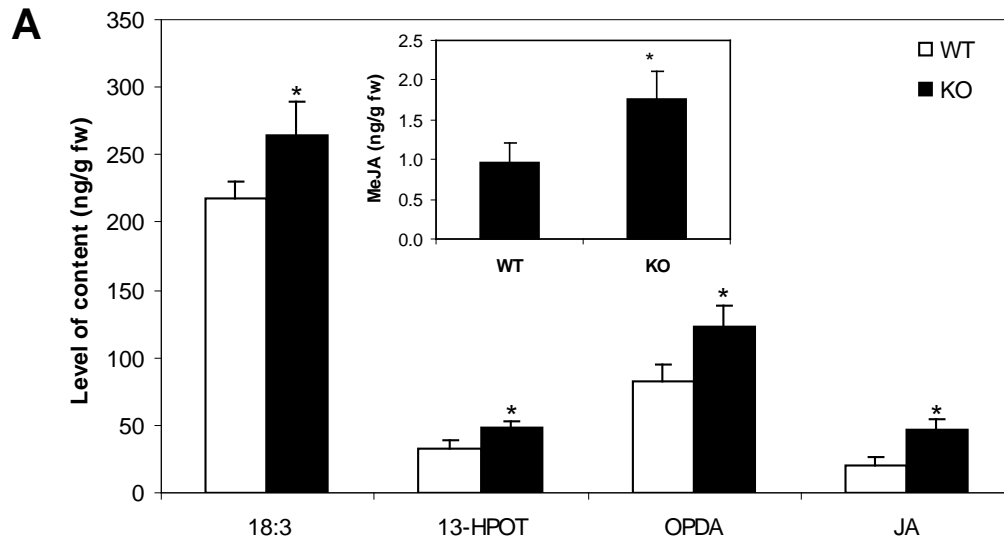


Fig. 5

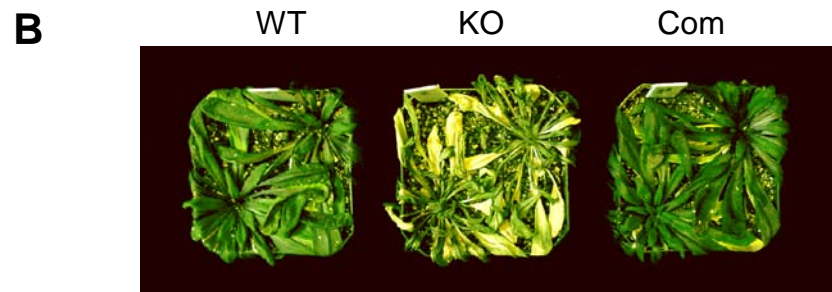
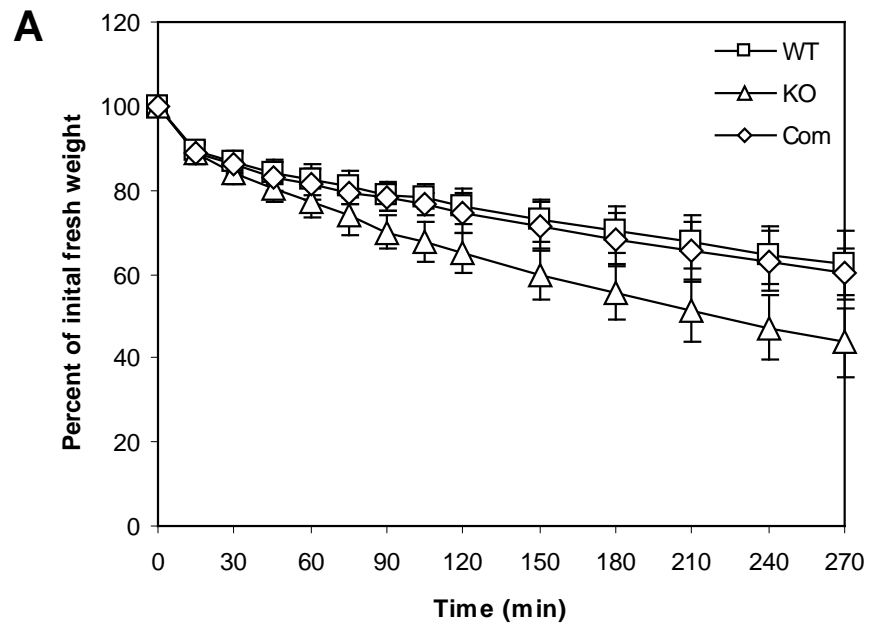


Fig. 6

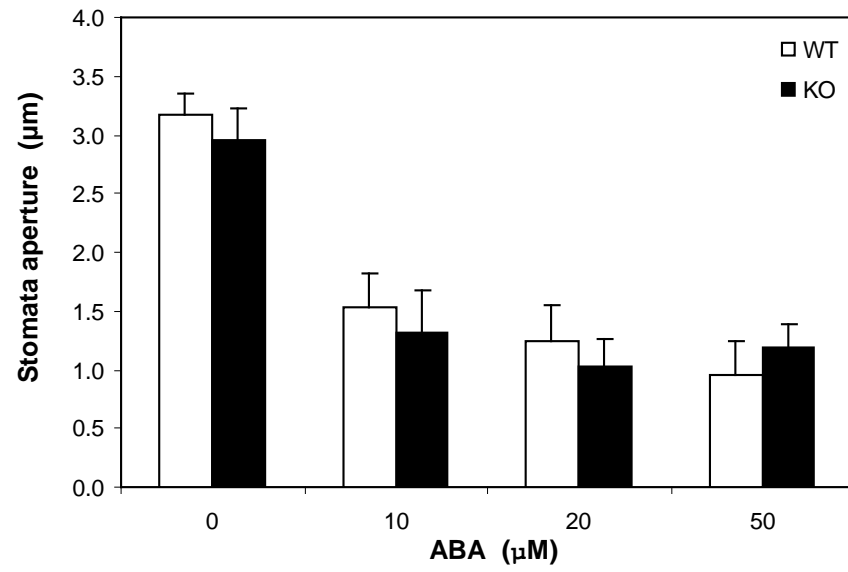


Fig. 7

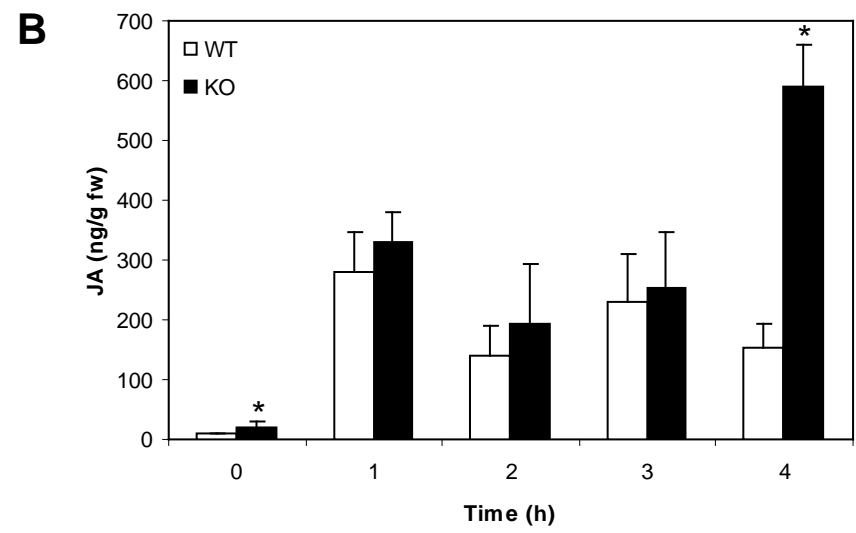
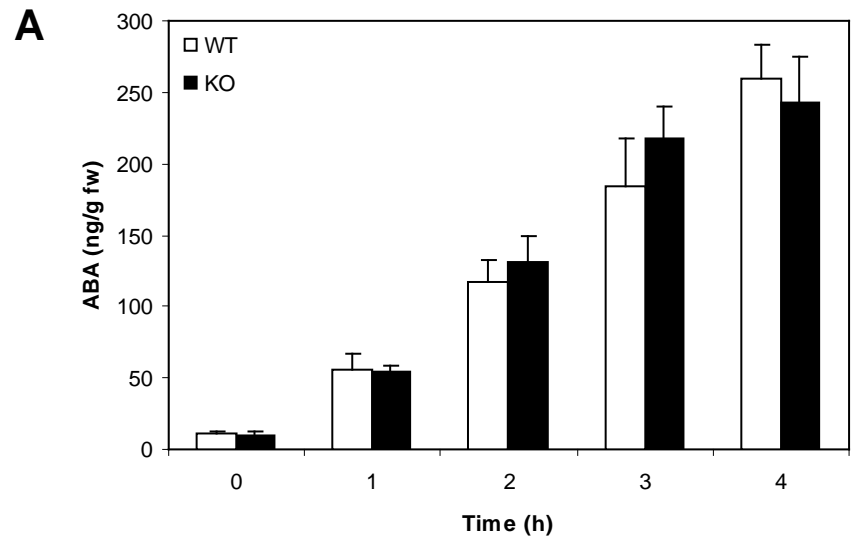


Fig. 8