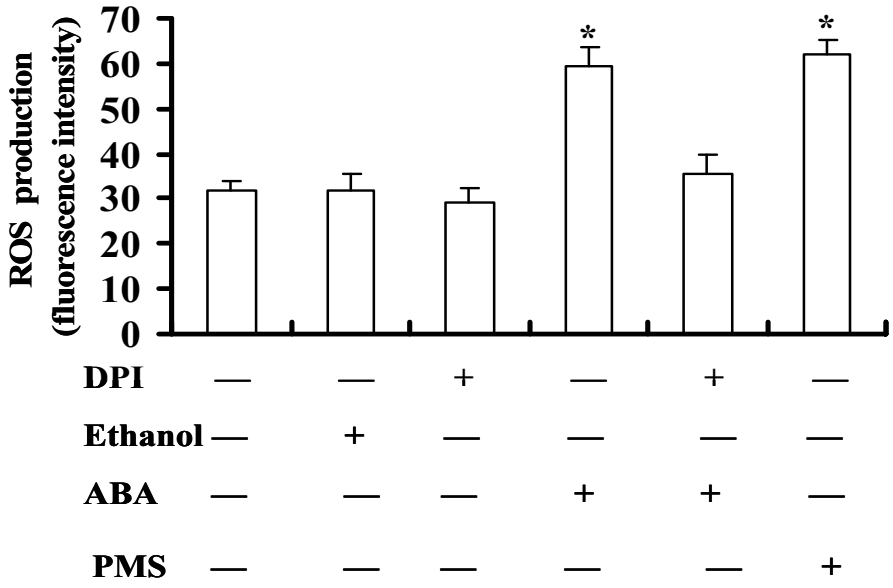
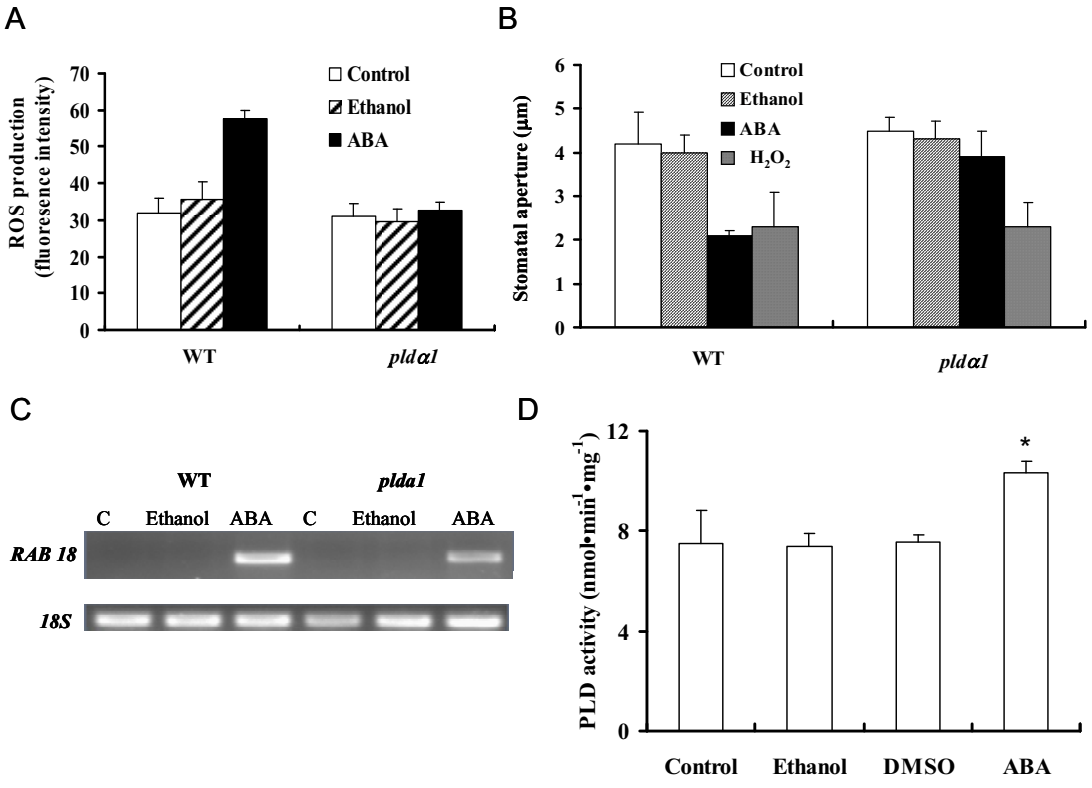
Supplemental Data. Zhang et al. (2009). Phospholipase Dα1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in *Arabidopsis*.



Supplemental Figure 1. ROS Assay with NADPH Oxidase Inhibitor and Superoxide Donor.

ABA-induced ROS production in WT guard cells in epidermal peels was measured with and/or without 50 μM (±) ABA and/or the NADPH oxidase inhibitor DPI (10 μM). The superoxide donor, phenazime methosulfate (PMS, 5 μM) was used as a positive control for ROS production, and ethanol (0.1%, vol/vol) as a negative control. One asterisk indicates that the mean value is significantly different from that of the control, without any treatment ($p < 0.05$).



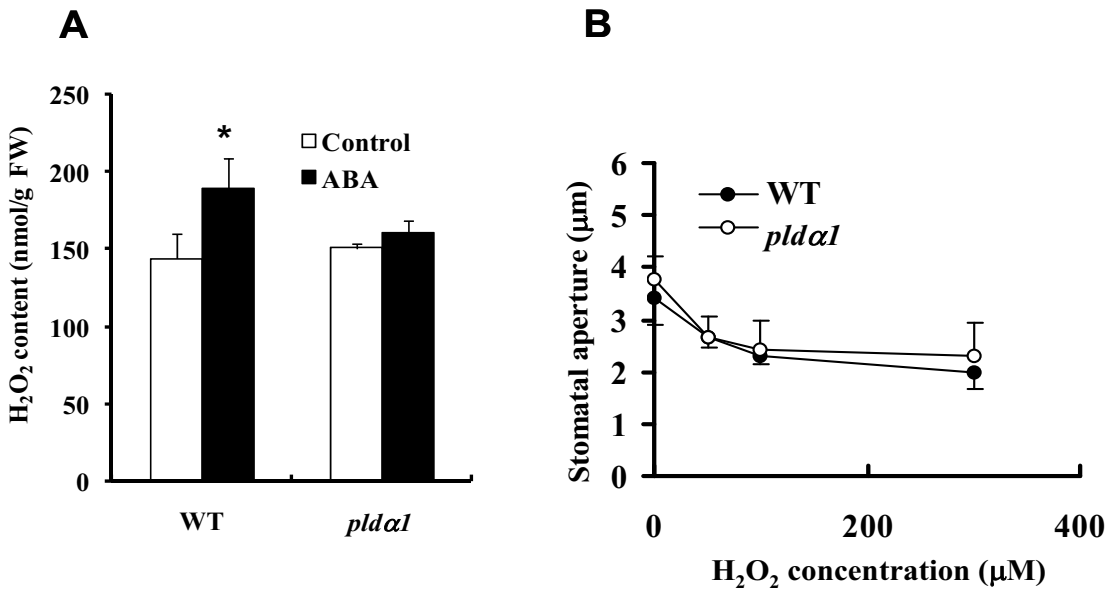
Supplemental Figure 2. Vehicle Effects on ROS production, Stomatal Closure, *RAB 18* Expression and PLDα activity.

(A) Effect of ABA and ethanol on ROS production in WT and *pldα1* plants. The epidermal peels were pre-loaded with H_2DCFDA for 10 min before treatment with ABA (50 μM) or 0.1% (vol/vol) ethanol for 5 min. Confocal images were quantified as mean pixel intensities. Data are means ± SE; n = 50 of each genotype per experiment.

(B) Ethanol effects on stomatal closure in epidermal strips. 50 μM (±) ABA and 0.1% (vol/vol) ethanol was incubated with the epidermal peels after stomata opening. Stomatal apertures were measured after 2.5 h of treatment. Data are means ± SE; n = 60 of each genotype per experiment.

(C) Effects of ABA and ethanol on the expression of *RAB18* in WT and *plda1*. Expression of the *RAB18* gene was assayed by RT- PCR using RNA isolated from leaves of wild-type and the *plda1* mutant. 100 μM (±) ABA, 0.1% ethanol（vol/vol）, or H_2O (C) was sprayed on leaves, and RNA was extracted 4 h later. The expression of 18S was used as a control for RNA loading. The photograph represents one of two experiments with the same results.

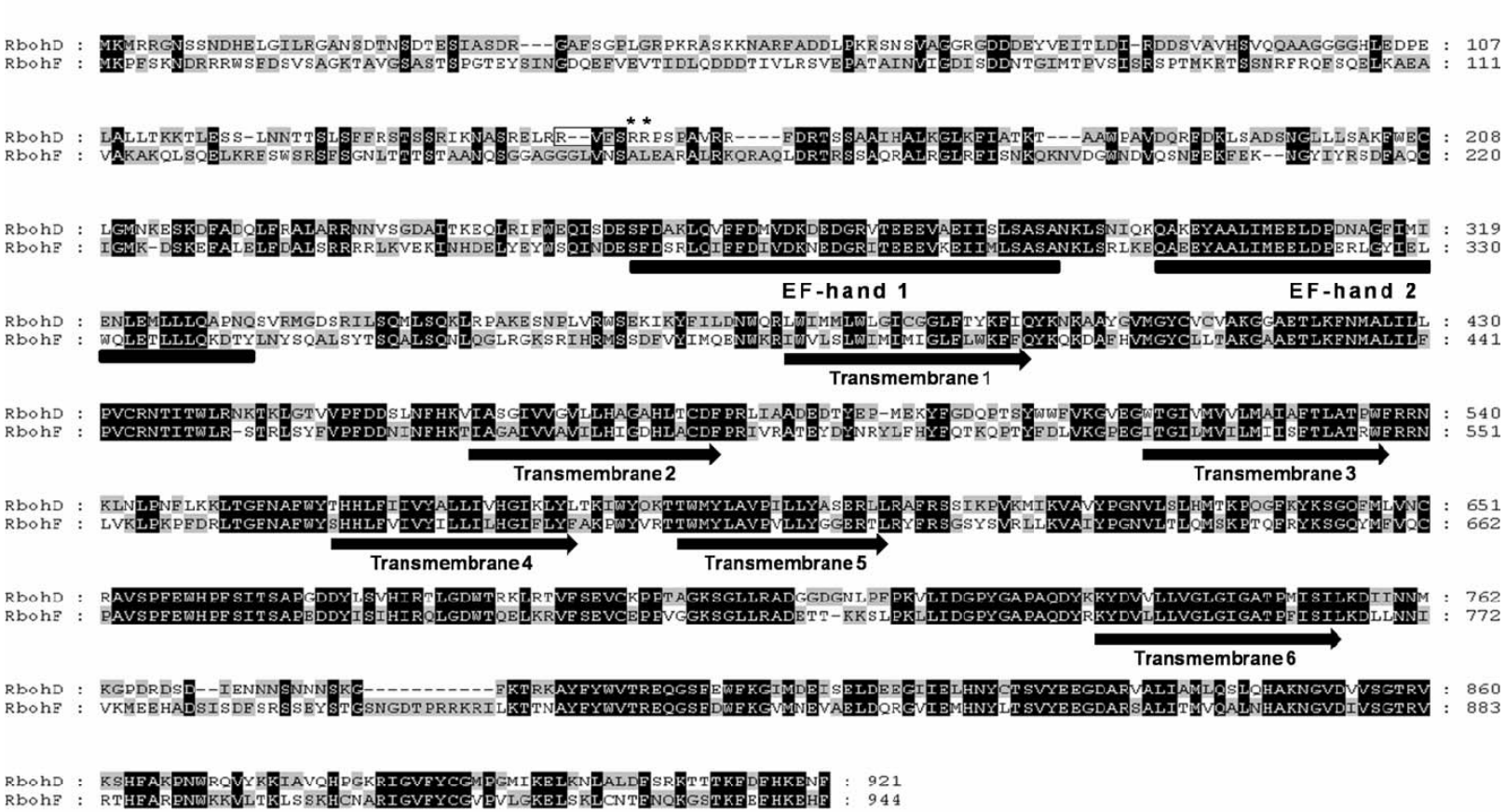
(D) Effects of ABA, ethanol, and DMSO on PLDα activity. The chemicals were sprayed on WT leaves. After 10 min, leaves were excised to determine PLD activity. Data are means ± SE of five replicates. One asterisk indicates the mean value is significantly different from that of the control ($p < 0.05$).



Supplemental Figure 3. H_2O_2 Content in WT and *pldα1* Leaves, and the Effect of Applied H_2O_2 on Stomatal Closure.

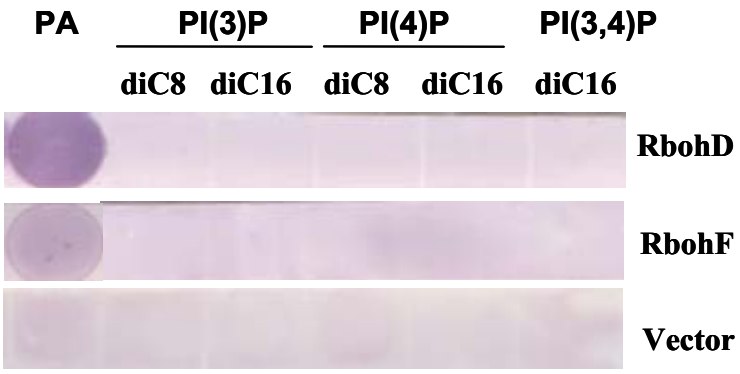
(A) ABA-induced H_2O_2 production in leaves of WT and *pldα1* plants treated with 100 μM (±) ABA. The H_2O_2 content was assayed with an Amplex red hydrogen peroxide/peroxidase assay kit after ABA treatment for 10 min. Data are the means ± SE of five replicates. One asterisk indicates that the mean value is significantly different from that of the control ($p < 0.05$).

(B) H_2O_2-induced stomatal closure in WT and *pldα1* plants. The epidermal peels with fully opened stomata were incubated with 50, 100, or 200 μM H_2O_2 for 2 h. Values are means ± SE (n =120) of three independent experiments.



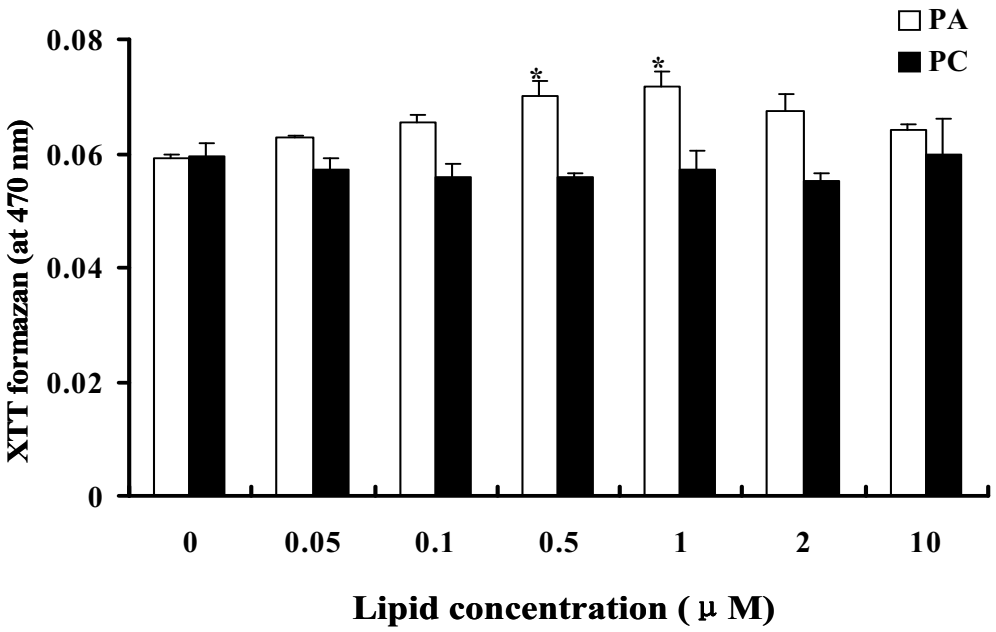
Supplemental Figure 4. Sequence Alignment of RbohD and RbohF Proteins.

The major PA binding residues in RbohD are denoted by asterisks. The B-X-X-S motif is boxed. Two EF-hands are marked with thick lines. Six predicated transmembrane structures are indicated by arrows.



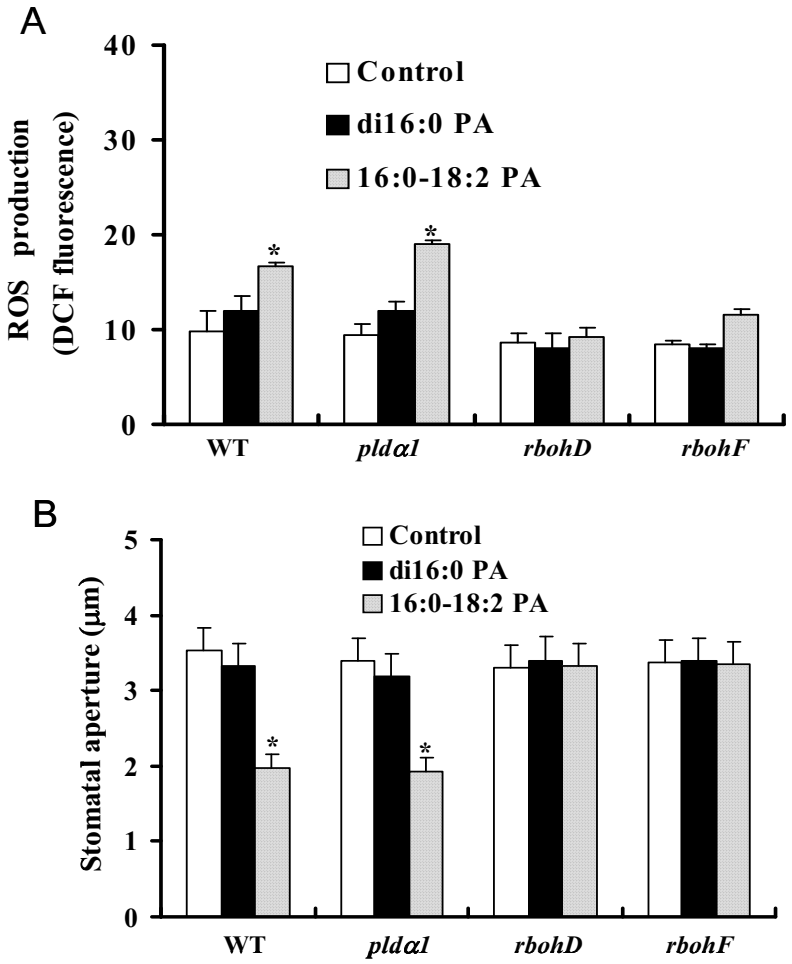
Supplemental Figure 5. Detection of Potential PtdInsPs Binding to Rbohs on Filter.

PI(3)P, PI(4)P, and PI(3,4)P (10 μg) with different fatty acids were spotted on to nitrocellulose and then incubated with His-tag RbohD or RbohF protein fusions. Binding was visualized by staining for alkaline phosphatase activity.



Supplemental Figure 6. PA Activation of NADPH Oxidase Activity in a Dose-Dependent Manner.

Effect of different concentrations of PA and PC on NADPH oxidase activity in wild-type MCPs. The MCPs were incubated with 16:0-18:2 PA or 16:0-18:2 PC before XTT assay. Values are means ± SE of three replicates. The asterisk indicates that the mean value is significantly different from that of the control (no lipid addition) ($p < 0.05$).

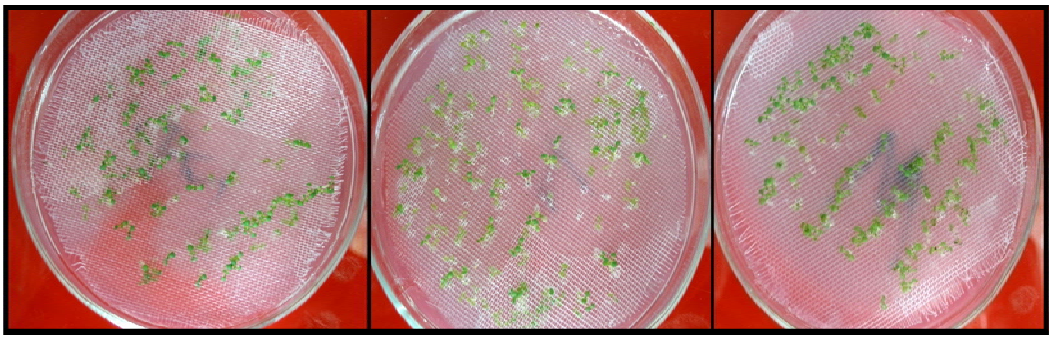


Supplemental Figure 7. Effect of di16:0 PA on ROS Production and Stomatal Closure.

(A) Effect of di16:0 PA and 16:0-18:2 PA on ROS production in the guard cells of epidermal peels. DCF fluorescence pixel intensity indicates ROS production after 10 min of PA treatment.

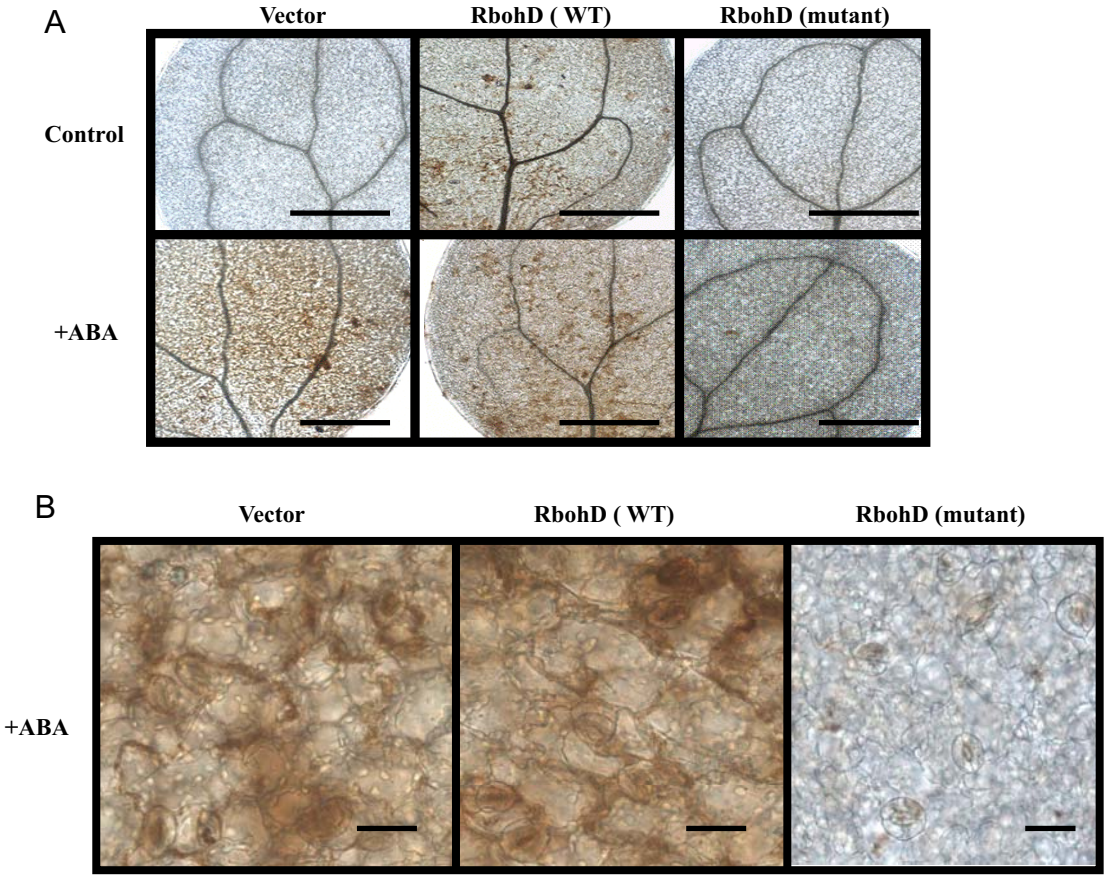
(B) Effect of di16:0 PA and 16:0-18:2 PA on stoma closure.

Values in **(A)** and **(B)** are means ± SE; n=55 of each genotype per experiment. Data are from three experiments. An asterisk indicates that the mean value is significantly different from that of the control ($p < 0.05$).



Vector **RbohD(WT)** **RbohD(mutant)**

Supplemental Figure 8. Phenotypes of Seedlings Transiently Expressing RbohD. *rbohD* seedlings were transformed with wild-type *RbohD-GFP* (WT) and the non-PA binding *RbohD-GFP* mutant (mutant) as indicated in the Methods section. Photographs were taken three days after the transformation. Cotyledons became yellow in seedlings transiently expressing wild-type RbohD.

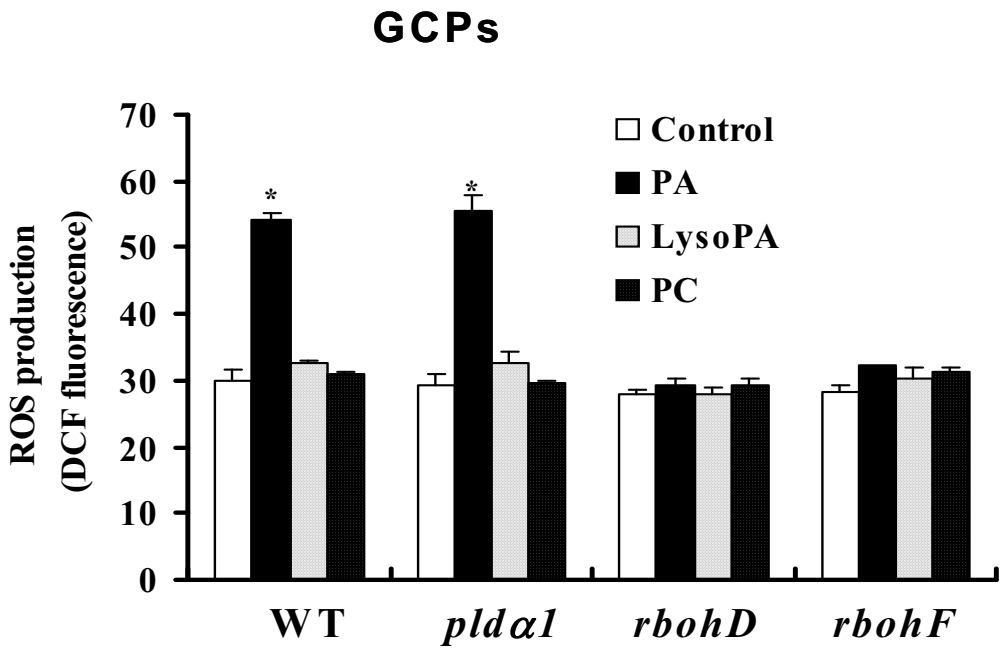


Supplemental Figure 9. DAB Staining for ROS in Cotyledons Transiently Expressing RbohD.

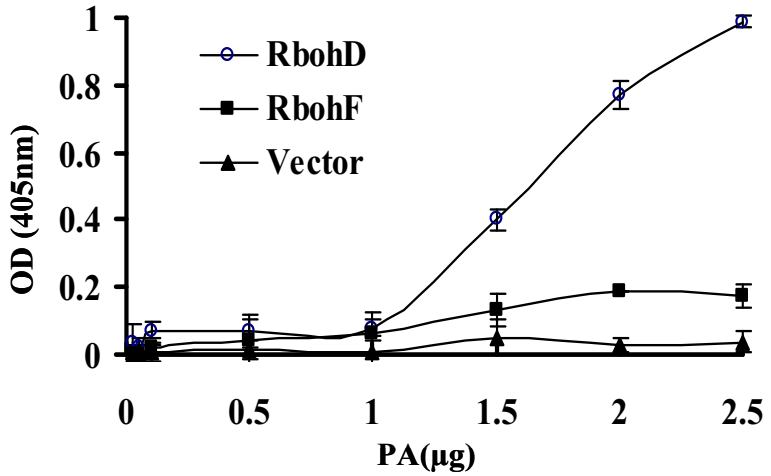
(A) DAB staining of cotyledons transiently expressing wild-type and non-PA-binding RbohD fused to GFP in *rbohD* seedlings. The cotyledons were treated with 2 μM (±) ABA. Scale bar = 1 mm.

(B) Enlarged images showing DAB staining of ROS in guard cells treated with 2 μM (±) ABA. Scale bar = 10 μm.

The brown color in **(A)** and **(B)** shows ROS accumulation.

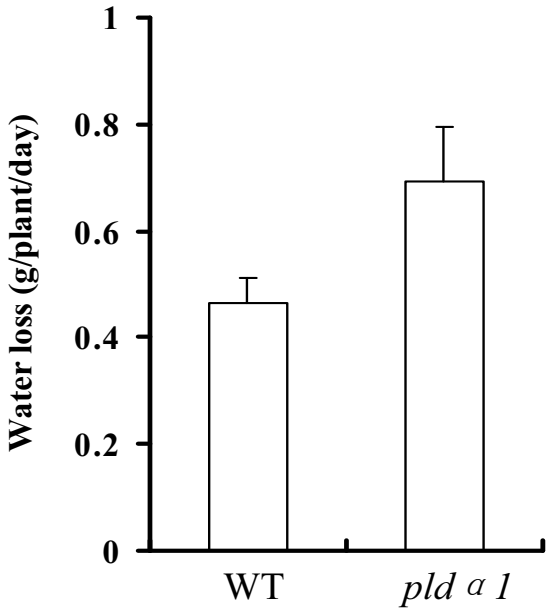


Supplemental Figure 10. Lipid Specificity for ROS Generation in GCPs.
16:0-18:2 PA (1 μM) was added to GCPs from WT, *pldα1*, *rbohD,* or *rbohF* plants for 10 min. ROS was monitored by DCF. The images were quantified as mean pixel intensities. LysoPA and 16:0-18:2 PC were compared with 16:0-18:2 PA at the same concentration. Data are means ± SE from three independent experiments; n = 50 per genotype per experiment. An asterisk indicates that the mean value is significantly different from that of the control ($p < 0.05$).



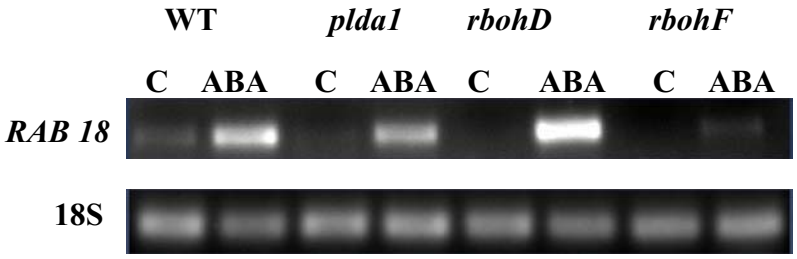
Supplemental Figure 11. Detecting PA Concentration-Dependent Rboh Binding by the ELISA Assay.

RbohD and RbohF proteins were expressed in *E. coli*. 16:0-18:2 PA in serial dilution from 0.025 to 2.5 µg was loaded onto a 96-well plate, and incubated with His-tagged proteins (4 µg/well). Details of the experiment have been described in the text. Data were means ± SE from five replicates.



Supplemental Figure 12. Water Loss from WT and *pldα1* Plants.

Four-week-old wild-type and *pldα1* mutant plants grown in culture solution were transferred to Hoagland solution (pH 5.7) for three days. Transpirational water loss was determined by weighing the solution before and after the treatment. Data were the means ± SE of five replicates.



Supplemental Figure 13. *RAB18* Expression in WT, *pldα1*, *rbohD*, and *rbohF*.
Expression of *RAB18* gene was assayed by RT-PCR. The leaves were treated with 100 μM (±) ABA for 4 h. 0.1% ethanol (vol/vol） treatment was set as a control (C). The expression of 18S rRNA was used as an internal control.

Supplemental Table 1. PCR Primers Used in the Paper.

Primer name	Sequences
RbohD-SALK-F	5’-AGAGGCTGCTCCGTGCTT-3’
RbohD-SALK-R	5’-CCTATTCTTTTGCCGGGATG-3’
LB3.1	5’-ATTTTGCCGATTTCGGAAC-3’
RbohD-C-F	5’-CGATGAAAATGAGACGAGGCAATTCAAG -3’
RbohD-C-R	5’-TAATCTAGAAGTTCTCTTTGTGGAAGTC-3’
RbohF-C-F	5’-ATATACTTCCGATATCCTTCAACCA-3’
RbohF-C-R	5’-CGCTCGAGGAAATGCTCCTTGTGAAAT TC -3’
RbohD-RT-F	5’-ACCATGAACTTGGGATTCTACGAGGAGC-3’
RbohD-RT-R	5’-GGAAGTCAAACTTGGTAGTTGTCTTTCGAG-3’
Actin8-F	5’-TACTGGAATGGTTAAGGCTGGAT-3’
Actin8-R	5’-GACTTCTGGGCACCTGAATCTC-3’
RbohD921F	5’- CGGAATTCATGAAAATGAGACGAGGCAAT-3’
RbohD921R	5’-AAGCGGCCGCGAAGTTCTCTTTGTGGAAGTCAAAC-3’
RbohD713R	5’-AAGCGGCCGCTCCATCTCCTCCGTCT-3’
RbohD600R	5’-AAGCGGCCGCATATAGAAGGATGGGTACA-3’
RbohD450R	5’-AAGCGGCCGCAACGACAGTACCAAGCTTAG-3’
RbohD330R	5’-AAGCGGCCGCCGACTGGTTTGGTGCTTG-3’
RbohD160R	5’-AAGCGGCCGCGCGGTCAAACCGCCGC-3’
RbohD150R	5’-AAGCGGCCGCACGTCTAGAGAACACGCG-3’
RbohD140R	5’-AAGCGGCCGCGGAGGCGTTCTTGATGC-3’
RbohD100R	5’-AAGCGGCCGCTCCACCTCCAGCTGCTTG-3’
R141A-F	5’-CAAGAACGCCTCCGCCGAGCTCCGCCGCG-3’
R141A-R	5’-CGCGGCGGAGCTCGGCGGAGGCGTTCTTG-3’
R(144,145)A-F	5’- GCCTCCCGCGAGCTCGCCGCCGTGTTCTCTAGACG-3’
R(144,145)A-R	5’- CGTCTAGAGAACACGGCGGCGAGCTCGCGGGAGGC-3’
R(149,150)G-F	5’- CGCCGCGTGTTCTCTGGAGGTCCCTCCCCGGCCG-3’
R(149,150)G-R	5’- CGGCCGGGGAGGGACCTCCAGAGAACACGCGGCG-3’
R(149,150,156,157)A-F	5’-CGTGTTCTCTGGAGGTCCCTCCCCGGCCGTGGCGGCGTTTGACCGCG-3’
R(149,150,156,157)A-R	5’-CGCGGTCAAACGCCGCCACGGCCGGGGAGGGACCTCCAGAGAACACG -3’
R(156,157)A-F	5’- CCTCCCCGGCCGTGGCGGCGTTTGACCGCGCGGC-3’
R(156,157)A-R	5’-GCCGCGCGGTCAAACGCCGCCACGGCCGGGGAGG-3’
R160A-F	5’- GCGGCGGTTTGACGCCGCGGCCGCACTCGAGC-3’
R160A-R	5’- GCTCGAGTGCGGCCGCGGCGTCAAACCGCCGC-3’
D-160-HA-F	5’- CGGGATCCATGAAAATGAGACGAGGCAAT-3’
D-160-HA-R	5’- GAAGGCCTGCGGTCAAACCGCCGCACGGC-3’
D-R(149,150,156,157)A-HA-R	5’- GAAGGCCTGCGGTCAAACGCCGCCACGGC-3’
D-921-HA-R	5’- GAAGGCCTGAAGTTCTCTTTGTGGAAGTCAAAC-3’
D-GFP-F	5’- CGGAATTCATGAAAATGAGACGAGGCAATT-3’
D-GFP-R	5’- GGGGTACCGAAGTTCTCTTTGTGGAAGTC-3’
F- AL(156,157)RR-F	5’- GGTTTGGTGAACTCGCGTAGAGAAGCGCGAGCGTTGCG-3’
F- AL(156,157)RR-R	5’- CGCAACGCTCGCGCTTCTCTACGCGAGTTCACCAAACC-3’
RbohF171-HA -F	5’- CGGGATCCATGAAACCGTTCTCAAAGAA-3’

RbohF171-HA -R	5’- GAAGGCCTCCGATCTAACTGAGCACGTT-3’
RbohF944F	5’- CGCGGATCCATGAAACCGTTCTCAAAG-3’
RbohF944R	5’- CCGCTCGAGGAAATGCTCCTTGTGAAATTC-3’
RbohF723R	5’- CCGCTCGAGTGTTGTTTCGTCGGCTCTG-3’
RbohF555R	5’- CCGCTCGAGAAGCTTGACTAGGTTACGC-3’
RbohF341R	5’- CCGCTCGAGGTCTTTTTGTAGAAGCAAAG-3’
RbohF171R	5’- CCGCTCGAGCCGATCTAACTGAGCACG-3’
RbohF104R	5’- CCGCTCGAGTGAGAATTGTCGGAACCGATTAG-3’

Methods:

ROS Measurement with DAB Staining and Kit

Production of ROS in leaves was measured by staining with 3,3'-diaminobenzidine (DAB) and by assaying H_2O_2 concentrations in the leaves. For quantification, H_2O_2 was extracted from leaves treated with ABA for 10 min, and its concentration was measured with an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR) as previously described (Zhang et al., 2003).

Water Loss Measurement

For water loss measurement, four-week-old wild-type and *pldα1* plants grown in culture solution were transferred to Hoagland solution (pH 5.7) for three days. Transpirational water loss was determined by weighing the solution before and after the treatment. Five replicates were performed for each treatment.

PLDα Activity Assay

PLDα activity was assayed according to previous methods (Sang et al., 2001).

***RAB18* Transcript Analysis by RT-PCR**

RNA isolation was as indicated in the text. The *RAB18* transcript was amplified using the gene-specific primers 5′-ACGAGTACGGAAATCCGATG-3′ (forward) and 5′-TGTCCATCATCCCCTTCTTC-3′(reverse). Relative transcript levels in the samples were normalized using 18S rRNA as a constitutively expressed internal control, with primers 5′-CCTATCAACTTTCGATGGTAGGATA-3′ (forward) and 5′-CGTTAAGGGATTTAGATTGTACTCATT-3′ (reverse).

References:

Sang, Y., Zheng, S., Li, W., Huang, B., and Wang, X. (2001). Regulation of plant water loss by manipulating the expression of phospholipase Dα. Plant J. **28:** 135–144.

Zhang, W., Wang, C., Qin, C., Wood, T., Olafsdottir, G., Welti, R., and Wang, X. (2003). The oleate-stimulated phospholipase D, PLDδ, and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*. Plant Cell **15:** 2285-2295.