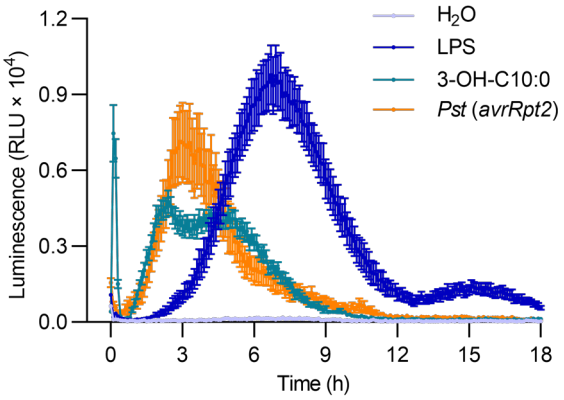
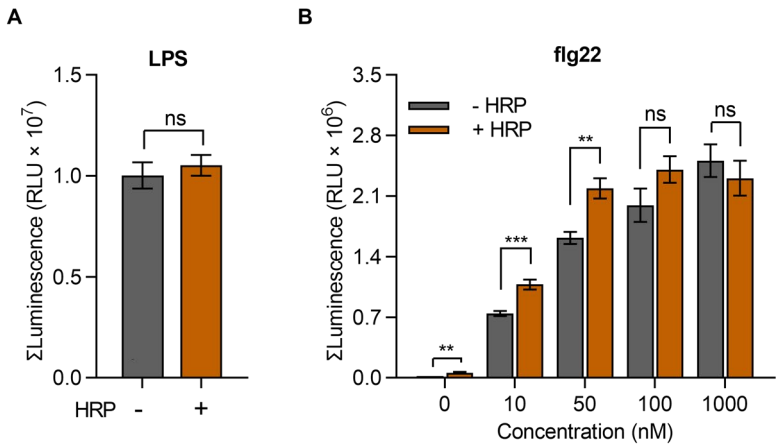
Ascorbate peroxidase 1 allows monitoring cytosolic accumulation of effector-triggered reactive oxygen species using a luminol-based assay

Supplemental figures and table



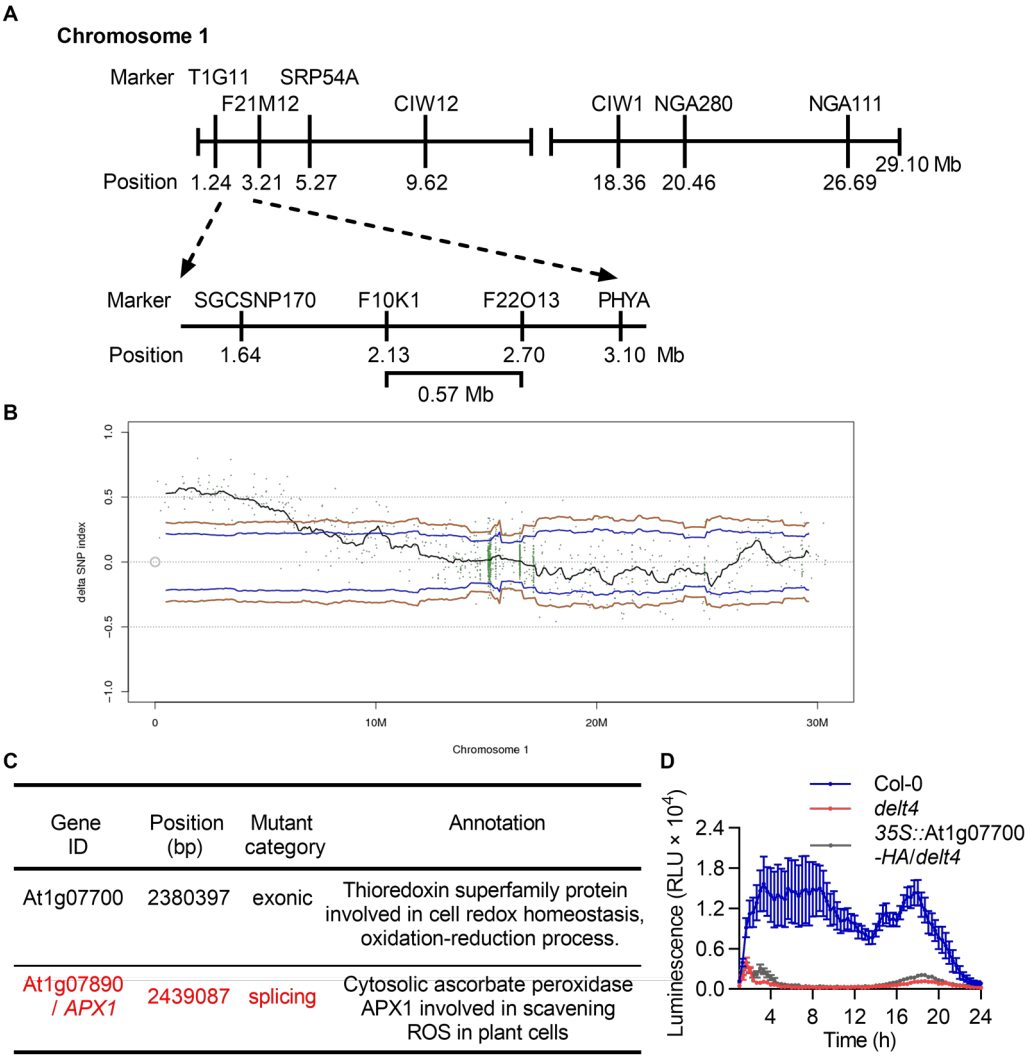
Supplemental Figure S1. The kinetics of ROS burst triggered by LPS/3-OH-C10:0/*Pst* (*avrRpt2*).

The kinetics of LPS (50 μg/mL) or 3-OH-C10:0 (5 μM)-triggered long-lasting ROS burst is highly similar to that triggered by avirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (*avrRpt2*) at OD_{600} of 0.003. ROS signals were monitored for 18 h using a chemiluminescence assay with luminol as a substrate. Data are presented as the mean ± SE (n = 8). The experiment was repeated three times with similar trends.



Supplemental Figure S2. HRP is not indispensable when ROS level is high enough to oxidize luminol

ROS were measured using a luminol-based chemiluminescent assay with or without HRP (20 mg/L). The bar graph indicates the total integrated photon counts within 21 h after LPS (50 μg/mL) treatment (**A**) or 30 min after different concentrations of flg22 treatment (**B**). Data are presented as the mean ± SE (n = 10 in **A**, 7 in **B**). Student's *t* test; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns, no significant differences. The experiments were repeated at least three times with similar trends.



Gene ID	Position (bp)	Mutant category	Annotation
At1g07700	2380397	exonic	Thioredoxin superfamily protein involved in cell redox homeostasis, oxidation-reduction process.
At1g07890 / *APX1*	2439087	splicing	Cytosolic ascorbate peroxidase APX1 involved in scavening ROS in plant cells

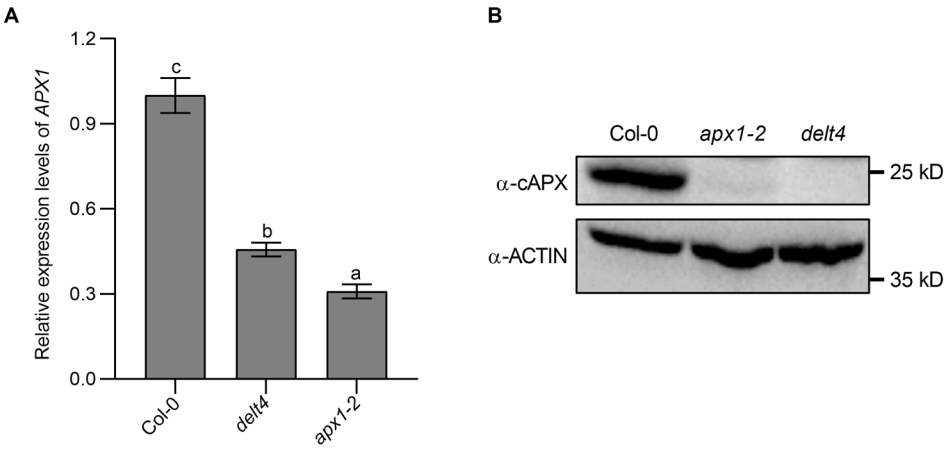
Supplemental Figure S3. Molecular characterization of the *DELT4* gene

A. The *DELT4* mutation mapped to chromosome 1 (Chr1). The two molecular markers F10K1 and F22O13 were 0.57 Mb apart.

B. ΔSNP-index plot of Chr1 generated by whole-genome sequencing. Green dots correspond to each ΔSNP-index, and the black line represents the average values of the ΔSNP-index at 1 Mb intervals with a 100-kb increment. The candidate region (0–5 Mb) is above the orange line indicating cutoff values.

C. Two nonsynonymous SNPs in the At1g07890 and At1g07700 genes were identified in the candidate regions as indicated by genetic mapping in **A**.

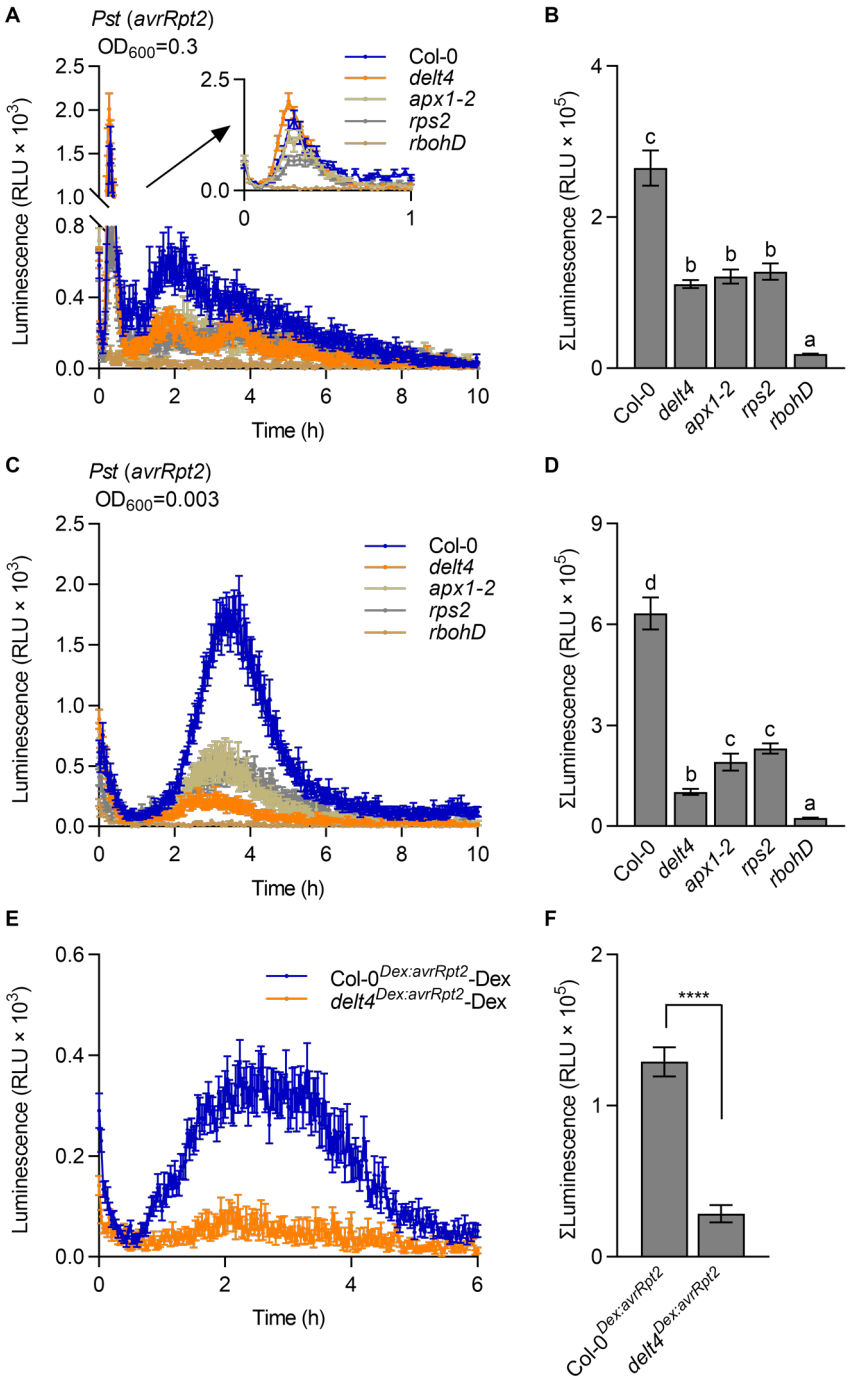
D. At1g07700 failed to rescue the luminescent signals of *delt4* mutants. Luminescent signals were measured using a luminol-based chemiluminescent assay after treatment with LPS (50 μg/mL). Data are shown as the mean ± SE (n = 8).



Supplemental Figure S4. *APX1* transcripts and protein abundance in *apx1* mutants

A. *APX1* transcript levels. Total RNA was prepared from 8-day-old seedlings. *APX1* expression in Col-0, *apx1-2*, and *delt4* mutants was quantified by RT-qPCR. Data are shown as the mean ± SE (n = 4). Means with different letters denote significant differences ($P \leq 0.05$, one-way ANOVA).

B. APX1 protein abundance. Proteins were extracted from 8-day-old seedlings and detected in Col-0, *apx1-2*, and *delt4* mutants by immunoblot analysis using an α-cAPX antibody. Actin was used as the loading control. All experiments were repeated at least three times with similar results.



Supplemental Figure S5. The *apx1* mutants show reduced effector-triggered long-lasting luminescent signals

A. The *delt4* and *apx1-2* mutants showed reduced long-lasting luminescent signals after treatment with *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (*avrRpt2*) at an OD_{600} of 0.3. Luminescent signals were monitored for 10 h using a luminol-based chemiluminescent assay. The first peak within 1 h is magnified in the upper right corner of the figure. The *rps2* and *rbohD* mutants were used as negative controls. Data are shown as the mean ± SE (n = 9).

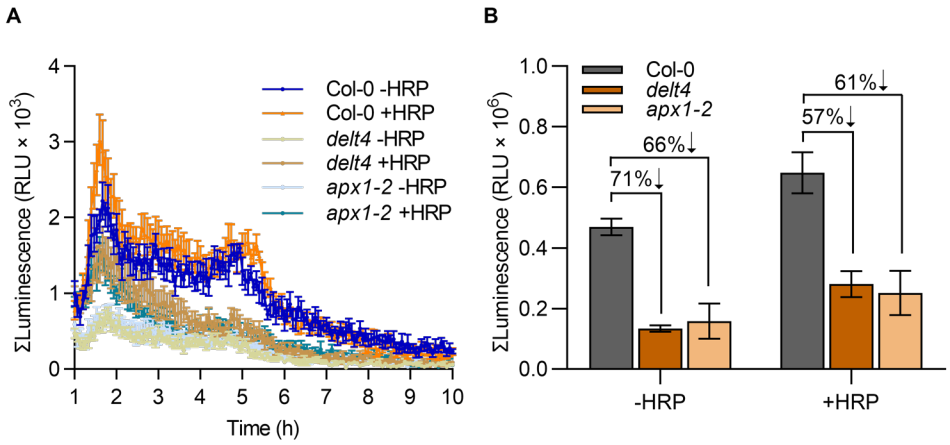
B. Total luminescent signals within 1–10 h from **A**. Data are shown as the mean ± SE (n = 9). Different letters above the bars indicate significant differences between different genotypes ($P \leq 0.05$, one-way ANOVA).

C. The *delt4* and *apx1-2* mutants showed reduced long-lasting luminescent signals after treatment with *Pst* (*avrRpt2*) at an OD_{600} of 0.003. Luminescent signals were monitored for 10 h using a luminol-based chemiluminescent assay. The *rps2* and *rbohD* mutants were used as negative controls. Data are shown as the mean ± SE (n = 9).

D. Total luminescent signals within 1–10 h from **C**. Data are shown as the mean ± SE (n = 9). Different letters above the bars indicate significant differences between different genotypes ($P \leq 0.05$, one-way ANOVA).

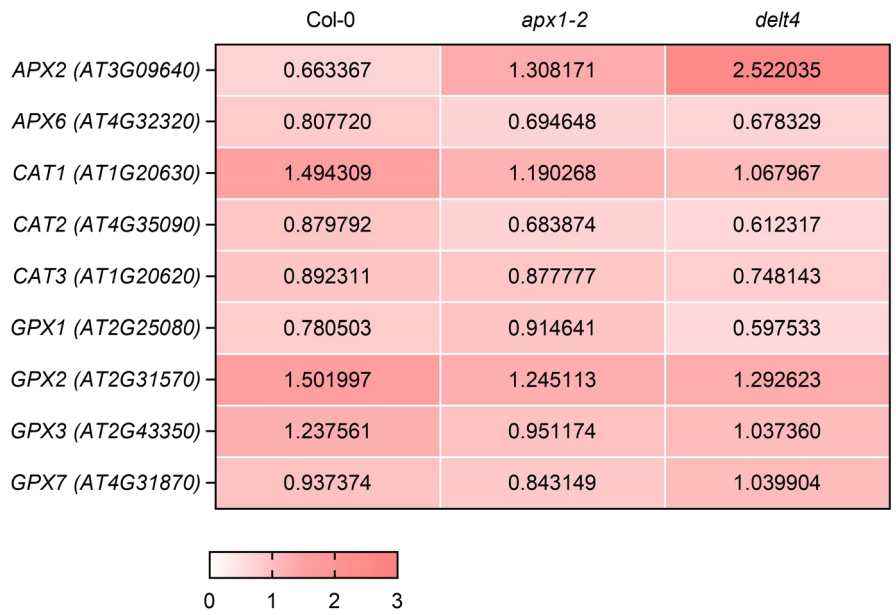
E. The dynamics of luminescent signals after treatment with 10 μM dexamethasone (Dex). Luminescent signals were monitored using a luminol-based chemiluminescent assay in the leaf disks of Col-$0^{Dex:avrRpt2}$ and *delt4*$^{Dex:avrRpt2}$ transgenic plants. Data are shown as the mean ± SE (n = 18).

F. Total luminescent signals within 0.5–6.0 h from **E**. Data are shown as the mean ± SE (n = 18, ****$P \leq 0.0001$, *t*-test). Experiments in this figure were repeated at least three times with similar trends.



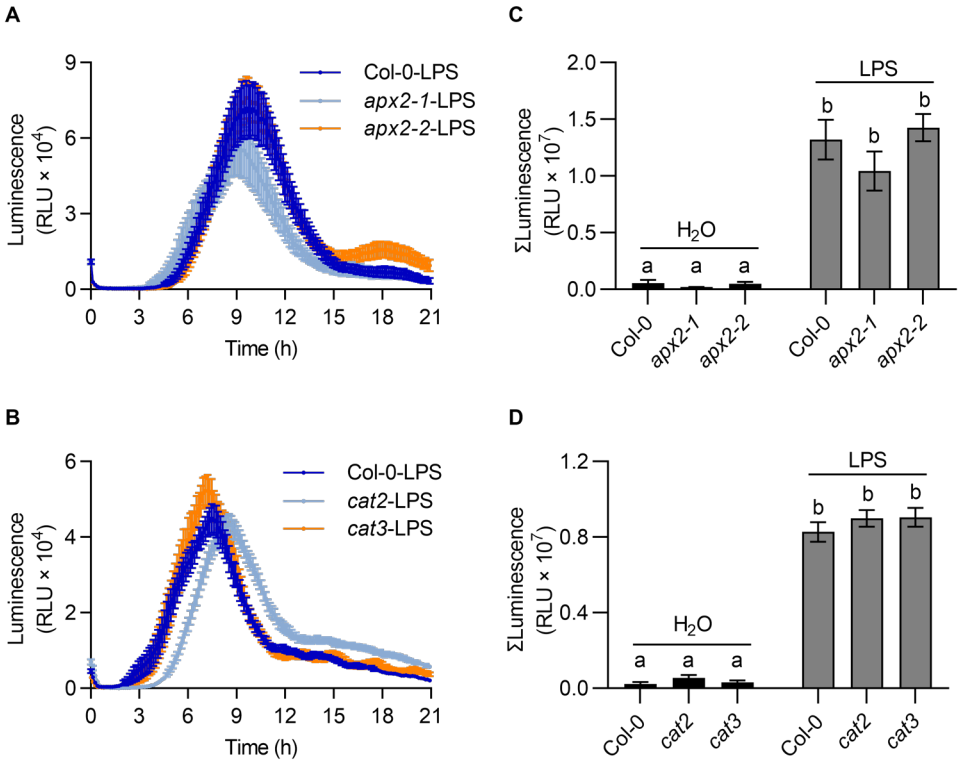
Supplemental Figure S6. The exogenous addition of HRP cannot restore the reduced ETI-ROS signals in *apx1* mutants

HRP (20 mg/L) was exogenously applied for the luminol-based chemiluminescent assay. ROS signals were recorded for 10 h after treatment with *Pst* (*avrRpt2*) (OD_{600} = 0.03). Total photon counts (**B**) within 1-10 h were calculated from (**A**). Data are presented as the mean ± SE (n = 7-8 in **A**, **B**). Values above the bars indicate the different percentage between groups, and arrows indicate a decrease in mutants compared with the wild-type (Col-0). The experiment was repeated three times with similar trends.



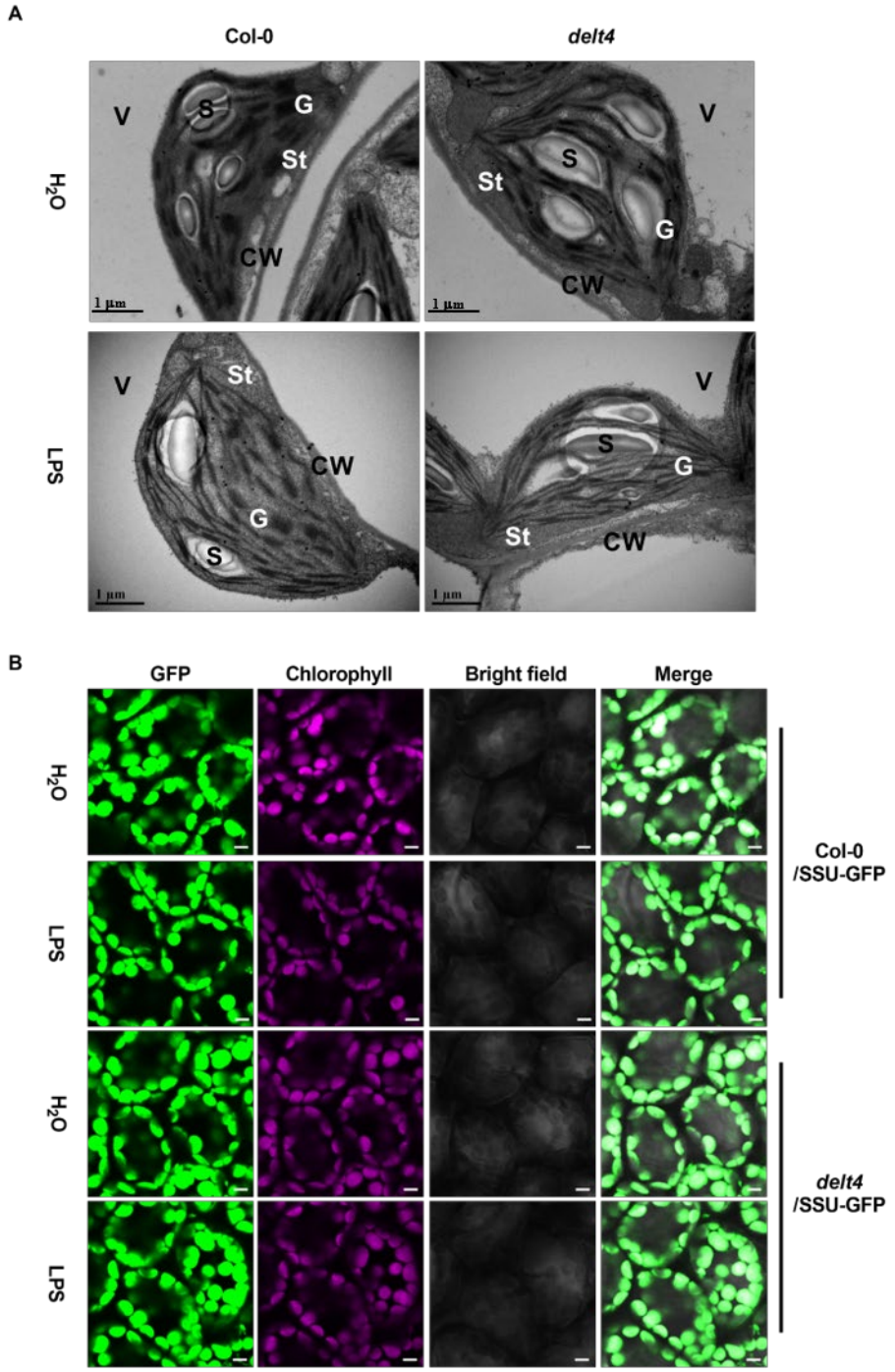
Supplemental Figure S7. Expression of genes involved in ROS scavenging

Total RNA was extracted from wild type plants, *apx1-2* and *delt4* mutants 4 h after treatment with ddH_2O or 50 µg/mL LPS. Relative gene expression was quantified by RT-qPCR. The heatmap shown is based on log_2-transformed fold changes in gene expression (normalized against expression of the corresponding genes treated with mock control (ddH_2O). APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase.



Supplemental Figure S8. *apx2*, *cat2,* and *cat3* mutants are not impaired in LPS-triggered long-lasting ROS accumulation

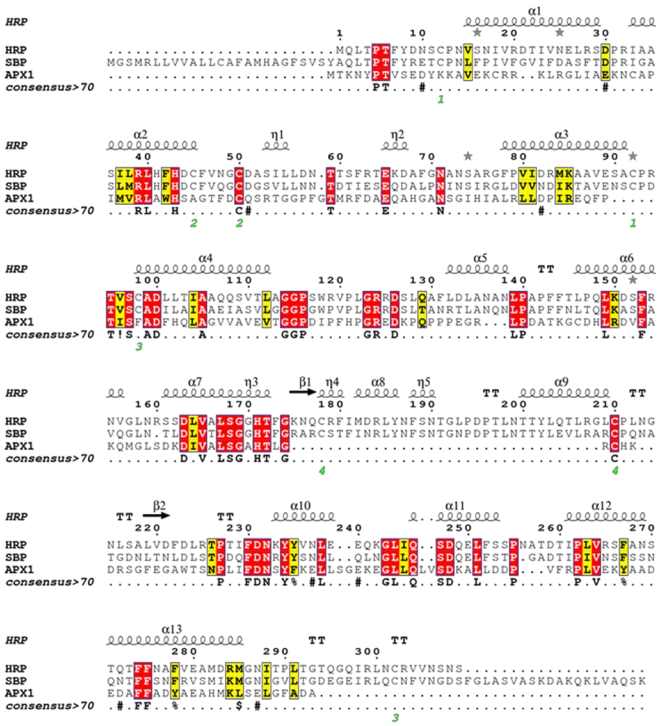
ROS signals were monitored using a luminol-based chemiluminescent assay. Signals were recorded for 21 h after treatment with 50 µg/mL LPS. ROS kinetics (**A** and **B**) and total ROS levels within 1–21 h (**C** and **D**) were compared between wild-type and the indicated mutants. Data are shown as the mean ± SE (n = 8 in **A** and **C**; 12 in **B** and **D**). Means with different letters are significantly different ($P \leq 0.05$, one-way ANOVA), whereas those with the same letters are not. All experiments were repeated three times with similar results.



Supplemental Figure S9. Chloroplasts of *delt4* mutants are not impaired after LPS treatment

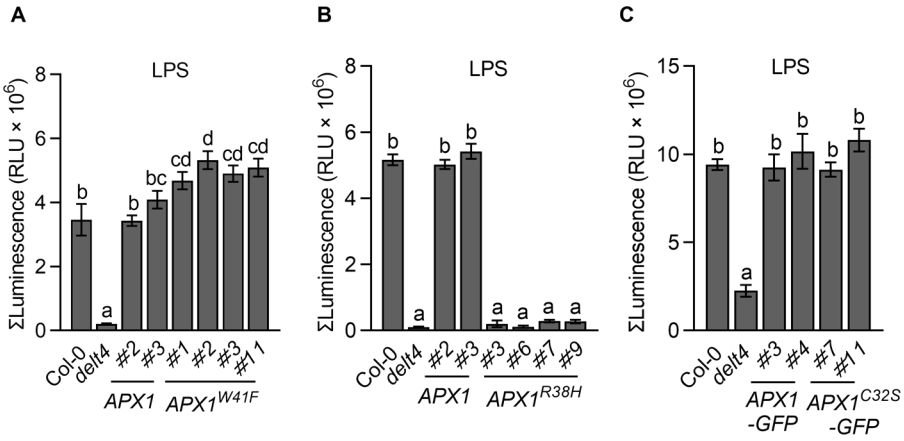
A. *delt4* mutants showed a chloroplast structure similar to that of the wild-type. Four-week-old leaves of Col-0 and *delt4* mutants were harvested 4 h after infiltration with LPS (100 µg/mL) or ddH_2O, and fixed and sectioned for observation by transmission electron microscopy. Two leaves were prepared for each treatment, with three sections for each leaf. Representative images of the ultrastructure of chloroplasts are shown. G, granum; St, chloroplast stroma; S, starch; V, vacuoles; CW cell wall. Scale bars, 1 µm.

B. *delt4* mutants did not exhibit the release of the small subunit of ribulose-1.5-bisphosphate carboxylase (SSU) proteins from the chloroplasts before and after treated with LPS (100 μg/mL) or ddH_2O for 4 h. Confocal microscopy shows signals from green fluorescent protein (GFP)-tagged SSU (SSU-GFP) proteins and chlorophyll (Chl) autofluorescence. Scale bars, 5 μm.



Supplemental Figure S10. Sequence alignment of APX1 with horseradish peroxidase (HRP) and soybean peroxidase (SBP)

Sequence alignment was performed using the web server, ESPript 3.0 (http://espript.ibcp.fr). Residues conserved between groups are indicated by bold white letters in red boxes (e.g., R38). Residues conserved within a group while significantly different between groups are indicated by bold black letters on a yellow background (e.g., W41). The symbols above the blocks of sequences correspond to the secondary structure of the HRP protein.



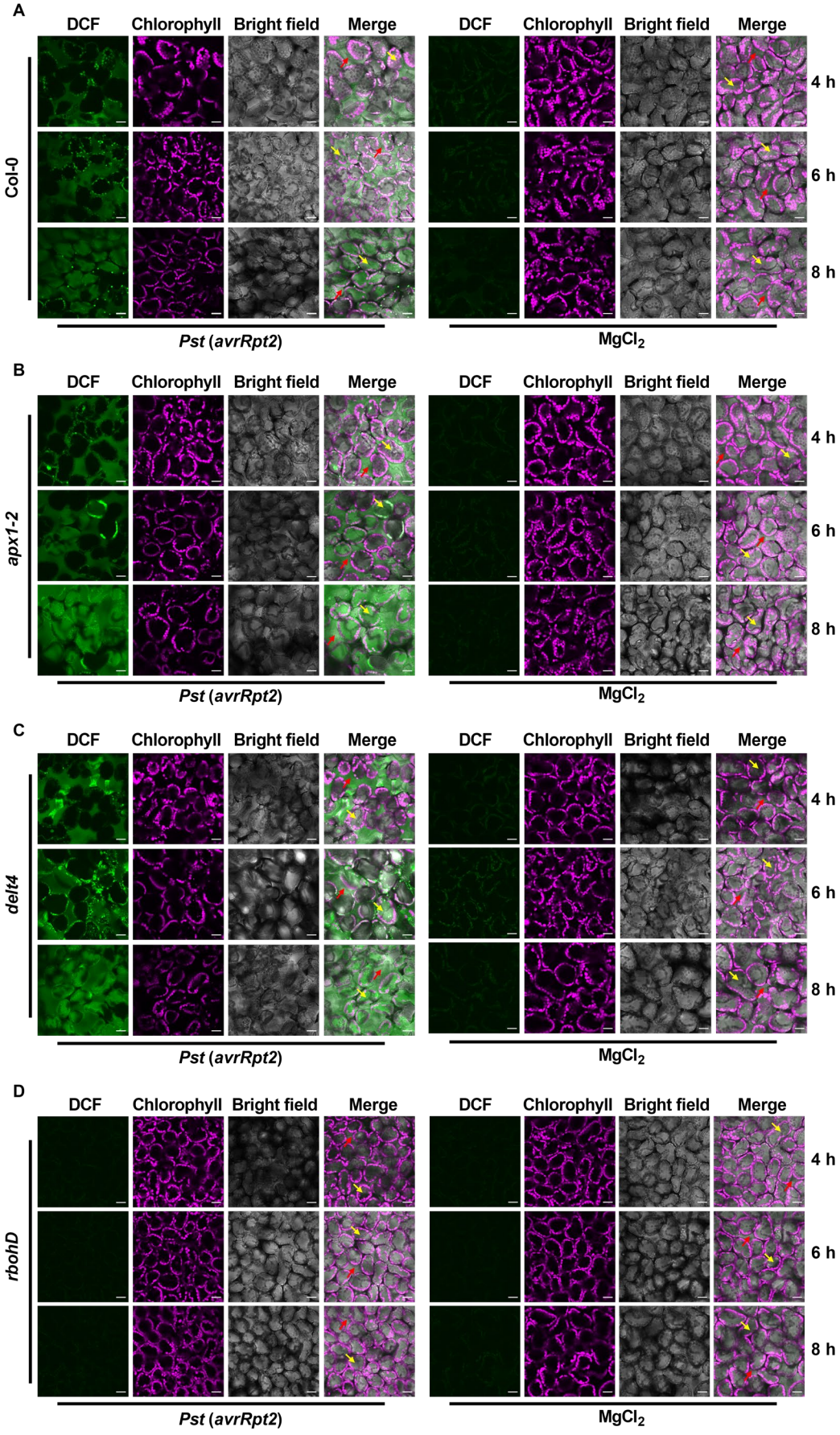
Supplemental Figure S11. Effect of *APX1* mutation on luminol-based light signals triggered by LPS

A. Transformation of *APX1*^{W41F} complemented the luminol-emitted long-lasting light signals of *delt4* mutants in response to LPS. The bar graph indicates the total integrated photon counts within 1-21 h using a luminol-based chemiluminescent assay after LPS (50 μg/mL) treatment.

B. Transformation *APX1*^{R38H} failed to complement the luminol-emitted long-lasting light signals of *delt4* mutants in response to LPS. The experimental conditions were similar to those used in **A**.

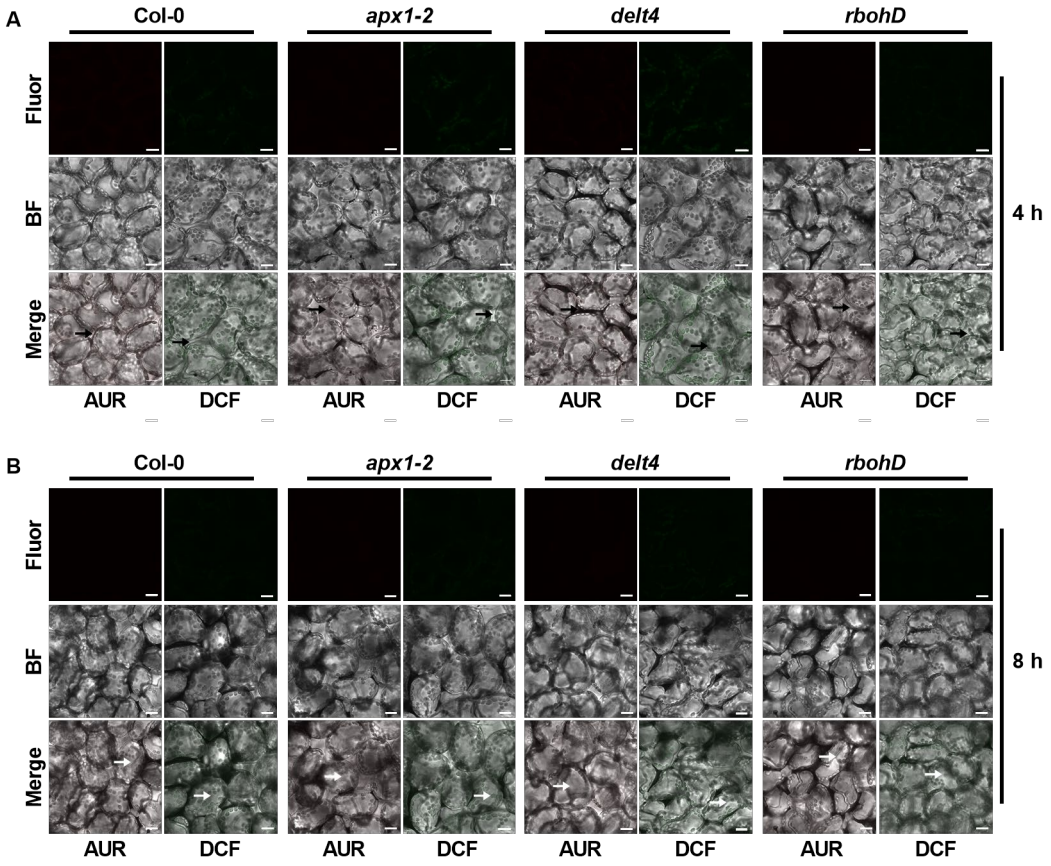
C. Transformation of *APX1*^{C32S} complemented the luminol-emitted long-lasting light signals of *delt4* mutants in response to LPS. The experimental conditions were similar to those used in **A**.

Data are shown as the mean ± SE (n = 8 in A, B, C). Different letters above the bars indicate significant differences between different genotypes ($P \leq 0.05$, one-way ANOVA). All experiments in this figure were repeated three times with similar trends.


A
DCF
Chlorophyll
Bright field
Merge
DCF
Chlorophyll
Bright field
Merge
Col-0
4 h
6 h
8 h
Pst (avrRpt2)
MgCl2
B
DCF
Chlorophyll
Bright field
Merge
DCF
Chlorophyll
Bright field
Merge
apx1-2
4 h
6 h
8 h
Pst (avrRpt2)
MgCl2
C
DCF
Chlorophyll
Bright field
Merge
DCF
Chlorophyll
Bright field
Merge
delt4
4 h
6 h
8 h
Pst (avrRpt2)
MgCl2
D
DCF
Chlorophyll
Bright field
Merge
DCF
Chlorophyll
Bright field
Merge
rbohD
4 h
6 h
8 h
Pst (avrRpt2)
MgCl2

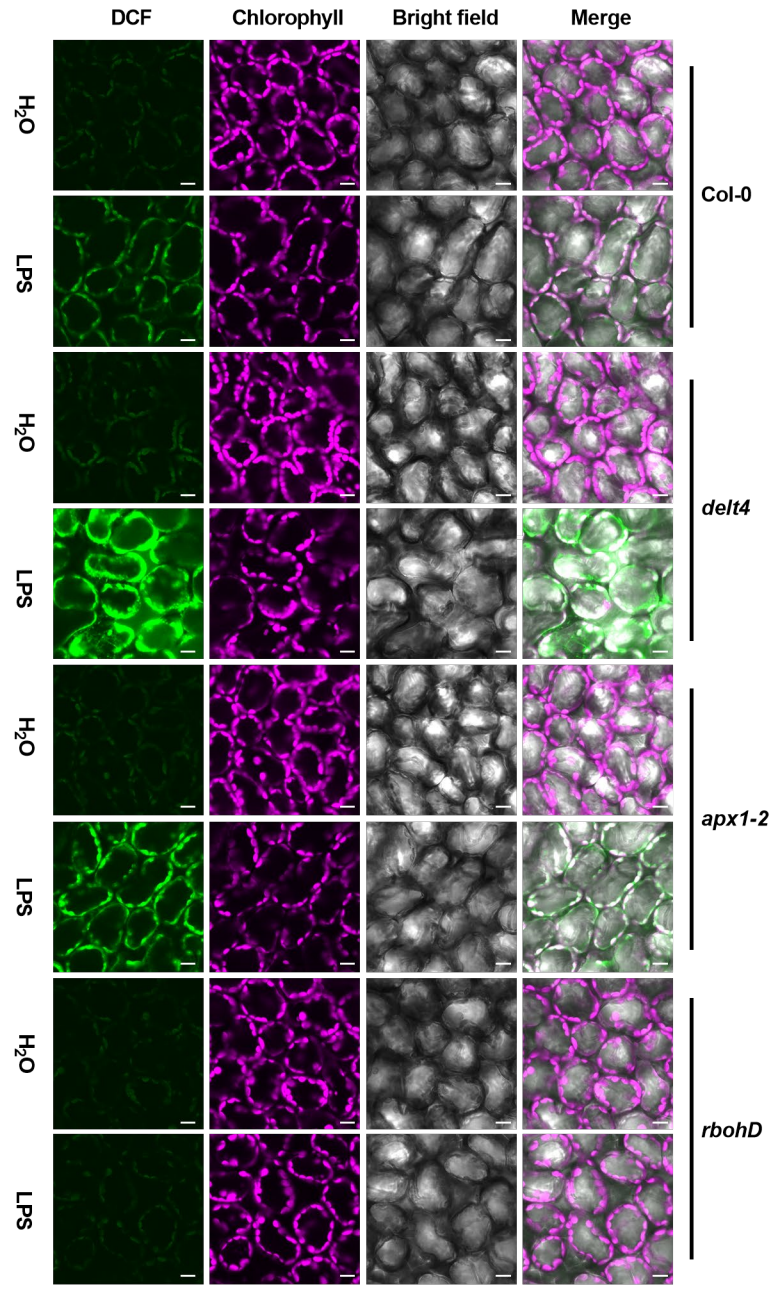
Supplemental Figure S12. The temporal and spatial ETI-ROS detected by H_2DCFDA staining

ROS burst was detected in four-week-old Col-0 (**A**), *apx1-2* (**B**), *delt4* (**C**) and *rbohD* (**D**) plants at 4, 6, 8 h after bacterial infiltration. The abaxial side of leaves of the indicated plants were infiltrated with *Pst* (*avrRpt2*) at an OD_{600} of 0.03, with $MgCl_2$ as the control. Before ROS observation, the H_2DCFDA solution (10 μM) was infiltrated into leaves for 10-15 min. ROS fluorescence (green) and chlorophyll autofluorescence (magenta) were analyzed under a confocal microscope. At least six leaves were observed for each treatment, and representative images were shown. Red arrowheads indicate the apoplast space, while yellow arrowheads indicate the cytoplasm. Scale bars, 20 μm. The experiment was repeated three times with similar results.



Supplemental Figure S13. Detection of ROS accumulation in leaves infiltrated with $MgCl_2$ by AUR and H_2DCFDA staining.

The abaxial side of four-week-old leaves from Col-0, *apx1-2*, *delt4* and *rbohD* plants were infiltrated with $MgCl_2$, which was used as the control for *Pst* (*avrRpt2*) in Figure **7**, and the experimental conditions were same as those used in Figure **7**. Fluorescence was observed at 4 (**A**) and 8 h (**B**) after infiltration. Before microscopic observation, leaves were infiltrated with a solution of AUR or H_2DCFDA and incubated for 15-30 min. At least six leaves were observed for each treatment, and representative images were shown. Black arrowheads indicate the apoplast space, while white arrowheads indicate the cytoplasm. Scale bars, 15 μm. Fluor, Fluorescence; BF, Bright Field. The experiment was repeated three times with similar results.



Supplemental Figure S14. *apx1* mutants show increased cytosolic ROS accumulation after LPS treatment, as detected by H_2DCFDA staining

The seventh to ninth leaves of four-week-old Col-0, *delt4*, *apx1-2*, and *rbohD* plants were infiltrated with 100 μg/mL LPS, with ddH_2O as the control. ROS was detected at 4 h post infiltration. Before detection under confocal microscopy, the collected leaves were immersed in 10 μM H_2DCFDA solution for at least 30 min and then washed with ddH_2O several times. Green colors indicate ROS fluorescence, and magenta colors indicate chlorophyll autofluorescence. At least six leaves were observed for each treatment, and representative images were shown. Scale bars, 10 μm. The experiment was repeated three times with similar results.

Supplemental Table S1. Primers used in this study

Primer	Sequences (5' to 3')	Experiments
Salk_000249-LP	CCACCCTGGAAGAGAGGTTAG	Genotyping
Salk_000249-RP	CAACGGATGTGTTCAAATCG	Genotyping
Salk_091880-LP	GCTTTCATTTGTTTACCATC	Genotyping
Salk_091880-RP	GAATTGGACATGAGCTGAATTAG	Genotyping
Salk_057686-LP	TGGTCCTCCAGTGATCTCAAC	Genotyping
Salk_057686-RP	CACGTGGTGTGTATCTGTTGG	Genotyping
Salk_057998-LP	AGAGGCAAGATATCCTCAGGC	Genotyping
Salk_057998-RP	TCTGGTGCTCCTGTATGGAAC	Genotyping
Salk_092911-LP	TAACCGGAGTTTGAACACCAG	Genotyping
Salk_092911-RP	TCGATTGTTTAGACGTCCGAC	Genotyping
AvrRpt2-F	ATGAAAATTGCTCCAGTTGCC	Genotyping
AvrRpt2-R	GCGGTAGAGCATTGCGTGTGG	Genotyping
rps2-F	AGGTTTAGGCGTCGGGAACAGA	Genotyping
rps2-R	GCTAGTGGCAATCCTCCACA	Genotyping
delt4-F	CACACTCTGGTTCGTTTCTCTC	Genotyping
delt4-R	GATGTGTTCAAATCGCAACC	Genotyping
T1G11-F	AATTGCATAAGGCACTTGAAAG	Genetic mapping
T1G11-R	GAAGACAAAGCTCTGCAGTAATG	Genetic mapping
SRP54A-F	AAAAGGAACCCTACCAAAAACA	Genetic mapping

SRP54A-R	TGAATTATGGAATCAATGTTCG	Genetic mapping
SGCSNP170-F	CACCAAGAGGAGAAGAGGAGGA	Genetic mapping
SGCSNP170-R	AAACCAACAACCAAGCCTTG	Genetic mapping
PHYA-F	CGTCATGCAAACTATCAGTGCTC	Genetic mapping
PHYA-R	GATTACTCAACCTCAGTGCG	Genetic mapping
F10K1-F	ATCATAGCAAAGTTGCTGGTCA	Genetic mapping
F10K1-R	TCTCTACGGCTCTTTCCCTT	Genetic mapping
F22O13-F	GTCTTGATTCTATTGGTCATTGTGC	Genetic mapping
F22O13-R	CGTCTAATGCTTCGATAGCCTGT	Genetic mapping
NGA111-F	TGTTTTTTAGGACAAATGGCG	Genetic mapping
NGA111-R	CTCCAGTTGGAAGCTAAAGGG	Genetic mapping
NGA280-F	CTGATCTCACGGACAATAGTGC	Genetic mapping
NGA280-R	GGCTCCATAAAAAGTGCACC	Genetic mapping
CIW1-F	ACATTTTCTCAATCCTTACTC	Genetic mapping
CIW1-R	GAGAGCTTCTTTATTTGTGAT	Genetic mapping
CIW12-F	AGGTTTTATTGCTTTTCACA	Genetic mapping
CIW12-R	CTTTCAAAAGCACATCACA	Genetic mapping
F21M12-F	GGCTTTCTCGAAATCTGTCC	Genetic mapping
F21M12-R	TTACTTTTTGCCTCTTGTCATTG	Genetic mapping
APX1-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAC CACGACTTCCGTCGAGTAGATTC	Gene cloning

APX1-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCA GCATCAGCAAACCTGCAACCAAC	Gene cloning
APX1-CDS-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAC ATGACGAAGAACTACCCAAC	Gene cloning
APX1-CDS-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCA GCATCAGCAAACCCAAGCT	Gene cloning
gAt1g07700-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAC GGCTGACAAAGTCGATACCA	Gene cloning
gAt1g07700-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTA ACCAAAACTAGTAAGCTG	Gene cloning
APX1^{C32S}-F	ATCGCTGAGAAGAACTCTGCACCCATC ATGGTC	Gene cloning
APX1^{C32S}-R	GACCATGATGGGTGCAGAGTTCTTCTC AGCGAT	Gene cloning
cAPX1^{W41F}-F	TCCGACTCGCATTCCACTCTGCT	Gene cloning
cAPX1^{W41F}-R	AGCAGAGTGGAATGCGAGTCGGA	Gene cloning
cAPX1^{R38H}-F	ATCATGGTCCATCTCGCATGGCA	Gene cloning
cAPX1^{R38H}-R	TGCCATGCGAGATGGACCATGAT	Gene cloning
gAPX1^{W41F}-F	TGGCTAAACTGCAGATTCCACTCTGCTGGAAC	Gene cloning
gAPX1^{W41F}-R	GTTCCAGCAGAGTGGAATCTGCAGTTTAGCCA	Gene cloning
gAPX1^{R38H}-F	ATCATGGTCCATCTCGCGTAAG	Gene cloning
gAPX1^{R38H}-R	CTTACGCGAGATGGACCATGAT	Gene cloning
Actin7-F	ATGGCCGATGGTGAGGATAT	RT-PCR
Actin7-R	TCTGCGGTAGTGGTGAACAT	RT-PCR

EF-1α-F	TGAGCACGCTCTTCTTGCTTTCA	RT-qPCR
EF-1α-R	GGTGGTGGCATCCATCTTGTTACA	RT-qPCR
APX1-F	GTCCATTCGGAACAATGAGGTTTGAC	RT-qPCR
APX1-R	GTGGGCACCAGATAAAGCGACAAT	RT-qPCR
GPX1-F	GTCTCCGGTAACCAAAAATG	RT-qPCR
GPX1-R	GACGAGAAAGGTTGCTGAGG	RT-qPCR
GPX2-F	AAACTGCGTTGGGACAGG	RT-qPCR
GPX2-R	CCCATGAAAAGACATCGAATAC	RT-qPCR
GPX3-F	GGGTCAATCAGCGAGCTAC	RT-qPCR
GPX3-R	CGATGGCGAAGAAGGGTATC	RT-qPCR
GPX7-F	TCGGCCCATCATTGAGATTC	RT-qPCR
GPX7-R	CTGCAGCCCTTGCATAGAC	RT-qPCR
APX2-F	CTGGTGGACACACCTTGGG	RT-qPCR
APX2-R	GCAGCATATTTTTCAACAAATGGGA	RT-qPCR
APX6-F	CGGCCCAACAATTCCAGTAGT	RT-qPCR
APX6-R	GAGGTAGCTTGCCTTCTGGATC	RT-qPCR
CAT1-F	ATGACTCGATCGCAGCCG	RT-qPCR
CAT1-R	TCTTCATGGGCAGGATCCAT	RT-qPCR
CAT2-F	TGTCCGGTTCTCCACCGTTA	RT-qPCR
CAT2-R	AACCACGAGGGTCTCTCAAGGT	RT-qPCR
CAT3-F	CTACATGTCCCACTTGCCTG	RT-qPCR

CAT3-R	CTCGGGGTAGTTGCCAGAT	RT-qPCR

Supplemental Table S2. Constructs used in this study

Construct name	Vectors	Usage
pAPX1::APX1	PGWB1	Transgenic plants
pAPX1::APX1-GFP	PGWB4	Transgenic plants
pAPX1::APX1^{W41F}	PGWB1	Transgenic plants
pAPX1::APX1^{R38H}	PGWB1	Transgenic plants
pAPX1::APX1^{C32S}-GFP	PGWB4	Transgenic plants
35S::At1g07700-HA	PGWB14	Transgenic plants
MBP-APX1	pMAL-c2x-GW	Prokaryotic expression
MBP-APX1^{W41F}	pMAL-c2x-GW	Prokaryotic expression
MBP-APX1^{R38H}	pMAL-c2x-GW	Prokaryotic expression
MBP-APX1^{C32S}	pMAL-c2x-GW	Prokaryotic expression