Supplementary information

The malectin-like receptor-like kinase LETUM1 modulates NLR protein SUMM2 activation via MEKK2 scaffolding

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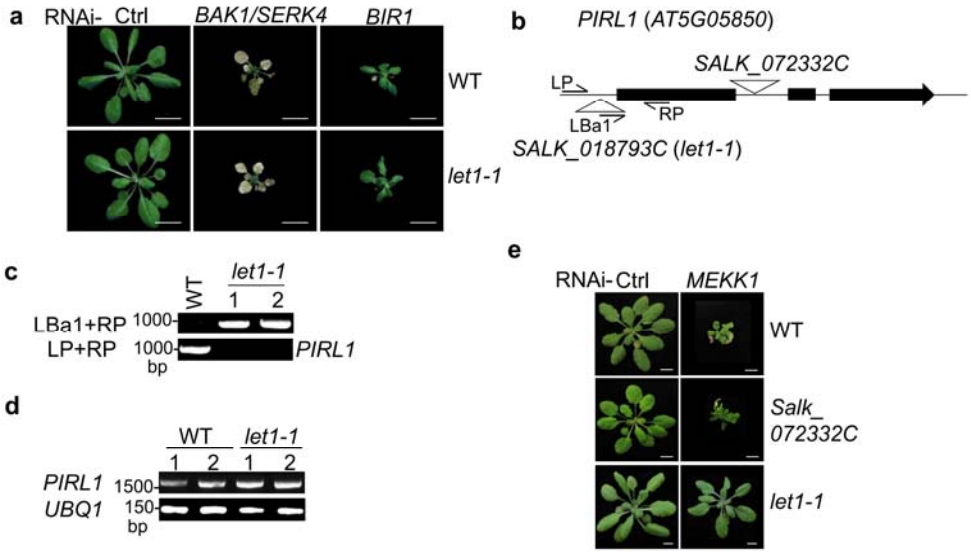
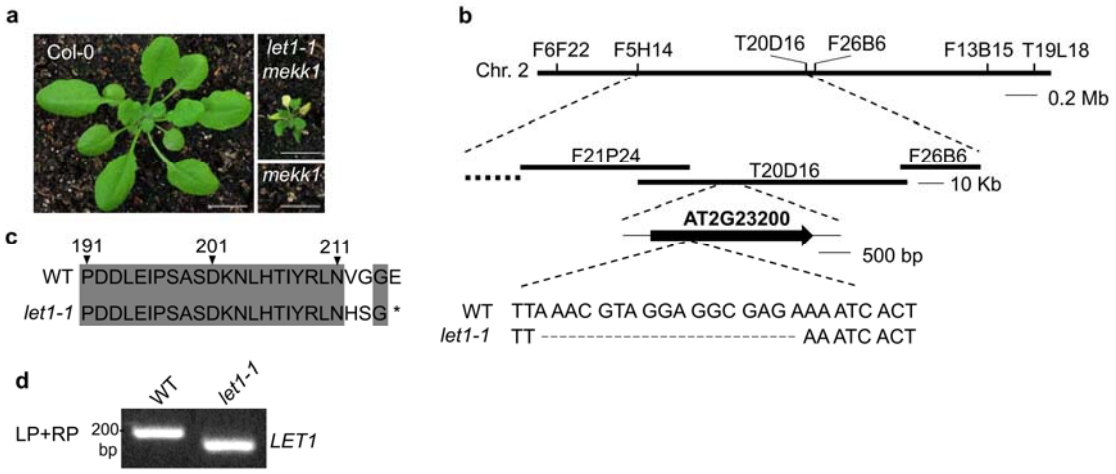


Supplementary Fig. 1 LET1 is not involved in RNAi-*BAK1/SERK4* and -*BIR1* cell death, and *PIRL1* is not the causal gene in *let1-1*.

a, The *let1-1* mutant does not affect plant growth defects caused by RNAi-*BAK1/SERK4* and -*BIR1*. Silencing *BAK1/SERK4* by VIGS caused severe growth defects and leaf chlorosis to a similar level in WT and *let1-1*. Silencing *BIR1* by VIGS triggered plant dwarfism and curling leaves similarly in WT and *let1-1*. Plants of WT and *let1-1* are shown three weeks after the inoculation of *Agrobacterium* carrying the VIGS vector (Ctrl), VIGS-*BAK1/SERK4*, or VIGS-*BIR1*. Scale bar, 1 cm. **b,** Schematic diagram of *PIRL1* with annotated T-DNA insertions in different SALK line mutants. Solid bars indicate exons, and lines indicate introns. Arrows indicate the primers used for genotyping PCR analysis in C. **c,** PCR confirmation of homozygous T-DNA insertion at *PIRL1* in *let1-1*. The primer pair of LP and RP amplified the genomic DNA fragment of *PIRL1* and the primer pair of LBa1 and RP amplified the T-DNA insertion. 1 and 2 are two individual plants. **d,** The *let1-1* mutant does not affect the transcript level of *PIRL1*. The transcripts of *PIRL1* from WT and *let1-1* cDNA were PCR-amplified. *UBQ1* was used as an internal control. **e,** Unlike *let1-1*, SALK_072332C, another T-DNA insertion allele of *PIRL1,* did not suppress growth defects by RNAi-*MEKK1*. Plant phenotypes are shown three weeks after VIGS of *MEKK1*. Scale bar, 1 cm.

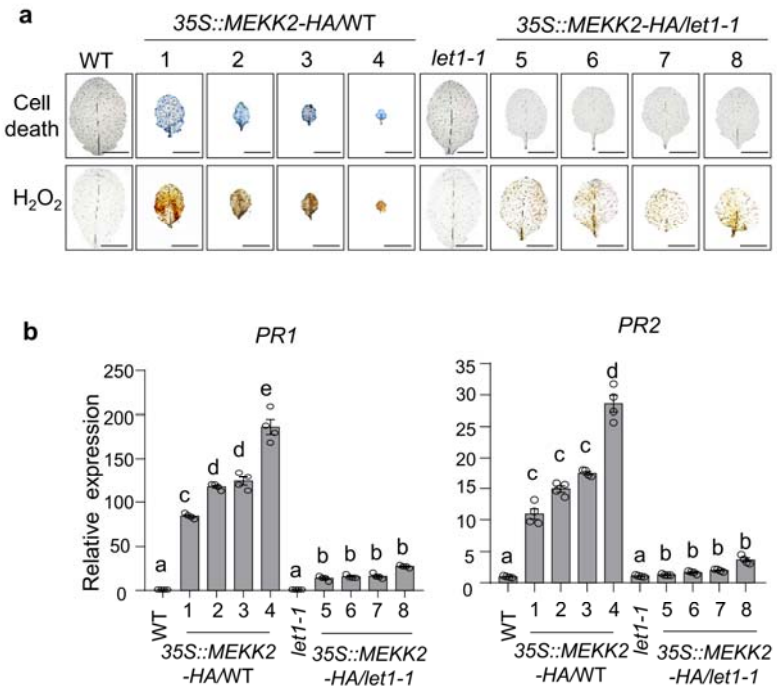
The above experiments were repeated at least three times with similar results.



Supplementary Fig. 2 Map-based cloning of *LET1*.

a, The *let1-1* mutant partially suppresses *mekk1* growth defects when plants are grown on soil. In the *mekk1/let1-1* F_2 population grown on soil, at three-week-old stage, in addition to WT- and *mekk1*-looking plants, ~1/16 plants showed dwarfism and early senescence, which are likely resulted from the partial suppression of *mekk1* cell death by the *let1* mutation. PCR genotyping for *mekk1* confirmed those plants were *mekk1* homozygous. **b,** Schematic diagram of markers on Chromosome 2 for map-based cloning. *AT2G23200* was identified as the causal gene with a 17-bp deletion in *let1-1* by next-generation sequencing and confirmed by Sanger-sequencing. **c,** The 17-bp deletion results in a truncated protein with an early stop codon (*) in the annotated malectin-like domain of AT2G23200 in *let1-1*. Partial amino acid sequences of WT and *let1-1* close to the deletion are shown, and the amino acid positions are labeled on the top. **d,** PCR fragments using primers spanning the deletion site (203 bp in total) of *LET1* are separated by agarose gel electrophoresis. The picture shows that the PCR fragment in *let1-1* is smaller than that in WT, confirming the deletion in *let1-1*.

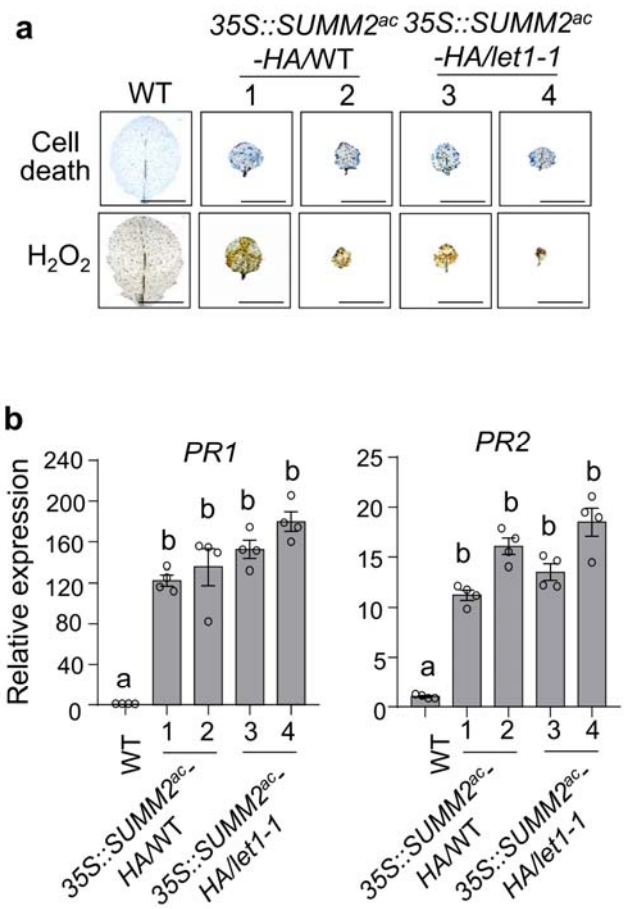
The experiment in d was repeated at least three times with similar results.



Supplementary Fig. 3 The *let1-1* mutant suppresses autoimmunity triggered by overexpression of *MEKK2*.

a, Overexpression of *MEKK2* triggers cell death and H_2O_2 accumulation in WT, which are reduced in *let1-1*. Cell death and H_2O_2 are shown with true leaves of four-week-old plants stained with trypan blue and DAB, respectively. Scale bar, 0.5 cm. **b,** Elevated *PR1* and *PR2* expression caused by overexpression of *MEKK2* in WT is ameliorated in *let1-1*. The expression of *PR1* and *PR2* in four-week-old plants was normalized to the expression of *UBQ10*. The data are shown as mean ± SE from four independent repeats. The different letters denote statistically significant differences according to one-way ANOVA followed by the Tukey test (P<0.05).

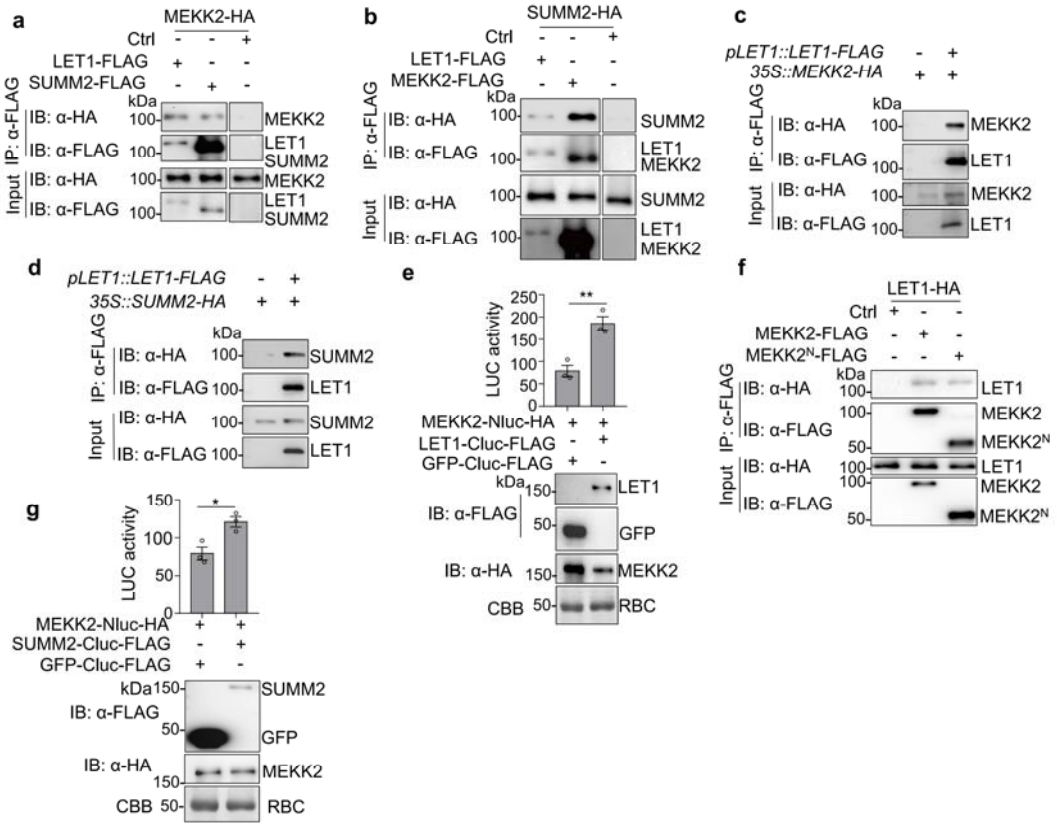
The above experiments were repeated three times with similar results.



Supplementary Fig. 4 The *let1-1* mutant does not suppress autoimmunity triggered by overexpression of *SUMM2^{ac}*.

a, Overexpression of the active form of SUMM2 (*SUMM2^{ac}*) triggers cell death and H_2O_2 accumulation to a similar level in WT and *let1-1*. Cell death and H_2O_2 are shown with true leaves of four-week-old plants stained by trypan blue (upper panel) and DAB (lower panel), respectively. Scale bar, 0.5 cm. **b,** Overexpression of *SUMM2^{ac}* induces *PR1* and *PR2* expression to a similar level in WT and *let1-1*. The expression of *PR1* and *PR2* in four-week-old plants was normalized to the expression of *UBQ10*. The data are shown as mean ± SE from four independent repeats. The different letters denote statistically significant differences according to one-way ANOVA followed by the Tukey test (P<0.05).

The above experiments were repeated three times with similar results.

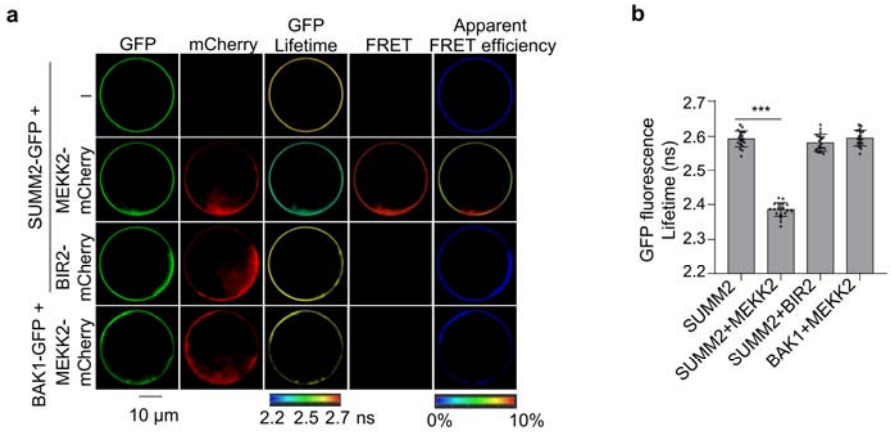


Supplementary Fig. 5 MEKK2, SUMM2 and LET1 associate in a complex.

a, MEKK2 associates with LET1 and SUMM2. *MEKK2-HA* was co-expressed with *LET1-FLAG* or *SUMM2-FLAG* in WT Col-0 protoplasts. Total proteins were immunoprecipitated with α-FLAG affinity beads and then immunoblotted by an α-HA or α-FLAG antibody (top two panels). Proteins before immunoprecipitation are shown as input controls (bottom two panels). **b,** SUMM2 associates with LET1 and MEKK2. *SUMM2-HA* was co-expressed with *LET1-FLAG* or *MEKK2-FLAG* in protoplasts. Co-IP and IB were done as in **a**. **c,** LET1 associates with MEKK2 in transgenic plants. The *pMDC-35S::MEKK2-HA* construct was transformed into WT or *pCB302-pLET1::LET1-FLAG* transgenic plants. Total proteins were extracted from leaves of four-week-old plants. Co-IP and IB were performed as in **a**. **d,** LET1 associates with SUMM2 in transgenic plants. The *pMDC-35S::SUMM2-HA* construct was transformed into WT or *pCB302-pLET1::LET1-FLAG* transgenic plants. Total proteins were extracted from leaves of four-week-old plants. Co-IP and IB were performed as in **a**. **e,** LET1 associates with MEKK2 with a split-luciferase assay. The LET1-Cluc-FLAG or GFP-Cluc-FLAG (Ctrl) was co-expressed with MEKK2-Nluc-HA in protoplasts, and luciferase activities were detected 12 hr after transfection. Expression of the

corresponding proteins is shown on the bottom with immunoblots. Data are shown as mean ± SE from three independent repeats, $P = 5.90 \times 10^{-3}$. The asterisk indicates statistical significance by using two-sided two-tailed Student's *t*-test (** $P<0.01$). **f,** LET1 associates with the N-terminal MEKK2 (MEKK2^{N}). LET1-HA was co-expressed with MEKK2-FLAG or MEKK2^{N}-FLAG in protoplasts. Co-IP and IB were done as in **a**. **g,** SUMM2 associates with MEKK2 with a split-luciferase assay. The SUMM2-Cluc-FLAG or GFP-Cluc-FLAG (Ctrl) was co-expressed with MEKK2-Nluc-HA in protoplasts. Assays were done as in **e**. Data are shown as mean ± SE from three independent repeats, $P = 1.98 \times 10^{-2}$. The asterisk indicates statistical significance by using two-sided two-tailed Student's *t*-test (* $P<0.05$).

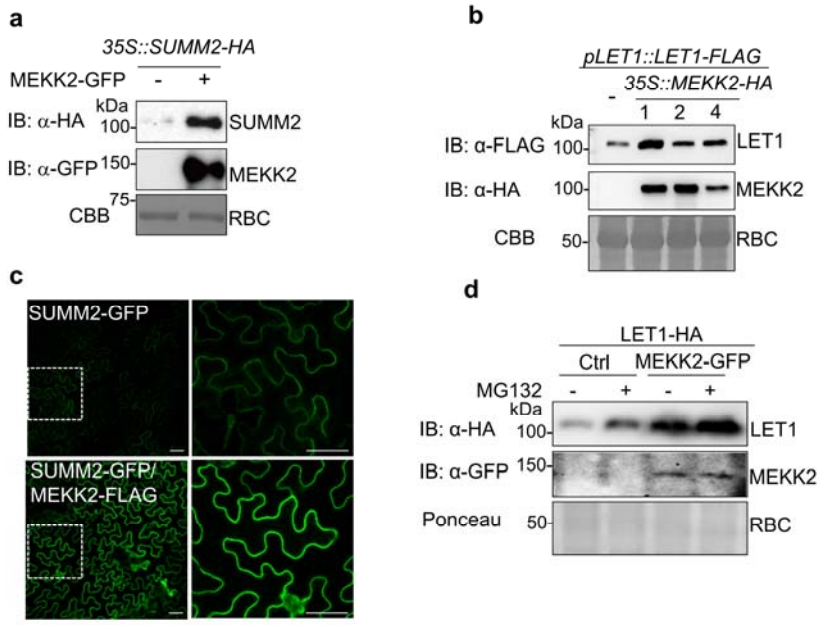
The above experiments were repeated at least three times with similar results.



Supplementary Fig. 6 FRET-FLIM analysis of SUMM2 and MEKK2 interaction in *Arabidopsis* protoplasts.

a and **b,** The indicated proteins were transiently expressed in protoplasts for 16 hr, and FRET-FLIM was visualized using a confocal laser scanning microscopy (**a**). Localization of the SUMM2-GFP/BAK1-GFP and MEKK2-mCherry/BIR2-mCherry is shown with the first (Green) and second column (Red), respectively. The lifetime (τ) distribution (third column), FRET (fourth column), and apparent FRET efficiency (fifth column) are presented as pseudo-color images according to the scale. The GFP mean fluorescence lifetime (τ) values, ranging from 2.2 to 2.7 nanoseconds (ns), were statistically analyzed and data are shown as mean ± SE from twenty independent cells (**b**). The pairs of BAK1-GFP/MEKK2-mCherry and SUMM-GFP/BIR2-mCherry were used as a negative control. $P < 1.00 \times 10^{-15}$ (column 1 and 2). Asterisks represent significant differences by using two-sided two-tailed Student's *t*-test (***, $P < 0.001$). Scale bar, 10 µm.

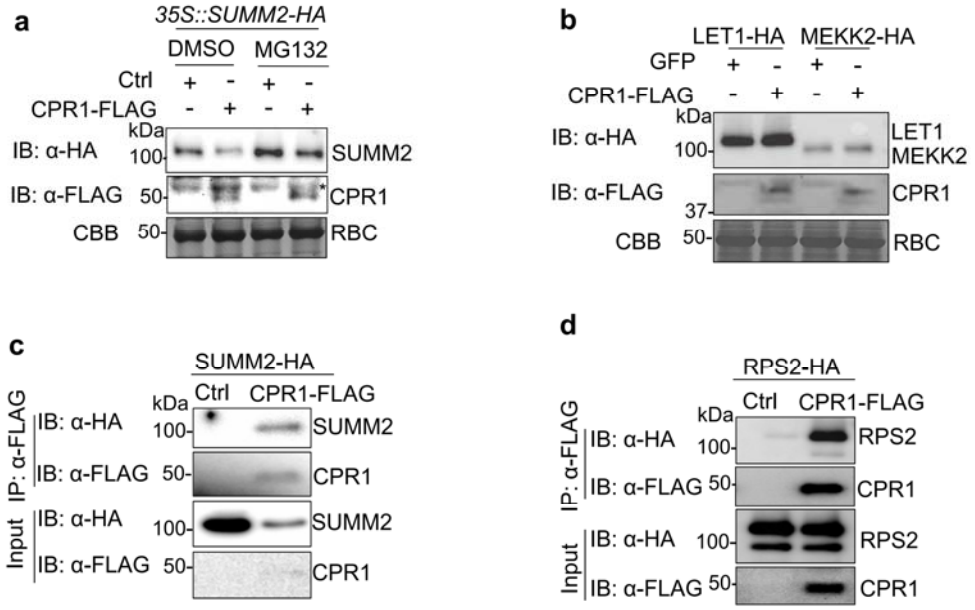
The above experiments were repeated at least three times with similar results.



Supplementary Fig. 7 MEKK2 stabilizes SUMM2 and LET1.

a, MEKK2 stabilizes SUMM2. MEKK2-GFP was expressed in protoplasts from *35S::SUMM2-HA* transgenic plants. Total proteins were immunoblotted with an α-HA antibody for SUMM2-HA or an α-GFP antibody for MEKK2-GFP. CBB was used as a loading control. **b,** MEKK2 enhances LET1 protein accumulation in transgenic plants. The *pMDC-35S::MEKK2-HA* construct was transformed into *pLET1::LET1-FLAG* transgenic plants. LET1 and MEKK2 protein levels were examined by α-FLAG and α-HA antibodies in T_2 generation of *pLET1::LET1-FLAG/35S::MEKK2-HA* transgenic plants (Line #1, #2 and #4). The LET1 protein level in parental *pLET1::LET1-FLAG* line was used as a control (lane 1). The protein loading is shown by CBB staining. **c,** MEKK2 stabilizes SUMM2-GFP in *N. benthamiana*. The Agrobacterial strain GV3101 carrying *35S::SUMM2-GFP* without or with *35S::MEKK2-FLAG* was infiltrated into *N. benthamiana*. Multiple infiltrated leaves were observed with a Leica SP8 confocal microscope two days after infiltration. Images were captured at 472 nm laser. The gating technology was applied to remove autofluorescence. The dashed boxes were expanded in the right panels. Scale bar, 10 μm. **d,** MEKK2 stabilizes LET1 in *N. benthamiana*. LET1-HA was co-expressed with or without MEKK2-GFP in *N. benthamiana* with or without 10 μM MG132 pre-treatment for 2 hr. Total proteins were immunoblotted with an α-HA antibody for LET1-HA or an α-GFP antibody for MEKK2-GFP. Ponceau staining was used as a loading control.

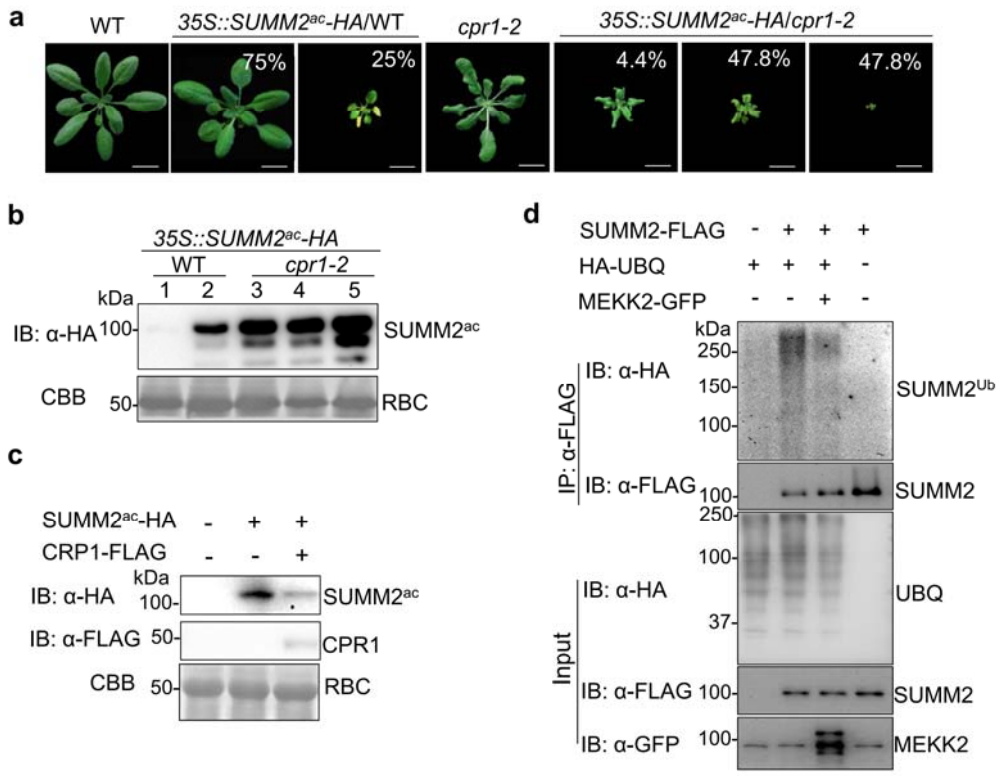
The above experiments were repeated three times with similar results.



Supplementary Fig. 8 CPR1 promotes SUMM2 degradation.

a, MG132 treatment blocks CPR1-mediated SUMM2 degradation. Protoplasts from *35S::SUMM2-HA* transgenic lines were transfected with a vector (Ctrl) or CPR1-FLAG with or without 10 μM MG132 treatment. Total proteins were immunoblotted with an α-HA or α-FLAG antibody to detect SUMM2-HA or CPR1-FLAG, respectively. * indicates nonspecific bands. CBB was used as a loading control. DMSO is the control for MG132. **b,** CPR1 does not affect the protein accumulation of MEKK2 and LET1. LET1-HA or MEKK2-HA was coexpressed with or without CPR1-FLAG in *N. benthamiana*. Total proteins were immunoblotted with an α-HA or α-FLAG antibody. CBB was used as a loading control. **c,** SUMM2 associates with CPR1. SUMM2-HA was co-expressed with or without CPR1-FLAG in *N. benthamiana*. Co-IP and IB were done as in Supplementary Fig. 5a. **d,** RPS2 associates with CPR1. RPS2-HA was coexpressed with or without CPR1-FLAG in WT protoplasts. Co-IP and IB were done as in Supplementary Fig. 5a.

The above experiments were repeated three times with similar results.

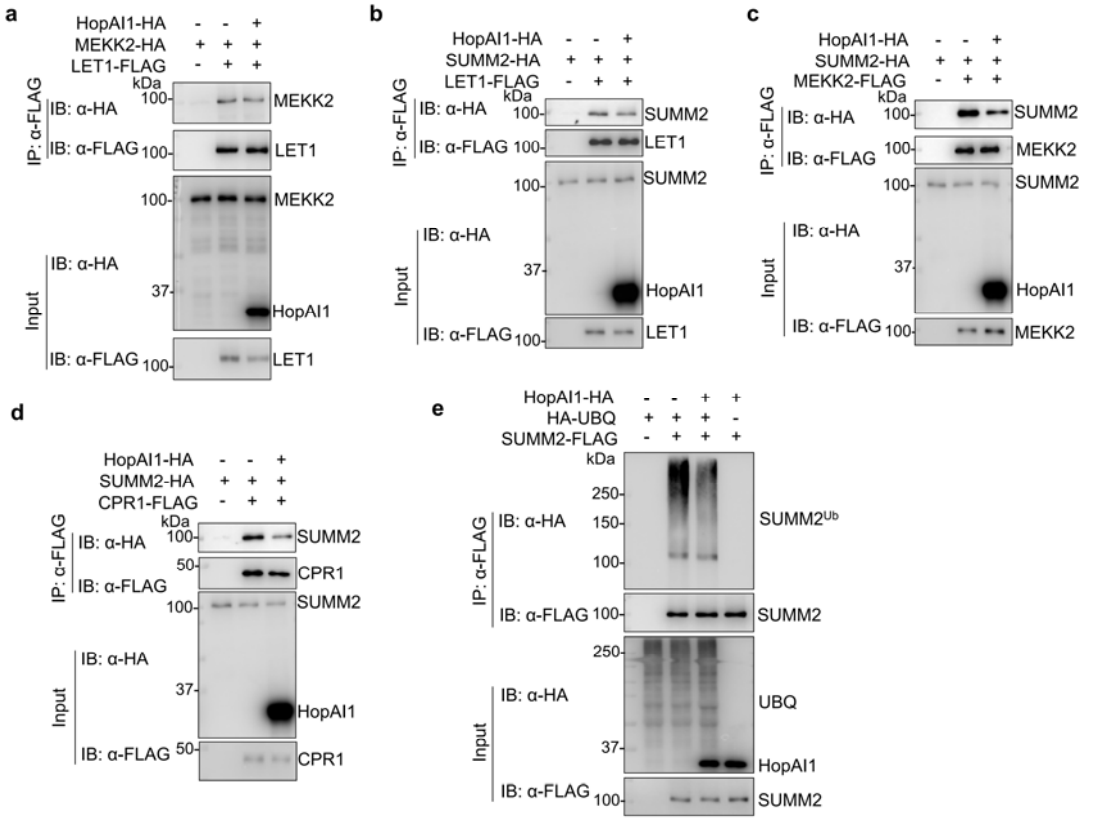


Supplementary Fig. 9 CPR1 promotes SUMM2^{ac} degradation, and MEKK2 blocks SUMM2 ubiquitination.

a, Overexpression of *SUMM2^{ac}* aggravates growth defects in *cpr1-2*. The *35S::SUMM2^{ac}-HA* construct was transformed into WT and *cpr1-2*. Plants were grown at 26 ℃ to reduce the growth defects of *cpr1-2*. About ~25% of *35S::SUMM2^{ac}-HA/*WT transgenic plants showed growth defects and dwarfism (*n*=84). The transgenic plants of *35S::SUMM2^{ac}-HA/cpr1-2* were grouped into three categories (the ratio of each category is indicated) based on the severity of growth defects (*n*=159). Pictures were taken from soil-grown plants four weeks after germination. Scale bar, 1cm. **b,** SUMM2^{ac}-HA proteins accumulate to a higher level in *cpr1-2* than those in WT plants. Transgenic plants in **a** were analyzed using immunoblots with an α-HA antibody to detect SUMM2^{ac}-HA protein, and CBB was used as a loading control. The SUMM2^{ac}-HA protein level is positively correlated with the severity of growth defects of transgenic plants shown in **a**. **c,** CPR1 reduces SUMM2^{ac} protein level. SUMM2^{ac}-HA was co-expressed with or without CPR1-FLAG in *N. benthamiana*, and total proteins were assayed 60 hr after infiltration by immunoblotting with an α-HA or α-FLAG antibody. CBB was used as a loading control. **d,** MEKK2 antagonizes SUMM2 ubiquitination. Protoplasts from WT plants were transfected with SUMM2-FLAG and HA-UBQ

with or without MEKK2-GFP. Total proteins were immunoprecipitated with α-FLAG affinity beads and immunoblotted with an α-HA or α-FLAG antibody (top two panels). Immunoblots using total proteins before immunoprecipitation are shown as protein inputs (bottom three panels). The input for SUMM2 was adjusted to a similar level for Co-IP and IB.

The above experiments were repeated three times with similar results.



Supplementary Fig. 10 HopAI1 regulates SUMM2 association with MEKK2 and CPR1.

a and **b,** HopAI1 doesn't affect the association of LET1 with MEKK2 (**a)** or SUMM2 (**b)**. *MEKK2-HA* or *SUMM2-HA* was co-expressed with *LET1-FLAG* (second lane), or *LET1-FLAG* and *HopAI1-HA* (third lane) in WT Col-0 protoplasts. Total proteins were immunoprecipitated with α-FLAG affinity beads and immunoblotted by α-HA or α-FLAG antibody (top two panels). Proteins before immunoprecipitation are shown as input controls (bottom two panels). **c,** HopAI1 reduces the association of MEKK2 and SUMM2. *SUMM2-HA* was co-expressed with *MEKK2-FLAG* (second lane), or *MEKK2-FLAG* and *HopAI1-HA* (third lane) in protoplasts. Co-IP and IB were done as in **a**. **d,** HopAI1 reduces the association of SUMM2 and CPR1. *SUMM2-HA* was co-expressed with *CPR1-FLAG* (second lane), or *CRP1-FLAG* and *HopAI1-HA* (third lane) in protoplasts. Co-IP and IB were done as in **a**. **e,** HopAI1 reduces SUMM2 ubiquitination. *SUMM2-FLAG* was co-expressed with *HA-UBQ* with or without *HopAI1-HA* in protoplasts. Co-IP and IB were done as in **a**.

The experiments were repeated three times with similar results.

Supplementary Table 1 Primers used in this study.

Cloning primers

Primer	Sequence
BamHI-At2g23200-F	CGGGATCCATGGAGAATTTCTGTTTTCAAGAC
StuI-At2g23200-R	GAAGGCCTTCTTGCATCAGAGATCTTCAACT
BamHI-LET1 CD-F	GGAGGATCCATGGTATTCTTGAAGCGG
XhoI-LET1pro-F	CCGCTCGAGCTCCATGTGCAAAATGTAGATTGTAGACG
BamHI-LET1pro-R	CATGCCATGGATCCAGCTTTGATGCAGAGAAGGAGAAAG
At2g23200-no BamHI-F	CGGTTACTTGGACCCAGAATATCTCC
At2g23200-no BamHI-R	GGAGATATTCTGGGTCCAAGTAACCG
BamHI-MPK4-F	GCCGGATCCATGTCGGCGGAGAGTTGTTT C
StuI-MPK4-R	GAAGGCCTCACTGAGTCTTGAGGATTGAAC
BamHI-MEKK2-F	CGGGATCCATGAAGAAGTCGTCGGATAA
StuI-MEKK2-R	GAAGGCCTTCTACGGATTAGCGGAGATG
StuI-MEKK2N-R	CAGAGGCCTCCAACTGGATAAACATATCTC
BamHI-SUMM2-F	CGGGATCCATGGGAGCTTGTTTAACACTCTCG
StuI-SUMM2-R	GAAGGCCTCCGCACATAACTAACTTGCCATTC
StuI-SUMM2 CC-NBS-R	ACCAGGCCTTTCATTTTCATCTATGAATCC
BamHI-SUMM2 LRR-F	GGAGGATCCAGTAGAGAAAGAGCATTAAG
XhoI-SUMM2pro-F	GGGAAACGACAATCTGAGCTCTATGCCAAGGCTAGTTGT
BamHI-SUMM2pro-R	TGCGGATCCTGCTGGAGATGGAAAGAAG
BamHI-CPR1-F	CGGGATCCATGGCGACGATTCCAATGGA
StuI-CPR1-R	GAAGGCCTTAAGACCAGCTTGAATCCTT
LET1-K516E-F	ACCAAAGCCGCTATCGAACGAGGCAAAACC
LET1-K516E-R	GGTTTTGCCTCGTTCGATAGCGGCTTTGGT
MEKK2 K529M-F	GACTTCTTTGCTGTCATGGAAGTTTCACTTCTT
MEKK2 K529M-R	AAGAAGTGAAACTTCCATGACAGCAAAGAAGTC
SUMM2-D478V-F	GTTAAAATGCATGTTGTGG TTCGG
SUMM2-D478V-R	CCGAACCACAACATGCATTTTAAC
StuI-LET1-CD-F	GAAGGCCTCATGGTATTCTTGAAGCGG
KpnI-LET1-R	CGGGGTACCTCATCTTGCATCAGAGATCT
BamHI-HopAI1-F	CGGGATCCATGCTCGCTTTGAAGCTGAAC
StuI-HopAI1-R	GAAGGCCTGCGAGTCCAGGGCGGTGGC

The restriction enzyme sites are underlined.

Split-luciferase assay primers

Primer	Sequence
pHBT-FLAG-StuI-Cluc-R	GTCGTCCTTGTAGTCAGAAGGCCTCACGGCGATCTTTCCGCCC
pHBT-HA-StuI-Nluc-R	TGGAACGTCGTATGGGTAAGGCCTTCCATCCTTGTCAATCAAG
pHBT-SUMM2-StuI-Nluc-F	GTTAGTTATGTGCGGAGGCCTATGGAAGACGCCAAAAACATAAAG
pHBT-SUMM2-StuI-Cluc-F	GTTAGTTATGTGCGGAGGCCTATGTCCGGTTATGTAAACAATC
pHBT-MEKK2-StuI-Nluc-F	TCCGCTAATCCGTAGAAGGCCTATGGAAGACGCCAAAAACATAAAG
pHBT-LET1-StuI-Cluc-F	AGATCTCTGATGCAAGAAGGCCTATGTCCGGTTATGTAAACAATC
GFP-BamHI-F	TGCGGATCCATGGTGAGCAAGGGCGAGGAG
GFP-SpeI-F	GGACTAGTCTTGTACAGCTCGTCCATG

The restriction enzyme sites are underlined.

Genotyping primers

Primer	Sequence
SALK-52557-LP	AATCGGAACCTCGAGATGAAG
SALK-52557-RP	TTCACTCAAATCCTGACCCTG
SALK_018793-LP	CGAAAACAGAGTCTCCATTGC
SALK_018793-RP	ACAAATCAACTCGGTCCAATG
SALK_072332C-LP	TTGGGAAAATTTAATTATAAGTGCG
SALK_072332C-RP	TGCATGGTTGTATTTGTACGA
SALK_020561C-LP	AAAGTCATCATCGTCAGGCAG
SALK_020561C-RP	GTTTCTCGATGCTTTTGCTTG
SALK_112949C-LP	ACTCATTGTGGATGGAACTCG
SALK_112949C-RP	ATCAGATGTTTACGCATTCGG
SALK_150039-LP	CGCCGGACTAGTCTTATCTCC
SALK_150039-RP	TACATTTTTGCAGCCACTTTG
AT2g23200-genotyping-F	CTCTTTAGCTCTCATCAATGCCA
AT2g23200-genotyping-R	TGTTCCTCGCTGAATCTTTTC
LBb1.3	ATTTTGCCGATTTCGGAAC

qRT-PCR primers

UBQ10-qRT-F	5’-AGATCCAGGACAAGGAAGGTATTC-3’
UBQ10-qRT-R	5’-CGCAGGACCAAGTGAAGAGTAG-3’
PR1-F	5’-GGTTAGCGAGAAGGCTAACTAC-3’
PR1-R	5’-CATCCGAGTCTCACTGACTTTC-3’

PR2-F	5’-GCATTCGCTGGATGTTTTG-3’
PR2-R	5’-CTTCAACCACACCAGCTTGGAC-3’