A trimeric CrRLK1L-LLG1 complex genetically modulates SUMM2-mediated autoimmunity

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Cell death is intrinsically linked with immunity. Disruption of an immune-activated MAPK cascade, consisting of MEKK1, MKK1/2, and MPK4, triggers cell death and autoimmunity through the nucleotide-binding leucine-rich repeat (NLR) protein SUMM2 and the MAPK kinase kinase MEKK2. In this study, we identify a Catharanthus roseus receptor-like kinase 1-like (CrRLK1L), named LETUM2/MEDOS1 (LET2/MDS1), and the glycosylphosphatidylinositol (GPI)-anchored protein LLG1 as regulators of mekk1-mkk1/2-mpk4 cell death. LET2/MDS1 functions additively with LET1, another CrRLK1L, and acts genetically downstream of MEKK2 in regulating SUMM2 activation. LET2/MDS1 complexes with LET1 and promotes LET1 phosphorylation, revealing an intertwined regulation between different CrRLK1Ls. LLG1 interacts with the ectodomain of LET1/2 and mediates LET1/2 transport to the plasma membrane, corroborating its function as a co-receptor of LET1/2 in the mekk1-mkk1/2-mpk4 cell death pathway. Thus, our data suggest that a trimeric complex consisting of two CrRLK1Ls LET1, LET2/MDS1, and a GPI-anchored protein LLG1 that regulates the activation of NLR SUMM2 for initiating cell death and autoimmunity.
Being sessile and lacking the adaptive immunity, plants have evolved two-tiered immune receptors to detect infections. The plasma membrane-associated immune receptors, termed pattern-recognition receptors (PRRs), sense pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), or host-derived danger-associated molecular patterns (DAMPs) that trigger immune responses against a broad spectrum of pathogens, including non-adapted pathogens. PRRs are often receptor-like kinases (RLKs) and receptor-like proteins (RLPs) in plants. The intracellular immune receptors, which are often nucleotide-binding domain leucine-rich repeat proteins (NLRs), recognize directly or indirectly pathogen-delivered effectors and trigger race-specific resistance against adapted pathogens carrying the cognate effectors. The NLR-mediated immune response is usually associated with a rapid and localized cell death at the infection site, known as the hypersensitive response (HR), to restrict pathogen spread.

Plants have evolved a largely expanded number of RLKs. The most well-studied RLKs contain an extracellular leucine-rich repeat (LRR) domain, called LRR-RLKs, which play important roles not only in regulating plant immunity by sensing MAMPs/DAMPs, but also in modulating plant growth and development by perceiving endogenous signals or environmental cues. RLKs with an extracellular malectin-like domain, also called Catharanthus roseus RLK1-like kinases (CrRLK1Ls), have long been known to be key regulators in various developmental processes including cell elongation, polarized growth, and fertilization. Among 17 members in Arabidopsis, FERONIA (FER) is involved in a myriad of biological processes including fertilization, root hair growth, plant hormone signaling, and immunity. AXNUR1 (ANX1) and ANX2, close homologs of FER, play redundant roles in cell wall integrity during pollen tube growth. BUDHA’S PAPER SEAL 1 (BUPS1) and BUPS2 interact with ANX1/ANX2 in plant immunity.

Although the recognition of pathogens by the innate immune system differs, common responses and signaling components converge at multiple levels. The mitogen-activated protein kinase (MAPK or MAP kinase) cascades are among essential modules regulating both PRR and NLR-mediated immune responses in plants. The classical MAPK cascades consist of three sequentially phosphorylated kinases, including MAPK kinase kinases (MAPKKKs, MKKks, or MEKKs), MAPK kinases (MAPKks, or MKks), and MAP kinases (MPKks). Two parallel MAPK cascades, MKK3/5-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4, play important roles in PRR signaling. Plants with deficiency in MEKK1, MKK1/2 or MPK4 display autoimmunodeficiency and are seedling lethal. The autoimmunity in mkk1, mkk1/2, and mpk4 mutants is due to the activation of the NLR protein SUPPRESSOR OF mkk1 mkk2 (SUMM2)-mediated defense. Intriguingly, the MEKK1-MKK1/2-MPK4 cascade negatively regulates another MAPKKK MEK2, which interacts with MPK4, and positively regulates the NLR SUMM2-triggered autoimmunity. Furthermore, the transcript and protein abundance of MEK2 is positively correlated with its ability to trigger autoimmunity. Another kinase, CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASTIC KINASE 3 (CRCK3), which is phosphorylated by MPK4, is also required for SUMM2-activated autoimmunity. Apparently, a PRR-activated MAPK cascade, consisting of MEKK1-MKK1/2-MPK4, functions genetically upstream of SUMM2 in regulating autoimmunity.

To gain insights into the mechanisms underlying SUMM2-mediated defense, which is otherwise suppressed by a PRR-activated MAPK cascade, we deployed a transient RNAi-based genetic screen by virus-induced gene silencing (VIGS) and screened for suppressors of mkk1 cell death from a collection of T-DNA insertion mutants. We identified lethality suppressor of mkk1 1 (letum1 or let1) that largely suppressed the autoimmunity in mkk1, mkk1/2, and mpk4. LET1 is a member of uncharacterized CrRLK1Ls. In this study, we have screened additional CrRLK1Ls and identified LET2/MEDOSI1 (MDS1) in regulating mkk1, mkk1/2, and mpk4 autoimmunity. Both let1 and let2 single mutants suppressed mkk1, mkk1/2, and mpk4 cell death, however, the let1let2 double mutant showed further suppression, indicating the additive function of LET1 and LET2/MDS1 in modulating SUMM2 activation. Interestingly, LET1 and LET2/MDS1 heteromerize, and LET2/MDS1 promotes LET1 phosphorylation, suggesting a phosphoregulation between different CrRLK1Ls. Similar to LET1 and LET2/MDS1, the GPI-anchored protein LLG1, but not LLG2, LLG3, nor LRE, plays a role in modulating mkk1, mkk1/2, and mpk4 cell death, and functions genetically downstream of MEKK2 and upstream of SUMM2. Likely as a co-receptor, LLG1 interacts with the ectodomain of LET1 and likely LET2/MDS1 and mediates LET1/2 transport to the plasma membrane. Thus, our results suggest that a specific trimeric CrRLK1L module consisting of LET1, LET2/MDS1, and the GPI-anchored LLG1 modulates SUMM2-mediated autoimmunity.

Results

The mutations in the CrRLK1L gene, LET2/MDS1, suppress RNAi-MEKK1 cell death. There are 17 CrRLK1L genes in the Arabidopsis genome (Fig. 1a). Among them, LET1 (AT2G23200) was identified as a modulator of autoimmunity in mkk1, mkk1/2, and mpk4. To systematically investigate the CrRLK1L gene family members in this process, we collected the T-DNA insertion lines of individual CrRLK1L genes and determined their roles on silencing MEKK1-triggered cell death through a VIGS approach (Supplementary Fig. 1a). Among 20 T-DNA insertion lines, including the herk1-1the1-4 double mutant, five lines of three genes, AT5G24010 (two lines), AT4G39110 (BUPS1, two lines), and AT2G21480 (BUPS2, one line), do not bear T-DNA insertions in the annotated sites and were characterized as wild type (WT; Supplementary Fig. 1a). It has been shown that the bups mutants have defects in pollen tube growth. The remaining 15 T-DNA insertion lines are homozygous mutants (Supplementary Fig. 1a). Among them, two mutants, SALK_139579 and SALK_066322, but not the other 13 mutants of 12 CrRLK1Ls, suppressed the growth defects and cell death caused by RNAi-MEKK1 (Fig. 1b, c and Supplementary Fig. 1b). SALK_139579 bears a T-DNA insertion in the signal peptide (SP) motif, and SALK_066322 has a T-DNA insertion in the malectin-like domain of AT5G38990, respectively (Fig. 1b and Supplementary Fig. 1a). Since they suppressed RNAi-MEKK1-mediated cell death, AT5G38990 was named as LET2, and the corresponding mutants SALK_139579 and SALK_066322 were named as let2-1 and let2-2.

LET2 has been previously named as MEDOSI1 (MDS1) and is involved in growth responses to metal ions. Notably, LET2/
**Fig. 1** LET2/MDS1 is involved in RNAi-MEK1 cell death. a A phylogenetic tree of CrRLK1L family proteins. The full-length protein sequences were used to generate the phylogenetic tree by the UPGMA method with 1000 bootstrap replicates in MEGA-X. b A schematics depicting LET2/MDS1 protein motifs and T-DNA insertion sites in the let2 mutants. LET2/MDS1 consists of the N-terminal signal peptide (SP), a malectin-like domain, a transmembrane domain (TM), and a cytokinase kinase domain. The arrows indicate the T-DNA insertion sites of the indicated mutant alleles. c The let2 mutants suppress plant dwarfism and leaf chlorosis induced by silencing MEKK1. The plant images were photographed at 3 weeks after inoculation with Agrobacterium carrying the indicated VIGS vectors. Ctrl is the vector containing GFP. Scale bar, 1 cm. d The LET2/MDS1, but not MDS2, MDS3, nor MDS4, is involved in RNAi-MEK1 cell death. The mds1/2/3/4, mds5/2, and mds2/3/4 CRISPR/Cas knockout plants were inoculated with Agrobacterium carrying the indicated VIGS vectors. The plant images were photographed at 3 weeks after inoculation. The chromosome locations and Indels of MDS1, MDS2, MDS3, and MDS4 on individual mutants are shown on the top. Scale bar, 1 cm. e The let2-1 mutant suppresses cell death and H2O2 accumulation triggered by silencing MEKK1. The leaves were detached from plants in c and stained by trypan blue for cell death (left panel) and DAB for H2O2 accumulation (right panel). Scale bar, 0.5 cm. f The let2-1 mutant suppresses the expression of PR genes triggered by silencing MEKK1. The expression of PR1 and PR2 from plants in c was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). P = 3.00 × 10^-14 (PR1, column 1 and 2), P = 1.60 × 10^-7 (PR1, column 3 and 4), P = 4.40 × 10^-14 (PR1, column 2 and 4), and P = 3.00 × 10^-14 (PR2, column 1 and 2), P = 3.45 × 10^-11 (PR2, column 3 and 4), and P = 5.10 × 10^-14 (PR2, column 2 and 4). The different letters indicate a significant difference determined by one-way analysis of variance (ANOVA) followed by the Tukey test (P < 0.05). g Expression of LET2/MDS1 in let2-1 restores the cell death triggered by silencing MEKK1. The plant images were taken at 3 weeks after inoculation with Agrobacterium carrying the indicated VIGS vectors. #1 and #2 are two independent 35S::LET2-HA transgenic lines in let2-1. Scale bar, 1 cm. Protein expression of LET2-HA in transgenic lines is shown on the bottom. The total proteins were immunoblotted by an α-HA antibody (upper panel). Coomassie Brilliant Blue (CBB) staining of Rubisco (RBC) is shown as a loading control (lower panel). The molecular weight (MW) was labeled on the left of immunoblots as kDa. h The LET2/MDS1 kinase mutant cannot complement let2-1. Two lines of transgenic plants carrying the kinase-inactive mutant of LET2KM (K554E) driven by a 35S promoter in let2-1 are shown. Scale bar, 1 cm. Protein expression of LET2KM-HA in transgenic lines is shown on the bottom. i LET2/MDS1 kinase activity in vitro. GST and the LET2/MDS1 cytosolic kinase domain (HIS-SUMO-LET2CD) proteins were purified from E. coli. The LET1 cytokinase kinase domain (HIS-GST-LET1CD) proteins were purified from insect cells. The kinase assay was performed with [γ-32P] ATP. CBB staining was used as a loading control. The above experiments were repeated three times with similar results.

MDS1 belongs to the MDS1-4 subfamily which resides in a tandem repeat region with three additional CrRLK1Ls, MDS2, MDS3, and MDS4 (Fig. 1a, d). MDS3 and MDS4 genes have redundant function in plant growth adaptation upon exposure to excess nickel ions. We tested whether MDS genes also have redundant function in regulating RNAi-MEK1 cell death with the CRISPR/Cas9-generated double, triple, and quadruple mds mutants. The mds1/2/3/4 mutants #1 (mds4GQ) and #2 (mds12NN) contain large deletions from MDS1 to MDS4; the mds1/2/3/4 #3 (mds13CDQ) contains deletions in four individual MDS genes; the mds2/3/4 mutant (mds22cQ1) has Indels in MDS2, MDS3, and MDS4; and the mds1/2 mutant (mds13BS4) has deletions in MDS1 and MDS2 (Fig. 1d). The individual mutants of mds2, mds3, and mds4 did not affect RNAi-MEK1 cell death.
at the 4-week-old stage (Fig. 2d, e). In addition, the double mutant suppressed and with HIS-tagged SUMO enzyme target peptide (HIS-SUMO-(CD) consisting of the juxtamembrane and kinase domains fused let2mekk1 double mutant by crossing the function of mekk1 MPK4 in the (Fig. 2d, e). The let1/2mekk1 mutant was slightly smaller than let2mekk1 (Fig. 2c). These data indicate that let1 and LET2/MS1 are not involved in MPK4-regulated root development.

**LET2/MS1 functions genetically downstream of MEKK2 and upstream of SUMM2.** Since both LET2/MS1 and MEKK2 are required for SUMM2 activation, we tested the genetic relationship of LET2/MS1 with MEKK2 and SUMM2. Overexpression of MEKK2 under a constitutive 35S promoter induced growth defects, cell death, H2O2 accumulation, and expression of PR genes in WT background, which were positively correlated to the MEKK2 protein level (Fig. 3a-d). We have obtained 75 independent transgenic plants carrying 35S::MEKK2-HA at the T1 generation with positive signals by α-HA immunoblots. We further classified them into four categories according to the growth defect severity: 16% (12 out of 75) plants exhibited severe dwarfism and cell death; 25.3% (19 out of 75) showed moderate dwarf and cell death; 26.7% (20 out of 75) exhibited further alleviated dwarfism with relatively big leaves and 32% (24 out of 75) exhibited weak dwarfism (Fig. 3a). We also generated 70 independent transgenic plants at the T1 generation expressing 35S::MEKK2-HA in the let2-1 background with immunoblot positive signals for MEKK2-HA. Overall, the plant dwarfism and growth defects triggered by overexpression MEKK2 in WT were alleviated in let2-1 with 4.3% (3 out of 70) of plants showing severe dwarfism and cell death, 7.1% (5 out of 70) showing moderate dwarfism, 34.3% (24 out of 70) showing weak dwarfism, and 54.3% (38 out of 70) showing slightly smaller size than let2-1 (Fig. 3a). The cell death, H2O2 accumulation, and expression of PR genes caused by overexpression MEKK2 were also reduced in let2-1 compared to WT plants (Fig. 3c, d). Notably, the protein expression level of MEKK2 was similar in let2-1 and WT plants (Fig. 3b). The data indicate that LET2/MS1 is required for overexpressing MEKK2-activated cell death and functions genetically downstream of MEKK2.

It has been reported that the active SUMM2 (SUMM2*), which bears an aspartate-to-valine mutation at the 478th amino acid residue in the methionine-histidine-aspartic acid (MHD) motif triggers cell death in Nicotiana benthamiana. To delineate the genetic relationship of SUMM2 and LET2/MS1 in cell death regulation, we generated 35S::SUMM2ac-HA transgenic plants in WT and let2-1. About 52.9% (36 out of 68) of 35S::SUMM2ac-HA transgenic plants in WT showed growth defects, cell death, H2O2 accumulation, and elevated expression of PR genes (Fig. 3e–h). The 35S::SUMM2ac-HA transgenic plants in let2-1 showed a similar level of plant growth defects and dwarfism with 53.5% (38 out of 71) of plants (Fig. 3e–h). The protein expression level of

**LET1 and LET2/MS1 function additively in modulating mekk1, mkk1/2, and mpk4 cell death.** To genetically confirm the function of LET2/MS1 in mekk1 cell death, we generated the let2mekk1 double mutant by crossing the let2-1 and mekk1**+/−** (mekk1 is heterozygous) mutants. The let2mekk1 double mutant significantly alleviated the growth defects and dwarfism of mekk1 when grown on ½MS plates (Fig. 2a, b). Since the let1mekk1 double mutant also suppressed the growth defects of mekk1, we compared the phenotype of let2mekk1 and let1mekk1. The let2mekk1 mutant was slightly smaller than let1mekk1 at 2-week-old stage (Fig. 2a, b). At the reproductive stage when grown on soil, the let2mekk1 mutant is obviously smaller than let1mekk1, displaying stronger cell death and failing to bolt (Fig. 2c). Interestingly, the let1/2mekk1 triple mutant grew bigger and had more fresh weight than the let1mekk1 and let2mekk1 mutants at both seedling (Fig. 2a, b) and the reproductive (Fig. 2c) stages. The let1/2mekk1 mutant normally bolted and produced seeds (Fig. 2c). These data indicate that let2 suppresses cell death caused by either silencing or mutation of MEKK1, and LET1 and LET2/MS1 function additively in modulating mekk1 cell death.

The MEKK1 pathway is mediated through MKK1/2 and MPK4. Similar as mekk1, the mkk1/2 double mutant and the mpk4 mutant are seedling lethal. We tested whether the let2 mutant interferes with mkk1/2 and mpk4 cell death by generating the let2mkk1/2 double mutant, and the let2mpk4 double mutant. The let2mkk1/2 mutant largely alleviated mkk1/2 cell death (Fig. 2d, e). The let2mkk1/2 quadrapl mutant grew better than let2mkk1/2 and let1mkk1/2 triple mutants, with fewer dead leaves at the 4-week-old stage (Fig. 2d, e). In addition, the let2mpk4 double mutant suppressed mpk4 cell death (Fig. 2f, g). The data indicate that LET2/MS1 functions genetically downstream of MPK4 in the mekk1-mkk1/2-mpk4 cell death pathway. The

let2mpk4, let1mpk4, and let1/2mpk4 mutants were in the ascending order of plant size and fresh weight (Fig. 2f, g), corroborating the notion that LET2/MS1 acts additively with LET1 in modulating the mekk1-mkk1/2-mpk4 cell death. MEKK1 belongs to a tandemly duplicated gene family with MEKK2 and MEKK3. The mekk2 mutant suppressed mekk1, mkk1/2, and mpk4 cell death. Notably, the plant size and fresh weight of let1/2mpk4 were similar with those of mekk2mpk4 (Fig. 2f, g). To dissect whether LET1/2 and MEKK2 function independently or in a same pathway in regulating the mekk1-mkk1/2-mpk4 cell death, we generated the let2let1/2mpk4 quadruple mutant. The plant size and fresh weight of let2let1/2mpk4 are not significantly different from those of let1/2mpk4 or mekk2mpk4 (Fig. 2f, g), suggesting that LET1 and LET2/MS1 function genetically in the same pathway with MEKK2. The mpk4 mutant displays the increased root width, which is independent of MEKK2. Similarly, the increased root width in mpk4 was not suppressed in the let1mpk4, let2mpk4, let1/2mpk4, or let2let1/2mpk4 mutants (Supplementary Fig. 2), suggesting that LET1 and LET2/MS1 are not involved in MPK4-regulated root development.
SUMM2 is comparable in WT and let1–1. The data indicate that LET2/MDS1 is not required for active SUMM2-triggered cell death and might act independently or upstream of SUMM2. Taken together, our results suggest that MEKK2 modulates LET2/MDS1 protein homeostasis and might act independently or upstream of SUMM2.

LET2/MDS1 promotes LET1 phosphorylation and heterimerizes with LET1. Consistent with the genetic data, a co-immunoprecipitation (Co-IP) assay with HA-tagged LET2/MDS1, and FLAG-tagged MEKK2, SUMM2, or MPK4 co-expressing in Arabidopsis protoplasts indicated that LET2/MDS1 associate with MEKK2 and SUMM2, but not MPK4 (Fig. 4a). We observed an increased protein accumulation of LET2-HA when co-expressing with MEKK2-GFP, but not GFP alone, in N. benthamiana (Fig. 4b). Notably, MEKK2 did not affect GFP protein level (Fig. 4b). The data suggest that MEKK2 might stabilize LET2/MDS1 in modulating SUMM2 activation. Consistently, LET2/MDS1 proteins were stabilized by the treatment of MG132, a proteasome-dependent protein degradation inhibitor, in 35S::LET2-HA transgenic plants and in N. benthamiana (Fig. 4c, d). Notably, the effect of MG132 was less pronounced in the presence of MEKK2, suggesting that MEKK2 had a similar effect with MG132 on the stabilization of LET2-HA (Fig. 4d). The defect of MEKK1-MKK1/2-MPK4 pathway induced accumulation of MEKK2 transcripts and proteins47, which might lead to the stabilization of LET2/MDS1. Consistent with this hypothesis, the amount of LET2-HA protein was increased in three independent 35S::LET2-HA transgenic plants upon silencing MEKK1 by VIGS (Fig. 4e). Collectively, these results suggest that MEKK2 modulates LET2/MDS1 protein homeostasis.

Significantly, we observed a mobility shift of LET1 in the presence of LET2/MDS1, but not its kinase mutant LET2KM (Fig. 4f). The mobility shift of LET1 induced by LET2/MDS1 could be removed by the λ-phosphatase treatment (Fig. 4g), suggesting that LET2/MDS1 promotes LET1 phosphorylation in a kinase activity-dependent manner. Apparently, LET2/MDS1 did not induce mobility shift of FER (Supplementary Fig. 3a), and FER also did not affect LET1 mobility (Supplementary Fig. 3b). We also did not observe any mobility shift of LET2/MDS1 in the
Fig. 3 LET2/MDS1 is required for MEKK2-, but not SUMM2ac-mediated autoimmunity. a Plant dwarfsisms and growth defects triggered by overexpressing MEKK2 in WT are alleviated in the let2-1 mutant. 75 and 70 independent primary (T1) transgenic plants carrying 35S::MEKK2-HA in WT and let2-1 were characterized, respectively. Four-week-old plants representing different levels of dwarfsisms labeled with the percentage of the cognate category are shown. Scale bar, 1 cm. b Protein expression of MEKK2-HA in transgenic plants. Total proteins were isolated from plants in a and immunoblotted using an α-HA antibody (top panel). CBB staining for RBC is shown as the loading control (bottom panel). The expression of MEKK2-HA in WT are reduced in the let2-1 mutant. Leaves from plants in a were stained by trypan blue for cell death (upper panel) and DAB for H2O2 (lower panel). Scale bar, 0.5 cm. d The elevated expression of PR1 and PR2 triggered by overexpressing MEKK2 in WT is reduced in let2-1. The expression of PR1 and PR2 was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). The plants 1–4 are 35S::MEKK2-HA/WT, and 5–8 are 35S::MEKK2-HA/let2-1 (a–d). e Plant dwarfsisms and growth defects triggered by overexpressing SUMM2ac are similar in WT and let2-1. In all, 68 and 71 independent primary (T1) transgenic plants carrying 35S::SUMM2ac-HA in WT and let2-1 were characterized respectively. Two representative 3-week-old plants, which showed the growth defects, and their controls, are shown in the figure. Scale bar, 1 cm. f Protein expression of SUMM2ac-HA in transgenic plants. g The cell death and H2O2 accumulation triggered by overexpressing SUMM2ac in WT and let2-1. The expression levels of PR1 and PR2 triggered by overexpressing SUMM2ac in WT and let2-1. The expression of PR1 and PR2 was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). P < 1.00 × 10−15 (PR1, column 1 and 2), P < 1.00 × 10−15 (PR1, column 1 and 3), P = 2.62 × 10−12 (PR1, column 1 and 4), P < 1.00 × 10−15 (PR1, column 1 and 5), P = 1.24 × 10−7 (PR2, column 1 and 2), P = 1.04 × 10−10 (PR2, column 1 and 3), P = 5.86 × 10−6 (PR2, column 1 and 4), P < 1.00 × 10−15 (PR2, column 1 and 5). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). The plants 1–2 are 35S::SUMM2ac-HA/WT, and 3–4 are 35S::SUMM2ac-HA/let2-1 (e–h). The above experiments were repeated three times with similar results.
Phosphatase (Sigma) for 1 h at 30 °C. LET1-HA was separated by 10% SDS-PAGE and detected by an CBB staining protoplasts. The FLAG-tagged proteins were immunoprecipitated from the cell lysates with LET1-HA was immunoprecipitated by the FLAG-HA antibody (top two panels). b MEKK2 stabilizes LET2/MDS1 protein accumulation in N. benthamiana. LET2-HA or GFP was co-expressed with MEKK2-GFP or GFP in N. benthamiana for 3 days. The proteins were immunoblotted with an α-HA or α-GFP antibody. CBB staining of RBC was used as a loading control. c MG132 treatment increases LET2/MDS1 protein accumulation in transgenic plants. The 10-day-old seedings of 35S::LET2-HA/let2-1 (Line #2 and #3) transgenic plants were treated with DMSO (Ctrl) or 5 μM MG132 for 6 h. Proteins were immunoblotted using an α-HA antibody, and CBB was used as a loading control. d MG132 treatment increases LET2/MDS1 protein accumulation in N. benthamiana. The leaves of N. benthamiana were inoculated with Agrobacterium carrying LET1-HA and GFP, or LET1-HA and MEKK2-GFP for 12 h, and then treated with DMSO (Ctrl) or 5 μM MG132 for 36 h. Proteins were immunoblotted with an α-HA or α-GFP antibody. CBB was used as a loading control. e Silencing MEKK1 increases LET2/MDS1 protein accumulation. MEKK1 was silenced in the 35S::LET2-HA/let2-1 transgenic plants (Line #1, #2 and #3) by VIGS. Total proteins were extracted 2 weeks after VIGS, and immunoblotted using an α-HA antibody, CBB was used as a loading control. f LET2/MDS1 increases LET2/MDS1 protein accumulation. LET1-HA was co-expressed with LET1-FLAG in protoplasts for 12 h. LET1-FLAG was separated by 7.5% SDS-PAGE. CBB staining of RBC was used as a loading control. g LET2/MDS1 induces LET1 phosphorylation. LET1-HA was co-expressed with Ctrl or LET2-FLAG in protoplasts for 12 h. LET1-HA was immunoprecipitated by an α-HA antibody. The immunoprecipitated LET1-HA protein was incubated without or with 0.5 μL (200 U) λ-phosphatase (Sigma) for 1 h at 30 °C. LET1-HA was separated by 10% SDS-PAGE and detected with an α-HA antibody (top panel). LET1-HA and LET2-HA before immunoprecipitation were detected by the corresponding antibody (middle two panels). CBB staining of RBC was used as a loading control (bottom panel). h LET2/MDS1 increases LET1 kinase activity. LET1-FLAG or LET1FLAG-McC was co-expressed with the vector control, LET2-HA or LET2-HA, in protoplasts. The FLAG-tagged proteins were immunoprecipitated from the cell lysates with α-FLAG affinity beads and used in a kinase assay with [γ-32P] ATP. The GFP-FLAG was used as a negative control. The proteins were immunoblotted by an α-FLAG or α-HA antibody for input controls. i LET1 associates with LET2/MDS1. LET1-HA was co-expressed with Ctrl or LET2-FLAG in protoplasts for 12 h. The LET2-FLAG proteins were immunoprecipitated by α-FLAG affinity beads, and then immunoblotted with an α-HA or α-FLAG antibody (top two panels). The proteins before immunoprecipitation were immunoblotted by an α-HA or α-FLAG antibody as inputs (bottom two panels). j FRET-FLIM analysis of LET1 and LET2/MDS1 interaction in Arabidopsis protoplasts. The indicated proteins were transiently expressed in protoplasts for 16 h, and FRET-FLIM was visualized using a confocal laser scanning microscopy. k Localization of the LET1-GFP and LET2-mCherry/BR2-mCherry is shown with the first (Green) and second column (Red), respectively. The lifetime (τ) distribution (third column), and apparent FRET efficiency (fourth 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 are shown as mean ± SD (n = 15) (k). P = 1.07 × 10−12 (column 1 and 2), P = 1.08 × 10−12 (column 2 and 3). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). Scale bar, 10 μm. f LET2 associates with LET1 in a pull-down assay. Arabidopsis protoplasts expressing LET1-FLAG were incubated with purified His-SUMO-LET2ex proteins. The interaction between LET1 and LET2ex was detected by an α-FLAG immuno blot after pull-down with Ni-NTA agarose. HIS-SUMO-LET2ex proteins were stained by CBB. The above experiments were repeated three times with similar results.

Fig. 4 LET1 and LET2/MDS1 regulation and heteromerization. a LET2/MDS1 associates with MEK2 and SUMM2, but not MPK4. LET2-HA was co-expressed with Ctrl, MEK2-FLAG, SUMM2-FLAG, or MPK4-FLAG in protoplasts for 12 h. The FLAG-tagged proteins were immunoprecipitated by α-FLAG affinity beads, and then immunoblotted by an α-HA or α-FLAG antibody (top two panels). The proteins before immunoprecipitation were immunoblotted by an α-HA or α-FLAG antibody as inputs (bottom two panels). b MEKK2 stabilizes LET2/MDS1 protein accumulation in N. benthamiana. LET2-HA or GFP was co-expressed with MEKK2-GFP or GFP in N. benthamiana for 3 days. The proteins were immunoblotted with an α-HA or α-GFP antibody. CBB staining of RBC was used as a loading control. c MG132 treatment increases LET2/MDS1 protein accumulation in transgenic plants. The 10-day-old seedings of 35S::LET2-HA/let2-1 (Line #2 and #3) transgenic plants were treated with DMSO (Ctrl) or 5 μM MG132 for 6 h. Proteins were immunoblotted using an α-HA antibody, and CBB was used as a loading control. d MG132 treatment increases LET2/MDS1 protein accumulation in N. benthamiana. The leaves of N. benthamiana were inoculated with Agrobacterium carrying LET1-HA and GFP, or LET1-HA and MEKK2-GFP for 12 h, and then treated with DMSO (Ctrl) or 5 μM MG132 for 36 h. Proteins were immunoblotted with an α-HA or α-GFP antibody. CBB was used as a loading control. e Silencing MEKK1 increases LET2/MDS1 protein accumulation. MEKK1 was silenced in the 35S::LET2-HA/let2-1 transgenic plants (Line #1, #2 and #3) by VIGS. Total proteins were extracted 2 weeks after VIGS, and immunoblotted using an α-HA antibody, CBB was used as a loading control. f LET2/MDS1 increases LET2/MDS1 protein accumulation. LET1-HA was co-expressed with LET1-FLAG in protoplasts for 12 h. LET1-FLAG was separated by 7.5% SDS-PAGE. CBB staining of RBC was used as a loading control. g LET2/MDS1 induces LET1 phosphorylation. LET1-HA was co-expressed with Ctrl or LET2-FLAG in protoplasts for 12 h. LET1-HA was immunoprecipitated by an α-HA antibody. The immunoprecipitated LET1-HA protein was incubated without or with 0.5 μL (200 U) λ-phosphatase (Sigma) for 1 h at 30 °C. LET1-HA was separated by 10% SDS-PAGE and detected with an α-HA antibody (top panel). LET1-HA and LET2-HA before immunoprecipitation were detected by the corresponding antibody (middle two panels). CBB staining of RBC was used as a loading control (bottom panel). h LET2/MDS1 increases LET1 kinase activity. LET1-FLAG or LET1FLAG-McC was co-expressed with the vector control, LET2-HA or LET2-HA, in protoplasts. The FLAG-tagged proteins were immunoprecipitated from the cell lysates with α-FLAG affinity beads and used in a kinase assay with [γ-32P] ATP. The GFP-FLAG was used as a negative control. The proteins were immunoblotted by an α-FLAG or α-HA antibody for input controls. i LET1 associates with LET2/MDS1. LET1-HA was co-expressed with Ctrl or LET2-FLAG in protoplasts for 12 h. The LET2-FLAG proteins were immunoprecipitated by α-FLAG affinity beads, and then immunoblotted with an α-HA or α-FLAG antibody (top two panels). The proteins before immunoprecipitation were immunoblotted by an α-HA or α-FLAG antibody as inputs (bottom two panels). j FRET-FLIM analysis of LET1 and LET2/MDS1 interaction in Arabidopsis protoplasts. The indicated proteins were transiently expressed in protoplasts for 16 h, and FRET-FLIM was visualized using a confocal laser scanning microscopy. k Localization of the LET1-GFP and LET2-mCherry/BR2-mCherry is shown with the first (Green) and second column (Red), respectively. The lifetime (τ) distribution (third column), and apparent FRET efficiency (fourth 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 are shown as mean ± SD (n = 15) (k). P = 1.07 × 10−12 (column 1 and 2), P = 1.08 × 10−12 (column 2 and 3). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). Scale bar, 10 μm. f LET2 associates with LET1 in a pull-down assay. Arabidopsis protoplasts expressing LET1-FLAG were incubated with purified His-SUMO-LET2ex proteins. The interaction between LET1 and LET2ex was detected by an α-FLAG immuno blot after pull-down with Ni-NTA agarose. HIS-SUMO-LET2ex proteins were stained by CBB. The above experiments were repeated three times with similar results.
The *llg1* mutants specifically suppress RNAi-MEKK1 cell death. The GPI-anchored proteins LRE, LLG1, LLG2, and LLG3, function as adapters/co-receptors for CrRLK1Ls FER and BUPS/AXIN28-31. LLG2 and LLG3 function redundantly in regulating pollen tube integrity30,31. We tested whether LRE/LLGs are involved in LET1/2-mediated *mekk1* cell death by silencing MEKK1 in the corresponding single and double mutants, including two *re* mutant alleles ( *re*-3 and *re*-6), two *llg1* mutant alleles ( *llg1*-1 and *llg1*-2), *llg2*-1, and *llg3*-1 single mutants, and *llg2*-*llg3*-1 double mutant. The *llg1* mutants, *llg1*-1 and *llg1*-2, but not other mutants, suppressed the growth defects (Fig. 5a and Supplementary Fig. 4a), cell death, H2O2 accumulation (Fig. 5b), and constitutive expression of PR genes (Supplementary Fig. 4b) caused by silencing MEKK1, suggesting a specific role of LLG1 in controlling *mekk1* cell death. Both *llg1*-1 and *llg1*-2 mutants display certain growth defects with reduced plant size (Fig. 5a). The *llg1*-3 mutant, which bears a mutation in glycine at the 114th amino acid to arginine, grew similarly as WT plants (Supplementary Fig. 4c). We tested whether the mutation in *llg1* affects growth defects, reduced fresh weight and *MEKK1* regulated growth and MEKK1 cell death are uncoupled. We further tested whether the mutation in *llg1* affects *mkk1* and *mpk4* cell death by generating the *llg1*-1*mpk4* mutant (Supplementary Fig. 6a), and the *llg1*-1*mpk4* ( *llg1*-*mpk4*) double mutant (Supplementary Fig. 6b). The *llg1*-1*mpk4* mutant partially suppressed the cell death in the *mkk1* mutant when grown on ½MS plates (Fig. 6a). The *llg1mkk1* mutant was bigger than *mkk1* in size and had significantly increased fresh weight compared to *mkk1* (Fig. 6b). The true leaves of *llg1mkk1* were also larger than those of *mkk1*. At the 2-week-old seedling stage, the first pair of true leaves already senesced in *mkk1* but still kept green in *llg1mkk1* (Fig. 6a). Notably, the *llg1*+/- *mkk1* mutant, in which *LLG1* was heterozygous, had no effect on *mkk1* cell death, suggesting that *llg1* is a complete recessive mutation in regulating *mkk1* cell death. The rosette size of the *llg1mpk4* mutant was also bigger than *mpk4* when grown on soil (Fig. 6d). The fresh weight of *llg1mpk4* was significantly higher than that of *mpk4* (Fig. 6e), and the increased *PR1* expression in *mpk4* was partially reduced in *llg1mpk4* (Fig. 6f). Interestingly, the *llg1*+/- *mpk4* mutant behaved in between *mpk4* and *llg1mpk4* in terms of rosette size, fresh weight, and *PR1* gene expression (Fig. 6d–f), indicating that *LLG1* regulates *mpk4* cell death in a dosage-dependent manner. It was reported that the *mkk2* mutant rescued the *mpk4* cell death in a dosage-dependent manner47. Altogether, similar as the *let2* mutants, the *llg1* mutants suppressed *mkk1* and *mpk4* cell death, suggesting that *LLG1* functions genetically downstream of *MPK4* in regulating *mkk1*-*mpk4* cell death.

The *llg1-1* mutation suppresses *mkk1* and *mpk4* cell death. We further tested whether the mutation in *LLG1* affects *mkk1* and *mpk4* cell death by generating the *llg1-1*-*mkk1* mutant ( *llg1mkk1*) triple mutant (Supplementary Fig. 6a), and the *llg1-1*-*mpk4* ( *llg1mpk4*) double mutant (Supplementary Fig. 6b). The *llg1-1*-*mpk4* mutant partially suppressed the cell death in the *mkk1* mutant when grown on ½MS plates (Fig. 6a). The *llg1mkk1* mutant was bigger than *mkk1* in size and had significantly increased fresh weight compared to *mkk1* (Fig. 6b). The true leaves of *llg1mkk1* were also larger than those of *mkk1*. At the 2-week-old seedling stage, the first pair of true leaves already senesced in *mkk1* but still kept green in *llg1mkk1* (Fig. 6a). Compared with *mkk1*, the expression of *PR1* was partially suppressed in *llg1mkk1* (Fig. 6c). Notably, the *llg1*+/- *mkk1* mutant, in which *LLG1* was heterozygous, had no effect on *mkk1* cell death, suggesting that *llg1* is a complete recessive mutation in regulating *mkk1* cell death. The mutations in *LLG1* block MEKK2-, but not SUMM2ac-, triggered cell death. To delineate the genetic position of *LLG1* with MEKK2 and SUMM2 in the regulation of cell death, we examined whether *llg1* mutants exerted an effect on over-expressing MEKK2 or active SUMM2 (SUMM2ac-) triggered cell death by expressing 35S::MEKK2-HA or 35S::SUMM2ac-HA in *llg1* mutants. As shown previously (Fig. 3a), overexpressing MEKK2-HA in WT caused growth defects and elevated *PR1* expression in a dosage-dependent manner (Fig. 6g–i). However, unlike *mkk1*, the enhanced growth defects of *llg1mkk1* are temperature-independent, which is different from that of *mekk1*.

The above data point to an intriguing and apparently contradict observation: the *llg1* mutants aggravated the growth defects caused by genetic lesions in *mekk1*, whereas suppressed cell death triggered by RNAi-mediated silencing of MEKK1. Notably, VIGS was performed using 12-day-old seedlings, and the silence effects of MEKK1 were apparent after 20-days post-germination. Compared to the genetic mutations, VIGS-mediated silencing bypasses the defects associated with embryonic and early seedling development51. The opposing effects of LLG1 on silencing and null mutations of MEKK1 suggest that LLG1 plays one role in regulating initial seedling development in concert with MEKK1, and another role in regulating *mekk1* cell death at a later stage. This is in line with the notion that LLG1 acts through interactions with different CrRLK1Ls as an adapter/co-receptor and regulates various biological processes. The mutations of
plants (Supplementary Fig. 7c, d). Taken together, similar to LET1 and LET2/MD51, LLG1 functions downstream of MEKK2 and upstream of SUMM2 in the mekk1-mkk1/2-mpk4 cell death pathway.

LLG1 associates with LET proteins and is required for their plasma membrane localization. LLGs directly interacts with the extracellular juxtamembrane region of some CrRLK1Ls and function as co-receptors/adapters of CrRLK1Ls in regulating plant growth, reproduction, and immunity. We hypothesized that LLG1 functions in the mekk1-mkk1/2-mpk4 cell death pathway through interaction and modulation of CrRLK1Ls LET1 and LET2/MD51. To test this, we performed Co-IP assays between LLG1 and LET1 or LET2/MD51. When N-terminal HA-tagged LLG1 (HA-LLG1) was co-expressed with C-terminal

**Fig. 5 LLG1 regulates mekk1 cell death.** a The llg1 mutants suppress growth defects triggered by silencing MEKK1. The plant images were photographed at 3-weeks after inoculation with Agrobacterium carrying the indicated VIGS vectors. Ctrl is the vector containing GFP. Scale bar, 1 cm. b The llg1 mutants suppress cell death and H2O2 accumulation induced by silencing MEKK1. The leaves from plants in a were stained by trypan blue for cell death and DAB for H2O2 accumulation. Scale bar, 0.5 cm. c Expression of HA-LLG1 in llg1-2 restores the cell death triggered by silencing MEKK1. #1 and #2 are two representative plLG1::HA-LLG1 transgenic lines in llg1-2. Scale bar, 0.5 cm. d Protein expression of HA-LLG1 in plLG1::HA-LLG1/llg1-2 transgenic plants. e The llg1-1 mekk1 mutant enhances growth defects of mekk1. The seedlings grown on 1/2MS plate at 22 °C were photographed at 2-weeks post-germination. Scale bar, 0.5 cm. f The fresh weight of llg1-1 mekk1 is less than mekk1. The data are shown as mean ± SE (n = 3). P = 3.63 × 10^{-5} (column 3 and 5). The asterisk indicates statistical significance by using two-sided two-tailed Student's t test (***P < 0.001). g llg1-1 mutant enhances the expression of PR1 in mekk1. The expression of PR1 was determined with the plants in e and normalized to the expression of UBQ10. The data are shown as the mean ± SE of four biological repeats (n = 4). P = 1.04 × 10^{-7} (column 3 and 4). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). h The fer-4 mekk1 mutant enhances growth defects of mekk1. The seedlings grown on 1/2MS plate at 22 °C were photographed at 2-weeks post-germination. Scale bar, 1 cm. i The fresh weight of fer-4 mekk1 mutant is less than mekk1. The data are shown as mean ± SE (n = 3). P = 6.92 × 10^{-4} (column 3 and 4). The asterisk indicates statistical significance by using two-sided two-tailed Student's t test (***P < 0.001). j fer-4 mutant enhances the expression of PR1 in mekk1. The expression of PR1 was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). P = 1.87 × 10^{-6} (column 3 and 4). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). The assay was performed as in g. k High temperature did not alleviate fer-4 mekk1 growth defects. The seedlings grown on 1/2MS plate at 28 °C were photographed at 2-weeks post-germination. Scale bar, 1 cm. l The fresh weight of fer-4 mekk1 mutant is less than mekk1 at 28 °C. The seedlings in k were used for measuring fresh weight. The data are shown as mean ± SE (n = 3). P = 1.81 × 10^{-6} (column 3 and 4). The asterisk indicates statistical significance by using two-sided two-tailed Student's t test (***P < 0.001). To measure the weight of mekk1, llg1 mekk1, and fer-4 mekk1 mutants, 10 plants were pooled and the weight of individual plants was averaged. The above experiments were repeated 3-4 times with similar results.
The asterisk indicates statistical significance by using two-sided two-tailed Student’s t test (*P < 0.05). ** The Ilg1-1 mutant reduces the expression of PR1 in mkkl/2. The expression of PR1 was determined with the plants in a and normalized to the expression of UBQ10. The data are shown as the mean ± SE of four biological repeats (n = 4). For the different letters indicate the significance difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). c The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. e The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. e The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. f The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. f The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. f The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. 

The elevated expression of PR1 triggered by overexpressing MEKK2 in WT (Lines 1, 2, and 3) is reduced in Ilg1-1 (Lines 4, 5, and 6). The expression of PR1 was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). For the different letters indicate the significance difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). To measure the weight of mkkl/2 and mpk4 mutants, 10 plants were pooled and the weight of individual plants was averaged. The above experiments were repeated three times with similar results.

Fig. 6 LLG1 genetically functions downstream of MEKK2. a The Ilg1-1 mutant partially suppresses the mkkl/2 cell death. The seedlings grown on ½ MS plate were photographed at 2-weeks post-germination. The leaves from the individual plants were placed with the order of age (from oldest to youngest, right panels). Scale bar, 0.5 cm. b The Ilg1-1 increases the fresh weight of mkkl/2. The data are shown as mean ± SE (n = 4), P = 1.46 × 10⁻² (column 3 and 5). The asterisk indicates statistical significance by using two-sided two-tailed Student’s t test (*P < 0.05). c The Ilg1-1 mutant decreases the expression of PR1 in mkkl/2. The expression of PR1 was determined with the plants in a and normalized to the expression of UBQ10. The data are shown as the mean ± SE of four biological repeats (n = 4), P = 2.06 × 10⁻⁵ (column 3 and 5). The different letters indicate the significant difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). d The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. e The Ilg1-1 mutant partially suppresses the mpk4 cell death. The plants grown on soil were photographed at 4-weeks after germination. Scale bar, 1 cm. 

The elevated expression of PR1 triggered by overexpressing MEKK2 in WT (Lines 1, 2, and 3) is reduced in Ilg1-1 (Lines 4, 5, and 6). The expression of PR1 was normalized to the expression of UBQ10 and the data are shown as the mean ± SE of four biological repeats (n = 4). For the different letters indicate the significance difference determined by one-way ANOVA followed by the Tukey test (P < 0.05). To measure the weight of mkkl/2 and mpk4 mutants, 10 plants were pooled and the weight of individual plants was averaged. The above experiments were repeated three times with similar results.

FLAG-tagged LET1 (LET1-FLAG) or LET2/MD51 (LET2-FLAG) in Arabidopsis protoplasts, both LET1 and LET2/MD51 immunoprecipitated LLG1 (Fig. 7a). We further tested whether LLG1 interacted with the extracellular maltein-like domains (ECD) of LET1 (LET1ECD). When co-expressed in Arabidopsis protoplasts, LET1ECD-FLAG could co-immunoprecipitate HA-LLG1 (Fig. 7b). We then determined whether LLG1 directly interacted with the extracellular juxtamembrane (exJ) domain of LET1 with an in vitro pull-down assay. To do this, we purified the exJ domain of LET1 (amino acid 337–400) fused with glutathione S-transferase (GST-LET1exJ), and the LLG1 truncation without the signal peptide (SP; amino acid 24–149) fused with the maltose-binding protein (MBP-LLG1) from E. coli and performed an in vitro pull-down assay with glutathione agarose beads. As shown in Fig. 7c, GST-LET1exJ, but not GST alone, pulled down MBP-LLG1, indicating a direct interaction between LLG1 and the exJ domain of LET1. Taken together, LLG1 likely functions as an adapter/co-receptor of LET1 and LET2/MD51 in cell death regulation.

LLG1 functions as a chaperone assisting FER protein delivery from the endoplasmic reticulum (ER) to the plasma membrane (PM), which is essential for extracellular signal perception and signaling initiation. To test where LET1 and LET2/MD51 are localized and whether the localization is mediated by LLG1, we
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independent transfections (Fig. 7f). These data indicate that LLG1 is important for LET1 and LET2/MDS1 cell death suppression (Fig. 2), suggesting that LLG1 interacts with LET1 fused with green fluorescent protein (LET1-GFP) and LET2-GFP in protoplasts of WT and the llg1–1 mutant. In WT protoplasts, the majority of cells with LET1-GFP signals (~80%) displayed PM localization. The majority of LET2-GFP signals in llg1–1 co-localized with the ER marker (Fig. 7d). Similarly, the ratio of LET2-GFP signals in PM was reduced from ~45% in WT to ~8% in llg1–1, and the ER localization of LET2-GFP was significantly increased in llg1–1 (Fig. 7d, e). The llg1–1 mutant did not affect free GFP localization (Fig. 7f). These data indicate that LLG1 is important for LET1 and LET2/MDS1 transport from ER to PM (Fig. 7g).

Discussion

CrrlK1Ls that carry an extracellular maelectin-like domain are key regulators in various developmental processes and plant defense responses to pathogens. In a parallel study of using a VIGS-based RNAi screen of mkk1 cell death suppressors with a collection of Arabidopsis T-DNA insertion lines, we identified the uncharacterized CrrlK1L, LET1, as a specific regulator of mkk1-mkk1/2-mpk4 autoimmunity. In this study, by screening individual CrrlK1Ls and revealed that LET2/MDS1 plays an additive role with LET1 in regulating mkk1-mkk1/2-mpk4 autoimmunity. The LET2/MDS1 closest tandemly arrayed homologs, MDS2, MDS3, and MDS4, had little contribution in modulating this process despite of their partially redundant role in regulating plant responses to ion metal (Fig. 1d).

Similar with LET1, LET2/MDS1 acts genetically downstream of Mekk2 and upstream of Summ2 (Fig. 3). We also show that LET2/MDS1 interacts with Mekk2 and Summ2 and its stability is regulated by Mekk2 (Fig. 4a–e). Thus, LET1 and LET2/MDS1 might have similar and additive function in modulating mkk1-mkk1/2-mpk4 autoimmunity. This is supported by the observation that the let1/2 double mutant further alleviated mkk1-mkk1/2, and mpk4 autoimmunity compared to let1 or let2 single mutants (Fig. 2). Notably, the single mutants of let1 or let2 clearly suppressed mkk1-mkk1/2-mpk4 cell death (Fig. 2), suggesting...
that LET1 and LET2/MDS1 might not simply function redundantly in regulating SUMM2 activation. Indeed, we observed that LET1 interacts with LET2/MDS1, and importantly, expression of LET2/MDS1 promotes LET1 phosphorylation (Fig. 4). Consistent with this observation, mutations of either LET1 or LET2/MDS1 in the let1 or let2 single mutants lead to the inactivation of SUMM2 in mekk1-1/mkk1-1/mpk4 cell death. The additive effect of LET1 and LET2/MDS1 in regulating mekk1-1/mkk1-1/mpk4 cell death also suggests that LET1 and LET2/MDS1 might have independent functions in this pathway. This could be due to that LET1 and LET2 might also form LET1 or LET2/MDS1 homodimers, in addition to LET1/2 heterodimer. In addition, LET1 and LET2/MDS1 might be activated by different ligands in modulating mekk1-1/mkk1-1/mpk4 cell death.

We have shown that MEKK2 likely plays a structural role, rather than functions as a kinase, in regulating SUMM2 activation32. Consistently, MEKK2 scaffolds LET1 and SUMM2 for signaling activation49. However, both LET1 and LET2/MDS1 have autophosphorylation activity, and their kinase activity is required for their functions (Fig. 1g–i)39. Thus LET1 and LET2/MDS1 are kinases in the activation of SUMM2. It has been proposed that NLRs are kept in an inactive form by intramolecular interaction (such as interaction between NBS and LRR domains), and disruption of intramolecular interaction activates NLRs6,8. LET1 interacts with LET2/MDS1, and importantly, expression of LET1 or LET2/MDS1 in the absence of MEKK1-MKK1/2-MPK4 cascade. LLG1 likely disrupts its intramolecular interaction. LET1 phosphorylation was shown to regulate plant immunity via modulating PRR complex, and we determined the significance of LLG1 and SUMM2 by ANOVA followed by the Tukey test (P < 0.05). Number of replicates is shown in the figure legends.

Methods

Quantity and statistical analysis. For data quantification analyses are presented as mean ± standard error (SE) or standard deviation (SD). The different letters indicate the significant difference as determined by Bonferroni multiple comparison test followed by the Tukey test (P < 0.05).

Plants

The Arabidopsis thaliana ecotype Col-0 was used as wild type (WT). The T-DNA insertion lines, SALK_139579 (let1-2, AT5G39890), SALK_066322 (let2-2, AT3G89900), SALK_076706C (mdd1-1, AT5G39920), SALK_007613C (mdd4-1, AT5G9300), SALK_029056C (AT3G1550), SALK_135057C (mdd2-2, AT2G52680), SALK_016179C (aux1-1, AT5G64690), SALK_105055C (herk2, AT1G30570), SALK_083346C (opt-1, AT5G43550), SALK_018977C (carry1, AT2G39360), SALK_116667C (any-1, AT5G97900), SALK_008043C (herk-1, AT3G46290), SALK_0007108 (mdd2-1, AT5G93000), SAIL_907_G02 (AT3G24010), SAIL_809_D01 (AT3G49110), and SAIL_448_D02 (AT2G22480), of different CRNLK1 family members, SAIL_47_G04 (llg1-1) for LLG1, SALK_000289 (ire-3) and C566103 (ire-6) for LORELEI, SALK_018793C (let1-1), SALK_052557 (mekk1), and SALK_150039C (mekk2) were obtained from Arabidopsis Biological Resource Center (ABRC). The seeds of herk1-1let1-4 were obtained from Dr. Yanyai Yin22. The seeds of fer-4 (SALK_086056) and plLGI1::HA-LGI1 (plLGI1::LGI1-2) were obtained from Dr. Alice Cheung28. The seeds of llg1-1 were obtained from Dr. Dingzhong Tang32. The seeds of SAIL_105056C, SAIL_809_D01, SAIL_448_D02, SAIL_47_G04, SALK_000289, SALK_052557, and SALK_150039C were previously reported28. The genotype of all the mutants was confirmed with PCR using the primers listed in Supplementary Table 1.

Growth conditions. Plants were grown in the growth rooms with 22 °C, 50–60% relative humidity, 70 µE m−2 s−1 light under 10/14 h light/dark cycles, except where indicated. The seedlings were grown on 1/2 MS plates supplemented with 0.5% sucrose, 0.8% agar, and 2.5 mM MES at pH 5.7. The seeds were cold treated for two days at 4 °C before moving to a growth room. For investigating regulated cell death at high temperature, the seedlings were grown on a 1/2 MS plates in a 22 °C growth room with for 3 days after cold treatment, and then transferred to a 28 °C growth room with the indicated time.

Plasmid constructs

The constructs of pTRV–RNA1 and pTRV–RNA2 of pYL156-GFP and pYL156-MEKK1 have been reported55. The DNA sequence of LET2/MDS1 contains the restriction enzyme sites of BamH1 and Stul, which are commonly used in our plant expression and binary vectors. The LET2/MDS1 cDNA was generated from Ncol-BglII and Ncol-SnaBI site, the LET1 PCR products by PCR and digested by Ncol and SnaBI. The digested fragment was cloned into the linearized pHBT-HA vector digested by Ncol and Stul to get the intermediate construct of pHBT-LE1T2-2HA. The fragment of LET2/MDS1 digested by BglII and Stul from the pHBT-LE1T2-2HA were cloned into the linearized vectors of pHBT-HA, pHBT-GFP, pHBT-GFP-ir, pHBT-FLAG, pHBT-LE1T2-2HA, and pHBT-LE1T2-2HA-LGI1-2 by BamH1 and Stul or SmaI to generate the constructs of pHBT-LE1T2-2HA, pHBT-LE1T2-FLAG, pHBT-LE1T2-GFP, pHCB302-LE1T2-2HA, and pHBT-LE1T2-2HA-LGI1-2. The fragment of pHBT-LGI1-2 digested by BglII and SmaI from the pHBT-LE1T2-2HA was cloned into linearized vectors of pHCB302-LE1T2-2HA digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2.

The LET2 (AT2G35200) gene (2502 bp) was amplified by PCR from Col-0 cDNA using the primers containing BamH1 at the 5’ end and Stul at the 3’ end. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul.

The LE1T2 (AT1G074200) gene (2502 bp) was amplified by PCR from Col-0 cDNA using the primers containing BamH1 at the 5’ end and Stul at the 3’ end. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul. The PCR products were digested by BamH1 and Stul and cloned into the expression vector pHBT-HA digested by BamH1 and Stul.

The fragment of pHBT-GFP was digested by BglII and SmaI from the pHBT-LGI1-2/HA digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2. The fragment of pHBT-LGI1-2 digested by BglII and SmaI from the pHBT-LE1T2-2HA-LGI1-2 was cloned into linearized vectors of pHCB302-LE1T2-2HA digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2. The fragment of pHCB302-LE1T2-2HA-LGI1-2 was cloned into linearized vectors of pHCB302-LE1T2-2HA digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2.

The fragment of pHCB302-LE1T2-2HA digested by BamH1 and Stul. The fragment of pHCB302-LE1T2-2HA-LGI1-2 digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2. The fragment of pHCB302-LE1T2-2HA-LGI1-2 was cloned into linearized vectors of pHCB302-LE1T2-2HA digested by BamH1 and Stul to generate the construct of pHCB302-LE1T2-2HA-LGI1-2.
338-400aa) were amplified by PCR and cloned into the vector of pHBT-HA or a modified Glutathione S-transferase (GST) fusion protein expression vector pGSTu by BamHI and SalI digestion to generate the D1-thiogalactoside (PRT)-LETL1CD-HA and pGSTu-LET1ECD. The p35S:HA-LETL1 construct in plant expression vector was obtained from Dr. Alice Cheung.28 The fragment of LLLG1 without signal peptide (∆SSP, 25-168aa) was amplified by PCR and digested by BglII and PstI, then ligated with a linearized maltose-binding protein (MBP) fusion protein expression vector pMAL (New England Biolabs, USA) by BamHI and PstI digestion to generate pMAL-LLLG1-SSP, LET1CD (1273-2502 bp) was amplified by PCR from pHBTT-35S::LETL1-HA using the primers containing StuI at the 5′ end and KpnI at the 3′ end and cloned into an insect cell expression vector pBACE1 (Thermo Scientific, USA) and cloned into an insect cell expression vector pHBT-HA by electroporation. Positive transformants were selected on LB plates containing Glufosinate-ammonium (Basta, 50 g/mL) for 7 days. The recombinant plasmids were transferred into Agrobacterium tumefaciens strain GV3101 and introduced into Arabidopsis using the floral dipping method. Transgenic plants were selected by GUS staining (Basta, 50 g/mL) for the pCB302 vector and hygromycin (50 μg/mL) for the pHDC32 vector. Multiple transgenic lines were analyzed by immunoblot (IB) for protein expression.

**Agrobacterium-mediated virus-induced gene silencing assay.** The binary TRV vector pTRV-RNA1 and pTRV-RNA2 derivatives, pTRV-MEK1, pTRV-CLA1, and pTRV-PCR (439-1899 bp) were transferred into Agrobacterium tumefaciens strain GV3101 by electroporation. Positive transformants were selected on LB plates containing 50 μg/mL kanamycin and 25 μg/mL gentamicin by incubating at 28°C for 36 h. An individual transformant was transferred into 2 mL LB liquid medium containing 50 μg/mL kanamycin and 25 μg/mL gentamicin in 20 mL glass culture tubes for overnight at 28°C in a roller drum, and sub-cultured in 100 times of volume of fresh LB liquid medium containing 50 μg/mL kanamycin, 25 μg/mL gentamicin, 10 mM MES, and 20 μM acetylsyringone for overnight at 28°C with 200 rpm shaking. Cells were pelleted by 1300 g centrifugation, re-suspended in buffer containing 10 mM MgCl₂, 10 mM MES, and 200 μM acetylsyringone, adjusted to OD₆₀₀ of 1.5 and incubated at 25°C for at least 3 h. Bacterial cultures containing pTRV-RNA2 and pTRV-RNA2 derivatives were mixed at a 1:1 ratio and inoculated into the leaves for 3 days. Protoplasts were lysed by vortexing (2 × 10⁵ cells/mL) for the PEG-mediated transfection. 2 μL of plasmid DNA (2 μg/μL) was mixed with 1 mL of protoplasts and incubated at 25°C for at least 3 h. Bacterial cultures containing pTRV-RNA1 derivatives were mixed at a 1:1 ratio and inoculated into the leaves for 3 days. Protoplasts were lysed by vortexing (2 × 10⁵ cells/mL) for the PEG-mediated transfection. After centrifugation at 12,500g at 4°C for 15 min, 250 μL of extraction buffer were added to dissolve pellets, and 20 μL of supernatant were collected for input controls, and the remaining was incubated with α-FLAG affinity beads (Sigma, USA) at 4°C for 2 h with gentle shaking. Beads were collected and washed three times with washing buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 10 mM imidazole). The pull-down proteins were eluted by 50 mL of 2x SDS protein loading buffer for 10 min and detected by immunoblotting with an α-MBP or α-GST antibody. For His fusion protein pull-down assay, about 10 μg of pHis-SUMO-LET2CD or HIS-SUMO-LET2CD proteins were mixed with the LET1-FLAG cell lysates in the IP buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.5% Triton X-100) at 4°C for 30 min with gentle shaking, subsequently incubated with 20 μL of glutathione agarose beads at 4°C for another 2 h with gentle shaking. Beads were washed five times with pull-down buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100). Beads were boiled in 50 μL of 2x SDS protein loading buffer for 10 min and detected by immunoblotting with an α-MBP or α-GST antibody. For His fusion protein pull-down assay, about 10 μg of His-SUMO-LET2CD or HIS-SUMO-LET2CD proteins were mixed with the LET2-FLAG cell lysates in the IP buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.5% Triton X-100) at 4°C for another 30 min with gentle shaking. Beads were harvested by centrifugation and washed five times with the IP buffer and one time with washing buffer (20 mM Tris, pH 8.0, 500 mM NaCl, and 10 mM imidazole). The pull-down proteins were eluted by 50 μL of elution buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 250 mM imidazole) and detected by an immunoblot with an α-FLAG antibody.

**Transient expression in Arabidopsis protoplasts.** The indicated pHBT constructs were used for protoplast transfection following the protocol.29 Briefly, for Co-IP assay, 100 μL of plasmid DNA (2 μg/μL) was mixed with 1 mL of protoplasts (2 x 10⁵ cells/mL) for the PEG-mediated transfection.

**Co-immunoprecipitation assay.** Proteins were expressed overnight in Arabidopsis protoplasts or N. benthamiana leaves for 3 days. Protoplasts were lysed by vortexing and leaves were ground in the extraction buffer (100 mM NaCl, 1 mM EDTA, 10 mM HEPES, pH 7.5, 2 mM NaF, 2 mM EGTA, 1% Triton X-100, 10% glycerol, and 1 x protease inhibitor). After centrifugation at 12,500g at 4°C for 15 min, 250 μL of extraction buffer were added to dissolve pellets, and 20 μL of supernatant were collected for input controls, and the remaining was incubated with α-FLAG affinity beads (Sigma, USA) at 4°C for 2 h with gentle shaking. Beads were collected and washed three times with washing buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 50 μM Tris-HCl, pH 7.5). Proteins were eluted by 2 x SDS-PAGE loading buffer and boiled at 94°C for 5 min. Immunoprecipitation and input proteins were analyzed by immunoblot with indicated antibodies.

**Trypan blue and DAB staining.** For investigating cell death and H₂O₂ accumulation in leaves, the cotyledons or leaves were detached and soaked into 2.5 mg/mL DAB dissolved in ddH₂O, pH 3.8) for overnight incubation. Samples from the corresponding author upon request. Source data are provided with this paper.

**RNA isolation and qRT-PCR analysis.** Plant total RNAs were extracted by TRIzol reagent (Sigma/Invitrogen, USA). Genomic DNA was degraded by treatment with RNase-free DNase I (NEB, USA). Complementary DNAs (cDNAs) were synthesized with M-MuLV Reverse Transcriptase (NEB, USA) and oligo(dT) primers. Quantitative RT-PCR analysis was performed by iTaq Universal SYBR green Supermix (Bio-Rad, USA) with a Bio-Rad CFX384 Real-Time PCR System (Bio-Rad, USA). UBQ10 was used as an internal reference.

**Confocal Microscopy and FLIM-FRET assays.** The GFP and mCherry fusion proteins were detected using a Leica TCS SP8 confocal laser scanning microscope (Germany). The GFP fluorescence was excited at 488 nm and emissions were detected between 490 and 530 nm. The mCherry fluorescence was excited at 587 nm, and emissions were detected between 590 and 620 nm. The pinhole was set at 1 Airy unit. Images and FLIM/FRET analyses were performed by using Leica Application Suite X (LAS X) software as described.31 Briefly, FRET measurements were done with a pair of GFP/mCherry fusion proteins. The image of GFP donor fluorescence was analyzed and scanned at 488 nm and detected between 490 and 530 nm. The fluorescence lifetime (τ) was calculated as the average of 20 values randomly measured in the protoplast cells. The values obtained for 15 protoplasts were used to determine the average value of τ for each pair of proteins analyzed. The relative fluorescence intensity (FI) was measured in a certain region of interest (ROI). Lifetime and FRET efficiency were measured by the Leica LAS X software. FRET efficiency (E) was calculated by using the formula E = 1 - (τD/τA) / τDA, in which the lifetimes of the donor in the presence of acceptor and τD is fluorescence lifetime of the donor.

**Data availability**

The source data for Figs. 1 and 3–7 and Supplementary Figs. 3 and 6–7 are provided as a Source Data File. Other original data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

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**References**

1. Couto, D. & Zipfel, C. Regulation of pattern recognition receptor signaling in plants. Nat. Rev. Immunol. 16, 537–552 (2016).
2. Yu, X., Feng, B., He, P. & Shan, L. From chaos to harmony: responses and signaling upon microbial pattern recognition. Annu. Rev. Phytopathol. 55, 109–137 (2017).
3. Gust, A.-A., Pratt, R. & Nurnberger, T. Sensing danger: key to activating plant immunity. Trends Plant Sci. 22, 779–791 (2017).
4. Jamieson, P. A., Shan, L. B. & He, P. Plant cell surface molecular cypher: receptor-like proteins and their roles in immunity and development. Plant Sci. 274, 242–251 (2018).

5. Bohm, H., Albert, I., Fan, L., Reinhard, A. & Nurnberger, T. Immune receptor complexes at the plant cell surface. Curr. Opin. Plant Biol. 20, 47–54 (2014).

6. Cui, H., Tsuda, K. & Parker, J. E. Effector-triggered immunity: from pathogen perception to robust defense. Annu. Rev. Plant Biol. 66, 487–511 (2015).

7. Elmore, J. M., Lin, J. M. & Coaker, G. Plant NB-LRR signaling: upstreams and downstreams. Curr. Opin. Plant Biol. 14, 365–371 (2011).

8. DeYoung, B. J. & Innes, R. W. Plant NBS-LRR proteins in pathogen sensing and host defense. Nat. Immunol. 7, 1243–1249 (2006).

9. Shiu, S. H. & Bleecker, A. B. Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. Plant Physiol. 132, 530–543 (2003).

10. Belkhadir, Y., Yang, L., Hetzel, J., Dangl, J. L. & Chory, J. The growth-defense pivot: crisis management in plants mediated by LRR-RK surface receptors. Trends Biochem. Sci. 39, 447–456 (2014).

11. Hohmann, U., Lau, K. & Hothorn, M. The structural basis of ligand perception and signal activation by receptor kinases. Annu. Rev. Plant Biol. 68, 109–137 (2017).

12. Nissen, K. S., Willats, W. G. & Malinovsky, F. G. Understanding CrRLK1L family and receptor-like proteins in Arabidopsis. Nature 509, 669–670 (2014).

13. Richter, J. et al. Multiplex mutagenesis of four clustered CrRLK1L with acquired resistance. Cell 103, 1111–1120 (2001).

14. Kong, Q. et al. The MEKK1-MMK1/MKK2-MPK4 kinase cascade negatively regulates immunity mediated by a mitogen-activated protein kinase receptor SUMM2. Cell Host Microbe 11, 253–263 (2012).

15. Rodriguez, M. C., Petersen, M. & Mundy, J. Mitogen-activated protein kinase signaling in plants. Annu. Rev. Plant Biol. 61, 621–649 (2010).

16. Mende, X. & Zhang, S. MAPK cascades in plant disease resistance signaling. Annu. Rev. Phytopathol. 51, 245–266 (2013).

17. Ten, G., Boudsocq, M. & Sheen, J. Protein kinase signaling networks in plant innate immunity. Curr. Opin. Plant Biol. 14, 519–529 (2011).

18. Zhang, M., Su, J., Zhang, Y., Xu & Zhang, S. Conveying endogenous and exogenous signals: MAPK cascades in plant growth and defense. Curr. Opin. Plant Biol. 45, 1–10 (2018).

19. Sun, T. et al. Antagonistic interactions between two MAP kinase cascades in plant development and immune signaling. EMBO Rep. 19, e45324 (2018).

20. Bi, G. et al. Receptor-like cytoplasmic kinases directly link diverse pattern recognition receptors to the activation of mitogen-activated protein kinase cascades in Arabidopsis. Plant Cell 30, 1543–1561 (2018).

21. Assi, T. et al. MAP kinase signaling cascade in Arabidopsis innate immunity. Nature 415, 977–983 (2002).

22. Ichimura, K., Casais, C., Peck, S. C., Shinozaki, K. & Hiratsu, K. MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. 281, 36899–36976 (2006).

23. Xia, Y. et al. Mechanisms of RALF peptide perception by a heterotypic receptor activator mediates plant immunity regulated by the NBLRR protein SUMM2. Cell Host Microbe 11, 253–263 (2012).

24. Gao, M. et al. MEKK1-MKK1/MKK2-MPK4 function together in a mitogen-activated protein kinase cascade to regulate innate immunity in plants. Cell Res. 18, 1190–1198 (2008).

25. Zhang, Z. et al. Disruption of PAMP-induced MAP kinase cascade by a pseudokinase suppresses jasmonic acid signaling in Arabidopsis thaliana. Plant Physiol. 174, 478–488 (2017).

26. de Oliveira, M. V. V. et al. Specific control of Arabidopsis BAK1/SEK1-411 signaling regulates cell death by protein glycosylation. Plant Physiol. 174, 1521–1530 (2017).

27. Yang, Y. et al. RNAi-based screen reveals concerted functions of MEKK2 and CRCK3. EMBO Rep. 18, 292–307 (2017).

28. Liu, J. et al. The maleic-in-like-receptor-like kinase LETUM1 modulates NLR disease resistance by CRCK2 scaffolding. Nat. Plants 6, 125–133 (2020).

29. Richter, J. et al. Multiplex mutagenesis of four clustered CrRLK1L with CRISP/Cas9 exposes their growth regulatory roles in response to metal ions. Sci. Rep. 8, 12182 (2018).

30. de Oliveira, M. V. V. et al. Specific control of Arabidopsis BAK1/SEK1-411 signaling regulates cell death by protein glycosylation. Plant Physiol. 174, 1521–1530 (2017).

31. Yang, Y. et al. RNAi-based screen reveals concerted functions of MEKK2 and CRCK3 in plant cell death regulation. Plant Physiol. https://doi.org/10.1104/pp.1119.01555 (2020).

32. Wang, J. et al. Reconstitution and structure of a plant NLR resistant conferring immunity. Science 364, eaav5870 (2019).

33. Tsukamoto, T., Qin, Y., Honma, Y., Furuya, D. & Palanivelu, R. A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development. Plant J. 62, 571–588 (2010).

34. Gonme, M. & Reiter kinase THEEUS1 is a rapid alkalization factor 34 receptor in Arabidopsis. Curr. Biol. 28, 2452–245 (2018).

35. Haruta, M., Sabat, G., Stecker, K., Minkoff, B. B. & Sussman, M. R. A peptide hormone and its receptor protein kinase regulate plant cell expansion. Science 343, 408–411 (2014).

36. Guo, H. et al. Receptor-like kinase THEEUS1 is a rapid alkalization factor 34 receptor in Arabidopsis. Curr. Biol. 28, 2452–245 (2018).

37. Fujita, A., Aker, J., de Vries, S. & Borst, J. W. Probing protein-protein interactions using a combined biochemical and computational approach. Plant Cell 132, 355–367 (2020).

38. Sasaki, J., Hase, K., Kato, K., Kubo, C. & Nakamura, Y. An auxin-stable receptor-like kinase regulates cell division and cell size. EMBO J. 23, 1379–1388 (2004).

39. Niu, S. & Noh, S. K. The E3 ubiquitin ligase DUB1 is required for Arabidopsis sexual reproduction. Annu. Rev. Plant Biol. 70, 247–277 (2019).

40. Guo, H. et al. LORELEI-LIKE GPI-ANCHORED PROTEINS 2/3 regulate pollen tube growth as chaperones and coreceptors for ANXUK/BUFS receptor kinases in Arabidopsis. Mol. Plant 12, 1612–1623 (2019).

41. Shen, Q., Bourdais, G., Pan, H., Robatek, S. & Tang, D. Arabidopsis glycosylphosphatidylinositol-anchored protein LIG1 associates with and modulates FLS2 to regulate innate immunity. Proc. Natl. Acad. Sci. USA 114, 5749–5754 (2017).
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Author contributions
Y.H., C.Y., J.L., L.S., and P.H. conceived the project, designed experiments and analyzed data. Y.H., C.Y., J.L., B.F., D.G., L.K. and F.A.O.M. performed experiments and analyzed data. J.R., and M.T.H. generated mds CRISPR/Cas lines. W.M.W. analyzed data and provided critical feedback. Y.H., L.S., and P.H. wrote the manuscript with inputs from all co-authors.

Competing interests
The authors declare no competing interests.

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