Title: Plant mixed lineage kinase domain-like proteins limit biotrophic pathogen growth

One Sentence Summary: Plants have a protein family that is structurally homologous to vertebrate mixed lineage kinase domain-like protein, which induces necroptotic cell death, but these plant proteins can confer immunity without host cell death.

Short title: Plant MLKLs confer disease resistance

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Abstract:

Mixed lineage kinase domain-like (MLKL) protein mediates necroptotic cell death in vertebrates. We report here the discovery of a conserved protein family across seed plants that is structurally homologous to vertebrate MLKL. The *Arabidopsis thaliana* genome encodes three MLKLs with overlapping functions in limiting growth of obligate biotrophic fungal and oomycete pathogens. Although displaying a cell death activity mediated by N-terminal helical bundles, termed HeLo domain, AtMLKL-dependent immunity can be separated from host cell death. Cryo-electron microscopy structures of AtMLKLs reveal a tetrameric configuration, in which the pseudokinase domain and brace region bury the HeLo-domains, indicative of an auto-repressed complex. We also show the association of two AtMLKLs with microtubules. These findings, coupled with resistance-enhancing activity and altered microtubule association of a phosphomimetic mutation in the pseudokinase domain of AtMLKL1, point to a cell death-independent immunity mechanism.

Main text:

Regulated cell death (RCD) is intimately connected with innate immunity in plants and animals (1-3). A shared feature of several proteins involved in RCD in plants, animals and fungi is a four-helical bundle structure called the HeLo-domain (4). HeLo domain-containing MLKL (mixed lineage kinase domain-like protein) mediates necroptosis in animals (5, 6), a form of RCD that is proposed to combat pathogens by releasing pro-inflammatory molecules (7, 8). Necroptosis is initiated by the plasma membrane (PM)-resident death receptors, with downstream activation of receptor-interacting serine/threonine-protein kinases (RIPKs) leading to the phosphorylation of the pseudokinase domain of the terminal pathway effector, MLKL (7, 8). This process assembles monomeric MLKL into pro-necroptotic oligomers (9) that translocate to the PM where oligomerized HeLo-domains interfere with membrane integrity (10-14). The extent to which RCD in plants and animals is directly responsible for disease resistance is under debate (3, 15-17).

To identify novel immune regulators in plants, we searched for HeLo domain-containing proteins in plant genomes by comparing Hidden Markov Models. This analysis identified a protein family that is highly conserved across plants (Fig. 1, Table S1, Supplemental file 1), with a modular structure resembling MLKL (Fig. 1A). The kinase-like domain lacks canonical residues known to underlie phosphoryl transfer (18) (Fig. 1B), suggestive of a catalytically inactive pseudokinase (8). Hereafter, we refer to these proteins as plant MLKLs. Plant MLKLs additionally possess an extended serine-rich region of varying length after the pseudokinase domain without similarity to any known

domain (Fig. 1A, Supplemental file 1). Plant MLKLs separate into two subgroups based on sequence similarity (Fig. 1D). *Arabidopsis thaliana* harbours three *MLKL* genes with *AtMLKL1* and *AtMLKL2* in subfamily I and *AtMLKL3* belonging to subfamily II (Fig. 1D).

To explore similarities between plant and animal MLKLs, we expressed *AtMLKL2* and *AtMLKL3* for structural analysis using cryo-electron microscopy (Fig. 2, Fig. S1 and S2). In gel filtration, both proteins eluted at an estimated molecular weight corresponding to tetramers (Fig. 2A, Fig. S2A). This contrasts with the vertebrate MLKL protein, which displayed heterogeneity in a similar assay (13). Representative 2D projection views indicate that both *AtMLKL* oligomers form a triangle-like architecture with a 2-fold symmetry (Fig. S1AB, Fig. S2BC). Further 3D classification and refinement generated density maps of oligomeric *AtMLKL2* and *AtMLKL3* with a global resolution of 4.1Å (Fig. S2E) and 3.4Å (Fig. S1D) respectively, based on the gold Fourier Shell Correlation standard (Fig. S1C, Fig. S2D). The 3D reconstructions show that both *AtMLKL2* and *AtMLKL3* oligomers are composed of four MLKL molecules (Fig. 2C, Fig. S3A), confirming that these form tetramers. Tetramerization of *AtMLKL2* or *AtMLKL3* results in formation of a pyramid-like structure. Structure alignment of the two final models indicates that tetramers of two subfamily members are nearly identical (Fig. S3AB). As the quality of the density of *AtMLKL3* is superior for model building, we limited our structural analysis to *AtMLKL3*.

The N-terminal HeLo domain of *At*MLKL3 forms a four-helix bundle (Fig. S3C) which superimposed well with the HeLo domains of mouse and human MLKL (mMLKL and hMLKL, respectively; Fig. 2D). This observation supports the idea that *At*MLKL3 is a bona fide homolog of vertebrate MLKLs. Nevertheless, compared to mMLKL, the HeLo domain of *At*MLKL3 packs tightly against its pseudokinase domain (Fig. S3D). The packing is further strengthened by the brace region of *At*MLKL3, which contains a string of five helices that simultaneously interact with the HeLo and pseudokinase domains (Fig. S3C). The HeLo domains and the brace regions form the core of the *At*MLKL3 tetramer, whereas the pseudokinase domains are presented at the apices of the pyramid-like structure (Fig. 2C). Hydrophobic packing of $\alpha 1$ helices (Fig. S3E) from two *At*MLKL3 molecules contributes to formation of a homodimeric *At*MLKL3. In the tetrameric *At*MLKL3, the two $\alpha 1$ -mediated homodimers pack perpendicularly to each other (Fig. 2G). Four brace regions, which are positioned nearly in the same plane, are sandwiched between the two homodimers and exclusively mediate homodimer-homodimer interaction (Fig. 2F). The N-terminal halves of the four brace regions form two homodimer pairs, and the C-terminal halves another two pairs (Fig. S3E). The intermolecular interactions lead to further sequestering of the *At*MLKL3 N-terminal HeLo domain. Taken together, our observations indicate that *At*MLKL3 is a structural homolog of vertebrate MLKLs and its N-terminal HeLo domain is sequestered through both intra- and intermolecular interactions.

To determine the role of plant MLKLs in immunity, we challenged combinatorial loss-of-function mutants of *At*MLKLs (Fig. S4) with different

microbial pathogens. *Atmlkl* single mutants exhibited increased susceptibility to the obligate biotrophic fungus *Golovinomyces orontii* compared to wild-type plants and, strikingly, the triple mutant was as susceptible as an *eds1* mutant (Fig. 3A), which is hyper-susceptible to a number of pathogens including the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). The immune response restricting *Golovinomyces* growth in Col-0 wild-type plants is not associated with host cell death (19). In *A. thaliana*, immunity to *Hpa* isolate Emwa1 is mediated by RPP4, an NLR that requires EDS1 for function (20). RPP4-mediated disease resistance was partially compromised in the *Atmlkl* triple mutant, as measured by increased *Hpa* sporangiophore formation on true leaves (Fig. 3B). We did not detect marked differences between the susceptibility of wild-type and mutant plants to hemi-biotrophic *Pseudomonas syringae* pv. *tomato* DC3000 bacteria or the fungal necrotroph *Botrytis cinerea* (Fig. S5AB). Furthermore, disease resistance mediated by RPS2 and cell death mediated by RPM1 NLRs were largely retained in the *Atmlkl123* mutant (Fig. S5CD).

RNA-seq analysis using mock- and *G. orontii*-challenged leaves of wild-type, *Atmlkl23*, *Atmlkl123* and *eds1* lines showed that the *AtMLKL1* transcript was induced upon pathogen challenge (7.4 fold) and that induction was fully dependent on *EDS1* (Fig. 3C, Fig. S6). Further analysis revealed that *AtMLKLs* regulate transcriptional reprogramming both under unchallenged conditions and upon pathogen attack (Fig. 3C). These data imply a specific and partly redundant role of *AtMLKLs* in resistance to obligate biotrophic pathogens.

149

150 To test whether *At*MLKLs possess cell death activity, possibly regulated by
 151 phosphorylation, we introduced single phosphomimetic substitutions at serine
 152 residues in the activation loops of *At*MLKLs (Fig. 1B) and expressed in *A.*
 153 *thaliana* leaf protoplasts (see method). Upon overexpression, all wild-type
 154 *At*MLKLs elicited cell death, which was as potent as an N-terminal barley NLR
 155 cell death module (21)(Fig. S7). We found that a phosphomimetic substitution
 156 at serine³⁹³ but not serine³⁹⁵ in *At*MLKL1 enhanced its cell-killing activity (Fig.
 157 4A). However, an alanine substitution at serine³⁹³ did not compromise the cell
 158 death activity, suggesting that other serine or threonine residues in the
 159 activation loop (Fig. 1B) have a compensatory function. Using transgenic *A.*
 160 *thaliana* lines, we observed that the enhanced susceptibility of *Atmlkl1* and
 161 *Atmlkl2* mutants to *G. orontii* was restored by stable transformations of
 162 genomic fragments encompassing wild-type *AtMLKL1* and *AtMLKL2*,
 163 respectively (Fig. 4B,C and Fig. S8-9). In line with the protoplast assay (Fig.
 164 4A), *At*MLKL1 (S393D) transgenic plants exhibited enhanced resistance to *G.*
 165 *orontii* compared to Col-0 wild type or to plants expressing the transgenes
 166 *At*MLKL1 or *At*MLKL1 (S395D) (Fig. 4B,C). Unexpectedly, *At*MLKL1 (S393D)
 167 transgenic plants did not exhibit cell death lesions or apparent plant growth
 168 retardation indicative of autoimmunity (Fig. 4B, Fig. S8A). Collectively our
 169 results suggest that plant MLKL function is in part regulated by activation loop
 170 conformation induced by phosphorylation, similar to vertebrate MLKLs (22),
 171 but *At*MLKL1 activity needed to restrict *G. orontii* growth can be separated
 172 from host cell death.

173

174

175 We next examined whether the *At*MLKL N-terminal HeLo domain contributes
 176 to its cell death activity, as in animals (8, 12, 14, 23). Taking advantage of a
 177 chemically enforced oligomerization system (Fig. S10, see method), we found
 178 that expression of the HeLo domains with the brace region of *At*MLKL1 and
 179 *At*MLKL3 was sufficient for cytotoxic activity and this activity was further
 180 enhanced upon oligomerization (Fig. S10D). This finding mirrors the activity
 181 of the HeLo domains with the brace region of MLKL (14) and is consistent
 182 with our structure-based hypothesis that the full-length MLKL tetramer with
 183 buried HeLo domains represents an inactive form (Fig. 2).

184

185 As animal MLKL translocates to the PM upon activation (10-14) we examined
 186 whether GFP-tagged *At*MLKL variants associate with the PM using time-
 187 resolved confocal microscopy. We detected mobile punctate signals in the
 188 cytoplasmic space for all *At*MLKL1 variants (Fig. 4DE). Reduced mobility for a
 189 fraction of *At*MLKL1-GFP signals when proximal to the PM (Fig. 4E) is
 190 consistent with PM association. Intriguingly, maximum projection of the time-
 191 lapse images revealed that *At*MLKL1 (wild type)-GFP, the S395D mutant
 192 moved along filamentous structures (Fig. 4E, Supplemental movies 1 and 3;
 193 Fig. S11A). These filamentous structures specifically colocalized with the
 194 microtubule marker (24) mCherry-MAP4 (Fig. S11B-C), and association was
 195 detectable upon *G. orontii* invasion (Fig. S12). Intriguingly, no filamentous
 196 structures were detected in the S393D phosphomimetic line despite the
 197 presence of a microtubule array (Fig. S13). These data suggest that specific

198 phosphorylation modulates the intracellular localization and activities of
199 AtMLKL1 via cytoskeletal association.

200

201 The plant MLKL family described here is a first example of a non-receptor
202 immunity component consisting of multiple domains that is structurally shared
203 between animal and plants. MLKLs in these two kingdoms are likely products
204 of convergent evolution because their exon-intron structures are unrelated (~4
205 and 11 exons in AtMLKLs and hMLKL, respectively). Our work serves as a
206 template to test structural predictions implicating the presence of a HeLo-
207 domain fold in a number of plant modular proteins, including the ADR1 family
208 of NLRs (4, 25-27). Despite the cell death activity of a putative HeLo-domain
209 of ADR1 (28), this family confers disease resistance without apparent host cell
210 death (29). This resembles AtMLKL-mediated defense in wild-type plants to
211 *G. orontii* without host cell death, although AtMLKLs have the capacity to
212 elicit cell death. These results raise the question of whether host defence and
213 cell death functions can be further disentangled in animal necroptosis
214 signalling.

215

Fig. 1: Plant genomes encode structural homologs of MLKL.

A, A diagram of plant and animal MLKLs. A variably sized serine-rich region that has no similarity to known structures is directly C-terminal to the pseudokinase domain of plant MLKLs.

B, Alignments of the four helical bundles (HeLo domain) and the pseudokinase domains of *A. thaliana* MLKLs (AtMLKLs) and representative vertebrates. Invariant residues and conservative substitutions in > 50% of the sequences are shown in light blue and grey backgrounds, respectively. The residues responsible for the activation of human or mouse MLKL upon phosphorylation are indicated in the red box. The serine residues examined in this study are shown in red.

C, Plant MLKL-encoding genes across representative plant species. A full list of MLKL-encoding genes in the genomes of 48 seed plants is shown in Table S1.

D, Phylogenetic relationship of plant MLKLs. Neighbor-net analysis discriminates two subgroups of plant MLKLs colored in light blue and orange with bootstrap support of 0.85 (1,000 bootstrap replicates).

Fig. 2. Structure of the AtMLKL3 tetramer.

A, Top: Gel filtration profiles of AtMLKL3. Position of standard molecular weight is indicated by arrow. Bottom: Peak fractions on the top were verified by reducing SDS-PAGE with Coomassie Blue staining.

B, Final EM density map of the AtMLKL3 tetramer. Local resolution of the density map is shown on the right with scale indicated by colors, in angstroms.

C, Top: Final 3D reconstruction of AtMLKL3 tetramer shown in three orientations. Each monomer of AtMLKL3 is shown in different colors. Bottom: Cartoon shows the overall structure of AtMLKL3 tetramer in three orientations. Subdomains of AtMLKL3 are shown in different colors.

D Superposition of the HeLo domains of AtMLKL3 (yellow, PDB ID code 6KA4), human MLKL (blue, PDB ID code 2MSV) and mouse MLKL (Pink, PDB ID code 4BTF). RMSD between AtMLKL3 and mMLKL:3.745, RMSD between AtMLKL3 and hMLKL:4.010.

E, Electrostatic surface of HeLo domains of AtMLKL3, human MLKL and mouse MLKL in two orientations.

F, Cartoon shows the brace region of the AtMLKL3 tetramer. Each monomer of AtMLKL3 is shown in different colors.

G, Cartoon shows the HeLo domains of the AtMLKL3 tetramer in two orientations. Each monomer of AtMLKL3 is shown in different colors.

Fig. 3: Arabidopsis MLKLs confer resistance to powdery and downy mildew pathogens.

A, *Atmlkl* mutants are susceptible to the powdery mildew *Golovinomyces orontii* (arrowheads). Quantification of *G. orontii* DNA at seven days after inoculation is relative to the corresponding Col-0 samples. (n=3, Tukey HSD * $p < 0.05$).

B, The *Atmlkl* triple mutant is susceptible to the downy mildew *Hyaloperonospora arabidopsidis*. The number of *Hpa* spores per gram fresh weight of plants is presented. Data were obtained in three independent experiments, each including two biological replicates (n=6). Asterisks indicate significant differences (Tukey HSD $p < 0.05$). The 1st or 2nd true leaves were stained with trypan blue to visualise hyphae growth. Sporangiphores were formed on the true leaves of the *Atmlkl* triple mutant (black arrowheads).

C, Principal component analysis (PCA) of RNA-seq data from mock- and pathogen-challenged leaves collected at 48 hours post *G. orontii* inoculation.

*WT is a segregant line derived from the cross between *Atmlkl1* and *Atmlkl3*. The *eds1* mutant was used as a susceptible control.

Fig. 4: Cell death activity, disease resistance and subcellular localization of phosphomimetic AtMLKL1 variants.

A, A phosphomimetic substitution in AtMLKL1 elicited enhanced cell death in *Arabidopsis* mesophyll protoplasts. Luciferase and AtMLKL1 expression constructs were co-transfected into protoplasts and luciferase activity was measured as a proxy of cell viability at 16 hours post transfection. The positions of serine-to-aspartate residues are indicated in Fig. 1B. The C-terminally HA-tagged variants were expressed under the control of the constitutive cauliflower mosaic virus 35S promoter. (n=3, Tukey HSD * $p < 0.01$).

B, Macroscopic phenotype of transgenic *Arabidopsis* expressing phosphomimetic variants of AtMLKL1 in response to *G. orontii* (arrowheads). Plants were photographed seven days after pathogen challenge.

C, Quantification of *G. orontii* DNA in infected leaves of transgenic plants at 14 days after pathogen challenge (n=4, Tukey HSD * $p < 0.05$).

D, Schematic diagram of the confocal images shown in **B**. The white circle indicates GFP signal proximal to the PM.

E, Confocal images of the abaxial epidermis of the *Arabidopsis* transgenic lines expressing phosphomimetic and wild-type variants of AtMLKL1. GFP signals indicated by white circles were immobile in the examined time period. The corresponding movies are available as supplemental movies 1-3. The asterisk indicates plastidial autofluorescence. Scale bars = 5 μ m.

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Data and materials availability: All data underlying the study are deposited at Protein Data bank (PDB): <https://www.rcsb.org/>) or Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).

Author contributions: T.M. conceptualized the project; L.M., M.H., X.Z., R.T.N., L.B.K., I.M.L.S., F.J., V.K., D.L., K.H., T.M. performed the investigations. J.P. J.M.M., P.S-L. J.C. T.M. validated the data, J.P. J.M.M., P.S-L. J.C. T.M. supervised the work, J.C. and T.M. wrote the original draft of the manuscript, J.P. P.S-L., J.M.M. reviewed and edited the manuscript.

Competing interests: The authors declare no competing interests.

Data and materials availability: All data needed to replicate the work are deposited in the Protein Data Bank (PDB) or Gene Expression Omnibus (GEO) database. Plants and plasmids described in the manuscript are available upon request.

References

1. N. S. Coll, P. Epple, J. L. Dangl, Programmed cell death in the plant immune system. *Cell Death Differ* **18**, 1247-1256 (2011).
2. W. G. van Doorn, Classes of programmed cell death in plants, compared to those in animals. *J Exp Bot* **62**, 4749-4761 (2011).
3. F. K. Chan, N. F. Luz, K. Moriwaki, Programmed necrosis in the cross talk of cell death and inflammation. *Annu Rev Immunol* **33**, 79-106 (2015).
4. A. Daskalov *et al.*, Identification of a novel cell death-inducing domain reveals that fungal amyloid-controlled programmed cell death is related to necroptosis. *Proc Natl Acad Sci U S A* **113**, 2720-2725 (2016).
5. L. Sun *et al.*, Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* **148**, 213-227 (2012).
6. J. Zhao *et al.*, Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci U S A* **109**, 5322-5327 (2012).
7. T. Vanden Berghe, A. Linkermann, S. Jouan-Lanhuet, H. Walczak, P. Vandenabeele, Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol* **15**, 135-147 (2014).
8. E. J. Petrie, P. E. Czabotar, J. M. Murphy, The Structural Basis of Necroptotic Cell Death Signaling. *Trends Biochem Sci* **44**, 53-63 (2019).
9. E. J. Petrie, J. M. Hildebrand, J. M. Murphy, Insane in the membrane: a structural perspective of MLKL function in necroptosis. *Immunol Cell Biol* **95**, 152-159 (2017).
10. X. Chen *et al.*, Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. *Cell Res* **24**, 105-121 (2014).
11. Y. Dondelinger *et al.*, MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep* **7**, 971-981 (2014).
12. J. M. Hildebrand *et al.*, Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A* **111**, 15072-15077 (2014).
13. H. Wang *et al.*, Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell* **54**, 133-146 (2014).
14. G. Quarato *et al.*, Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. *Mol Cell* **61**, 589-601 (2016).
15. A. Bendahmane, K. Kanyuka, D. C. Baulcombe, The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell* **11**, 781-792 (1999).
16. N. S. Coll *et al.*, Arabidopsis type I metacaspases control cell death. *Science* **330**, 1393-1397 (2010).
17. J. S. Pearson, J. M. Murphy, Down the rabbit hole: Is necroptosis truly an innate response to infection? *Cell Microbiol* **19**, (2017).

- 371 18. G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The
372 protein kinase complement of the human genome. *Science* **298**, 1912-
373 1934 (2002).
- 374 19. W. Wang, Y. Wen, R. Berkey, S. Xiao, Specific targeting of the
375 Arabidopsis resistance protein RPW8.2 to the interfacial membrane
376 encasing the fungal Haustorium renders broad-spectrum resistance to
377 powdery mildew. *Plant Cell* **21**, 2898-2913 (2009).
- 378 20. E. A. van der Biezen, C. T. Freddie, K. Kahn, J. E. Parker, J. D. Jones,
379 Arabidopsis RPP4 is a member of the RPP5 multigene family of TIR-
380 NB-LRR genes and confers downy mildew resistance through multiple
381 signalling components. *Plant J* **29**, 439-451 (2002).
- 382 21. F. Jacob *et al.*, A dominant-interfering camta3 mutation compromises
383 primary transcriptional outputs mediated by both cell surface and
384 intracellular immune receptors in Arabidopsis thaliana. *New Phytol* **217**,
385 1667-1680 (2018).
- 386 22. J. M. Murphy *et al.*, The pseudokinase MLKL mediates necroptosis via
387 a molecular switch mechanism. *Immunity* **39**, 443-453 (2013).
- 388 23. M. C. Tanzer *et al.*, Evolutionary divergence of the necroptosis effector
389 MLKL. *Cell Death Differ* **23**, 1185-1197 (2016).
- 390 24. J. Marc *et al.*, A GFP-MAP4 reporter gene for visualizing cortical
391 microtubule rearrangements in living epidermal cells. *Plant Cell* **10**,
392 1927-1940 (1998).
- 393 25. A. R. Bentham, R. Zdrzalek, J. C. De la Concepcion, M. J. Banfield,
394 Uncoiling CNLs: Structure/Function Approaches to Understanding CC
395 Domain Function in Plant NLRs. *Plant Cell Physiol* **59**, 2398-2408
396 (2018).
- 397 26. C. Barragan *et al.*, RPW8/HR Repeats Predict NLR-dependent Hybrid
398 Performance. *bioRxiv*, 559864 (2019).
- 399 27. L. M. Jubic, S. Saile, O. J. Furzer, F. El Kasmi, J. L. Dangl, Help
400 wanted: helper NLRs and plant immune responses. *Curr Opin Plant*
401 *Biol* **50**, 82-94 (2019).
- 402 28. S. M. Collier, L. P. Hamel, P. Moffett, Cell death mediated by the N-
403 terminal domains of a unique and highly conserved class of NB-LRR
404 protein. *Mol Plant Microbe Interact* **24**, 918-931 (2011).
- 405 29. V. Bonardi *et al.*, Expanded functions for a family of plant intracellular
406 immune receptors beyond specific recognition of pathogen effectors.
407 *Proc Natl Acad Sci U S A* **108**, 16463-16468 (2011).

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Methods

Plant material and growth conditions

The *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) was used in this study. The T-DNA insertional mutants(1, 2) (SALK_041569c (*AtMLKL1*), SALK_124412c (*AtMLKL2*) and GABI_491E02 (*AtMLKL3*) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Double and triple mutants of *Atmlkl* were generated by crossing the T-DNA insertion lines. A segregant line derived from the cross between the *Atmlkl1* and *Atmlkl3* mutants were used as a wild-type line in addition to Col-0 wild-type. Each genotype was confirmed by PCR. The *eds1-2* mutant was described previously (3).

The transgenic lines expressing *AtMLKL1* variants or *AtMLKL2* fused to a monomeric green fluorescence protein were established in *Atmlkl1* and *Atmlkl2* mutant backgrounds, respectively. Genomic fragments including coding region and native *cis*-regulatory sequence were amplified by PCR from Col-0 genomic DNA and cloned into pENTR/D-TOPO (Thermo Fisher Scientific, Waltham, MA, USA). The phosphomimetic substitutions were introduced using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Resulting entry vectors were transferred into pGWB550(4) using LR clonase II (Thermo Fisher Scientific). Plants were transformed by the floral dip method (5) with *Agrobacterium tumefaciens* strain GV3101 harbouring pMP90RK (6). Plant growth conditions were described previously (7). Primer sequences for genotyping and plasmid construction are listed in Table S3.

Sequence analysis of plant and animal MLKLs

Sequence similarity within the animal and plant families was established by the generalized profile method (8). Sequences were aligned by the L-INS-I method of the MAFFT alignment software (9), followed by minor manual editing of ambiguously aligned regions. Sequence similarity between the animal and plant MLKL families was established by Hidden Markov Model (HMM)-to-HMM comparison using the HHSEARCH package (10). The 99,696 orthogroups (OGs) among 52 plant species have been established recently (11). An OG containing *At*MLKLs was used for the neighbor-net analysis of codon-aligned nucleotide sequence as described previously (12). The sequence from the papaya genome was excluded in this study.

Protein expression and purification

Full length *At*MLKL3 (residues 1-701) with an engineered C-terminal 6×His tag was generated by standard PCR-based cloning strategy and its identity was confirmed by sequencing. The protein was expressed in sf21 insect cells using the vector pFastBac 1 (Invitrogen). One litre of cells (2.5×10^6 cells ml⁻¹, medium from Expression Systems) was infected with 20 ml baculovirus at 28°C. After growth at 28°C for 48 hours, the cells were harvested, re-suspended in the buffer containing 50 mM Tris-HCl pH 8.0 and 300 mM NaCl, and lysed by sonication. The soluble fraction was purified from the cell lysate using Ni²⁺-nitrilotriacetate affinity resin (Ni-NTA, Qiagen). The protein was then further purified by further purified by gel filtration (Superose 6, 10/30; GE Healthcare). For cryo-EM investigation, the purified protein was concentrated to about 0.3 mg/mL in buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl and 3 mM DTT.

The construct of full length AtMLKL2 (residues 1-711) with N-terminal GST tag was cloned into the pGEX-6P-1 vector (GE Healthcare), and was expressed in *Escherichia coli* strain BL21(DE3; Novagen) at 16 °C. After isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma) induction for 12 h, cells were harvested and re-suspended in buffer containing 50 mM Tris-HCl pH 8.0 and 300 mM NaCl, and lysed by sonication. The soluble fraction was purified from the cell lysate using Glutathione Sepharose 4B beads (Invitrogen). The proteins were then digested with PreScission protease (GE Healthcare) to remove the GST tag and further purified by gel filtration (Superose 6, 10/30; GE Healthcare). For cryo-EM investigation, the purified protein was concentrated to about 0.3 mg/mL in buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl and 3 mM DTT.

Cryo-EM sample preparation and data collection

For cryo-EM analysis, an aliquot of 3.5 μ l AtMLKL2 or AtMLKL3 protein was applied to a holey carbon grids (Quantifoil Cu 1.2/1.3, 200 mesh) glow-discharged (Harrick Plasma) with a middle force for 30 s after evaluating for 2 min. The grids were blotted by a pair of 55 mm filter papers (TED PELLA, INC.) for 3-3.5 s at 8 °C with 100% humidity and flash-frozen in liquid ethane using a FEI Vitrobot Marked IV. Cryo-EM data were collected on Titan Krios electron microscope operated at 300 kV and a Gatan K2 Summit direct electron detection camera (Gatan) using eTas. Micrographs were recorded in super-resolution mode at a nominal magnification of 22500 $\times$, resulting in a physical pixel size of 1.30654 Å per pixel. Defocus values varied from -1.7 μ m to -2.3 μ m for data set. The dose rate was 10.6 electron per pixel per second.

Exposures of 8.0 s were dose-fractionated into 32 sub-frames, leading to a total accumulated dose of 50 electrons per Å². In total, two batches of data were collected, one for AtMLKL3 and another for AtMLKL2.

Image processing and 3D reconstruction

A total of 1,434 and 1,828 raw images stacks of AtMLKL3 and AtMLKL2 acquired under super-resolution mode, were 2x binned processed using MotionCor2 (13), generating aligned, dose-weighted and summed micrographs in a pixel size of 1.30654 Å per pixel. CTFFIND4 (14) was used to estimate the contrast transfer function (CTF) parameters. After the removal of bad micrographs via the evaluation of CTF parameters, remaining images were processed in RELION (15). Approximate 2,000 particles were manually picked and 2D-classified to generate templates for auto-picking. 983,779 and 1,135,463 autopicked particles for AtMLKL3 and AtMLKL2 respectively were then used for reference-free 2D classification, to remove contaminants and bad particles. The left good particles were subjected to 3D classification using initial 3D reference model obtained by *ab initio* calculation from Relion3.0. Particles from good classes that possess density map with better overall structure features were selected for the 3D refinement. The final 3D refinement using D2 symmetry resulted in reconstructions of AtMLKL3 and AtMLKL2 tetramer at resolution of 3.4 Å and 4.1 Å, the resolutions were determined by gold-standard Fourier shell correlation. Local resolution distribution was evaluated using Relion.

Model building and refinement

EM density map of AtMLKL3 was used to build the model de novo, as the overall resolution of map density was efficient to display side chains. The model of AtMLKL3 was manually built into the density in COOT(16), and was refined against the EM map by PHENIX(17) in real space with secondary structure and geometry restraints. The refined AtMLKL3 model was docked into the density of AtMLKL2. The sequence of the docked AtMLKL3 model was changed to that of AtMLKL2 under COOT and the AtMLKL2 model with corrected sequence was subjected to refinement by PHENIX. The C-terminal serine-rich region of AtMLKL2 or AtMLKL3 is much less well defined in the density and is not included in the models. Final model of AtMLKL3 and AtMLKL2 was validated using MolProbity and EMRinger in PHENIX package. The structures of human MLKL (18) and mouse MLKL (19) were used for the superposition of the HeLo domains as shown in Fig. 2D. Table S2 summarized the model statistics.

RNA sequencing

Total mRNA from leaves was obtained at 48 hours after challenge with conidia of *Golovinomyces orontii* using the RNeasy plant mini kit (Qiagen). RNA sequencing (RNA-Seq) libraries were prepared by the Max Planck Genome Centre Cologne (Cologne, Germany) using the Illumina TruSeq stranded RNA sample preparation kit (Illumina). The resulting libraries were subjected to 150-bp single-end sequencing using the Illumina HiSeq3000 (Illumina). Mapping of sequenced reads onto the *Arabidopsis thaliana* gene model (TAIR10), principal component analysis, and differential gene expression analysis were performed in the CLC Genomics Workbench

(Qiagen, ver. 10.1.2) using the tool 'RNA-Seq with the default parameter setting. The data derived from Col-0 and WT were pooled as data of wild-type lines in the analysis. The heat map of 93 genes differentially expressed between wild type lines and *Atmlkl* mutants ($|\log_2FC| > 1$ and false discovery rate (FDR) < 0.05) were generated using the R package (ver. 1.08) with the pheatmap function. Gene ontology enrichment analysis was performed using the PANTHER classification system (<http://pantherdb.org/>) with default settings for *Arabidopsis thaliana*. The RNA-Seq data generated in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE129011.

Transient gene expression in *Arabidopsis* protoplasts

Isolation, transfection and luciferase activity measurement of *Arabidopsis* protoplasts were performed as described previously (20). Protoplasts were isolated from the leaves of two-week-old *Arabidopsis* plants grown in liquid 1 x Murashige and Skoog medium. Coding sequences (CDS) of *AtMLKL1* and *AtMLKL2* without stop codons were initially cloned into pENTR/D-TOPO (Thermo Fisher Scientific). The CDS of *AtMLKL3* without a stop codon was chemically synthesized and cloned into pENTR221 (Thermo Fisher Scientific). Two synonymous substitutions (G1371T and A1413G) were introduced into *AtMLKL3* CDS to remove restriction sites that hamper the DNA synthesis. Entry clones were transferred into the gateway cloning-compatible pAMPAT-GW-mYFP, pAMPAT-GW-3xHA, or pAMPAT-GW expression vectors (21), which are derivatives of pAMPAT-MCS (accession number: AY436765). Primers sequences of for plasmid construction are listed in Table S3. pENTR-

tagRFP-T-Lifeact (22) was transferred into pAMPAT-GW. The expression vectors for HvMLA(1-160aa), MAP4 and Tub6 were described previously (21, 23, 24).

Protoplast viability assay

Following protoplast transfection and regeneration, Evans blue dye dissolved in water was added to the samples to a final concentration of 0.04% (w/v). The stained cells were examined under a standard microscope. For luciferase-based viability assay luciferase and AtMLKL expression constructs were co-transfected into protoplasts and luciferase activity was measured as a proxy of cell viability.

Induced oligomerization

Two domains of FKBP (F36V) tagged with HA without N-myristoylation signal were PCR amplified from pC4M-FV2E plasmid (ARIAD, Cambridge, MA, USA). *NcoI* and *HindIII* restriction sites were added to the 5' end of forward and reverse primers, respectively. The digested PCR fragment with *NcoI* and *HindIII* were ligated into the same restriction sites present between the attR2 and the terminator sequences of pAMPAT-GW expression vector. The resulting vector is named pAMPAT-GW-FV2E-HA. Coding regions corresponding to the N- and C-terminal luciferase fragments (nLUC and cLUC) were PCR amplified from nLUC and cLUC expression vectors (25) and cloned into pENTR/D-TOPO. Respective entry clones were transferred into pAMPAT-GW-FV2E-HA. B/B homodimerizer (also known as AP20187 ligand) were purchased from TakaraBio, Japan. After transfection, protoplasts in

incubation buffer (i.e. WI solution (26)) were separated into two tubes and added the same amount of incubation buffer supplemented with B/B homodimerizer (250 nM at the final concentration) or ethanol as solvent control. Primer sequences for the plasmid construction are listed in Table S2. We were able to reconstitute luciferase activity of co-expressed N- and C-terminal halves of luciferase fused to 2xDmrB domains in a ligand-specific manner (Fig. S8b) and the ligand itself did not affect the luciferase reporter assay in protoplasts (Fig. S8c).

Pathogen infection assay

The *G. orontii* infection assay was performed as described previously (27). *G. orontii* DNA was quantified by qPCR at indicated time points after inoculation of conidia and normalised using the amount of plant specific gene (AT3G21215). The *Hpa* infection assay was performed as previously described (28). Lactophenol-trypan blue staining was described previously (29). The *B. cinerea* strain B05.10 was used in this study. Droplet inoculation of six-week-old plants was performed as described previously (30), except that 2 µl of the spore solution were used on each side of the leaf and two leaves of similar age were used per plant. *B. cinerea* DNA was quantified by qPCR as previously described (31). *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and *Pst* DC3000 expressing *AvrRpt2* or *AvrRpm1* were used in this study. *Pst* growth assays and ion leakage measurement following bacterial infiltration were performed as described previously (7).

Immunoblot assays

Primary antibodies were monoclonal antibodies from mouse: α -GFP (JL-8, 1:5000, Takara, Shiga, Japan) or rat: α -HA (3F10, 1:1000, Sigma-Aldrich, St. Louis, MO, USA). Goat α -mouse IgG-HRP (1:10000, Santa Cruz Biotechnology, Dallas, TX, USA) or goat α -rat IgG-HRP (1:10000, Sigma-Aldrich) were used as secondary antibodies. The detailed procedure is described in (7).

Biolistic transient gene expression

Biolistic delivery of plasmid DNA into the abaxial epidermis of leaves was essentially performed as described previously (32). Leaves were detached immediately before bombardment and the bombarded leaves were transferred to 1% agar plates supplemented with 85 μ M benzimidazole and incubated at 20°C for 15 h before confocal microscopy.

Confocal microscopy

Transfected protoplasts in a chamber slide (Nunc Lab-Tek, Thermo Fisher Scientific) with incubation buffer (i.e. WI solution, (26)) or 2-5 mm leaf discs prepared from rosette leaves of 4-5-week-old plants were observed under a confocal microscope (LSM880, Carl Zeiss) equipped with a 40X water-immersion and a 63X oil-immersion objective. Lambda stack images were obtained for spectral imaging. Images were analyzed and processed with ZEN Software (Carl Zeiss) and ImageJ (NIH). In Fig. 4E, confocal images were acquired over time (for wild type, 124 seconds; for S393D, 194 seconds; for

629 S395D, 166 seconds) and used for maximum intensity projection (bottom
630 panels). Representative single frame images are shown (top panels).

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Supplemental data

Supplemental Fig. 1: 3D reconstruction of AtMLKL3 tetramer

A, Representative cryo-EM image of AtMLKL3 tetramer. Representative particles are indicated with black circles.

B, Representative top and side views of 2D class averages of AtMLKL3 tetramer.

C, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D reconstruction of AtMLKL3 tetramer.

D, Flowchart representing cryo-EM data processing and 3D reconstruction of AtMLKL3 tetramer.

Supplemental Fig. 2: Purification and 3D reconstruction of AtMLKL2 tetramer

A, Top: Gel filtration profile of AtMLKL2 protein. Position of standard molecular weight is indicated by arrow. Bottom: Peak fractions in the top were verified by SDS-PAGE with Coomassie Blue staining.

B, Representative cryo-EM images of the AtMLKL2 tetramer. Representative particles are indicated with black circles.

C, Representative top and side views of 2D class averages of AtMLKL2 tetramer.

D, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D reconstruction of AtMLKL2 tetramer.

E, Flowchart representing cryo-EM data processing and 3D reconstruction of AtMLKL2 tetramer.

Supplemental Fig. 3: Inter- and intra-domain interactions of AtMLKL3 tetramer and structural comparison of the AtMLKL2 and AtMLKL3

A, Superposition of AtMLKL2 (grey) and AtMLKL3 (blue). RMSD between monomer of AtMLKL2 and AtMLKL3 :0.985.

B, The sequence of AtMLKL3 model (blue) was docked into AtMLKL2 map (gray).

C, Top: Cartoon representation of AtMLKL3 monomer in two orientations. Subdomains of AtMLKL3 are shown in different colors, the interacting regions between domains are highlighted with open frames. Bottom left: Detailed interactions of HeLo domain and pseudokinase domain for the red-framed region. Bottom right: Detailed interactions of HeLo domain and brace region for the blue-framed region.

D, Structural comparison of the monomer AtMLKL2 (left), AtMLKL3 (middle, PDB ID code 6KA4) and mMLKL (right, PDB ID code 4BTF).

E, Top left: Cartoon showing HeLo domain and brace region of AtMLKL3 tetramer. Top right: Detailed interactions of HeLo domains for the green-

framed region. Bottom left: Detailed C-terminal interactions of brace regions for the blue-framed region. Bottom right: Detailed N-terminal interactions of brace regions for the blue-framed region.

Supplemental Fig. 4: *Atmlkl* mutants exhibit no obvious growth defect.

A, The Arabidopsis *MLKL* genes and T-DNA insertion sites of the *Atmlkl* mutants. White and black boxes indicate non-coding and coding exons, respectively. A gene model for *AtMLKL3* was deduced from other plant *MLKL* structures.

B, Representative images of four-week-old plants of Col-0, wild type*, single mutants of *Atmlkl1*, *Atmlkl2*, *Atmlkl3*, double mutants *Atmlkl1 2*, *Atmlkl1 3*, *Atmlkl2 3*, and the triple mutant *Atmlkl1 2 3*. *The wild type is a segregant line derived from the cross between *Atmlkl1* and *Atmlkl3*. The plants were initially grown on Murashige and Skoog-agar plates for two weeks and subsequently transferred to Jiffy pots rehydrated in water with a fertilizer. Plants were grown for an additional two weeks under short-day conditions.

Supplemental Fig. 5: Arabidopsis MLKLs do not confer resistance to *Botrytis cinerea* and *Pseudomonas syringae* DC3000.

A, *Botrytis cinerea* DNA was quantified by qPCR at three days after spore inoculation and normalized using the amount of plant specific gene (see method). Amounts are presented relative to the corresponding Col-0 samples. The wild type is a segregant line derived from the cross between *Atmlkl1* and *Atmlkl3* (see Fig. S4). Data were obtained in three independent experiments (n=3).

B, Log₁₀-transformed colony forming units of *Pseudomonas syringae* DC3000 per cm² of *A. thaliana* leaves at two days after pathogen infiltration. The *eds1* mutant was used as a susceptible control. Data were obtained in three independent experiments (n=3).

C, Log₁₀-transformed colony forming units of *P. syringae* DC3000 expressing AvrRpt2 per cm² of *A. thaliana* leaves at three days after pathogen infiltration. The *rpm1 rps2* mutant was used as a susceptible control. Data were obtained in three independent experiments (n=3).

D, Ion leakage assay in *A. thaliana* leaves upon infiltration with *P. syringae* DC3000 expressing AvrRpm1. Samples were collected 30 min post infiltration with the bacterial suspension (OD₆₀₀=0.1). The *rpm1 rps2* mutant was used as a negative control. Data were obtained in three independent experiments (n=3).

n.s. not significant.

Supplemental Fig. 6: Heat map of 93 genes differentially expressed in *Atmlkl123* in comparison to wild type. The gene ontology (GO) term biotic or abiotic stresses (GO:0006950, asterisks) was overrepresented in the down-regulated transcripts in *Atmlkl123* compared to wild type lines. Mock- and

pathogen-challenged leaves were collected at 48 hours post *G. orontii* inoculation. *WT is a segregant line derived from the cross between *Atmlk1* and *Atmlk3*.

Supplemental Fig. 7: AtMLKLs are capable of eliciting cell death in *Arabidopsis* mesophyll protoplasts.

A, C-terminally HA-tagged and **B**, C-terminally mYFP-tagged AtMLKLs were expressed under the constitutive cauliflower mosaic virus 35S promoter. Expression constructs for AtMLKLs were transfected into protoplasts and cells were stained with Evans blue at 16 hours post transfection. Unstained cells (Evans blue-negative cells) were counted as living cells. For **B**, The N-terminal signalling region of barley NLR protein (*HvMLA₁₋₁₆₀*: (7) was used as a positive control. Data were obtained in three independent transfections (n=3) and different letters indicate statistically significant differences (Tukey HSD, $p < 0.01$).

C. Phosphomimetic substitutions in AtMLKL2 or AtMLKL3 do not alter cell death in *Arabidopsis* mesophyll protoplasts.

Expression constructs for luciferase and each of the AtMLKL variants were co-transfected into protoplasts and luciferase activity was measured as a proxy for cell viability at 16 hours post transfection. The positions of serine-to-aspartate substitutions are indicated in Fig. 1B. The C-terminally HA-tagged variants were expressed under the 35S promoter. Data were obtained in three independent transfections (n=3). n.s. not significant.

D, Western blot analysis of expression of C-terminally HA-tagged AtMLKLs in *Arabidopsis* mesophyll protoplasts. Total protein extracts were collected at seven hours post transfection. Samples that were transfected with the corresponding empty vector or the expression construct for AtMLKL2-mYFP were used as negative control.

Supplemental Fig. 8: Genetic complementation of the *Atmlk1* mutant.

A, Macroscopic phenotype of transgenic *Arabidopsis* lines expressing wild-type or phosphomimetic variants of AtMLKL1-GFP under the native *cis*-regulatory sequence in *Atmlk1* in response to *G. orontii*. Plants were photographed seven days after pathogen challenge.

B, Western blot analysis of the C-terminally GFP-tagged AtMLKL1 variants in the stable transgenic lines. Total protein extracts were collected from two-week-old plants of the transgenic lines grown on sterile Murashige and Skoog solid media.

Supplemental Fig. 9: Genetic complementation of the *Atmlk2* mutant.

A, Macroscopic phenotype of transgenic *Arabidopsis* lines expressing AtMLKL2-GFP under the native *cis*-regulatory sequence in *Atmlk2*. Four-

week-old plants were inoculated with *G. orontii* spores and plants were photographed at 14 days after pathogen challenge.

B, Western blot analysis of C-terminally GFP-tagged AtMLKL2. Total protein extracts were collected from two-week-old plants of the transgenic lines grown on sterile Murashige and Skoog solid media.

The asterisk and arrow indicate non-specific and specific bands, respectively.

C, Quantification of *G. orontii* DNA in infected leaves. Four-week-old plants were inoculated with spores of the powdery mildew and plants were examined at 14 days after the pathogen challenge. Fungal DNA was quantified by qPCR and normalised using the amount of plant specific gene (AT3G21215). The relative amounts to corresponding Col-0 wild-type samples were presented. Experiments were repeated three times (n=3) and the asterisk indicates a statistically significant difference (Tukey HSD, $p < 0.05$).

Supplemental Fig. 10: N-terminal domains of AtMLKL1 and 3 were sufficient to elicit cell death and their enforced oligomerization potentiated the activities.

A, Schematic of chemically induced oligomerization. AP20187 (Dimerizer), a synthetic cell-permeable ligand, induces homodimerization of fusion proteins containing the DmrB domain. Dimerizer-dependent oligomerization is facilitated by a tandem fusion of DmrB domains.

B, Dimerizer-induced reconstitution of luciferase activity of the N- or C-terminal halves of luciferase fused with 2 x DmrB domains. Data were obtained with eight independent transfections (n=8). Luciferase activity was measured at 16 hours post transfection. The luciferase activity was statistically higher in the presence of dimerizer (one sample *t*-test, $p < 0.01$).

C, Dimerizer does not influence the luciferase reporter assay. Data were obtained with seven independent transfections (n=7). Luciferase activity was measured at 16 hours post transfection. No statistically significant differences were detected (one sample *t*-test).

D, HeLo domain plus brace region of AtMLKL1 and AtMLKL3 were sufficient to elicit cell death and their enforced oligomerization potentiated the activities. Expression constructs for luciferase and each of the AtMLKL fusion proteins indicated in the figure were co-transfected into protoplasts and luciferase activity was measured as a proxy of cell viability at 16 hours post transfection. Relative luciferase activities compared to those of the empty vector control with dimerizer were plotted. Data were obtained with eight independent transfections (n=9). Asterisks indicate statistically significant differences (Tukey HSD, $p < 0.01$).

E, Western blot analysis of the HeLo domain plus brace region constructs expressed in *Arabidopsis* mesophyll protoplasts. The fusion proteins were expressed as C-terminally HA-tagged proteins. Total protein extracts were collected at seven hours post-transfection.

B-E, Mesophyll protoplasts were prepared from the triple mutant *Atmlk1/123* and AP20187 (Dimerizer) was added after transfection (see method).

Supplemental Fig. 11: Subcellular localization of AtMLKLs in protoplasts.

A, Subcellular localization of AtMLKLs in *Arabidopsis* mesophyll protoplasts. The C-terminally monomeric YFP (mYFP)-tagged variants were expressed under the 35S promoter. Representative confocal images were taken at ten hours post transfection. Scale bars = 10 μ m.

B, Co-expression of AtMLKL1 and cytoskeleton markers in *Arabidopsis* mesophyll protoplasts. Co-expression of AtMLKL1-mYFP and mcherry-MAP4 (microtubule marker: (33) top panels), or tagRFP-T-Lifeact (Actin marker: (34) middle panels). Representative confocal images were taken at 10 hours post transfection. Scale bars = 10 μ m.

C, The filamentous structures of AtMLKL1-mYFP were undetectable at 5 minutes after application of 5 μ M of the microtubule inhibitor, oryzalin. Representative confocal image was taken at 10 hours post transfection. Scale bar = 10 μ m.

Supplemental Fig. 12: Confocal images of an epidermal cell that was infected by *G. orontii*. The maximum intensity projection was obtained from confocal Z-stack images and the autofluorescence of fungal structures were discriminated from AtMLKL1-GFP signals by spectral imaging. Arrowheads indicate filamentous structures associated with AtMLKL1-GFP. C; cytoplasm, Ha: haustorium, E, haustorial encasement. Scale bar = 5 μ m.

Supplemental Fig. 13: Cortical microtubule arrays in abaxial leaf epidermal cells of transgenic *A. thaliana* lines expressing phosphomimetic and wild type variants of AtMLKL1. Representative images were obtained one day after biolistic delivery of the expression construct for the Atubiquitin10 promoter driven by mCherry-TUB6 (microtubule marker (24)) into the indicated transgenic lines. Scale bars = 10 μ m.

Supplemental movie 1

Dynamics of AtMLKL1(wild-type)-GFP in a non-pathogen-challenged abaxial leaf epidermal cell. The corresponding projection image is shown in Fig. 4E. The white circle indicates a GFP signal proximal to the PM (see Fig. 4D) and which was less mobile compared to other signals.

Supplemental movie 2

Dynamics of AtMLKL1(S393D)-GFP in a non-pathogen-challenged abaxial leaf epidermal cell. The corresponding projection image is shown in Fig. 4E.

The white circle indicates a GFP signal proximal to the PM (see Fig. 4D) and which was less mobile compared to other signals.

Supplemental movie 3

Dynamics of *AtMLKL1*(S395D)-GFP in a non-pathogen-challenged abaxial leaf epidermal cell. The corresponding projection image is shown in Fig. 4E. The white circle indicates a GFP signal proximal to the PM (see Fig. 4D) and which was less mobile compared to other signals.

Supplemental file 1

108 coding sequences (CDS) of plant *MLKLs* belong to the same orthogroups (11). A CDS derived from *Arabidopsis halleri* is incomplete due to the ambiguous assembly of the corresponding genomic region.

Table S1. Number of MLKL-like genes in plant genomes

Plant species	NCBI Taxonomy ID	Family	Number of <i>MLKL</i> -like genes
<i>Coccomyxa subellipsoidea</i>	248742	Coccomyxaceae	0
<i>Ostreococcus lucimarinus</i>	242159	Bathycoccaceae	0
<i>Volvox carteri</i>	3067	Volvocaceae	0
<i>Physcomitrella patens</i>	3218	Funariaceae	0
<i>Selaginella moellendorffii</i>	88036	Selaginellaceae	0
<i>Actinidia chinensis</i>	3625	Actinidiaceae	3
<i>Amborella trichopoda</i>	13333	Amborellaceae	1
<i>Spirodela polyrhiza</i>	29656	Araceae	2
<i>Phoenix dactylifera</i>	42345	Arecaceae	3
<i>Arabidopsis halleri</i>	81970	Brassicaceae	2
<i>Arabidopsis lyrata</i>	59689	Brassicaceae	3
<i>Arabidopsis thaliana</i>	3702	Brassicaceae	2
<i>Brassica oleracea</i>	3712	Brassicaceae	3
<i>Brassica rapa</i>	3711	Brassicaceae	2
<i>Boechera stricta</i>	72658	Brassicaceae	3
<i>Capsella grandiflora</i>	264402	Brassicaceae	3
<i>Capsella rubella</i>	81985	Brassicaceae	3
<i>Eutrema salsugineum</i>	72664	Brassicaceae	4
<i>Schrenkiella parvula</i>	98039	Brassicaceae	3
<i>Beta vulgaris</i>	161934	Chenopodiaceae	2
<i>Cucumis sativus</i>	3659	Cucurbitaceae	2
<i>Ricinus communis</i>	3988	Euphorbiaceae	2
<i>Arachis ipaensis</i>	130454	Fabaceae	2
<i>Medicago truncatula</i>	3880	Fabaceae	2
<i>Phaseolus vulgaris</i>	3885	Fabaceae	2
<i>Linum usitatissimum</i>	4006	Linaceae	4
<i>Gossypium raimondii</i>	29730	Malvaceae	3
<i>Theobroma cacao</i>	3641	Malvaceae	2
<i>Musa acuminata</i>	4641	Musaceae	4
<i>Erythranthe guttata</i>	4155	Phrymaceae	2
<i>Pinus taeda</i>	3352	Pinaceae	1
<i>Brachypodium distachyon</i>	15368	Poaceae	2
<i>Oryza sativa</i>	4530	Poaceae	2
<i>Sorghum bicolor</i>	4558	Poaceae	2
<i>Setaria italica</i>	4555	Poaceae	2
<i>Zea mays</i>	4577	Poaceae	2
<i>Aquilegia coerulea</i>	218851	Ranunculaceae	1
<i>Fragaria vesca</i>	57918	Rosaceae	2
<i>Malus domestica</i>	3750	Rosaceae	5
<i>Prunus persica</i>	3760	Rosaceae	2

<i>Coffea canephora</i>	49390	Rubiaceae	2
<i>Citrus clementina</i>	85681	Rutaceae	2
<i>Citrus sinensis</i>	2711	Rutaceae	2
<i>Populus trichocarpa</i>	3694	Salicaceae	2
<i>Capsicum annuum</i>	4072	Solanaceae	3
<i>Solanum melongena</i>	4111	Solanaceae	3
<i>Solanum lycopersicum</i>	4081	Solanaceae	3
<i>Solanum tuberosum</i>	4113	Solanaceae	3
<i>Vitis vinifera</i>	29760	Vitaceae	2

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858 **Table S2. Cryo-EM statistics and model refinement for AtMLKL tetramer**

	AtMLKL3 tetramer	AtMLKL2 tetramer
Data collection and processing		
Microscope	FEI Titan Krios	FEI Titan Krios
Detector	Gatan K2 Summit	Gatan K2 Summit
Voltage(kV)	300	300
Magnification	22500x	22500x
Pixel size (Å)	1.30654	1.30654
Total electron dose (e ⁻ /Å ²)	50	50
Defocus range (μm)	-1.5 ~ -2.3	-1.6~-2.2
Micrographs collected	1,434	1,828
Reconstruction		
Total extraced particles	983,779	1,527,009
Number of particles used for 3D reconstruction	629,287	796,901
Number of particles used for refinement	123,472	154,008
Symmetry	D2	D2
Resolution (Å) 0.143 after refinement	3.84	4.51
Resolution (Å) 0.143 after post-processing	3.39	4.08
Map sharpening B-factor (Å ²)	-152	-222.79
Refinement		
Estimated Resolution (Å)	3.4	4.1
Model composition		
Number of protein atoms	16584	14238
Number of ligand atoms	0	0
MapCC (mask/box)	0.83/0.79	0.81/0.81
Rwork/Rfree (%)	34.35/34.35	39.4/39.4
R.M.S deviations		
Bonds lengths (Å)	0.011	0.010
Bonds angles (°)	0.941	0.797
Validation		
MolProbity overall score	2.22	2.56
All-atom clashscore	15.96	18.07
Rotamer outliers (%)	0.67	1.57
C-beta deviations	0.0	0.0
EMRinger score	1.56	1.42
Ramachandran plot statistics		
Preferred (%)	91.27	85.27
Allowed (%)	8.33	14.31
Outlier (%)	0.40	0.42

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862 **Table S3 Primer pairs used in this study.**

Forward primer	Sequence 5' -3'	Reverse primer	Sequence 5' -3'	Use, comments
SALK_041569c_RP	GAGAGACAG CGAAAACCT GTG	SALK_041569c_LP	CTTCTCTT GCAATGCC TCAAC	Genotyping of the wild type allele of <i>AtMLKL1</i>
SALK_041569c_RP	GAGAGACAG CGAAAACCT GTG	LBb1.3	ATTTTGCC GATTCGG AAC	Genotyping of the mutant allele of <i>AtMLKL1</i>
SALK_124412c_RP AT5G41730	TCAGCTTAG CTTTCAACA CGG	SALK_124412c_LP AT5G41730	GAAGATAC TCCATCCC CCTTG	Genotyping of the wild type allele of <i>AtMLKL2</i>
SALK_124412c_RP AT5G41730	TCAGCTTAG CTTTCAACA CGG	LBb1.3	ATTTTGCC GATTCGG AAC	Genotyping of the mutant allele of <i>AtMLKL2</i>
GABI_491E02_RP	TACACGAAG CCCATTAC TTC	GABI_491E02_LP	TATTACCT CTGCGGAT TCACG	Genotyping of the wild type allele of <i>AtMLKL3</i>
GABI_o3144/35St	GTGGATTGA TGTGATATC TCC	GABI_491E02_LP	TATTACCT CTGCGGAT TCACG	Genotyping of the mutant allele of <i>AtMLKL3</i>
AT1g64300-Fw1	CACCATGGA GCAATTCAG GCAAATCGG AGAGGTT	AT1g64300-Rv1	TGTAAGCT CAGAGTCT GAAGCAG GACCAGTT A	D-topo cloning of <i>AtMLKL1</i> cDNA without STOP codon
AT5G41730-Fw1	CACCATGGA GCAATTCAG ACAAATCGG AGAAGTTCT T	AT5G41730-Rv1	AGTAAGCT CAGAGTCT GAAGCATG ACCAGTCT GTT	D-topo cloning of <i>AtMLKL2</i> cDNA without STOP codon
D-topo- <i>AtMLKL3</i> -f	CACCATGGA TCAATTTTCG AGAGATAGG AGAGGTATT	D-topo- <i>AtMLKL3</i> -r_without_stop	GGAAAGCT CAGAGTCA GAGGCAT GT	D-topo cloning of <i>AtMLKL3</i> cDNA without STOP codon
<i>AtMLKL1</i> (S393D)-f	CCTCAGTTA AGAATCAGG ATTTTCTCG GGCTTCC	<i>AtMLKL1</i> (S393D)-r	GGAAGCC CGAGAAAA ATCCTGAT TCTTAACT GAGG	Site-directed mutagenesis to obtain <i>AtMLKL1</i> (S393D) variant
<i>AtMLKL1</i> (S395D)-f	GTTAAGAAT CAGTCTTT GATCGGGCT TCCTCTAGA C	<i>AtMLKL1</i> (S395D)-r	GTCTAGAG GAAGCCC GATCAAAA GACTGATT CTTAAC	Site-directed mutagenesis to obtain <i>AtMLKL1</i> (S395D) variant
AT5G41730_(S394D)-f	GTCGTTAAA GCGCAGGAT TCCTCGAAA CCAG	AT5G41730_(S394D)-r	CTGGTTTC GAGGAATC CTGCGCTT TAACGAC	Site-directed mutagenesis to obtain <i>AtMLKL2</i> (S394D) variant
AT5G41730_(S395D)-f	CGTTAAAGC GCAGTCTGA TTCGAAACC AGGCACTCC	AT5G41730_(S395D)-r	GGAGTGC CTGGTTTC GAATCAGA CTGCGCTT TAACG	Site-directed mutagenesis to obtain <i>AtMLKL2</i> (S395D) variant
<i>AtMLKL3</i> (S392D)-f	GTTGAATTC TGCAAGGG ATTCGATAG TAAAGTGC TTCTTTGAC G	<i>AtMLKL3</i> (S392D)-r	CGTCAAAG AAGCACTT TACTATC GAATCCCT TGACAGAA TTCAAC	Site-directed mutagenesis to obtain <i>AtMLKL3</i> (S392D) variant

AtMLKL3(SS393D)-f	CTGTCAAGG GATTCTCTG ATAAAAGTG CTTCTTTGA C	AtMLKL3(S393D)-r	GTCAAAGA AGCACTTT TATCAGAG AATCCCTT GACAG	Site-directed mutagenesis to obtain AtMLKL3(S393D) variant
Genomic of AT1G64300-2nd_f	CACCCCTTG AGCCCAAAG TTTCTC	Genomic of AT1G64300-3rd_r for without stop	TGTAAGCT CAGAGTCT GAAGCAG GACCAGTT AGTTG	D-topo cloning of genomic fragments including coding region and native <i>cis</i> -regulatory sequence of AtMLKL1
Genomic of AT5g41730-2nd_f_new	CACCGTCGA GGACCAAAG GCTAGT	Genomic of AT5g41730-3rd_r for w/o stop	AGTAAGCT CAGAGTCT GAAGCATG A	D-topo cloning of genomic fragments including coding region and native <i>cis</i> -regulatory sequence of AtMLKL2
Go_V1_Cont	TCTTGGTGG CACGAATGA C	Go_V1_Contig76-r	AGTGCGA GAGTGGG ACAGAC	qPCR quantification of the fungal DNA
At3g21215-f	GAATCCACC CATACCACC AG	At3g21215-r	GAGGAGG AGGATGGT GATGA	qPCR quantification of the Arabidopsis DNA

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Supplemental References

1. N. Kleinboelting, G. Huep, A. Kloetgen, P. Viehoveer, B. Weisshaar, GABI-Kat SimpleSearch: new features of the Arabidopsis thaliana T-DNA mutant database. *Nucleic Acids Res* **40**, D1211-1215 (2012).
2. J. M. Alonso *et al.*, Genome-wide insertional mutagenesis of Arabidopsis thaliana. *Science* **301**, 653-657 (2003).
3. M. Bartsch *et al.*, Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. *Plant Cell* **18**, 1038-1051 (2006).
4. T. Nakagawa *et al.*, Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci Biotechnol Biochem* **71**, 2095-2100 (2007).
5. S. J. Clough, A. F. Bent, Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* **16**, 735-743 (1998).
6. C. Koncz, J. Schell, The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. *Molecular and General Genetics MGG* **204**, 383-396 (1986).
7. F. Jacob *et al.*, A dominant-interfering camta3 mutation compromises primary transcriptional outputs mediated by both cell surface and intracellular immune receptors in Arabidopsis thaliana. *New Phytol* **217**, 1667-1680 (2018).
8. P. Bucher, K. Karplus, N. Moeri, K. Hofmann, A flexible motif search technique based on generalized profiles. *Comput Chem* **20**, 3-23 (1996).
9. K. Katoh, K. Misawa, K. Kuma, T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059-3066 (2002).
10. J. Soding, Protein homology detection by HMM-HMM comparison. *Bioinformatics* **21**, 951-960 (2005).
11. D. Lapin *et al.*, A coevolved EDS1-SAG101-NRG1 module mediates cell death signaling by TIR-domain immune receptors. *bioRxiv*, 572826 (2019).
12. T. Maekawa *et al.*, Subfamily-Specific Specialization of RGH1/MLA Immune Receptors in Wild Barley. *Mol Plant Microbe Interact* **32**, 107-119 (2019).
13. S. Q. Zheng *et al.*, MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* **14**, 331-332 (2017).
14. A. Rohou, N. Grigorieff, CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *J Struct Biol* **192**, 216-221 (2015).
15. J. Zivanov *et al.*, New tools for automated high-resolution cryo-EM structure determination in RELION-3. *Elife* **7**, (2018).
16. A. Brown *et al.*, Tools for macromolecular model building and refinement into electron cryo-microscopy reconstructions. *Acta Crystallogr D Biol Crystallogr* **71**, 136-153 (2015).

- 915 17. P. D. Adams *et al.*, PHENIX: a comprehensive Python-based system
916 for macromolecular structure solution. *Acta Crystallogr D Biol*
917 *Crystallogr* **66**, 213-221 (2010).
- 918 18. L. Su *et al.*, A plug release mechanism for membrane permeation by
919 MLKL. *Structure* **22**, 1489-1500 (2014).
- 920 19. J. M. Murphy *et al.*, The pseudokinase MLKL mediates necroptosis via
921 a molecular switch mechanism. *Immunity* **39**, 443-453 (2013).
- 922 20. I. M. Saur *et al.*, Multiple pairs of allelic MLA immune receptor-powdery
923 mildew AVRAs effectors argue for a direct recognition mechanism. *Elife*
924 **8**, (2019).
- 925 21. T. Maekawa *et al.*, Coiled-coil domain-dependent homodimerization of
926 intracellular barley immune receptors defines a minimal functional
927 module for triggering cell death. *Cell Host Microbe* **9**, 187-199 (2011).
- 928 22. F. Vogler, S. S. Konrad, S. Sprunck, Knockin' on pollen's door: live cell
929 imaging of early polarization events in germinating Arabidopsis pollen.
930 *Front Plant Sci* **6**, 246 (2015).
- 931 23. T. Hamada *et al.*, Purification and characterization of novel
932 microtubule-associated proteins from Arabidopsis cell suspension
933 cultures. *Plant Physiol* **163**, 1804-1816 (2013).
- 934 24. S. Fujita *et al.*, An atypical tubulin kinase mediates stress-induced
935 microtubule depolymerization in Arabidopsis. *Curr Biol* **23**, 1969-1978
936 (2013).
- 937 25. C. Gehl *et al.*, Quantitative analysis of dynamic protein-protein
938 interactions in planta by a floated-leaf luciferase complementation
939 imaging (FLuCI) assay using binary Gateway vectors. *Plant J* **67**, 542-
940 553 (2011).
- 941 26. S. D. Yoo, Y. H. Cho, J. Sheen, Arabidopsis mesophyll protoplasts: a
942 versatile cell system for transient gene expression analysis. *Nat Protoc*
943 **2**, 1565-1572 (2007).
- 944 27. R. Wessling, R. Panstruga, Rapid quantification of plant-powdery
945 mildew interactions by qPCR and conidiospore counts. *Plant Methods*
946 **8**, 35 (2012).
- 947 28. A. Cabral *et al.*, Identification of *Hyaloperonospora arabidopsidis*
948 transcript sequences expressed during infection reveals isolate-specific
949 effectors. *PLoS One* **6**, e19328 (2011).
- 950 29. S. Bai *et al.*, Structure-function analysis of barley NLR immune
951 receptor MLA10 reveals its cell compartment specific activity in cell
952 death and disease resistance. *PLoS Pathog* **8**, e1002752 (2012).
- 953 30. R. P. Birkenbihl, C. Diezel, I. E. Somssich, Arabidopsis WRKY33 is a
954 key transcriptional regulator of hormonal and metabolic responses
955 toward *Botrytis cinerea* infection. *Plant Physiol* **159**, 266-285 (2012).
- 956 31. C. Gachon, P. Saindrenan, Real-time PCR monitoring of fungal
957 development in Arabidopsis thaliana infected by *Alternaria brassicicola*
958 and *Botrytis cinerea*. *Plant Physiol Biochem* **42**, 367-371 (2004).
- 959 32. Q. H. Shen *et al.*, Recognition specificity and RAR1/SGT1 dependence
960 in barley Mla disease resistance genes to the powdery mildew fungus.
961 *Plant Cell* **15**, 732-744 (2003).
- 962 33. J. Marc *et al.*, A GFP-MAP4 reporter gene for visualizing cortical
963 microtubule rearrangements in living epidermal cells. *Plant Cell* **10**,
964 1927-1940 (1998).

- 965 34. J. Riedl *et al.*, Lifeact: a versatile marker to visualize F-actin. *Nat*
 966 *Methods* **5**, 605-607 (2008).

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Figure 1

Figure 2

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G. orontii

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H. arabidopsidis Emwa1

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Figure 3

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Figure 4

Figure S1

Figure S2

Figure S3

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Figure S4

Figure S5

Figure S6

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Figure S7

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Figure S8

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Figure S9

Figure S10

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Figure S11

Figure S12

Figure S13