1 **Title: Plant mixed lineage kinase domain-like proteins limit biotrophic**

2 **pathogen growth**

3

4 **One Sentence Summary: Plants have a protein family that is**

5 **structurally homologous to vertebrate mixed lineage kinase domain-**

6 **like protein, which induces necroptotic cell death, but these plant**

7 **proteins can confer immunity without host cell death.**

8

9 **Short title: Plant MLKLs confer disease resistance**

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34

34   **Abstract:**

35   Mixed lineage kinase domain-like (MLKL) protein mediates necroptotic cell

36   death in vertebrates. We report here the discovery of a conserved protein

37   family across seed plants that is structurally homologous to vertebrate

38   MLKL. The *Arabidopsis thaliana* genome encodes three MLKLs with

39   overlapping functions in limiting growth of obligate biotrophic fungal and

40   oomycete pathogens. Although displaying a cell death activity mediated by N-

41   terminal helical bundles, termed HeLo domain, *At*MLKL-dependent immunity

42   can be separated from host cell death. Cryo-electron microscopy structures of

43   *At*MLKLs reveal a tetrameric configuration, in which the pseudokinase domain

44   and brace region bury the HeLo-domains, indicative of an auto-repressed

45   complex. We also show the association of two *At*MLKLs with microtubules.

46   These findings, coupled with resistance-enhancing activity and altered

47   microtubule association of a phosphomimetic mutation in the pseudokinase

48   domain of *At*MLKL1, point to a cell death-independent immunity mechanism.

49

49 **Main text:**

50 Regulated cell death (RCD) is intimately connected with innate immunity in

51 plants and animals (*1-3*). A shared feature of several proteins involved in RCD

52 in plants, animals and fungi is a four-helical bundle structure called the HeLo-

53 domain (*4*). HeLo domain-containing MLKL (mixed lineage kinase domain-like

54 protein) mediates necroptosis in animals (*5, 6*), a form of RCD that is

55 proposed to combat pathogens by releasing pro-inflammatory molecules (*7,*

56 *8*). Necroptosis is initiated by the plasma membrane (PM)-resident death

57 receptors, with downstream activation of receptor-interacting

58 serine/threonine-protein kinases (RIPKs) leading to the phosphorylation of the

59 pseudokinase domain of the terminal pathway effector, MLKL (*7, 8*). This

60 process assembles monomeric MLKL into pro-necroptotic oligomers (*9*) that

61 translocate to the PM where oligomerized HeLo-domains interfere with

62 membrane integrity (*10-14*). The extent to which RCD in plants and animals is

63 directly responsible for disease resistance is under debate (*3, 15-17*).

64

65 To identify novel immune regulators in plants, we searched for HeLo domain-

66 containing proteins in plant genomes by comparing Hidden Markov Models.

67 This analysis identified a protein family that is highly conserved across plants

68 (Fig. 1, Table S1, Supplemental file 1), with a modular structure resembling

69 MLKL (Fig. 1A). The kinase-like domain lacks canonical residues known to

70 underlie phosphoryl transfer (*18*) (Fig. 1B), suggestive of a catalytically

71 inactive pseudokinase (*8*). Hereafter, we refer to these proteins as plant

72 MLKLs. Plant MLKLs additionally possess an extended serine-rich region of

73 varying length after the pseudokinase domain without similarity to any known

74   domain (Fig. 1A, Supplemental file 1). Plant MLKLs separate into two

75   subgroups based on sequence similarity (Fig. 1D). *Arabidopsis thaliana*

76   harbours three *MLKL* genes with *AtMLKL1* and *AtMLKL2* in subfamily I and

77   *AtMLKL3* belonging to subfamily II (Fig. 1D).

78

79   To explore similarities between plant and animal MLKLs, we expressed

80   *At*MLKL2 and *At*MLKL3 for structural analysis using cryo-electron microscopy

81   (Fig. 2, Fig. S1 and S2). In gel filtration, both proteins eluted at an estimated

82   molecular weight corresponding to tetramers (Fig. 2A, Fig. S2A). This

83   contrasts with the vertebrate MLKL protein, which displayed heterogeneity in

84   a similar assay (*13*). Representative 2D projection views indicate that both

85   *At*MLKL oligomers form a triangle-like architecture with a 2-fold symmetry (Fig.

86   S1AB, Fig. S2BC). Further 3D classification and refinement generated density

87   maps of oligomeric *At*MLKL2 and *At*MLKL3 with a global resolution of 4.1Å

88   (Fig. S2E) and 3.4Å (Fig. S1D) respectively, based on the gold Fourier Shell

89   Correlation standard (Fig. S1C, Fig. S2D). The 3D reconstructions show that

90   both *At*MLKL2 and *At*MLKL3 oligomers are composed of four MLKL

91   molecules (Fig. 2C, Fig. S3A), confirming that these form tetramers.

92   Tetramerization of *At*MLKL2 or *At*MLKL3 results in formation of a pyramid-like

93   structure. Structure alignment of the two final models indicates that tetramers

94   of two subfamily members are nearly identical (Fig. S3AB). As the quality of

95   the density of *At*MLKL3 is superior for model building, we limited our structural

96   analysis to *At*MLKL3.

97   The N-terminal HeLo domain of *At*MLKL3 forms a four-helix bundle (Fig. S3C)

98   which superimposed well with the HeLo domains of mouse and human MLKL

99   (mMLKL and hMLKL, respectively; Fig. 2D). This observation supports the

100   idea that *At*MLKL3 is a bona fide homolog of vertebrate MLKLs. Nevertheless,

101   compared to mMLKL, the HeLo domain of *At*MLKL3 packs tightly against its

102   pseudokinase domain (Fig. S3D). The packing is further strengthened by the

103   brace region of *At*MLKL3, which contains a string of five helices that

104   simultaneously interact with the HeLo and pseudokinase domains (Fig. S3C).

105   The HeLo domains and the brace regions form the core of the *At*MLKL3

106   tetramer, whereas the pseudokinase domains are presented at the apices of

107   the pyramid-like structure (Fig. 2C). Hydrophobic packing of α1 helices (Fig.

108   S3E) from two *At*MLKL3 molecules contributes to formation of a homodimeric

109   *At*MLKL3. In the tetrameric *At*MLKL3, the two α1-mediated homodimers pack

110   perpendicularly to each other (Fig. 2G). Four brace regions, which are

111   positioned nearly in the same plane, are sandwiched between the two

112   homodimers and exclusively mediate homodimer-homodimer interaction (Fig.

113   2F). The N-terminal halves of the four brace regions form two homodimer

114   pairs, and the C-terminal halves another two pairs (Fig. S3E). The

115   intermolecular interactions lead to further sequestering of the *At*MLKL3 N-

116   terminal HeLo domain. Taken together, our observations indicate that

117   *At*MLKL3 is a structural homolog of vertebrate MLKLs and its N-terminal HeLo

118   domain is sequestered through both intra- and intermolecular interactions.

119

120   To determine the role of plant MLKLs in immunity, we challenged

121   combinatorial loss-of-function mutants of *At*MLKLs (Fig. S4) with different

122   microbial pathogens. *Atmlkl* single mutants exhibited increased susceptibility

123   to the obligate biotrophic fungus *Golovinomyces orontii* compared to wild-type

124   plants and, strikingly, the triple mutant was as susceptible as an *eds1* mutant

125   (Fig. 3A), which is hyper-susceptible to a number of pathogens including the

126   obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). The

127   immune response restricting *Golovinomyces* growth in Col-0 wild-type plants

128   is not associated with host cell death (*19*). In *A. thaliana*, immunity to *Hpa*

129   isolate Emwa1 is mediated by RPP4, an NLR that requires EDS1 for function

130   (*20*). RPP4-mediated disease resistance was partially compromised in the

131   *Atmlkl* triple mutant, as measured by increased *Hpa* sporangiophore formation

132   on true leaves (Fig. 3B). We did not detect marked differences between the

133   susceptibility of wild-type and mutant plants to hemi-biotrophic *Pseudomonas*

134   *syringae* pv. *tomato* DC3000 bacteria or the fungal necrotroph *Botrytis cinerea*

135   (Fig. S5AB). Furthermore, disease resistance mediated by RPS2 and cell

136   death mediated by RPM1 NLRs were largely retained in the *Atmlkl123* mutant

137   (Fig. S5CD).

138

139   RNA-seq analysis using mock- and *G. orontii*-challenged leaves of wild-type,

140   *Atmlkl23, Atmlkl123* and *eds1* lines showed that the *AtMLKL1* transcript was

141   induced upon pathogen challenge (7.4 fold) and that induction was fully

142   dependent on *EDS1* (Fig. 3C, Fig. S6). Further analysis revealed that

143   *At*MLKLs regulate transcriptional reprogramming both under unchallenged

144   conditions and upon pathogen attack (Fig. 3C). These data imply a specific

145   and partly redundant role of *At*MLKLs in resistance to obligate biotrophic

146   pathogens.

6

147

148   To test whether *At*MLKLs possess cell death activity, possibly regulated by

149   phosphorylation, we introduced single phosphomimetic substitutions at serine

150   residues in the activation loops of *At*MLKLs (Fig. 1B) and expressed in *A.*

151   *thaliana* leaf protoplasts (see method). Upon overexpression, all wild-type

152   *At*MLKLs elicited cell death, which was as potent as an N-terminal barley NLR

153   cell death module (*21*)(Fig. S7). We found that a phosphomimetic substitution

154   at serine^{393} but not serine^{395} in *At*MLKL1 enhanced its cell-killing activity (Fig.

155   4A). However, an alanine substitution at serine^{393} did not compromise the cell

156   death activity, suggesting that other serine or threonine residues in the

157   activation loop (Fig. 1B) have a compensatory function. Using transgenic *A.*

158   *thaliana* lines, we observed that the enhanced susceptibility of *Atmlkl1* and

159   *Atmlkl2* mutants to *G. orontii* was restored by stable transformations of

160   genomic fragments encompassing wild-type *AtMLKL1* and *AtMLKL2*,

161   respectively (Fig. 4B,C and Fig. S8-9). In line with the protoplast assay (Fig.

162   4A), *At*MLKL1 (S393D) transgenic plants exhibited enhanced resistance to *G.*

163   *orontii* compared to Col-0 wild type, plants expressing the transgenes

164   *At*MLKL1 or *At*MLKL1 (S395D) (Fig. 4B,C). Unexpectedly, *At*MLKL1 (S393D)

165   transgenic plants did not exhibit cell death lesions or apparent plant growth

166   retardation indicative of autoimmunity (Fig. 4B, Fig. S8A). Collectively our

167   results suggest that plant MLKL function is in part regulated by activation loop

168   conformation, similar to vertebrate MLKLs (*22*), but *At*MLKL1 activity needed

169   to restrict *G. orontii* growth can be separated from host cell death.

170

171

172 We next examined whether the *At*MLKL N-terminal HeLo domain contributes

173 to its cell death activity, as in animals (*8, 12, 14, 23*). Taking advantage of a

174 chemically enforced oligomerization system (Fig. S10, see method), we found

175 that expression of the HeLo domains with the brace region of *At*MLKL1 and

176 *At*MLKL3 was sufficient for cytotoxic activity and this activity was further

177 enhanced upon oligomerization (Fig. S10D）. This finding mirrors the activity

178 of the HeLo domains with the brace region of MLKL (*14*) and is consistent

179 with our structure-based hypothesis that the full-length MLKL tetramer with

180 buried HeLo domains represents an inactive form (Fig. 2).

181

182 As animal MLKL translocates to the PM upon activation (*10-14*) we examined

183 whether GFP-tagged *At*MLKL variants associate with the PM using time-

184 resolved confocal microscopy. We detected mobile punctate signals in the

185 cytoplasmic space for all *At*MLKL1 variants (Fig. 4DE). Reduced mobility for a

186 fraction of *At*MLKL1-GFP signals when proximal to the PM (Fig. 4E) is

187 consistent with PM association. Intriguingly, maximum projection of the time-

188 lapse images revealed that *At*MLKL1 (wild type)-GFP, the S395D mutant

189 moved along filamentous structures (Fig. 4E, Supplemental movies 1 and 3;

190 Fig. S11A). These filamentous structures specifically colocalized with the

191 microtubule marker (*24*) mCherry-MAP4 (Fig. S11B-C), and association was

192 detectable upon *G. orontii* invasion (Fig. S12). Intriguingly, no filamentous

193 structures were detected in the S393D phosphomimetic line despite the

194 presence of a microtubule array (Fig. S13). These data suggest that specific

8

195   phosphorylation modulates the intracellular localization and activities of

196   *At*MLKL1 via cytoskeletal association.

197

198   The plant MLKL family described here is a first example of a non-receptor

199   immunity component consisting of multiple domains that is structurally shared

200   between animal and plants. MLKLs in these two kingdoms are likely products

201   of convergent evolution because their exon-intron structures are unrelated (~4

202   and 11 exons in *At*MLKLs and hMLKL, respectively). Our work serves as a

203   template to test structural predictions implicating the presence of a HeLo-

204   domain fold in a number of plant modular proteins, including the ADR1 family

205   of NLRs (*4, 25-27*). Despite the cell death activity of a putative HeLo-domain

206   of ADR1 (*28*), this family confers disease resistance without apparent host cell

207   death (*29*). This resembles *At*MLKL-mediated defense in wild-type plants to

208   *G. orontii* without host cell death, although *At*MLKLs have the capacity to

209   elicit cell death. These results raise the question of whether host defence and

210   cell death functions can be further disentangled in animal necroptosis

211   signalling.

212

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

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

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

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

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

229

230 **Fig. 2. Structure of the *At*MLKL3 tetramer.**

231 **A,** Top: Gel filtration profiles of *At*MLKL3. Position of standard molecular
232 weight is indicated by arrow. Bottom: Peak fractions on the top were verified
233 by reducing SDS-PAGE with Coomassie Blue staining.
234
235 **B**, Final EM density map of the *At*MLKL3 tetramer. Local resolution of the
236 density map is shown on the right with scale indicated by colors, in angstroms.
237
238 **C**, Top: Final 3D reconstruction of *At*MLKL3 tetramer shown in three
239 orientations. Each monomer of *At*MLKL3 is shown in different colors. Bottom:
240 Cartoon shows the overall structure of *At*MLKL3 tetramer in three orientations.
241 Subdomains of *At*MLKL3 are shown in different colors.
242
243 **D** Superposition of the HeLo domains of *At*MLKL3 (yellow, PDB ID code
244 6KA4), human MLKL (blue, PDB ID code 2MSV) and mouse MLKL (Pink,
245 PDB ID code 4BTF). RMSD between *At*MLKL3 and mMLKL:3.745, RMSD
246 between *At*MLKL3 and hMLKL:4.010.

247
248 **E**, Electrostatic surface of HeLo domains of *At*MLKL3, human MLKL and
249 mouse MLKL in two orientations.
250

251 **F**, Cartoon shows the brace region of the *At*MLKL3 tetramer. Each monomer
252 of *At*MLKL3 is shown in different colors.

253 **G**, Cartoon shows the HeLo domains of the *At*MLKL3 tetramer in two
254 orientations. Each monomer of *At*MLKL3 is shown in different colors.

10

255

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. Sporangiophores 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.

273

Fig. 4: Cell death activity, disease resistance and subcellular localization of phosphomimetic *At*MLKL1 variants.

A, A phosphomimetic substitution in *At*MLKL1 elicited enhanced cell death in *Arabidopsis* mesophyll protoplasts. Luciferase and *At*MLKL1 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 *At*MLKL1 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 *At*MLKL1. 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 μm.

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

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

311 **Competing interests:** The authors declare no competing interests.

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

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402

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.

15

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 descried 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 Sysems) 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.

16

452   The construct of full length *At*MLKL2 (residues 1-711) with N-terminal GST

453   tag was cloned into the pGEX-6P-1 vector (GE Healthcare), and was

454   expressed in *Escherichia coli* strain BL21(DE3; Novagen) at 16 °C. After

455   isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) induction for 12 h, cells

456   were harvested and re-suspended in buffer containing 50 mM Tris-HCl pH 8.0

457   and 300 mM NaCl, and lysed by sonication. The soluble fraction was purified

458   from the cell lysate using Glutathione Sepharose 4B beads (Invitrogen). The

459   proteins were then digested with PreScission protease (GE Healthcare) to

460   remove the GST tag and further purified by gel filtration (Superose 6, 10/30;

461   GE Healthcare). For cryo-EM investigation, the purified protein was

462   concentrated to about 0.3 mg/mL in buffer containing 50 mM Tris-HCl pH 8.0,

463   300 mM NaCl and 3 mM DTT.

464   **Cryo-EM sample preparation and data collection**

465   For cryo-EM analysis, an aliquot of 3.5 µl *At*MLKL2 or *At*MLKL3 protein was

466   applied to a holey carbon grids (Quantifoil Cu 1.2/1.3, 200 mesh) glow-

467   discharged (Harrick Plasma) with a middle force for 30 s after evaluating for 2

468   min. The grids were blotted by a pair of 55 mm filter papers (TED PELLA,

469   INC.) for 3-3.5 s at 8 °C with 100% humidity and flash-frozen in liquid ethane

470   using a FEI Vitrobot Marked IV. Cryo-EM data were collected on Titan Krios

471   electron microscope operated at 300 kV and a Gatan K2 Summit direct

472   electron detection camera (Gatan) using eTas. Micrographs were recorded in

473   super-resolution mode at a nominal magnification of 22500 ×, resulting in a

474   physical pixel size of 1.30654 Å per pixel. Defocus values varied from -1.7 µm

475   to -2.3 µm for data set. The dose rate was 10.6 electron per pixel per second.

476   Exposures of 8.0 s were dose-fractionated into 32 sub-frames, leading to a

477   total accumulated dose of 50 electrons per Å2. In total, two batches of data

478   were collected, one for *At*MLKL3 and another for *At*MLKL2.

479   **Image processing and 3D reconstruction**

480   A total of 1,434 and 1,828 raw images stacks of *At*MLKL3 and *At*MLKL2

481   acquired under super-resolution mode, were 2x binned processed using

482   MotionCor2 (*13*), generating aligned, dose-weighted and summed

483   micrographs in a pixel size of 1.30654 Å per pixel. CTFFIND4 (*14*) was used

484   to estimate the contrast transfer function (CTF) parameters. After the removal

485   of bad micrographs via the evaluation of CTF parameters, remaining images

486   were processed in RELION (*15*). Approximate 2,000 particles were manually

487   picked and 2D-classified to generate templates for auto-picking. 983,779 and

488   1,135,463 autopicked particles for *At*MLKL3 and *At*MLKL2 respectively were

489   then used for reference-free 2D classification, to remove contaminants and

490   bad particles. The left good particles were subjected to 3D classification using

491   initial 3D reference model obtained by *ab initio* calculation from Relion3.0.

492   Particles from good classes that possess density map with better overall

493   structure features were selected for the 3D refinement. The final 3D

494   refinement using D2 symmetry resulted in reconstructions of *At*MLKL3 and

495   *At*MLKL2 tetramer at resolution of 3.4 Å and 4.1 Å, the resolutions were

496   determined by gold-standard Fourier shell correlation. Local resolution

497   distribution was evaluated using Relion.

498

499

Model building and refinement

EM density map of *At*MLKL3 was used to build the model de novo, as the overall resolution of map density was efficient to display side chains. The model of *At*MLKL3 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 *At*MLKL3 model was docked into the density of *At*MLKL2. The sequence of the docked *At*MLKL3 model was changed to that of *At*MLKL2 under COOT and the *At*MLKL2 model with corrected sequence was subjected to refinement by PHENIX. The C-terminal serine-rich region of *At*MLKL2 or *At*MLKL3 is much less well defined in the density and is not included in the models. Final model of *At*MLKL3 and *At*MLKL2 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

19

525   (Qiagen, ver. 10.1.2) using the tool 'RNA-Seq with the default parameter

526   setting. The data derived from Col-0 and WT were pooled as data of wild-type

527   lines in the analysis. The heat map of 93 genes differentially expressed

528   between wild type lines and *Atmlkl* mutants (|log2FC|>1 and false discovery

529   rate (FDR) < 0.05) were generated using the R package (ver. 1.08) with the

530   pheatmap function. Gene ontology enrichment analysis was performed using

531   the PANTHER classification system (http://pantherdb.org/) with default

532   settings for *Arabidopsis thaliana*. The RNA-Seq data generated in this study

533   have been deposited in the Gene Expression Omnibus (GEO) database

534   under accession number GSE129011.

535

536   **Transient gene expression in *Arabidopsis* protoplasts**

537   Isolation, transfection and luciferase activity measurement of Arabidopsis

538   protoplasts were performed as described previously (*20*). Protoplasts were

539   isolated from the leaves of two-week-old *Arabidopsis* plants grown in liquid 1 x

540   Murashige and Skoog medium. Coding sequences (CDS) of *AtMLKL1* and

541   *AtMLKL2* without stop codons were initially cloned into pENTR/D-TOPO

542   (Thermo Fisher Scientific). The CDS of *AtMLKL3* without a stop codon was

543   chemically synthesized and cloned into pENTR221 (Thermo Fisher Scientific).

544   Two synonymous substitutions (G1371T and A1413G) were introduced into

545   *AtMLKL3* CDS to remove restriction sites that hamper the DNA synthesis.

546   Entry clones were transferred into the gateway cloning-compatible pAMPAT-

547   GW-mYFP, pAMPAT-GW-3xHA, or pAMPAT-GW expression vectors (*21*),

548   which are derivatives of pAMPAT-MCS (accession number: AY436765).

549   Primers sequences of for plasmid construction are listed in Table S3. pENTR-

20

550   tagRFP-T-Lifeact (*22*) was transferred into pAMPAT-GW. The expression

551   vectors for *Hv*MLA(1-160aa), MAP4 and Tub6 were described previously (*21,*

552   *23, 24*).

Protoplast viability assay

554   Following protoplast transfection and regeneration, Evans blue dye dissolved

555   in water was added to the samples to a final concentration of 0.04% (w/v).

556   The stained cells were examined under a standard microscope. For

557   luciferase-based viability assay luciferase and *At*MLKL expression constructs

558   were co-transfected into protoplasts and luciferase activity was measured as

559   a proxy of cell viability.

560

Induced oligomerization

562   Two domains of FKBP (F36V) tagged with HA without N-myristoylation signal

563   were PCR amplified from pC4M-FV2E plasmid (ARIAD, Cambridge, MA,

564   USA). *Nco*I and *Hind*III restriction sites were added to the 5' end of forward

565   and reverse primers, respectively. The digested PCR fragment with *Nco*I and

566   *Hind*III were ligated into the same restriction sites present between the attR2

567   and the terminator sequences of pAMPAT-GW expression vector. The

568   resulting vector is named pAMPAT-GW-FV2E-HA. Coding regions

569   corresponding to the N- and C-terminal luciferase fragments (nLUC and

570   cLUC) were PCR amplified from nLUC and cLUC expression vectors (*25*) and

571   cloned into pENTR/D-TOPO. Respective entry clones were transferred into

572   pAMPAT-GW-FV2E-HA. B/B homodimerizer (also known as AP20187 ligand)

573   were purchased from TakaraBio, Japan. After transfection, protoplasts in

21

574 incubation buffer (i.e. WI solution (*26*)) were separated into two tubes and

575 added the same amount of incubation buffer supplemented with B/B

576 homodimerizer (250 nM at the final concentration) or ethanol as solvent

577 control. Primer sequences for the plasmid construction are listed in Table S2.

578 We were able to reconstitute luciferase activity of co-expressed N- and C-

579 terminal halves of luciferase fused to 2xDmrB domains in a ligand-specific

580 manner (Fig. S8b) and the ligand itself did not affect the luciferase reporter

581 assay in protoplasts (Fig. S8c).

582 **Pathogen infection assay**

583 The *G. orontii* infection assay was performed as described previously (*27*). *G.*

584 *orontii* DNA was quantified by qPCR at indicated time points after inoculation

585 of conidia and normalised using the amount of plant specific gene

586 (AT3G21215). The *Hpa* infection assay was performed as previously

587 described (*28*). Lactophenol-trypan blue staining was described previously

588 (*29*). The *B. cinerea* strain B05.10 was used in this study. Droplet inoculation

589 of six-week-old plants was performed as described previously (*30*), except

590 that 2 µl of the spore solution were used on each side of the leaf and two

591 leaves of similar age were used per plant. *B. cinerea* DNA was quantified by

592 qPCR as previously described (*31*). *Pseudomonas syringae* pv. *tomato* (*Pst*)

593 DC3000 and *Pst* DC3000 expressing *AvrRpt2* or *AvrRpm1* were used in this

594 study. *Pst* growth assays and ion leakage measurement following bacterial

595 infiltration were performed as described previously (*7*).

596

597

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 µM 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 form rosette leave 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

23

622   S395D, 166 seconds) and used for maximum intensity projection (bottom

623   panels). Representative single frame images are shown (top panels).

624

Supplemental data

Supplemental Fig. 1: 3D reconstruction of *At*MLKL3 tetramer

A, Representative cryo-EM image of *At*MLKL3 tetramer. Representative particles are indicated with black circles.
B, Representative top and side views of 2D class averages of *At*MLKL3 tetramer.
C, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D reconstruction of *At*MLKL3 tetramer.
D, Flowchart representing cryo-EM data processing and 3D reconstruction of *At*MLKL3 tetramer.

Supplemental Fig. 2: Purification and 3D reconstruction of *At*MLKL2 tetramer

A, Top: Gel filtration profile of *At*MLKL2 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 *At*MLKL2 tetramer. Representative particles are indicated with black circles.
C, Representative top and side views of 2D class averages of *At*MLKL2 tetramer.
D, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D reconstruction of *At*MLKL2 tetramer.
E, Flowchart representing cryo-EM data processing and 3D reconstruction of *At*MLKL2 tetramer.

Supplemental Fig. 3: Inter- and intra-domain interactions of *At*MLKL3 tetramer and structural comparison of the *At*MLKL2 and *At*MLKL3

A, Superposition of *At*MLKL2 (grey) and *At*MLKL3 (blue). RMSD between monomer of *At*MLKL2 and *At*MLKL3 :0.985.

B, The sequence of *At*MLKL3 model (blue) was docked into *At*MLKL2 map (gray).

C, Top: Cartoon representation of *At*MLKL3 monomer in two orientations. Subdomains of *At*MLKL3 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 *At*MLKL2 (left), *At*MLKL3 (middle, PDB ID code 6KA4) and *m*MLKL (right, PDB ID code 4BTF).

E, Top left: Cartoon showing HeLo domain and brace region of *At*MLKL3 tetramer. Top right: Detailed interactions of HeLo domains for the green-

668   framed region. Bottom left: Detailed C-terminal interactions of brace regions
669   for the blue-framed region. Bottom right: Detailed N-terminal interactions of
670   brace regions for the blue-framed region.
671
672   **Supplemental Fig. 4: *Atmlkl* mutants exhibit no obvious growth defect.**

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

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

684

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

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

693   **B**, Log_{10}-transformed colony forming units of *Pseudomonas syringae* DC3000
694   per cm^2 of *A. thaliana* leaves at two days after pathogen infiltration. The *eds1*
695   mutant was used as a susceptible control. Data were obtained in three
696   independent experiments (n=3).

697   **C**, Log_{10}-transformed colony forming units of *P. syringae* DC3000 expressing
698   AvrRpt2 per cm^2 of *A. thaliana* leaves at three days after pathogen infiltration.
699   The *rpm1 rps2* mutant was used as a susceptible control. Data were obtained
700   in three independent experiments (n=3).

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

706   n.s. not significant.

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

711 pathogen-challenged leaves were collected at 48 hours post *G. orontii*
712 inoculation. *WT is a segregant line derived from the cross between *Atmlkl1*
713 and *Atmlkl3*.

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

716 **A**, C-terminally HA-tagged and **B**, C-terminally mYFP-tagged *At*MLKLs were
717 expressed under the constitutive cauliflower mosaic virus 35S promoter.
718 Expression constructs for *At*MLKLs were transfected into protoplasts and cells
719 were stained with Evans blue at 16 hours post transfection. Unstained cells
720 (Evans blue-negative cells) were counted as living cells.   For **B**, The N-
721 terminal signalling region of barley NLR protein (HvMLA$_{1-160}$: (7) was used as
722 a positive control. Data were obtained in three independent transfections
723 (n=3) and different letters indicate statistically significant differences (Tukey
724 HSD, $p < 0.01$).

725 **C**. Phosphomimetic substitutions in *At*MLKL2 or *At*MLKL3 do not alter cell
726 death in *Arabidopsis* mesophyll protoplasts.

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

733 **D**, Western blot analysis of expression of C-terminally HA-tagged *At*MLKLs in
734 *Arabidopsis* mesophyll protoplasts. Total protein extracts were collected at
735 seven hours post transfection. Samples that were transfected with the
736 corresponding empty vector or the expression construct for *At*MLKL2-mYFP
737 were used as negative control.

738

Supplemental Fig. 8: Genetic complementation of the *Atmlkl1* mutant.

740 **A,** Macroscopic phenotype of transgenic *Arabidopsis* lines expressing wild-
741 type or phosphomimetic variants of *At*MLKL1-GFP under the native *cis*-
742 regulatory sequence in in *Atmlkl1* in response to *G. orontii*. Plants were
743 photographed seven days after pathogen challenge.

744 **B,** Western blot analysis of the C-terminally GFP-tagged *At*MLKL1 variants in
745 the stable transgenic lines. Total protein extracts were collected from two-
746 week-old plants of the transgenic lines grown on sterile Murashige and Skoog
747 solid media.

748

Supplemental Fig. 9: Genetic complementation of the *Atmlkl2* mutant.

750 **A,** Macroscopic phenotype of transgenic *Arabidopsis* lines expressing
751 *At*MLKL2-GFP under the native *cis*-regulatory sequence in *Atmlkl2*. Four-

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

754   **B**, Western blot analysis of C-terminally GFP-tagged *At*MLKL2. Total protein
755   extracts were collected from two-week-old plants of the transgenic lines grown
756   on sterile Murashige and Skoog solid media.

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

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

765

766   **Supplemental Fig. 10: N-terminal domains of *At*MLKL1 and 3 were**
767   **sufficient to elicit cell death and their enforced oligomerization**
768   **potentiated the activities.**

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

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

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

782   **D**, HeLo domain plus brace region of *At*MLKL1 and *At*MLKL3 were sufficient
783   to elicit cell death and their enforced oligomerization potentiated the activities.
784   Expression constructs for luciferase and each of the *At*MLKL fusion proteins
785   indicated in the figure were co-transfected into protoplasts and luciferase
786   activity was measured as a proxy of cell viability at 16 hours post transfection.
787   Relative luciferase activities compared to those of the empty vector control
788   with dimerizer were plotted. Data were obtained with eight independent
789   transfections (n=9). Asterisks indicate statistically significant differences
790   (Tukey HSD, $p < 0.01$)

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

795 **B-E,** Mesophyll protoplasts were prepared from the triple mutant *Atmlkl123*
796 and AP20187 (Dimerizer) was added after transfection (see method).

797

798 **Supplemental Fig. 11: Subcellular localization of *At*MLKLs in protoplasts.**

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

803 **B**, Co-expression of *At*MLKL1 and cytoskeleton markers in *Arabidopsis*
804 mesophyll protoplasts. Co-expression of *At*MLKL1-mYFP and mcherry-MAP4
805 (microtubule marker: (*33*) top panels), or tagRFP-T-Lifeact (Actin marker: (*34*)
806 middle panels). Representative confocal images were taken at 10 hours post
807 transfection. Scale bars = 10 μm.

808 **C**, The filamentous structures of *At*MLKL1-mYFP were undetectable at 5
809 minutes after application of 5 uM of the microtubule inhibitor, oryzalin.
810 Representative confocal image was taken at 10 hours post transfection.
811 Scale bar = 10 μm.

812 **Supplemental Fig. 12: Confocal images of an epidermal cell that was**
813 **infected by *G. orontii*.** The maximum intensity projection was obtained from
814 confocal Z-stack images and the autofluorescence of fungal structures were
815 discriminated from *At*MLKL1-GFP signals by spectral imaging. Arrowheads
816 indicate filamentous structures associated with *At*MLKL1-GFP. C; cytoplasm,
817 Ha: haustorium, E, haustorial encasement. Scale bar = 5 μm.

818 **Supplemental Fig. 13: Cortical microtubule arrays in abaxial leaf**
819 **epidermal cells of *transgenic A. thaliana lines expressing***
820 **phosphomimetic and wild type variants of *At*MLKL1.** Representative
821 images were obtained one day after biolistic delivery of the expression
822 construct for the *At*ubiqutin10 promoter driven by mCherry-TUB6 (microtubule
823 marker (*24*)) into the indicated transgenic lines. Scale bars = 10 μm.

824

825 **Supplemental movie 1**

826 Dynamics of *At*MLKL1(wild-type)-GFP in a non-pathogen-challenged abaxial
827 leaf epidermal cell. The corresponding projection image is shown in Fig. 4E.
828 The white circle indicates a GFP signal proximal to the PM (see Fig. 4D) and
829 which was less mobile compared to other signals.

830

831 **Supplemental movie 2**

832 Dynamics of *At*MLKL1(S393D)-GFP in a non-pathogen-challenged abaxial
833 leaf epidermal cell. The corresponding projection image is shown in Fig. 4E.

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

836

837 **Supplemental movie 3**

838 Dynamics of *At*MLKL1(S395D)-GFP in a non-pathogen-challenged abaxial
839 leaf epidermal cell. The corresponding projection image is shown in Fig. 4E.
840 The white circle indicates a GFP signal proximal to the PM (see Fig. 4D) and
841 which was less mobile compared to other signals.

842

843 **Supplemental file 1**

844 108 coding sequences (CDS) of plant *MLKLs* belong to the same orthogroups
845 (*11*). A CDS derived from *Arabidopsis halleri* is incomplete due to the
846 ambiguous assembly of the corresponding genomic region.

847

847

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

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

Malus domestica	3750	Rosaceae	5
Prunus persica	3760	Rosaceae	2
Coffea canephora	49390	Rubiaceae	2
Citrus clementina	85681	Rutaceae	2
Citrus sinensis	2711	Rutaceae	2
Populus trichocarpa	3694	Salicaceae	2
Capsicum annuum	4072	Solanaceae	3
Solanum melongena	4111	Solanaceae	3
Solanum lycopersicum	4081	Solanaceae	3
Solanum tuberosum	4113	Solanaceae	3
Vitis vinifera	29760	Vitaceae	2

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849 **Table S2. Cryo-EM statistics and model refinement for *At*MLKL tetramer**

	*At*MLKL3 tetramer	*At*MLKL2 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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853 **Table S3 Primer pairs used in this study.**

| Forward primer	Sequence 5′-3′	Reverse primer	Sequence 5′-3′	Use, comments
SALK_041569c_RP	GAGAGACAGCGAAAACCTGTG	SALK_041569c_LP	CTTCTCTTGCAATGCCTCAAC	Genotyping of the wild type allele of *AtMLKL1*
SALK_041569c_RP	GAGAGACAGCGAAAACCTGTG	LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping of the mutant allele of *AtMLKL1*
SALK_124412c_RP AT5G41730	TCAGCTTAGCTTTCAACACGG	SALK_124412c_LP AT5G41730	GAAGATACTCCATCCCCCTTG	Genotyping of the wild type allele of *AtMLKL2*
SALK_124412c_RP AT5G41730	TCAGCTTAGCTTTCAACACGG	LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping of the mutant allele of *AtMLKL2*
GABI_491E02_RP	TACACGAAGCCCATTCACTTC	GABI_491E02_LP	TATTACCTCTGCGGATTCACG	Genotyping of the wild type allele of *AtMLKL3*
GABI_o3144/35St	GTGGATTGATGTGATATCTCC	GABI_491E02_LP	TATTACCTCTGCGGATTCACG	Genotyping of the mutant allele of *AtMLKL3*
AT1g64300-Fw1	CACCATGGAGCAATTCAGGCAAATCGGAGAGGTT	AT1g64300-Rv1	TGTAAGCTCAGAGTCTGAAGCAGGACCAGTTA	D-topo cloning of *At*MLKL1 cDNA without STOP codon
AT5G41730-Fw1	CACCATGGAGCAATTCAGACAAATCGGAGAAGTTCTT	AT5G41730-Rv1	AGTAAGCTCAGAGTCTGAAGCATGACCAGTCTGTT	D-topo cloning of *At*MLKL2 cDNA without STOP codon
D-topo-*At*MLKL3-f	CACCATGGATCAATTTCGAGAGATAGGAGAGGTATT	D-topo-*At*MLKL3-r_without_stop	GGAAAGCTCAGAGTCAGAGGCATGT	D-topo cloning of *At*MLKL3 cDNA without STOP codon
*At*MLKL1(S393D)-f	CCTCAGTTAAGAATCAGGATTTTTCTCGGGCTTCC	*At*MLKL1(S393D)-r	GGAAGCCCGAGAAAAATCCTGATTCTTAACTGAGG	Site-directed mutagenesis to obtain *At*MLKL1(S393D) variant
*At*MLKL1(S395D)-f	GTTAAGAATCAGTCTTTTGATCGGGCTTCCTCTAGAC	*At*MLKL1(S395D)-r	GTCTAGAGGAAGCCCGATCAAAAGACTGATTCTTAAC	Site-directed mutagenesis to obtain *At*MLKL1(S395D) variant
AT5G41730_(S394D)-f	GTCGTTAAAGCGCAGGATTCCTCGAAACCAG	AT5G41730_(S394D)-r	CTGGTTTCGAGGAATCCTGCGCTTTAACGAC	Site-directed mutagenesis to obtain *At*MLKL2(S394D) variant
AT5G41730_(S395D)-f	CGTTAAAGCGCAGTCTGATTCGAAACCAGGCACTCC	AT5G41730_(S395D)-r	GGAGTGCCTGGTTTCGAATCAGACTGCGCTTAACG	Site-directed mutagenesis to obtain *At*MLKL2(S395D) variant
*At*MLKL3(S392D)-f	GTTGAATTCTGTCAAGGGATTCGATAGTAAAAGTGCTTCTTTGACG	*At*MLKL3(S392D)-r	CGTCAAAGAAGCACTTTTACTATCGAATCCCTTGACAGAATTCAAC	Site-directed mutagenesis to obtain *At*MLKL3(S392D) variant
*At*MLKL3(SS393D)-f	CTGTCAAGGGATTCTCTGATAAAAGTGCTTCTTTGAC	*At*MLKL3(S393D)-r	GTCAAAGAAGCACTTTTATCAGAGAATCCCTTGACAG	Site-directed mutagenesis to obtain *At*MLKL3(S393D) variant
Genomic of	CACCCCTTG	Genomic of	TGTAAGCT	D-topo cloning of genomic fragments

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*At*3g21215-f	GAATCCACC CATACCACC AG	*At*3g21215-r	GAGGAGG AGGATGGT GATGA	qPCR quantification of the Arabidopsis DNA

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856   **Supplemental References**

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957

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959

960

Figure 1

A

B

13.5 14 14.5 15 15.5 16 16.5 17 M(kDa)

CBB staining

C

HeLo domain
Brace region
Pseudokinase domain

D

AtMLKL3
hMLKL
mMLKL

α2
α1
α4
α3

α1-α2
Loop helix

α3-α4
Loop helix

E

AtMLKL3

hMLKL

mMLKL

F

G
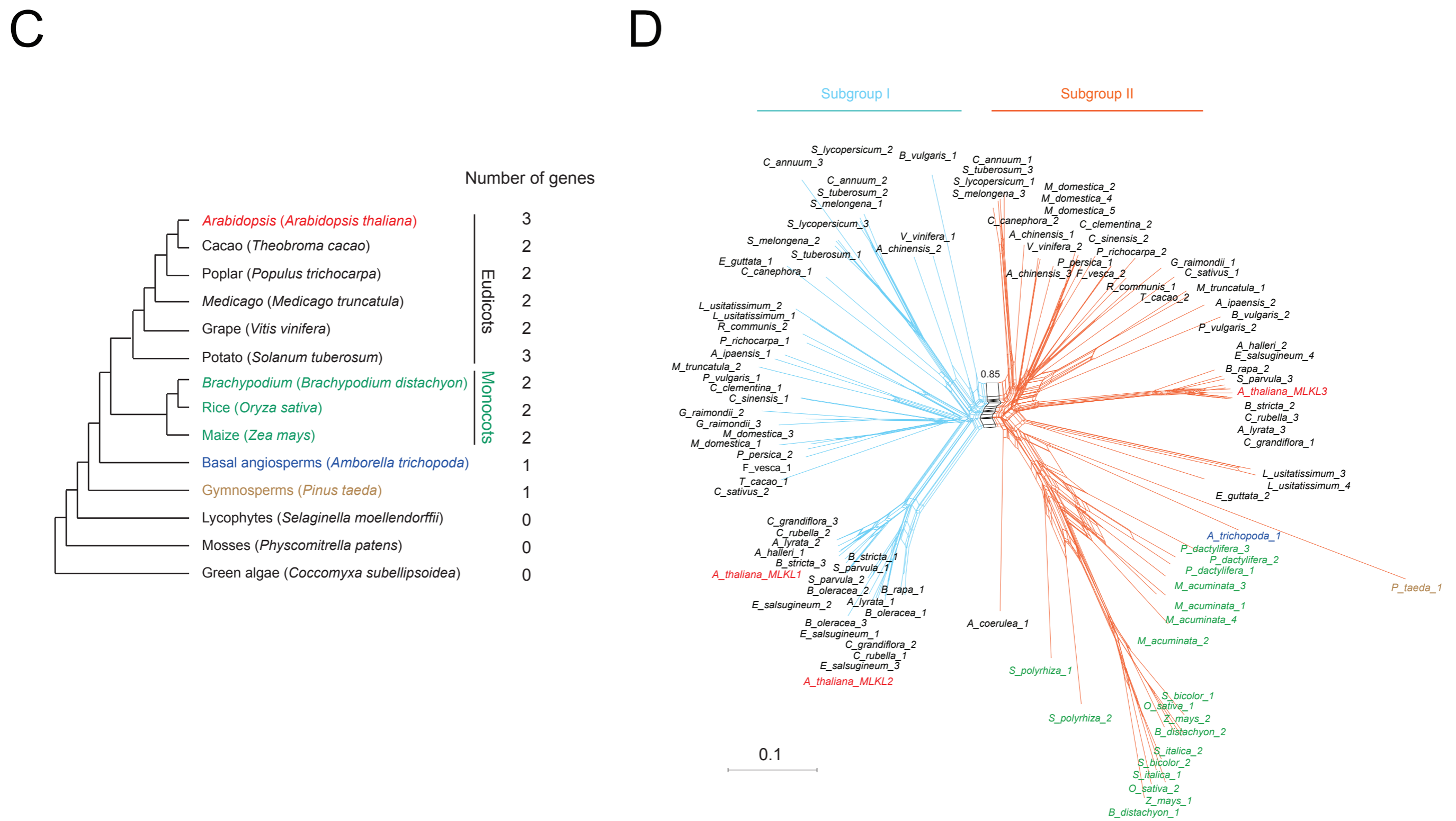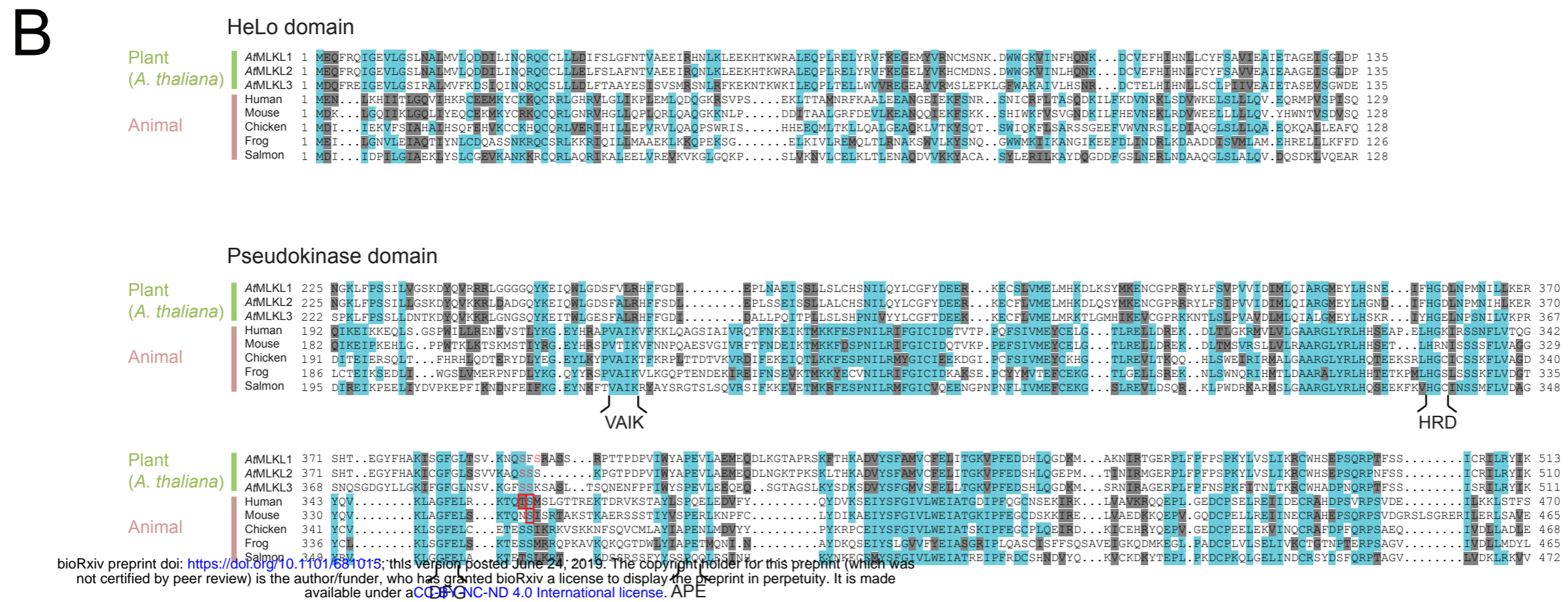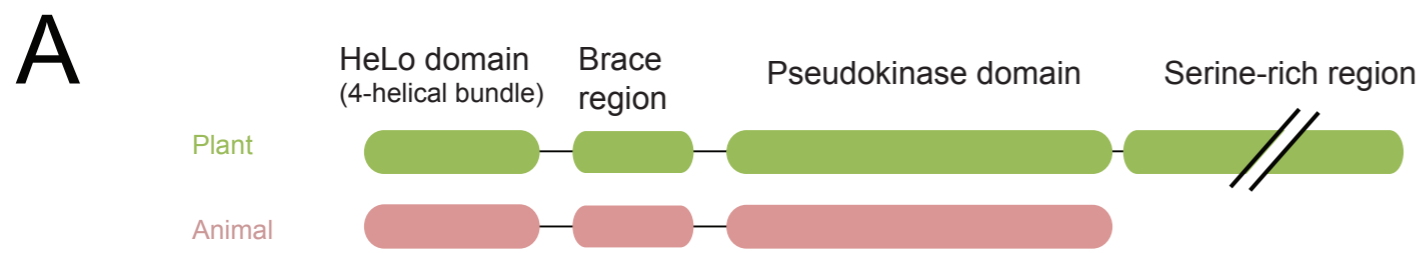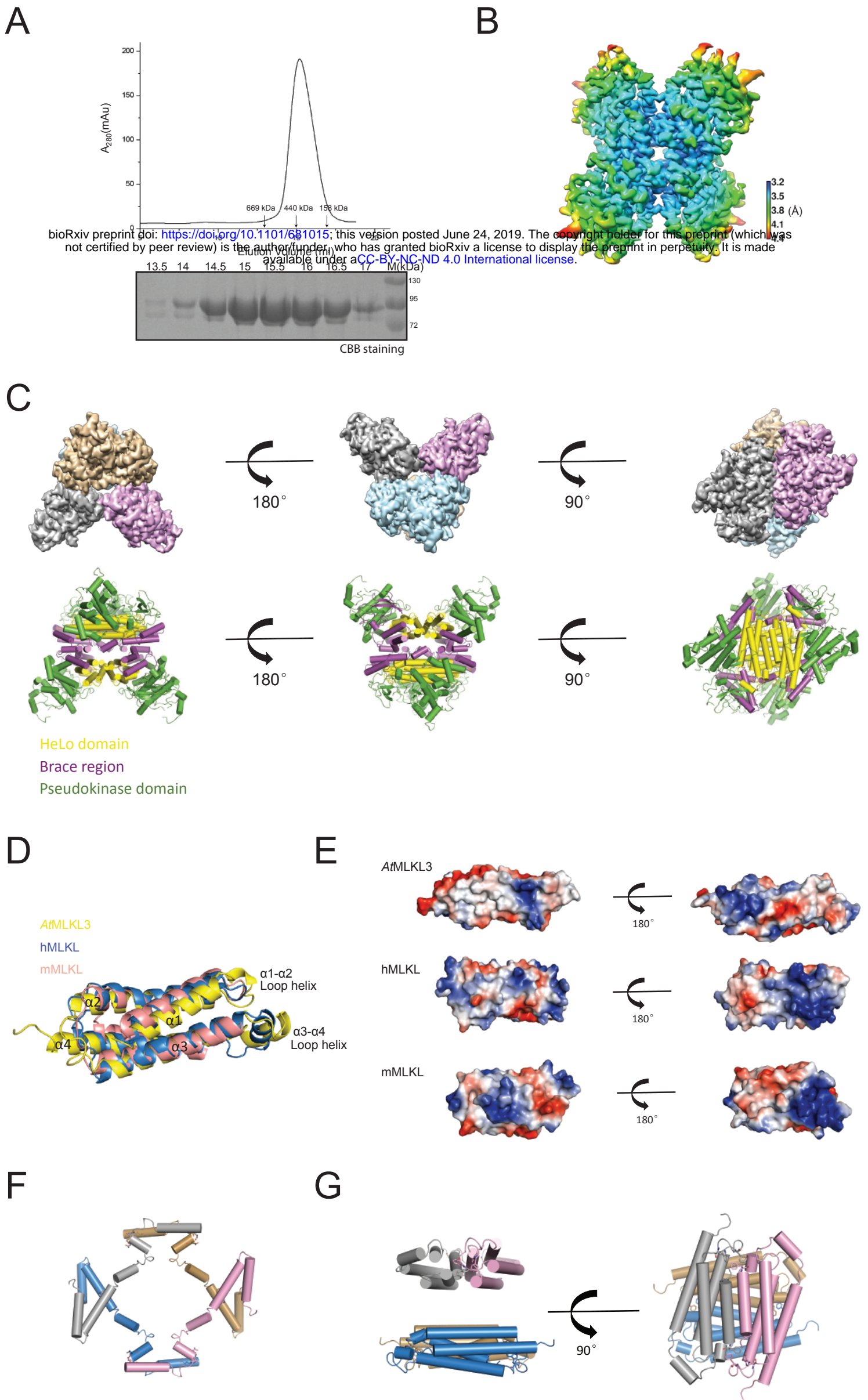
Figure 2

A

G. orontii
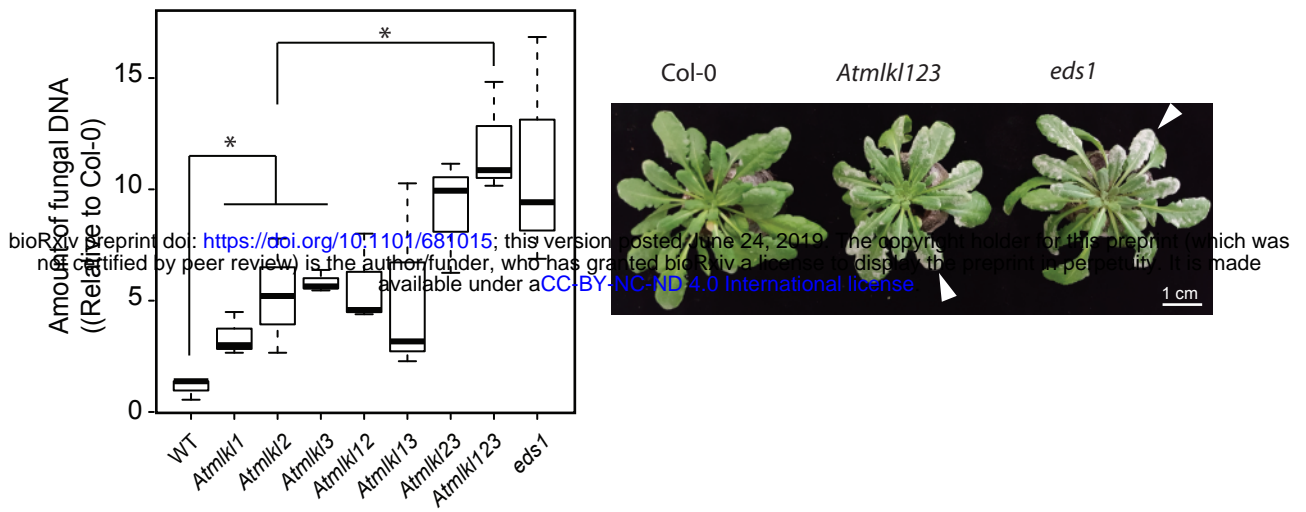
B

H. arabidopsidis Emwa1

C
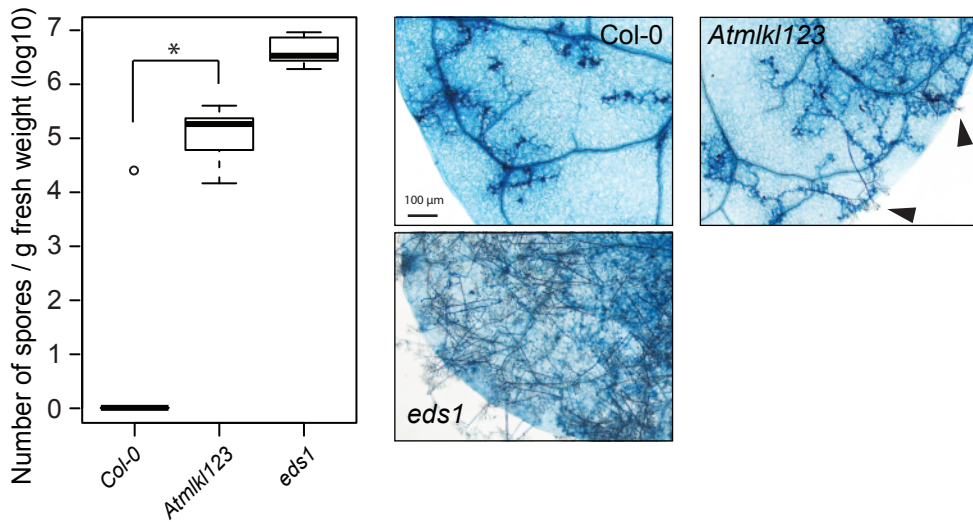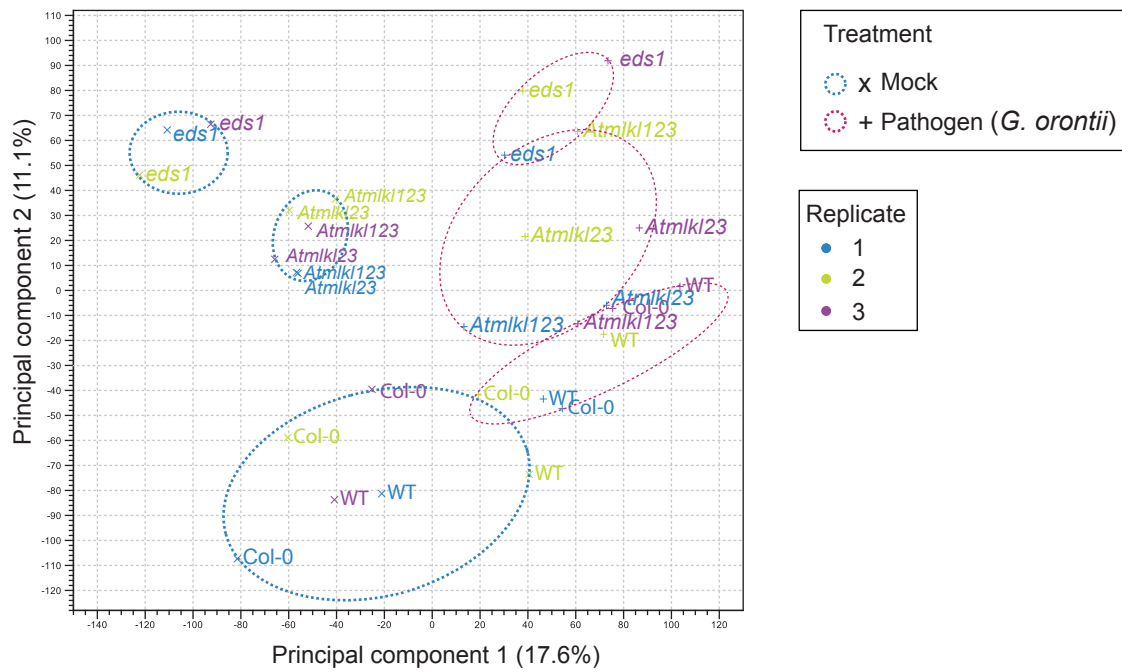
Figure 3
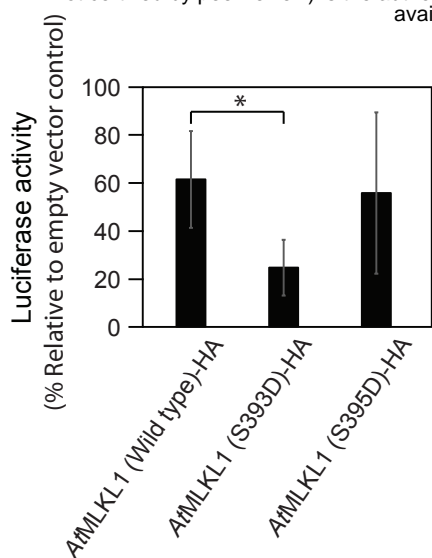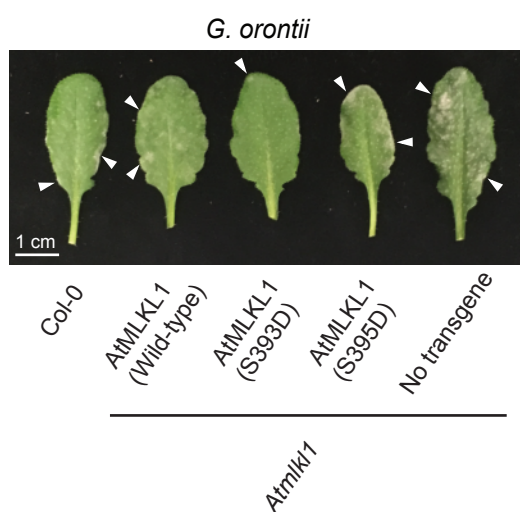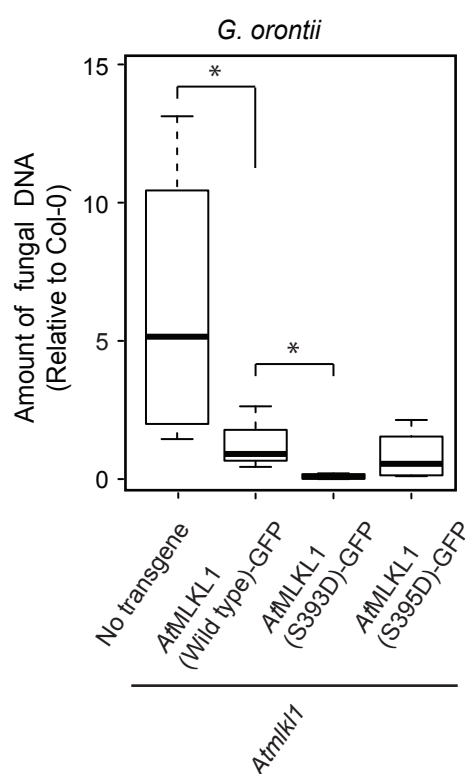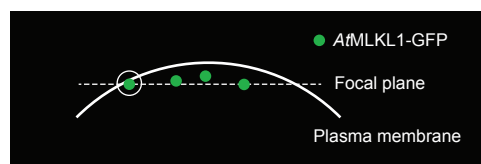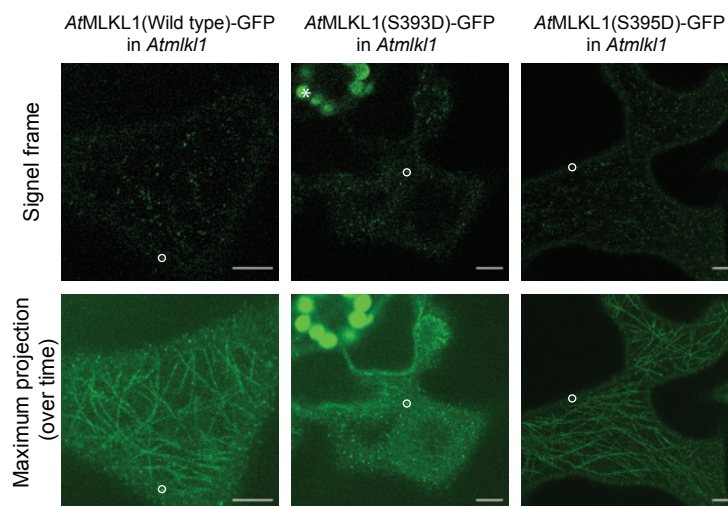
Figure 4

Figure S1

Figure S2

Figure S3

A

AT1G64300

LB | SALK_041569 | RB

-3

AtMLKL1

LB | SALK_124412 | RB

+95

AT5G41730

AtMLKL2

RB | GK-491E02 | LB

+1542

No gene model avaialbe in TAIR10

AtMLKL3

+911

AT1G01450

B

Col-0   Wild type   *Atmlkl1*   *Atmlkl2*   *Atmlkl3*

Atmlkl1 2   *Atmlkl1 3*   *Atmlkl2 3*   *Atmlkl1 2 3*

Figure S4

Figure S5

Figure S6

Figure S7

A

G. orontii

B

Col-0
No transgene
AtMLKL1 (Wild-type)-GFP
AtMLKL1 (S393D)-GFP
AtMLKL1 (S395D)-GFP

Atmlkl1

WB: anti-GFP

CBB staining

Figure S8

A

B

WB: anti-GFP

CBB staining

C

Figure S9

Figure S10

A

*At*MLKL1-mYFP   *At*MLKL2-mYFP   *At*MLKL3-mYFP

B

*At*MLKL1-mYFP   mCherry-MAP4   Overlay

*At*MLKL1-mYFP   tagRFP-T-Lifeact   Overlay

C

*At*MLKL1-mYFP
+ Oryzalin (5 min)

Figure S11

Figure S12

Figure S13