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, *At*MLKL-dependent immunity can be separated from host cell death. Cryo-electron microscopy structures of *At*MLKLs 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 *At*MLKLs with microtubules. These findings, coupled with resistance-enhancing activity and altered microtubule association of a phosphomimetic mutation in the pseudokinase domain of *At*MLKL1, 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 *At*MLKL2 and *A*tMLKL3 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 *At*MLKL 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 *At*MLKL2 and *At*MLKL3 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 *At*MLKL2 and *At*MLKL3 oligomers are composed of four MLKL molecules (Fig. 2C, Fig. S3A), confirming that these form tetramers. Tetramerization of *At*MLKL2 or *At*MLKL3 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 *At*MLKL3 is superior for model building, we limited our structural analysis to *At*MLKL3.

99 The N-terminal HeLo domain of *At*MLKL3 forms a four-helix bundle (Fig. S3C)
100 which superimposed well with the HeLo domains of mouse and human MLKL
101 (mMLKL and hMLKL, respectively; Fig. 2D). This observation supports the
102 idea that *At*MLKL3 is a bona fide homolog of vertebrate MLKLs. Nevertheless,
103 compared to mMLKL, the HeLo domain of *At*MLKL3 packs tightly against its
104 pseudokinase domain (Fig. S3D). The packing is further strengthened by the
105 brace region of *At*MLKL3, which contains a string of five helices that
106 simultaneously interact with the HeLo and pseudokinase domains (Fig. S3C).
107 The HeLo domains and the brace regions form the core of the *At*MLKL3
108 tetramer, whereas the pseudokinase domains are presented at the apices of
109 the pyramid-like structure (Fig. 2C). Hydrophobic packing of α1 helices (Fig.
110 S3E) from two *At*MLKL3 molecules contributes to formation of a homodimeric
111 *At*MLKL3. In the tetrameric *At*MLKL3, the two α1-mediated homodimers pack
112 perpendicularly to each other (Fig. 2G). Four brace regions, which are
113 positioned nearly in the same plane, are sandwiched between the two
114 homodimers and exclusively mediate homodimer-homodimer interaction (Fig.
115 2F). The N-terminal halves of the four brace regions form two homodimer
116 pairs, and the C-terminal halves another two pairs (Fig. S3E). The
117 intermolecular interactions lead to further sequestering of the *At*MLKL3 N-
118 terminal HeLo domain. Taken together, our observations indicate that
119 *At*MLKL3 is a structural homolog of vertebrate MLKLs and its N-terminal HeLo
120 domain is sequestered through both intra- and intermolecular interactions.

121

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

124 microbial pathogens. *Atmlkl* single mutants exhibited increased susceptibility
125 to the obligate biotrophic fungus *Golovinomyces orontii* compared to wild-type
126 plants and, strikingly, the triple mutant was as susceptible as an *eds1* mutant
127 (Fig. 3A), which is hyper-susceptible to a number of pathogens including the
128 obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*). The
129 immune response restricting *Golovinomyces* growth in Col-0 wild-type plants
130 is not associated with host cell death (*19*). In *A. thaliana*, immunity to *Hpa*
131 isolate Emwa1 is mediated by RPP4, an NLR that requires EDS1 for function
132 (*20*). RPP4-mediated disease resistance was partially compromised in the
133 *Atmlkl* triple mutant, as measured by increased *Hpa* sporangiophore formation
134 on true leaves (Fig. 3B). We did not detect marked differences between the
135 susceptibility of wild-type and mutant plants to hemi-biotrophic *Pseudomonas*
136 *syringae* pv. *tomato* DC3000 bacteria or the fungal necrotroph *Botrytis cinerea*
137 (Fig. S5AB). Furthermore, disease resistance mediated by RPS2 and cell
138 death mediated by RPM1 NLRs were largely retained in the *Atmlkl123* mutant
139 (Fig. S5CD).

140

141 RNA-seq analysis using mock- and *G. orontii*-challenged leaves of wild-type,
142 *Atmlkl23, Atmlkl123* and *eds1* lines showed that the *AtMLKL1* transcript was
143 induced upon pathogen challenge (7.4 fold) and that induction was fully
144 dependent on *EDS1* (Fig. 3C, Fig. S6). Further analysis revealed that
145 *At*MLKLs regulate transcriptional reprogramming both under unchallenged
146 conditions and upon pathogen attack (Fig. 3C). These data imply a specific
147 and partly redundant role of *At*MLKLs in resistance to obligate biotrophic
148 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 serine^{393} but not serine^{395} in *At*MLKL1 enhanced its cell-killing activity (Fig.
157 4A). However, an alanine substitution at serine^{393} 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 *At*MLKL1 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 *At*MLKLs 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 *At*MLKL-mediated defense in wild-type plants to
211 *G. orontii* without host cell death, although *At*MLKLs 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 (*At*MLKLs) 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 *At*MLKL3 tetramer.

A, Top: Gel filtration profiles of *At*MLKL3. 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 *At*MLKL3 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 *At*MLKL3 tetramer shown in three orientations. Each monomer of *At*MLKL3 is shown in different colors. Bottom: Cartoon shows the overall structure of *At*MLKL3 tetramer in three orientations. Subdomains of *At*MLKL3 are shown in different colors.

D Superposition of the HeLo domains of *At*MLKL3 (yellow, PDB ID code 6KA4), human MLKL (blue, PDB ID code 2MSV) and mouse MLKL (Pink, PDB ID code 4BTF). RMSD between *At*MLKL3 and mMLKL:3.745, RMSD between *At*MLKL3 and hMLKL:4.010.

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

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

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

259

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

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

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

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

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

277

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

280 **A,** A phosphomimetic substitution in *At*MLKL1 elicited enhanced cell death in
281 *Arabidopsis* mesophyll protoplasts. Luciferase and *At*MLKL1 expression
282 constructs were co-transfected into protoplasts and luciferase activity was
283 measured as a proxy of cell viability at 16 hours post transfection. The
284 positions of serine-to-aspartate residues are indicated in Fig. 1B. The C-
285 terminally HA-tagged variants were expressed under the control of the
286 constitutive cauliflower mosaic virus 35S promoter. (n=3, Tukey HSD *$p <$
287 0.01).

288 **B,** Macroscopic phenotype of transgenic *Arabidopsis* expressing
289 phosphomimetic variants of *At*MLKL1 in response to *G. orontii* (arrowheads).
290 Plants were photographed seven days after pathogen challenge.

291 **C,** Quantification of *G. orontii* DNA in infected leaves of transgenic plants at
292 14 days after pathogen challenge (n=4, Tukey HSD *$p < 0.05$).

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

295 **E**, Confocal images of the abaxial epidermis of the *Arabidopsis* transgenic
296 lines expressing phosphomimetic and wild-type variants of *At*MLKL1. GFP
297 signals indicated by white circles were immobile in the examined time period.
298 The corresponding movies are available as supplemental movies 1-3. The
299 asterisk indicates plastidial autofluorescence. Scale bars = 5 µm.

300

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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.

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408

409 **Methods**

410 **Plant material and growth conditions**

411 The *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) *was* used in
412 this study. The T-DNA insertional mutants(1, 2) (SALK_041569c (*At*MLKL1),
413 SALK_124412c (*At*MLKL2) and GABI_491E02 (*At*MLKL3) were obtained
414 from the Nottingham Arabidopsis Stock Centre (NASC). Double and triple
415 mutants of *Atmlkl* were generated by crossing the T-DNA insertion lines. A
416 segregant line derived from the cross between the *Atmlkl1* and *Atmlkl3*
417 mutants were used as a wild-type line in addition to Col-0 wild-type. Each
418 genotype was confirmed by PCR. The *eds1-2* mutant was described
419 previously (*3*).

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

433

434 **Sequence analysis of plant and animal MLKLs**

435 Sequence similarity within the animal and plant families was established by
436 the generalized profile method (*8*). Sequences were aligned by the L-INS-I
437 method of the MAFFT alignment software (*9*), followed by minor manual
438 editing of ambiguously aligned regions. Sequence similarity between the
439 animal and plant MLKL families was established by Hidden Markov Model
440 (HMM)-to-HMM comparison using the HHSEARCH package (*10*). The 99,696
441 orthogroups (OGs) among 52 plant species have been established recently
442 (*11*). An OG containing *At*MLKLs was used for the neighbor-net analysis of
443 codon-aligned nucleotide sequence as descried previously (*12*). The
444 sequence from the papaya genome was excluded in this study.

445 **Protein expression and purification**

446 Full length *At*MLKL3 (residues 1-701) with an engineered C-terminal 6×His
447 tag was generated by standard PCR-based cloning strategy and its identity
448 was confirmed by sequencing. The protein was expressed in sf21 insect cells
449 using the vector pFastBac 1 (Invitrogen). One litre of cells (2.5×10^6 cells ml^{-1},
450 medium from Expression Sysems) was infected with 20 ml baculovirus at
451 28°C. After growth at 28°C for 48 hours, the cells were harvested, re-
452 suspended in the buffer containing 50 mM Tris-HCl pH 8.0 and 300 mM NaCl,
453 and lysed by sonication. The soluble fraction was purified from the cell lysate
454 using Ni2+-nitrilotriacetate affinity resin (Ni-NTA, Qiagen). The protein was
455 then further purified by further purified by gel filtration (Superose 6, 10/30; GE
456 Healthcare). For cryo-EM investigation, the purified protein was concentrated
457 to about 0.3 mg/mL in buffer containing 50 mM Tris-HCl pH 8.0, 300 mM NaCl
458 and 3 mM DTT.

459 The construct of full length *At*MLKL2 (residues 1-711) with N-terminal GST
460 tag was cloned into the pGEX-6P-1 vector (GE Healthcare), and was
461 expressed in *Escherichia coli* strain BL21(DE3; Novagen) at 16 °C. After
462 isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) induction for 12 h, cells
463 were harvested and re-suspended in buffer containing 50 mM Tris-HCl pH 8.0
464 and 300 mM NaCl, and lysed by sonication. The soluble fraction was purified
465 from the cell lysate using Glutathione Sepharose 4B beads (Invitrogen). The
466 proteins were then digested with PreScission protease (GE Healthcare) to
467 remove the GST tag and further purified by gel filtration (Superose 6, 10/30;
468 GE Healthcare). For cryo-EM investigation, the purified protein was
469 concentrated to about 0.3 mg/mL in buffer containing 50 mM Tris-HCl pH 8.0,
470 300 mM NaCl and 3 mM DTT.

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

472 For cryo-EM analysis, an aliquot of 3.5 µl *At*MLKL2 or *At*MLKL3 protein was
473 applied to a holey carbon grids (Quantifoil Cu 1.2/1.3, 200 mesh) glow-
474 discharged (Harrick Plasma) with a middle force for 30 s after evaluating for 2
475 min. The grids were blotted by a pair of 55 mm filter papers (TED PELLA,
476 INC.) for 3-3.5 s at 8 °C with 100% humidity and flash-frozen in liquid ethane
477 using a FEI Vitrobot Marked IV. Cryo-EM data were collected on Titan Krios
478 electron microscope operated at 300 kV and a Gatan K2 Summit direct
479 electron detection camera (Gatan) using eTas. Micrographs were recorded in
480 super-resolution mode at a nominal magnification of 22500 ×, resulting in a
481 physical pixel size of 1.30654 Å per pixel. Defocus values varied from -1.7 µm
482 to -2.3 µm for data set. The dose rate was 10.6 electron per pixel per second.

483 Exposures of 8.0 s were dose-fractionated into 32 sub-frames, leading to a
484 total accumulated dose of 50 electrons per Å2. In total, two batches of data
485 were collected, one for *At*MLKL3 and another for *At*MLKL2.

486 **Image processing and 3D reconstruction**

487 A total of 1,434 and 1,828 raw images stacks of *At*MLKL3 and *At*MLKL2
488 acquired under super-resolution mode, were 2x binned processed using
489 MotionCor2 (*13*), generating aligned, dose-weighted and summed
490 micrographs in a pixel size of 1.30654 Å per pixel. CTFFIND4 (*14*) was used
491 to estimate the contrast transfer function (CTF) parameters. After the removal
492 of bad micrographs via the evaluation of CTF parameters, remaining images
493 were processed in RELION (*15*). Approximate 2,000 particles were manually
494 picked and 2D-classified to generate templates for auto-picking. 983,779 and
495 1,135,463 autopicked particles for *At*MLKL3 and *At*MLKL2 respectively were
496 then used for reference-free 2D classification, to remove contaminants and
497 bad particles. The left good particles were subjected to 3D classification using
498 initial 3D reference model obtained by *ab initio* calculation from Relion3.0.
499 Particles from good classes that possess density map with better overall
500 structure features were selected for the 3D refinement. The final 3D
501 refinement using D2 symmetry resulted in reconstructions of *At*MLKL3 and
502 *At*MLKL2 tetramer at resolution of 3.4 Å and 4.1 Å, the resolutions were
503 determined by gold-standard Fourier shell correlation. Local resolution
504 distribution was evaluated using Relion.

505

506

507 **Model building and refinement**

508 EM density map of *At*MLKL3 was used to build the model de novo, as the
509 overall resolution of map density was efficient to display side chains. The
510 model of *At*MLKL3 was manually built into the density in COOT(*16*), and was
511 refined against the EM map by PHENIX(*17*) in real space with secondary
512 structure and geometry restraints. The refined *At*MLKL3 model was docked
513 into the density of *At*MLKL2. The sequence of the docked *At*MLKL3 model
514 was changed to that of *At*MLKL2 under COOT and the *At*MLKL2 model with
515 corrected sequence was subjected to refinement by PHENIX. The C-terminal
516 serine-rich region of *At*MLKL2 or *At*MLKL3 is much less well defined in the
517 density and is not included in the models. Final model of *At*MLKL3 and
518 *At*MLKL2 was validated using MolProbity and EMRinger in PHENIX package.
519 The structures of human MLKL (*18*) and mouse MLKL (*19*) were used for the
520 superposition of the HeLo domains as shown in Fig. 2D.Table S2 summarized
521 the model statistics.

522 **RNA sequencing**

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

532 (Qiagen, ver. 10.1.2) using the tool 'RNA-Seq with the default parameter
533 setting. The data derived from Col-0 and WT were pooled as data of wild-type
534 lines in the analysis. The heat map of 93 genes differentially expressed
535 between wild type lines and *Atmlkl* mutants (|log2FC|>1 and false discovery
536 rate (FDR) < 0.05) were generated using the R package (ver. 1.08) with the
537 pheatmap function. Gene ontology enrichment analysis was performed using
538 the PANTHER classification system (http://pantherdb.org/) with default
539 settings for *Arabidopsis thaliana*. The RNA-Seq data generated in this study
540 have been deposited in the Gene Expression Omnibus (GEO) database
541 under accession number GSE129011.

542

543 **Transient gene expression in *Arabidopsis* protoplasts**

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

557 tagRFP-T-Lifeact (*22*) was transferred into pAMPAT-GW. The expression
558 vectors for *Hv*MLA(1-160aa), MAP4 and Tub6 were described previously (*21,*
559 *23, 24*).

560 **Protoplast viability assay**

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

567

568 **Induced oligomerization**

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

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

589 **Pathogen infection assay**

590 The *G. orontii* infection assay was performed as described previously (*27*). *G.*
591 *orontii* DNA was quantified by qPCR at indicated time points after inoculation
592 of conidia and normalised using the amount of plant specific gene
593 (AT3G21215). The *Hpa* infection assay was performed as previously
594 described (*28*). Lactophenol-trypan blue staining was described previously
595 (*29*). The *B. cinerea* strain B05.10 was used in this study. Droplet inoculation
596 of six-week-old plants was performed as described previously (*30*), except
597 that 2 µl of the spore solution were used on each side of the leaf and two
598 leaves of similar age were used per plant. *B. cinerea* DNA was quantified by
599 qPCR as previously described (*31*). *Pseudomonas syringae* pv. *tomato* (*Pst*)
600 DC3000 and *Pst* DC3000 expressing *AvrRpt2* or *AvrRpm1* were used in this
601 study. *Pst* growth assays and ion leakage measurement following bacterial
602 infiltration were performed as described previously (*7*).

603

604

605 **Immunoblot assays**

606 Primary antibodies were monoclonal antibodies from mouse: α-GFP (JL-8,
607 1:5000, Takara, Shiga, Japan) or rat: α-HA (3F10, 1:1000, Sigma-Aldrich, St.
608 Louis, MO, USA). Goat α-mouse IgG-HRP (1:10000, Santa Cruz
609 Biotechnology, Dallas, TX, USA) or goat α-rat IgG-HRP (1:10000, Sigma-
610 Aldrich) were used as secondary antibodies. The detailed procedure is
611 described in (*7*).

612

613 **Biolistic transient gene expression**

614 Biolistic delivery of plasmid DNA into the abaxial epidermis of leaves was
615 essentially performed as described previously (*32*). Leaves were detached
616 immediately before bombardment and the bombarded leaves were transferred
617 to 1% agar plates supplemented with 85 μM benzimidazole and incubated at
618 20°C for 15 h before confocal microscopy.

619

620 **Confocal microscopy**

621 Transfected protoplasts in a chamber slide (Nunc Lab-Tek, Thermo Fisher
622 Scientific) with incubation buffer (i.e. WI solution, (*26*)) or 2-5 mm leaf discs
623 prepared form rosette leave of 4-5-week-old plants were observed under a
624 confocal microscope (LSM880, Carl Zeiss) equipped with a 40X water-
625 immersion and a 63X oil-immersion objective. Lambda stack images were
626 obtained for spectral imaging. Images were analyzed and processed with ZEN
627 Software (Carl Zeiss) and ImageJ (NIH). In Fig. 4E, confocal images were
628 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).

631

632 **Supplemental data**

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

634 **A**, Representative cryo-EM image of *At*MLKL3 tetramer. Representative
635 particles are indicated with black circles.
636 **B**, Representative top and side views of 2D class averages of *At*MLKL3
637 tetramer.
638 **C**, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D
639 reconstruction of *At*MLKL3 tetramer.
640 **D**, Flowchart representing cryo-EM data processing and 3D reconstruction of
641 *At*MLKL3 tetramer.
642

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

645 **A**, Top: Gel filtration profile of *At*MLKL2 protein. Position of standard
646 molecular weight is indicated by arrow. Bottom: Peak fractions in the top were
647 verified by SDS-PAGE with Coomassie Blue staining.
648 **B**, Representative cryo-EM images of the *At*MLKL2 tetramer. Representative
649 particles are indicated with black circles.
650 **C**, Representative top and side views of 2D class averages of *At*MLKL2
651 tetramer.
652 **D**, Fourier shell correlation (FSC) curves at 0.5 and 0.143 of the 3D
653 reconstruction of *At*MLKL2 tetramer.
654 **E**, Flowchart representing cryo-EM data processing and 3D reconstruction of
655 *At*MLKL2 tetramer.
656

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

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

661 **B,** The sequence of *At*MLKL3 model (blue) was docked into *At*MLKL2 map
662 (gray).
663
664 **C**, Top: Cartoon representation of *At*MLKL3 monomer in two orientations.
665 Subdomains of *At*MLKL3 are shown in different colors, the interacting regions
666 between domains are highlighted with open frames. Bottom left: Detailed
667 interactions of HeLo domain and pseudokinase domain for the red-framed
668 region. Bottom right: Detailed interactions of HeLo domain and brace region
669 for the blue-framed region.
670
671 **D**, Structural comparison of the monomer *At*MLKL2 (left), *At*MLKL3 (middle,
672 PDB ID code 6KA4) and *m*MLKL (right, PDB ID code 4BTF).
673

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

676 framed region. Bottom left: Detailed C-terminal interactions of brace regions
677 for the blue-framed region. Bottom right: Detailed N-terminal interactions of
678 brace regions for the blue-framed region.
679
680 **Supplemental Fig. 4: *Atmlkl* mutants exhibit no obvious growth defect.**

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

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

692

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

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

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

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

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

714 n.s. not significant.

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

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

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

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

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

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

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

746

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

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

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

756

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

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

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

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

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

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

773

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

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

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

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

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

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

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

805

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

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

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

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

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

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

832

833 **Supplemental movie 1**

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

838

839 **Supplemental movie 2**

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

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

844

845 **Supplemental movie 3**

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

850

851 **Supplemental file 1**

852 108 coding sequences (CDS) of plant *MLKLs* belong to the same orthogroups
853 (*11*). A CDS derived from *Arabidopsis halleri* is incomplete due to the
854 ambiguous assembly of the corresponding genomic region.

855

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

858 **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

859

860

861

862 **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	GGAGTGCCTGGTTTCGAATCAGACTGCGCTTTAACG	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 AT1G64300-2nd_f	CACCCCTTGAGCCCAAAGTTTCTC	Genomic of AT1G64300-3rd_r for without stop	TGTAAGCTCAGAGTCTGAAGCAGGACCAGTTAGTTG	D-topo cloning of genomic fragments including coding region and native *cis*-regulatory sequence of *At*MLKL1
Genomic of AT5g41730-2nd_f_new	CACCGTCGAGGACCAAAGGCTAGT	Genomic of AT5g41730-3rd_r for w/o stop	AGTAAGCTCAGAGTCTGAAGCATGA	D-topo cloning of genomic fragments including coding region and native *cis*-regulatory sequence of *At*MLKL2
Go_V1_Cont	TCTTGGTGGCACGAATGAC	Go_V1_Contig76-r	AGTGCGAGAGTGGGACAGAC	qPCR quantification of the fungal DNA
*At*3g21215-f	GAATCCACCCATACCACCAG	*At*3g21215-r	GAGGAGGAGGATGGTGATGA	qPCR quantification of the Arabidopsis DNA

863

864

865

866 **Supplemental References**

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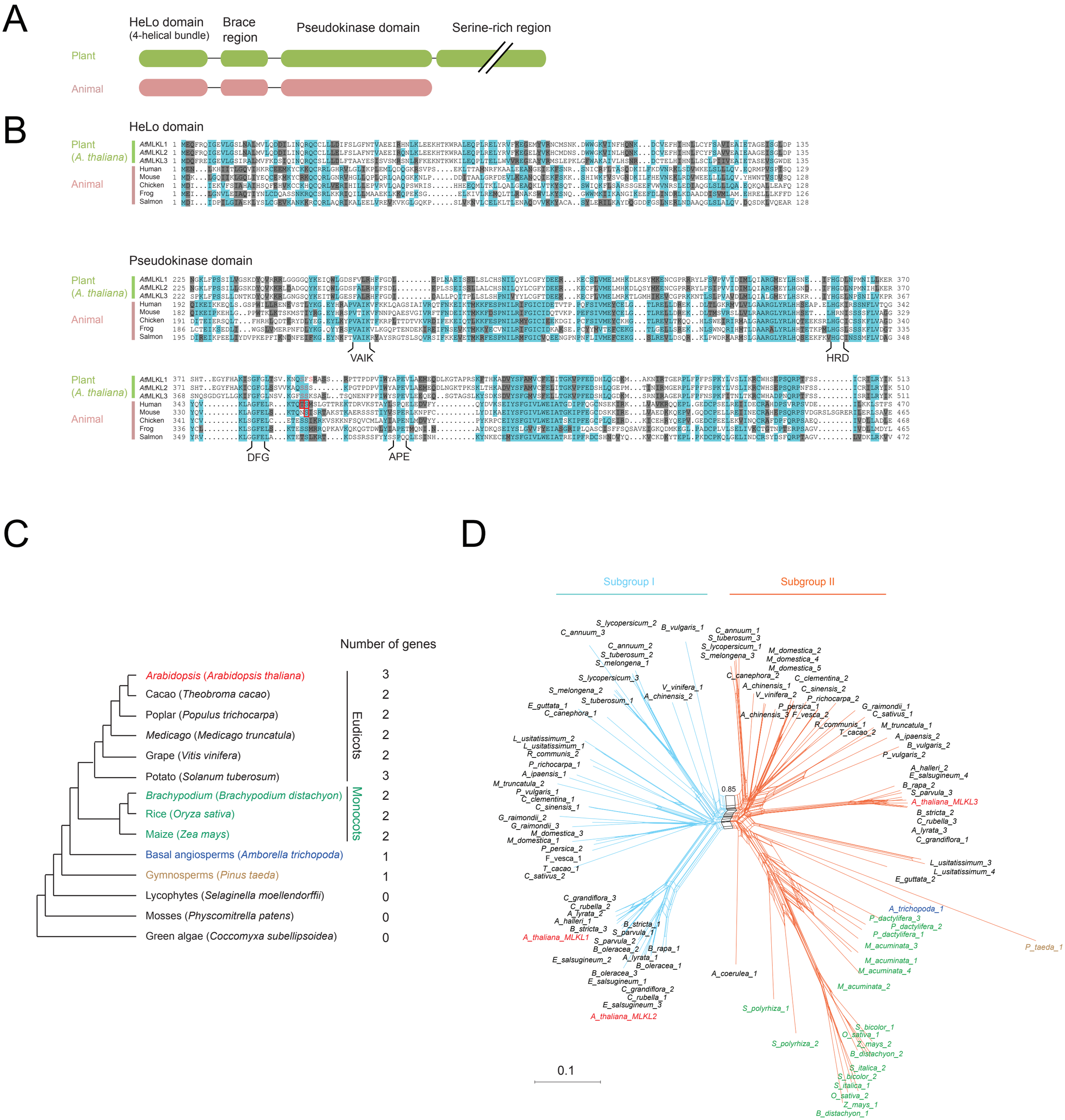

A
HeLo domain
(4-helical bundle)
Brace region
Pseudokinase domain
Serine-rich region
Plant
Animal
B
HeLo domain
Pseudokinase domain
Plant (A. thaliana)
Animal
VAIK
HRD
DFG
APE
C
Number of genes
Arabidopsis (Arabidopsis thaliana) 3
Cacao (Theobroma cacao) 2
Poplar (Populus trichocarpa) 2
Medicago (Medicago truncatula) 2
Grape (Vitis vinifera) 2
Potato (Solanum tuberosum) 3
Brachypodium (Brachypodium distachyon) 2
Rice (Oryza sativa) 2
Maize (Zea mays) 2
Basal angiosperms (Amborella trichopoda) 1
Gymnosperms (Pinus taeda) 1
Lycophytes (Selaginella moellendorffii) 0
Mosses (Physcomitrella patens) 0
Green algae (Coccomyxa subellipsoidea) 0
Eudicots
Monocots
D
Subgroup I
Subgroup II
0.85
0.1

Figure 1

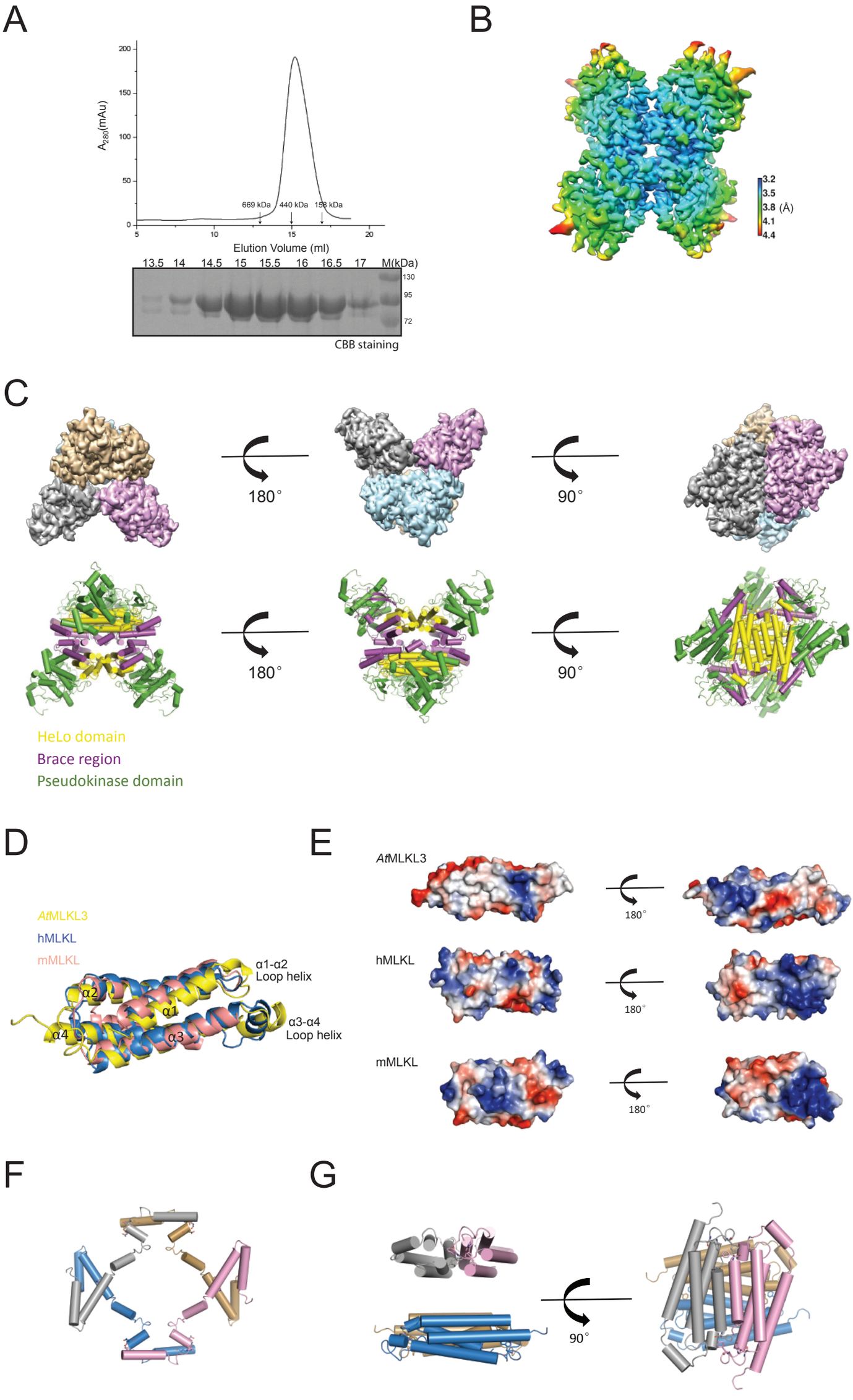

A
200
150
100
50
0
A_{280}(mAu)
669 kDa
440 kDa
158 kDa
5
10
15
20
Elution Volume (ml)
13.5 14 14.5 15 15.5 16 16.5 17 M(kDa)
130
95
72
CBB staining
B
3.2
3.5
3.8 (Å)
4.1
4.4
C
180°
90°
180°
90°
HeLo domain
Brace region
Pseudokinase domain
D
AtMLKL3
hMLKL
mMLKL
α1-α2
Loop helix
α2
α1
α3-α4
Loop helix
α4
α3
E
AtMLKL3
180°
hMLKL
180°
mMLKL
180°
F
G
90°

Figure 2

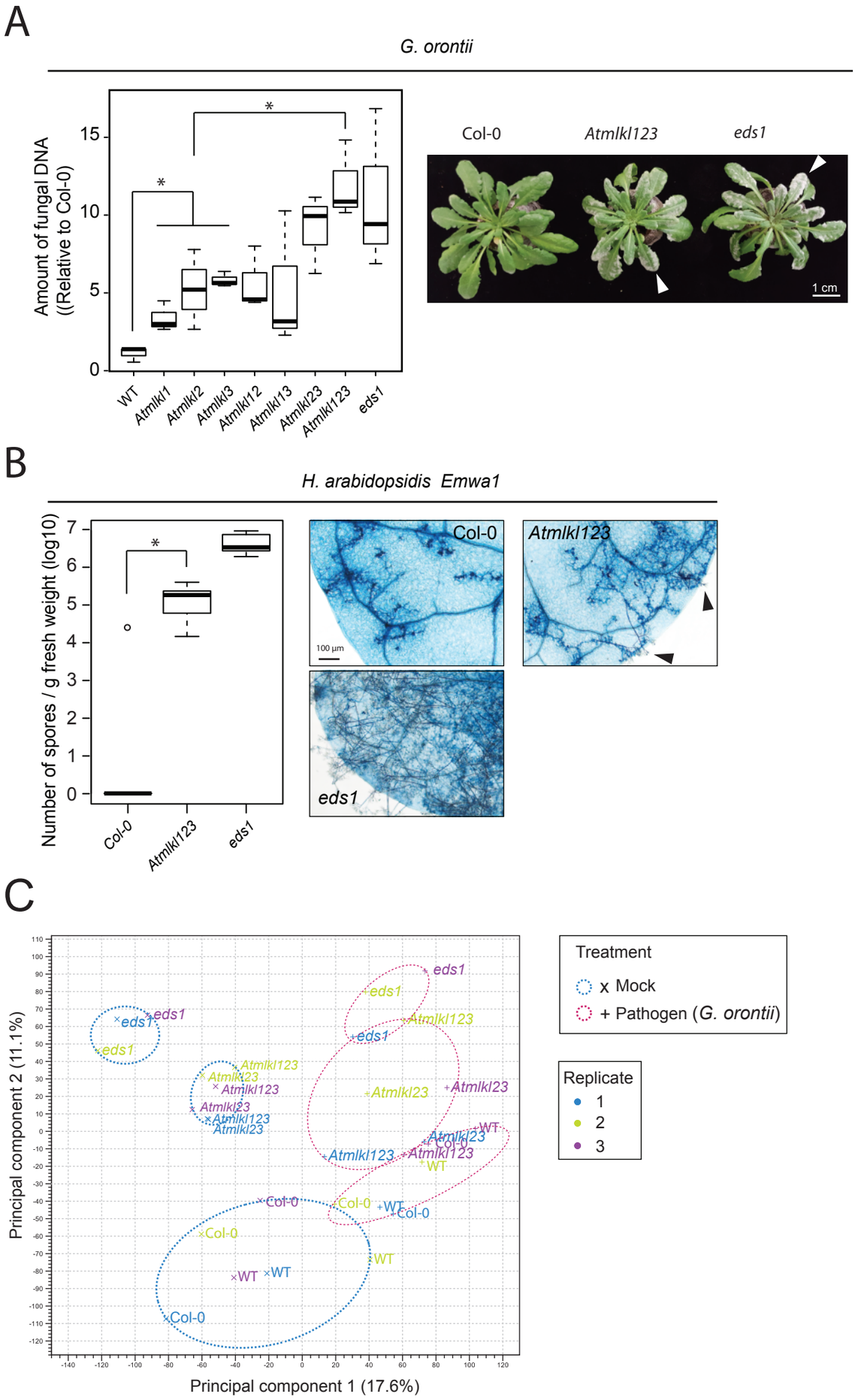

A
G. orontii
Amount of fungal DNA ((Relative to Col-0))
WT
Atmlkl1
Atmlkl2
Atmlkl3
Atmlkl12
Atmlkl13
Atmlkl23
Atmlkl123
eds1
Col-0
Atmlkl123
eds1
1 cm
B
H. arabidopsidis Emwa1
Number of spores / g fresh weight (log10)
Col-0
Atmlkl123
eds1
100 µm
C
Principal component 2 (11.1%)
Principal component 1 (17.6%)
Treatment
x Mock
+ Pathogen (G. orontii)
Replicate
1
2
3

Figure 3

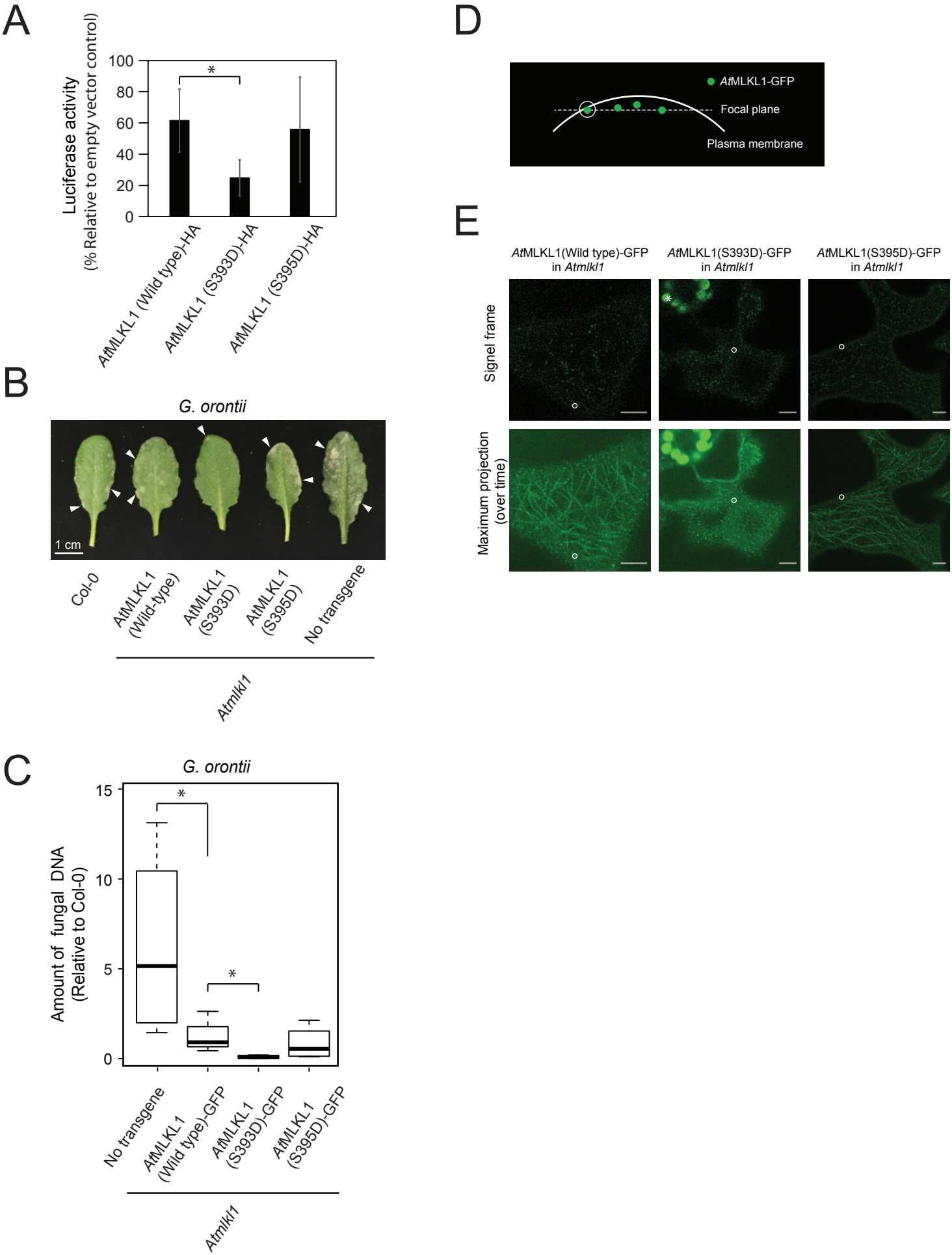

A
Luciferase activity
(% Relative to empty vector control)
100
80
60
40
20
0
*
AtMLKL1 (Wild type)-HA
AtMLKL1 (S393D)-HA
AtMLKL1 (S395D)-HA
B
G. orontii
1 cm
Col-0
AtMLKL1 (Wild-type)
AtMLKL1 (S393D)
AtMLKL1 (S395D)
No transgene
Atmlkl1
C
G. orontii
Amount of fungal DNA
(Relative to Col-0)
15
10
5
0
*
*
No transgene
AtMLKL1 (Wild type)-GFP
AtMLKL1 (S393D)-GFP
AtMLKL1 (S395D)-GFP
Atmlkl1
D
AtMLKL1-GFP
Focal plane
Plasma membrane
E
AtMLKL1(Wild type)-GFP in Atmlkl1
AtMLKL1(S393D)-GFP in Atmlkl1
AtMLKL1(S395D)-GFP in Atmlkl1
Signel frame
Maximum projection (over time)

Figure 4

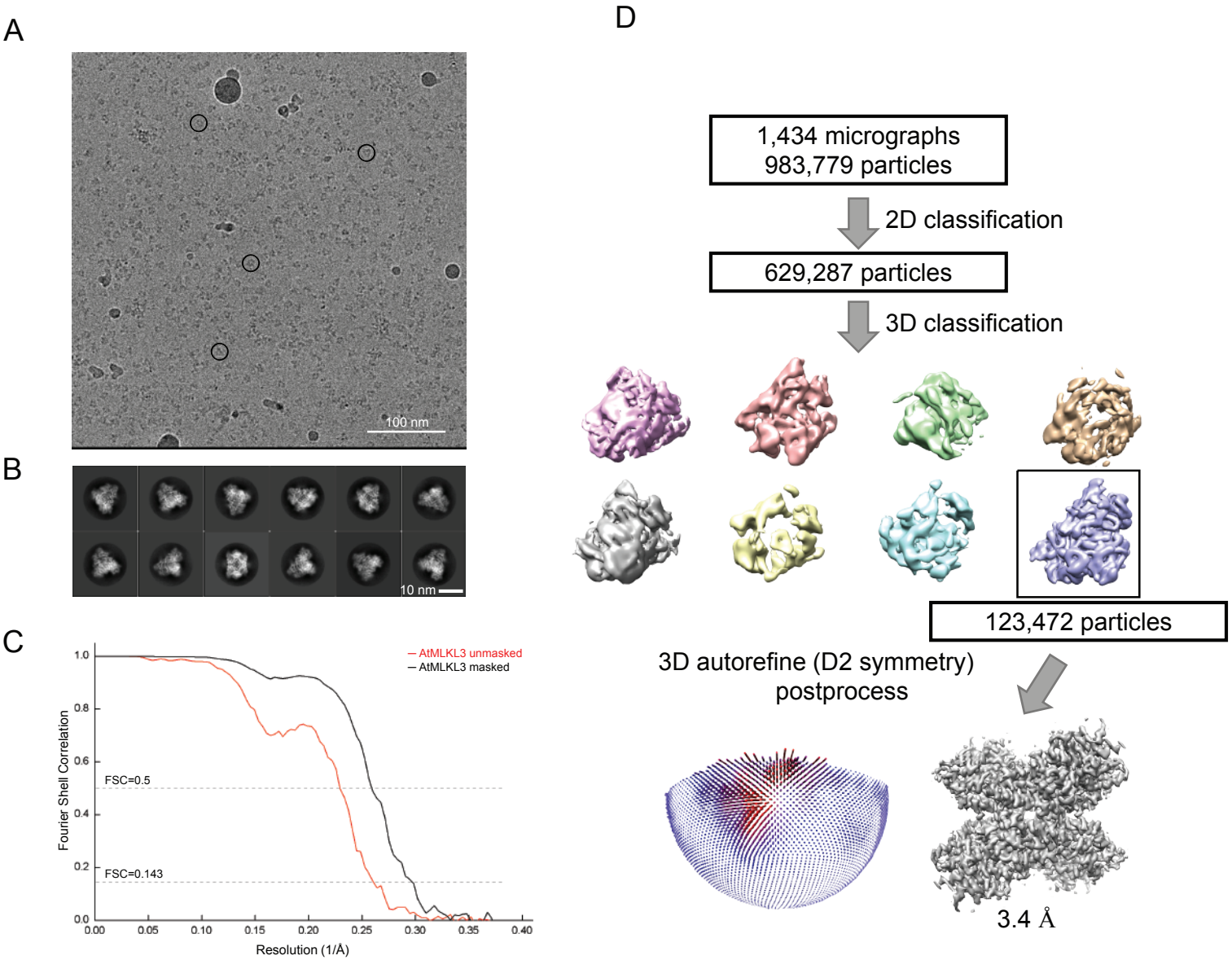

A
100 nm
B
10 nm
C
AtMLKL3 unmasked
AtMLKL3 masked
Fourier Shell Correlation
FSC=0.5
FSC=0.143
Resolution (1/Å)
D
1,434 micrographs
983,779 particles
2D classification
629,287 particles
3D classification
123,472 particles
3D autorefine (D2 symmetry)
postprocess
3.4 Å

Figure S1

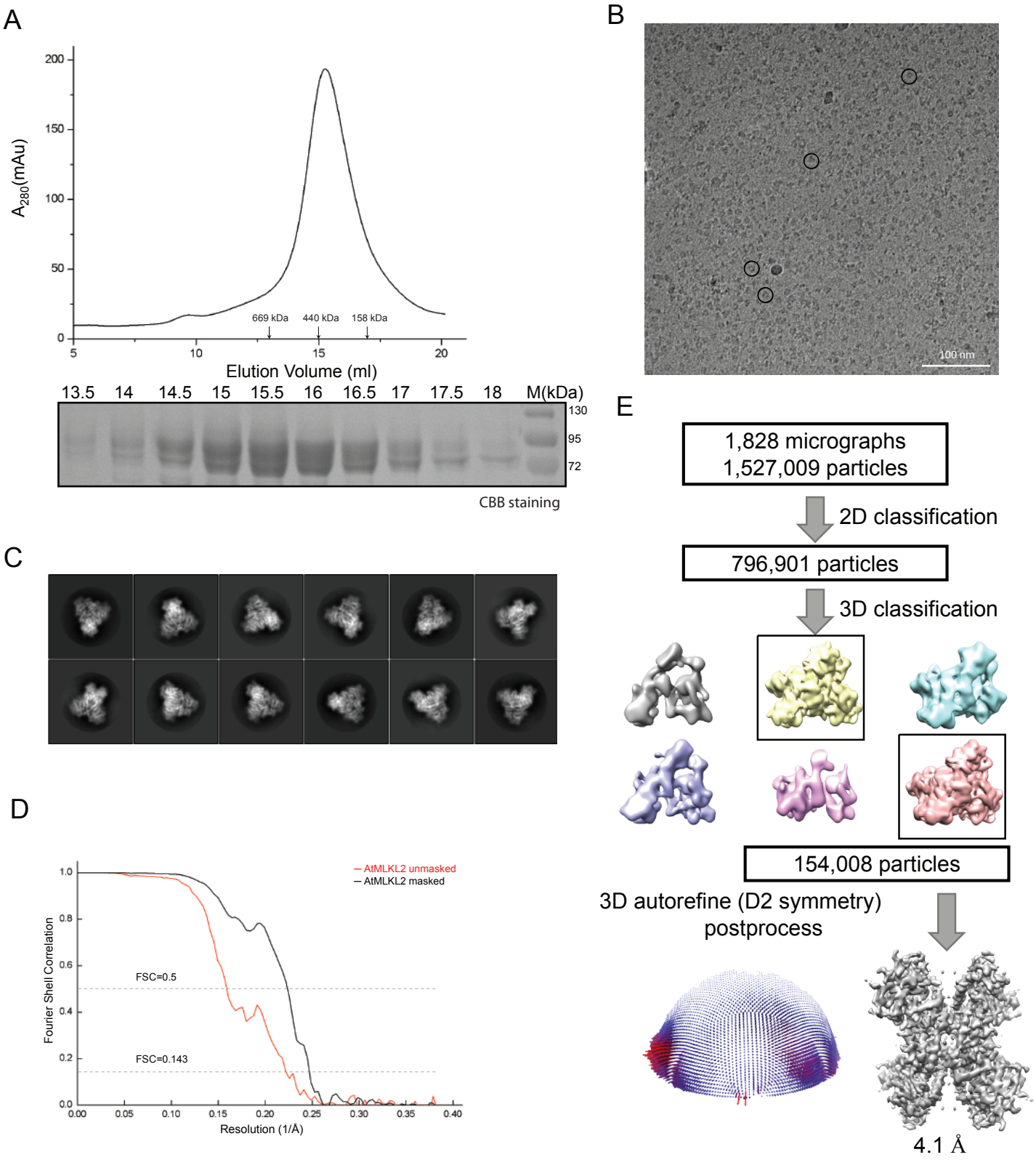

A
A_{280}(mAu)
200
150
100
50
0
5
10
15
20
669 kDa
440 kDa
158 kDa
Elution Volume (ml)
13.5
14
14.5
15
15.5
16
16.5
17
17.5
18
M(kDa)
130
95
72
CBB staining
B
100 nm
C
D
Fourier Shell Correlation
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
Resolution (1/Å)
AtMLKL2 unmasked
AtMLKL2 masked
FSC=0.5
FSC=0.143
E
1,828 micrographs
1,527,009 particles
2D classification
796,901 particles
3D classification
154,008 particles
3D autorefine (D2 symmetry)
postprocess
4.1 Å

Figure S2

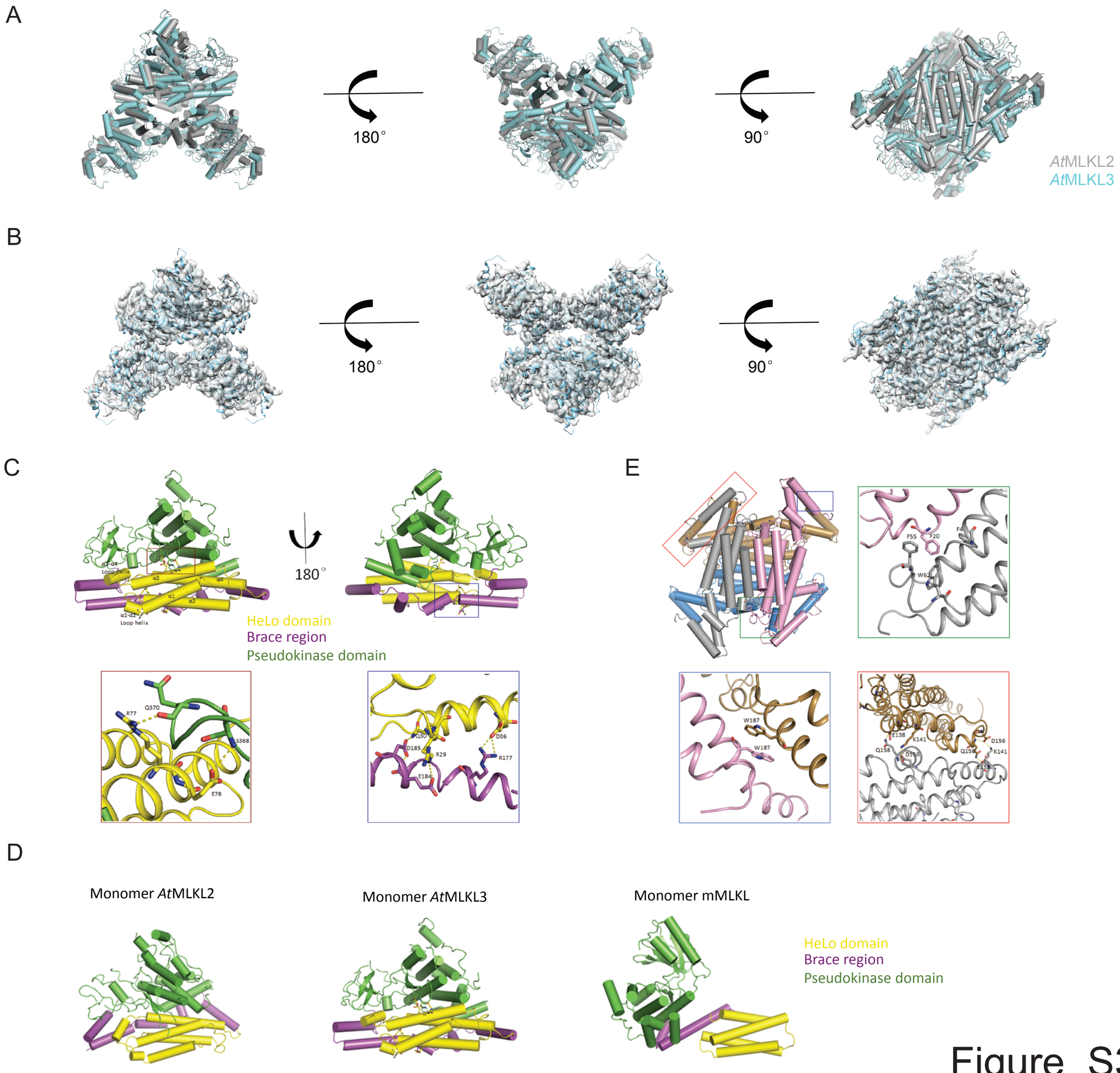
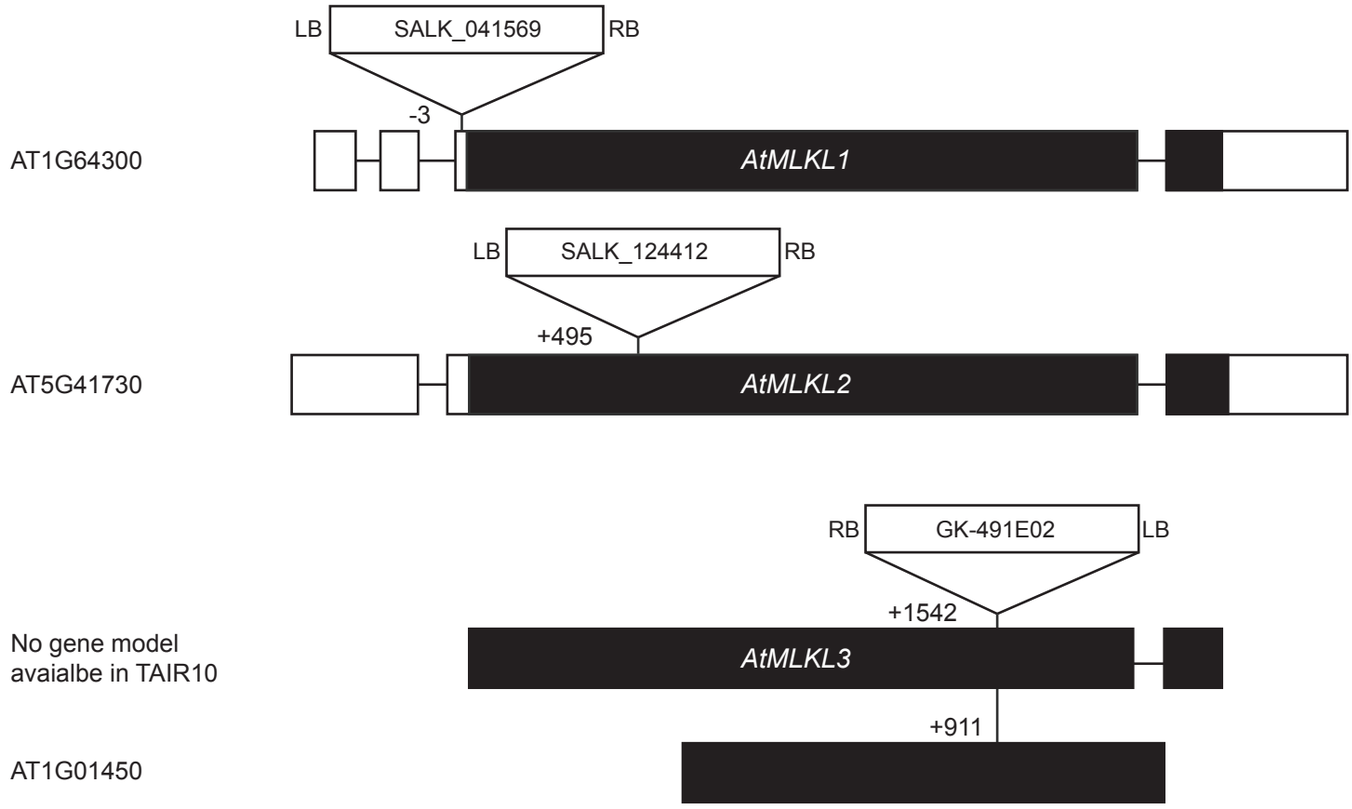

A
180°
90°
AtMLKL2
AtMLKL3
B
180°
90°
C
180°
HeLo domain
Brace region
Pseudokinase domain
E
D
Monomer AtMLKL2
Monomer AtMLKL3
Monomer mMLKL
HeLo domain
Brace region
Pseudokinase domain

Figure S3

A


LB
SALK_041569
RB
-3
AT1G64300
AtMLKL1
LB
SALK_124412
RB
+495
AT5G41730
AtMLKL2
RB
GK-491E02
LB
+1542
No gene model
avaialbe in TAIR10
AtMLKL3
+911
AT1G01450

B

1 cm
Col-0
Wild type
Atmlkl1
Atmlkl2
Atmlkl3
1 cm
Atmlkl1 2
Atmlkl1 3
Atmlkl2 3
Atmlkl1 2 3

Figure S4

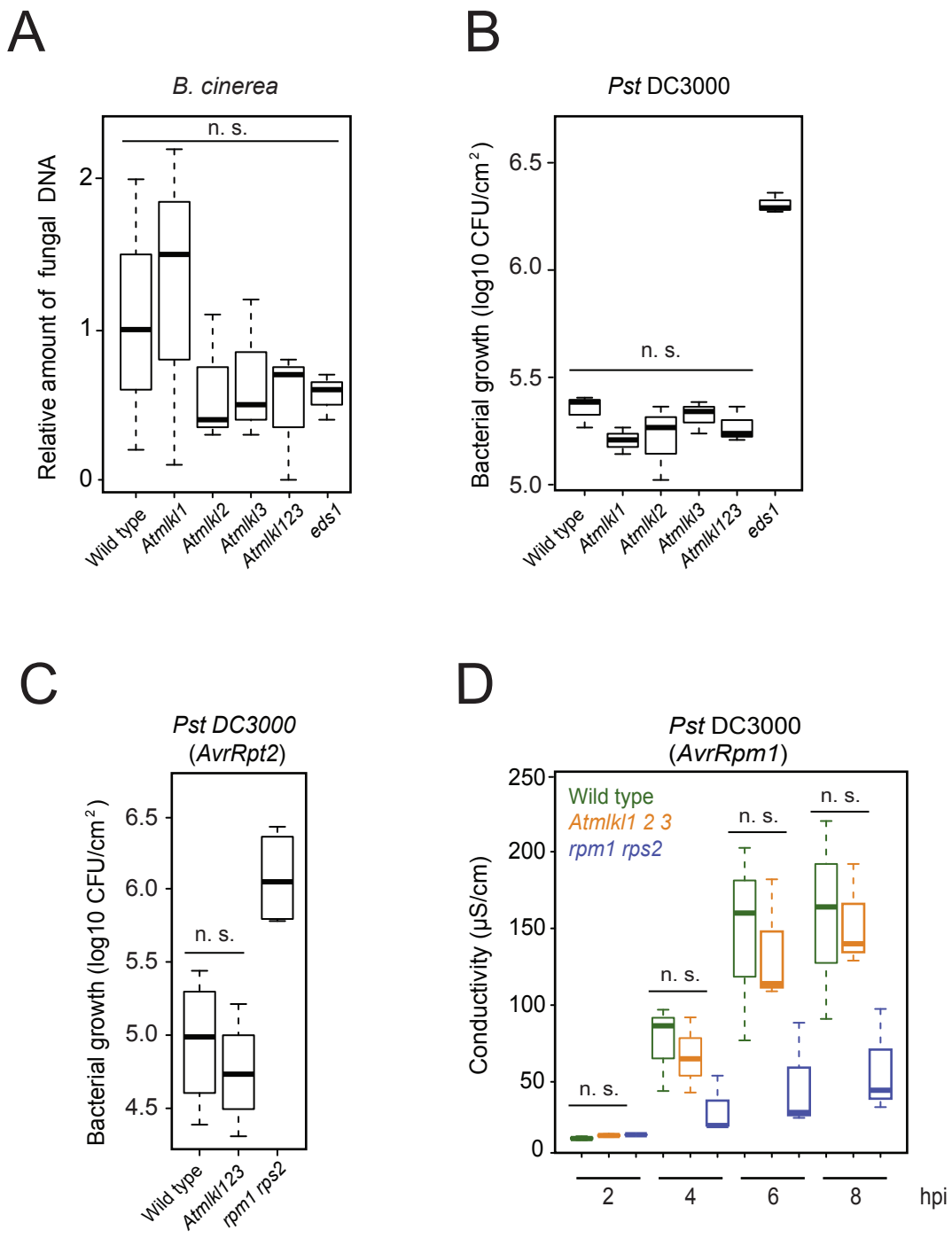

A
B. cinerea
Relative amount of fungal DNA
n. s.
0
1
2
Wild type
Atmlkl1
Atmlkl2
Atmlkl3
Atmlkl123
eds1
B
Pst DC3000
Bacterial growth (log10 CFU/cm2)
n. s.
5.0
5.5
6.0
6.5
Wild type
Atmlkl1
Atmlkl2
Atmlkl3
Atmlkl123
eds1
C
Pst DC3000
(AvrRpt2)
Bacterial growth (log10 CFU/cm2)
n. s.
4.5
5.0
5.5
6.0
6.5
Wild type
Atmlkl123
rpm1 rps2
D
Pst DC3000
(AvrRpm1)
Conductivity (µS/cm)
Wild type
Atmlkl1 2 3
rpm1 rps2
n. s.
n. s.
n. s.
n. s.
0
50
100
150
200
250
2
4
6
8
hpi

Figure S5

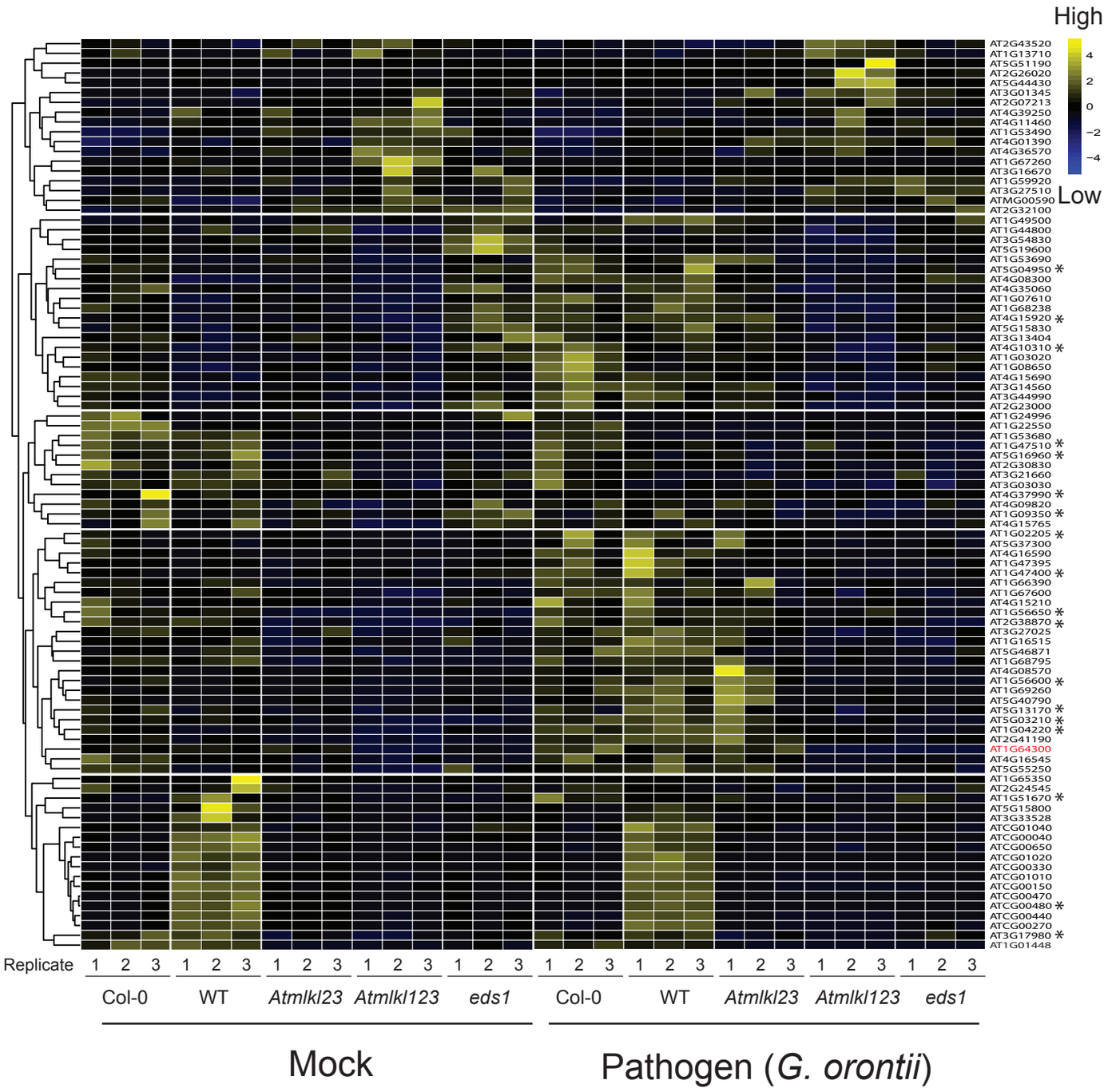

High
Low
4
2
0
−2
−4
Replicate
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Col-0
WT
Atmlkl23
Atmlkl123
eds1
Col-0
WT
Atmlkl23
Atmlkl123
eds1
Mock
Pathogen (G. orontii)

Figure S6

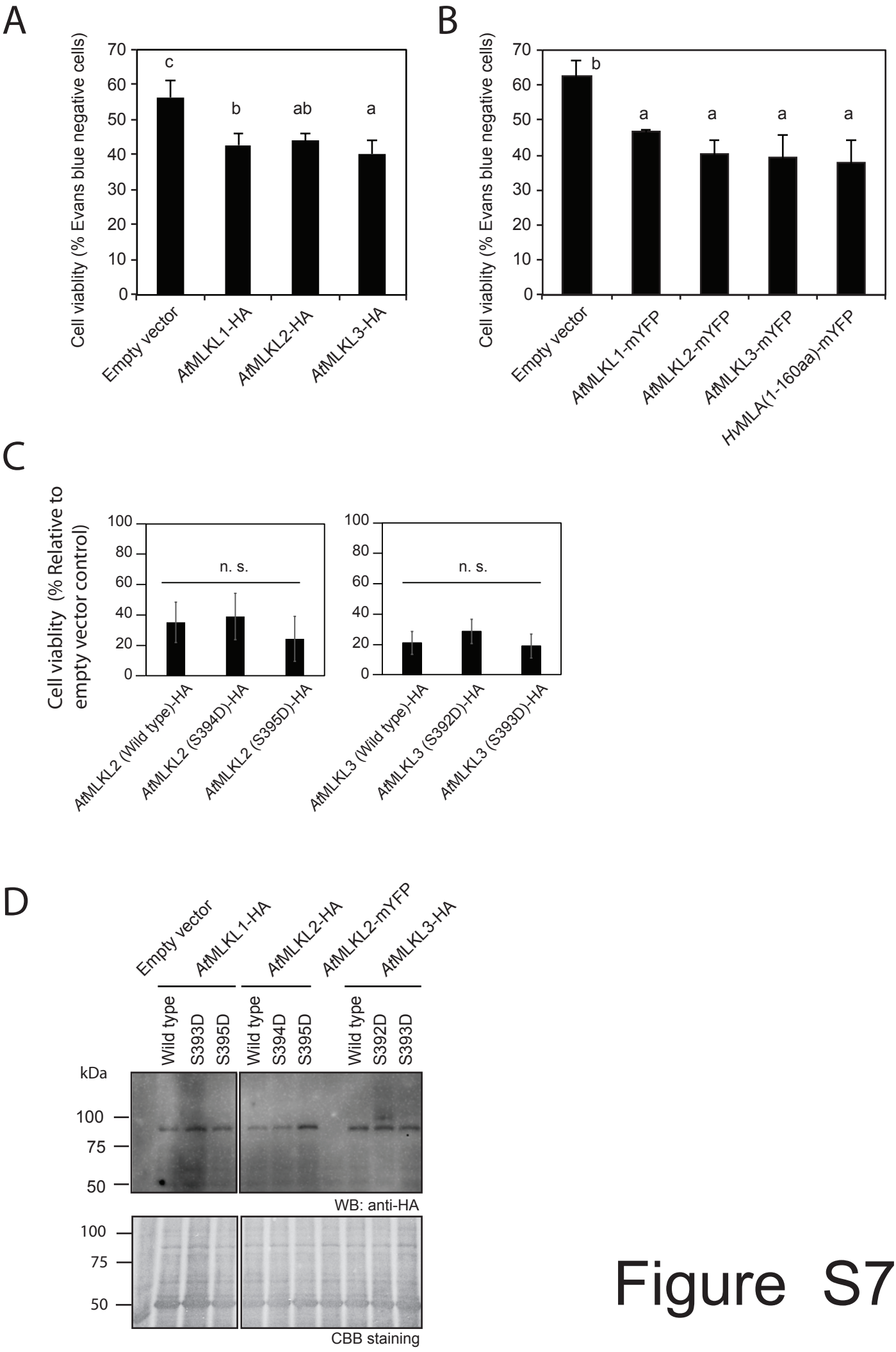
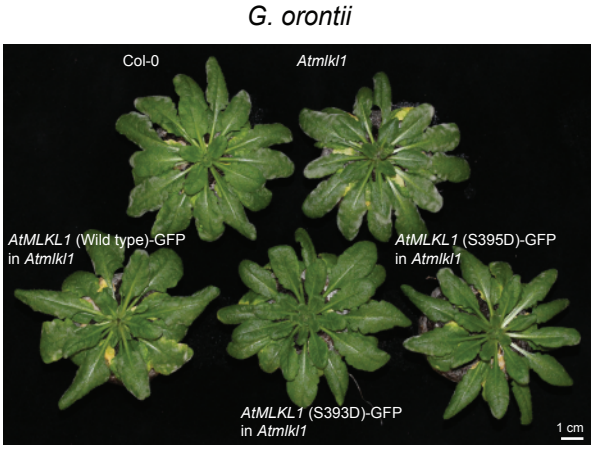

A
Cell viability (% Evans blue negative cells)
70
60
50
40
30
20
10
0
c
b
ab
a
Empty vector
AtMLKL1-HA
AtMLKL2-HA
AtMLKL3-HA
B
Cell viability (% Evans blue negative cells)
b
a
a
a
a
Empty vector
AtMLKL1-mYFP
AtMLKL2-mYFP
AtMLKL3-mYFP
HvMLA(1-160aa)-mYFP
C
Cell viablity (% Relative to empty vector control)
100
80
60
40
20
0
n. s.
AtMLKL2 (Wild type)-HA
AtMLKL2 (S394D)-HA
AtMLKL2 (S395D)-HA
AtMLKL3 (Wild type)-HA
AtMLKL3 (S392D)-HA
AtMLKL3 (S393D)-HA
D
Empty vector
AtMLKL1-HA
AtMLKL2-HA
AtMLKL2-mYFP
AtMLKL3-HA
Wild type
S393D
S395D
Wild type
S394D
S395D
Wild type
S392D
S393D
kDa
100
75
50
WB: anti-HA
CBB staining

Figure S7

A


G. orontii
Col-0
Atmlkl1
AtMLKL1 (Wild type)-GFP
in Atmlkl1
AtMLKL1 (S395D)-GFP
in Atmlkl1
AtMLKL1 (S393D)-GFP
in Atmlkl1
1 cm

B

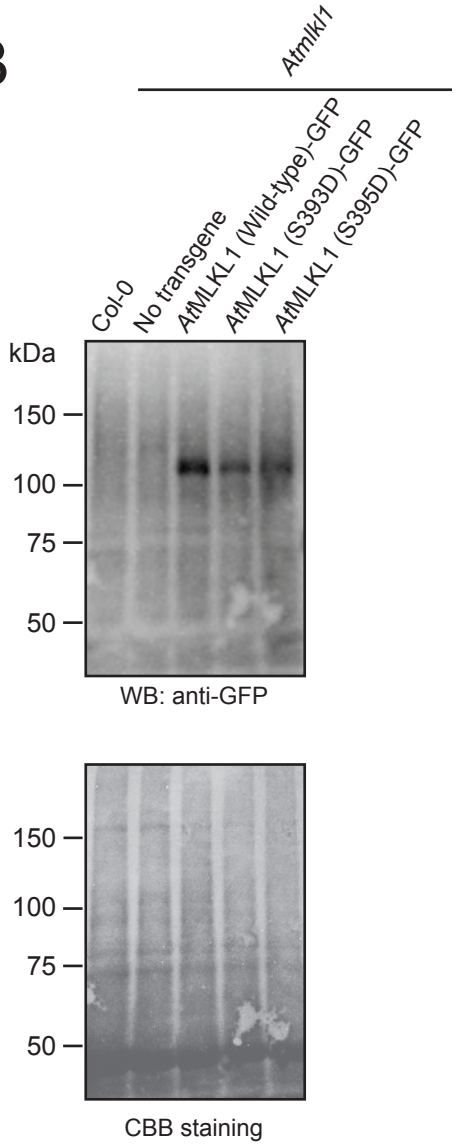
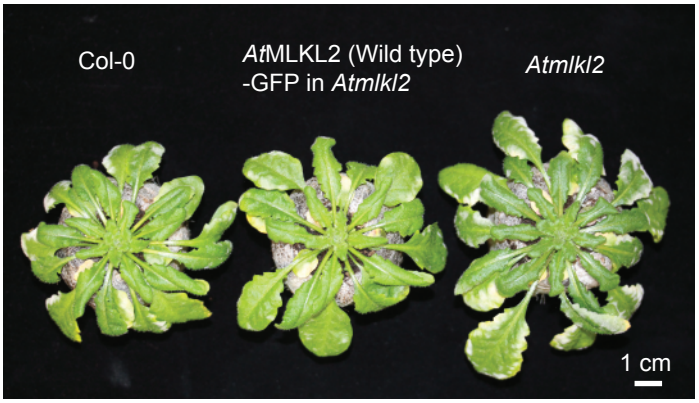
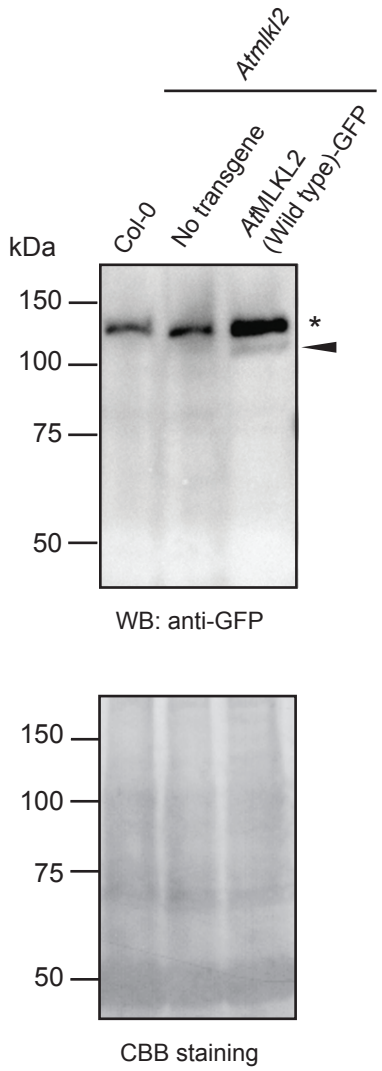

Atmlkl1
Col-0
No transgene
AtMLKL1 (Wild-type)-GFP
AtMLKL1 (S393D)-GFP
AtMLKL1 (S395D)-GFP
kDa
150
100
75
50
WB: anti-GFP
150
100
75
50
CBB staining

Figure S8


A
Col-0
AtMLKL2 (Wild type)
-GFP in Atmlkl2
Atmlkl2
1 cm


B
Atmlkl2
Col-0
No transgene
AtMLKL2
(Wild type)-GFP
kDa
150
100
75
50
*
WB: anti-GFP
150
100
75
50
CBB staining

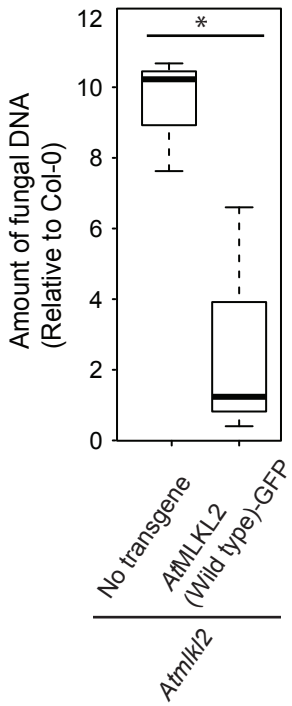

C
Amount of fungal DNA
(Relative to Col-0)
12
10
8
6
4
2
0
*
No transgene
AtMLKL2
(Wild type)-GFP
Atmlkl2

Figure S9

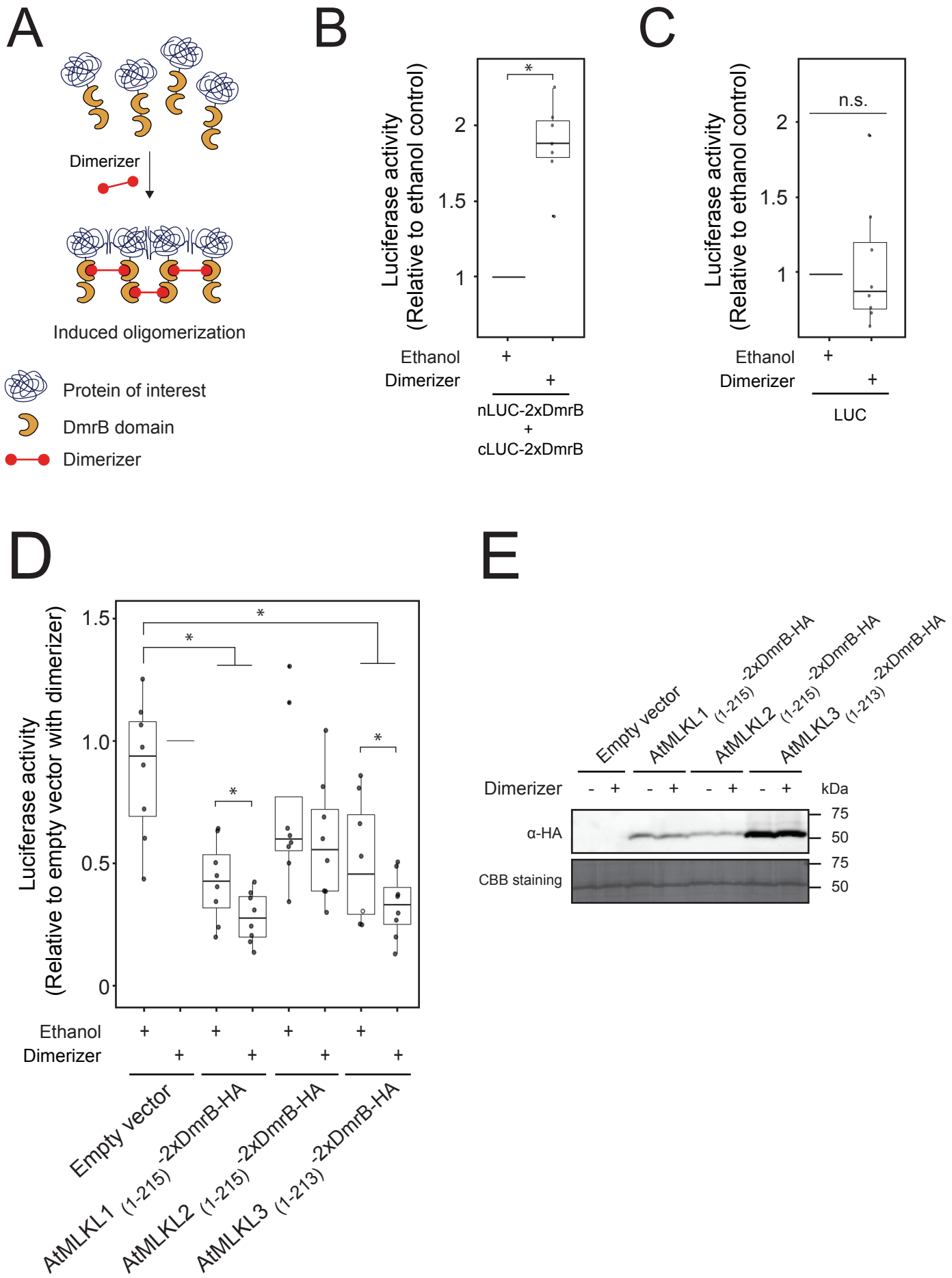
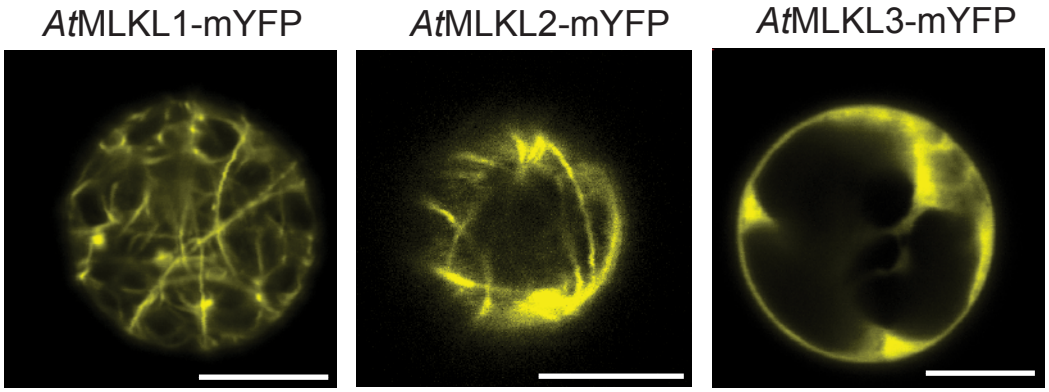
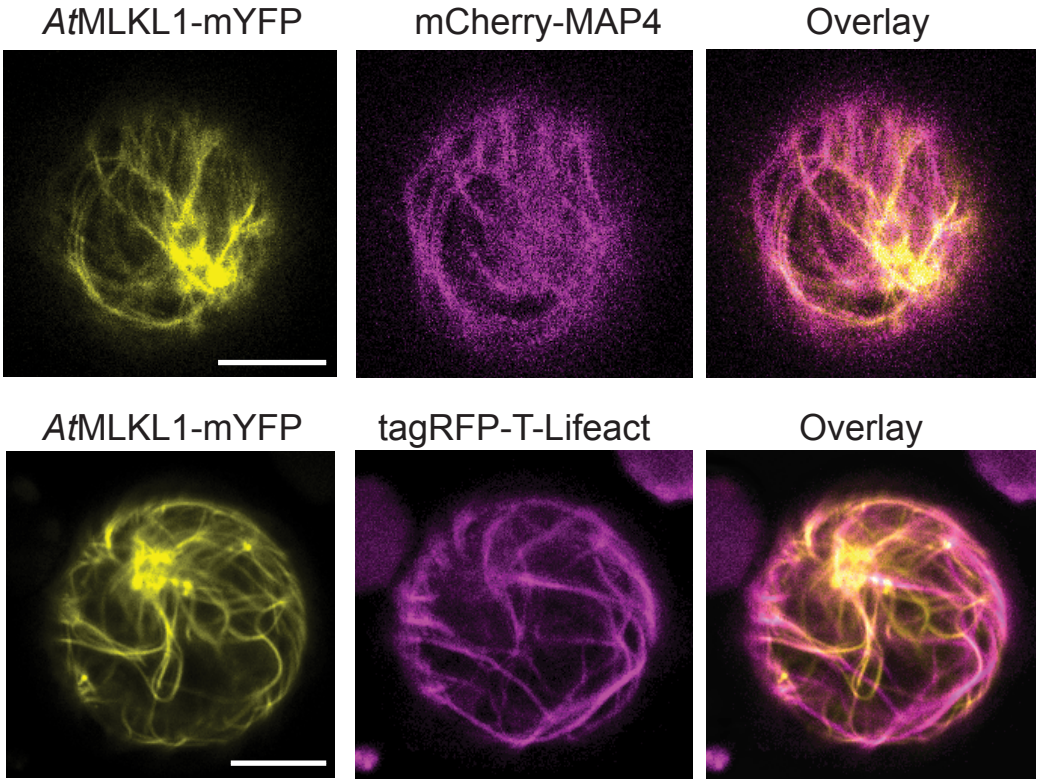

A
Dimerizer
Induced oligomerization
Protein of interest
DmrB domain
Dimerizer
B
Luciferase activity
(Relative to ethanol control)
2
1.5
1
*
Ethanol +
Dimerizer +
nLUC-2xDmrB
+
cLUC-2xDmrB
C
Luciferase activity
(Relative to ethanol control)
2
1.5
1
n.s.
Ethanol +
Dimerizer +
LUC
D
Luciferase activity
(Relative to empty vector with dimerizer)
1.5
1.0
0.5
0
*
Ethanol + + + +
Dimerizer + + + +
Empty vector
AtMLKL1 (1-215) -2xDmrB-HA
AtMLKL2 (1-215) -2xDmrB-HA
AtMLKL3 (1-213) -2xDmrB-HA
E
Empty vector
AtMLKL1 (1-215) -2xDmrB-HA
AtMLKL2 (1-215) -2xDmrB-HA
AtMLKL3 (1-213) -2xDmrB-HA
Dimerizer - + - + - + - +
kDa
α-HA
75
50
CBB staining
75
50

Figure S10

A



B



C

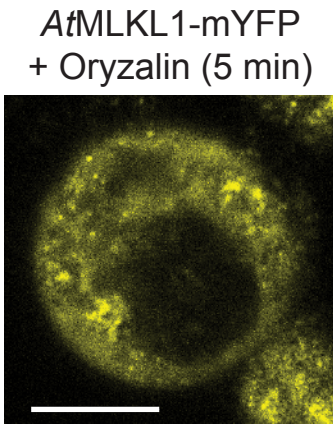
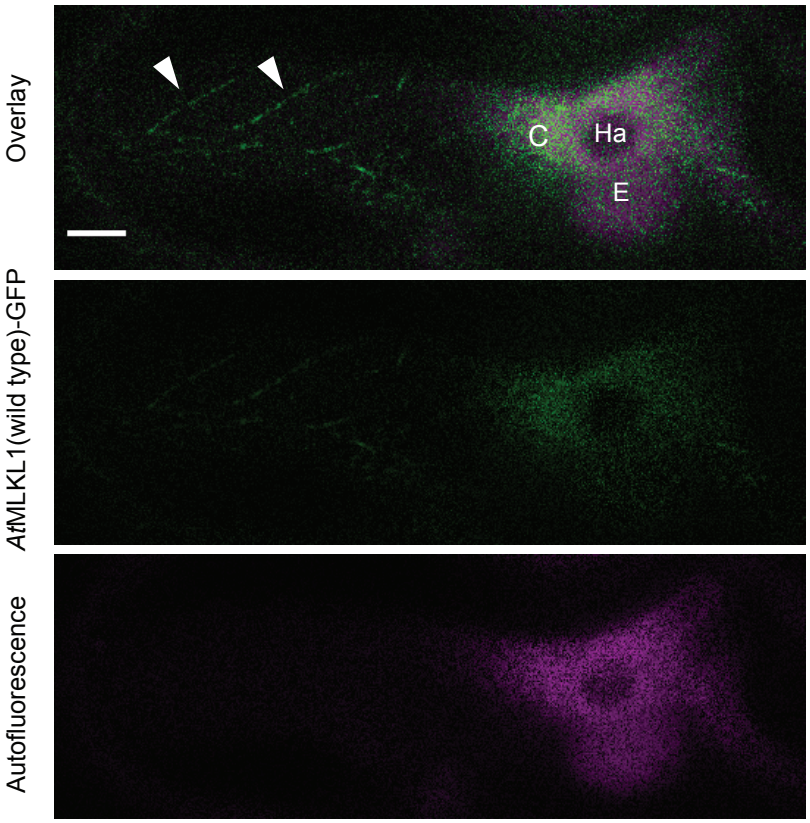


Figure S11

*At*MLKL1(Wild type)-GFP in *Atmlkl1*



Maximum projection (over Z)

Figure S12

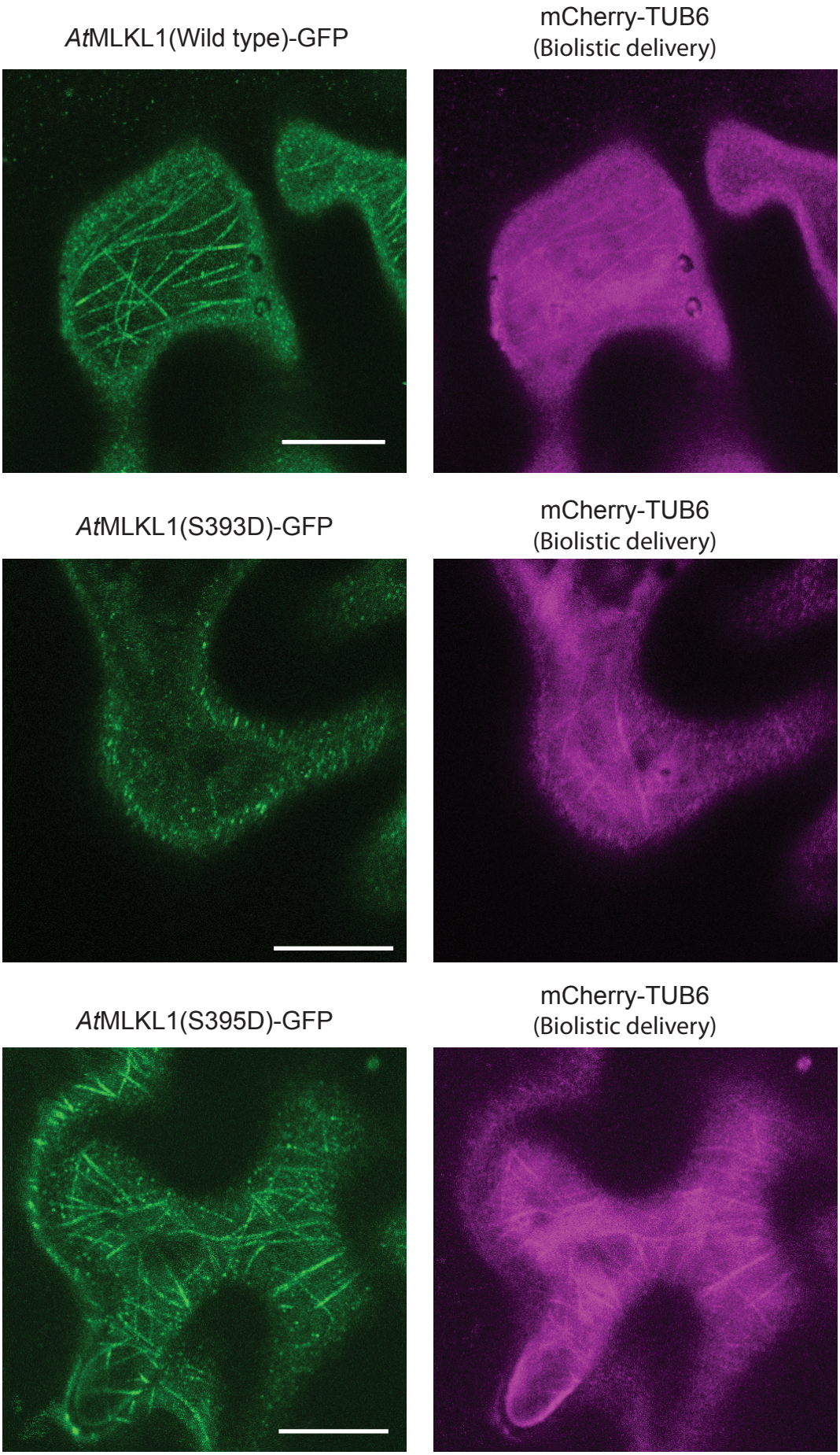

*At*MLKL1(Wild type)-GFP
mCherry-TUB6
(Biolistic delivery)
*At*MLKL1(S393D)-GFP
mCherry-TUB6
(Biolistic delivery)
*At*MLKL1(S395D)-GFP
mCherry-TUB6
(Biolistic delivery)

Figure S13