

1 **Coiled-coil and RPW8-type immune receptors function at the plasma membrane in a**
2 **phospholipid dependent manner**

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27 **Abstract**

28 Activation of intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) results in immunity
29 and a localized cell death response of infected cells. Cell death activity of many NLRs requires
30 oligomerization and in some cases plasma membrane (PM) localization. However, the exact
31 mechanisms underlying PM localization of NLRs lacking recognizable N- or C-terminal lipidation motifs
32 or predicted transmembrane domains remains elusive. Here we show that the PM localization and
33 stability of members of the RPW8-like coiled-coil (CC_R) domain NLRs (RNLs) and a CC-type NLR (CNL)
34 depend on the interaction with PM phospholipids. Depletion of phosphatidylinositol-4-phosphate (PI4P)
35 from the PM led to a mislocalization of the analyzed NLRs and consequently inhibited their cell death
36 activity. We further demonstrate activation-dependent self-association of cell death inducing RNLs. Our
37 results provide new insights into the molecular mechanism of NLR PM localization and defines an
38 important role of phospholipids for CNL and RNL activity during immunity.

39

40 **Introduction**

41 Plant intracellular immune receptors of the nucleotide-binding leucine-rich repeat receptor (NLR) family
42 mediate recognition of pathogen-derived effector proteins and the induction of a strong immune
43 response. In many cases, NLR activation leads to the hypersensitive response, a type of programmed
44 cell death of the infected cells¹⁻³. Based on their N-terminal domain architecture, three classes of NLRs
45 have been described in plants: Toll/Interleukin-1 receptor (TIR) NLRs (TNLs), coiled-coil (CC) NLRs
46 (CNLs) and the RPW8-like coiled-coil (CC_R) domain NLRs (RNLs)¹. In *Arabidopsis thaliana*
47 (*Arabidopsis*) the RNL subclass consists of two gene families, *ACTIVATED DISEASE RESISTANCE 1*
48 (*ADR1*) and *N REQUIREMENT GENE 1 (NRG1)*, both being required for immune signalling and cell
49 death induction of many other NLRs, particularly TNLs⁴⁻⁹. CNLs, TNLs and most likely RNLs induce
50 immune signalling and cell death by oligomerization¹⁰⁻¹³. CNL activation is speculated to result in the
51 formation of a pore-like or membrane disrupting structure of the CC domain (a so-called resistosome)
52 at the plasma membrane (PM)^{10,14-16}. PM localization was demonstrated to be required for cell death
53 and immune function of many CNLs, including *Arabidopsis* RPS5, RPM1 and ZAR1^{10,17-20}. The
54 subcellular localization of RNLs has not yet been analysed in detail. So far only a potential endoplasmic
55 reticulum (ER) localization as well as a partial PM localization of AtNRG1s was described^{6,7}.
56 Interestingly, many PM-localized CNLs and the RNLs have no predicted N- or C-terminal lipidation motif
57 or transmembrane domain/sequence and the mechanism that tethers them to the PM is unknown¹⁷.
58 Thus, the molecular determinants driving their localization and cell death function at the membrane are
59 not identified.

60

61 Homology modelling suggests that the CC_R domains of RNLs share structural similarities with the N-
62 terminal 4-helix bundle (HeLo domain) of mammalian mixed-lineage kinase domain-like (MLKL) proteins
63 and fungal HET-s/HELL proteins²¹⁻²³. HeLo domains mediate the cell death function of MLKL and HET-
64 s/HELL proteins and are proposed to oligomerize and disrupt or permeabilize the PM^{24,25}. Cell death
65 function and PM localization of MLKL proteins requires the interaction of their HeLo domain with specific
66 phospholipids at the PM^{26,27}.

67 Negatively charged, anionic phospholipids at membranes mediate electrostatic interactions with many
68 proteins that contain polybasic, basic hydrophobic or cationic domains or clusters^{28,29}.
69 Phosphatidylinositol-4-phosphate (PI4P) is one of the major phospholipids of the plant PM and a main
70 driver of PM electronegativity³⁰. Expression of the PM-anchored catalytic domain of the yeast
71 phospholipid-phosphatase Sac1p protein, which specifically dephosphorylates PI4P and therefore
72 reduces PI4P levels and the PM electronegativity^{30,31}, can be used to determine whether a protein
73 requires the presence of PI4P (or a high electronegativity) for localization and/or function at the PM.
74 Depletion of PI4P from the PM affects the localization and function of several proteins, including the
75 auxin transport regulator PINOID or the BRI1 kinase inhibitor 1, BKI1³⁰.

76

77 We show that decreasing PI4P abundance at the PM results in mis-localization and rapid degradation
78 of AtRPM1 (a CNL) and the three AtADR1s (RNLs). Further, depletion of PI4P also severely affected
79 cell death induction mediated by AtRPM1, as well as both AtRNL subfamilies.

80

81 Our results provide new insights into the molecular mechanism of NLR PM localization and defines an
82 important role of the PM phosphatidylinositol phosphate (PIP) pool in CNL- and RNL-mediated cell
83 death induction during plant immunity. Further, our work indicates that CNL- and RNLs deploy a lipid-
84 protein interaction similar to animal MLKL proteins for PM localization, which is likely necessary for cell
85 death execution at the PM.

86

87 **Results**

88 **Arabidopsis ADR1s localize at the plasma membrane in *Nicotiana benthamiana*.** The tentative
89 subcellular localization of two Arabidopsis full length RNLs, AtNRG1.1 and AtNRG1.2, was recently
90 described. Both proteins localize at ER membranes, partially at the PM and potentially in the cytosol
91 when transiently over-expressed in *Nicotiana benthamiana* (*N. benthamiana*) and measured by
92 confocal microscopy^{6,7}. Their intracellular localization was not changed upon effector-triggered and
93 TNL-mediated activation^{6,8}. However, there is no information on the localization of either pre- or post-
94 activated AtADR1s, the other RNL subfamily. To investigate the subcellular localization of the three
95 AtADR1 proteins pre- and post-activation we transiently expressed C-terminally EYFP- or Citrine-HA-
96 tagged wildtype ADR1, ADR1-L1 and ADR1-L2 in *N. benthamiana* leaves. We observed a strong co-
97 localization of all three wildtype AtADR1s with the PM-localized receptor-like kinase BRI1-mRFP (Fig.
98 1a,c,e; Supplementary Fig. S1a,e,g)³². In contrast to AtADR1-L1 and AtADR1-L2 the localization of
99 AtADR1 was not restricted to the PM. AtADR1 additionally localized to some puncta, of which some
100 might be PM and/or ER associated (Supplementary Fig. S1a and c), and the ER membrane, where it
101 co-localized with the ER-resident plant V-ATPase assembly factor AtVMA12-RFP (Supplementary Fig.
102 S1c)³³.

103

104 NLR localization may change when the receptor is activated¹⁰. To test this, we generated autoactive
105 alleles of ADR1s by mutating a conserved aspartic acid in the MHD motif to valine (QHD to QHV in
106 AtRNLs; cell death phenotype of autoactivated AtRNLs shown in Fig. 1g)³⁴⁻³⁶. The auto-activated

107 AtADR1^{DV} localization appeared more punctate compared to the wildtype AtADR1 (Fig. 1b and
108 Supplementary Fig. S1b and d), indicating that AtADR1 activation could result in a more clustered
109 localization. Similar to AtADR1-L1 and AtADR1-L2 wildtype proteins autoactivated AtADR1-L1^{DV} and
110 AtADR1-L2^{DV} localized to the PM (Fig. 1d,f; Supplementary Fig. S1f,h). We also noticed that AtADR1^{DV}
111 and AtADR1-L1^{DV} localized to BRI1-mRFP positive puncta (Fig. 1b,d; Supplementary Fig. S1b,f), most
112 likely endosomes. This potential endosomal localization was not observed for AtADR1-L2^{DV} (Fig. 1f;
113 Supplementary Fig. S1h).

114

115 These results demonstrate that three members of the Arabidopsis ADR1 subfamily localize at the plant
116 PM pre- and post-activation and further suggest that wildtype (steady-state) AtADR1 additionally
117 localizes to ER membranes and ER-associated dot-like structures, as observed for AtNRG1.1^{6,7}. The
118 PM localization of the (auto-)activated AtADR1s suggests that they could also execute their immune
119 (cell death) function at the PM.

120

121 **Self-association of cell death inducing Arabidopsis ADR1s.** NLR function in plants and animals is
122 proposed to require oligomerization for proper induction of cell death and immunity³⁷. However, self-
123 association of RNLs was only shown for *N. benthamiana* NRG1⁸. To test whether Arabidopsis ADR1s
124 self-associate and whether self-association is dependent on the activation status, we co-expressed
125 differently tagged wildtype and the QHV (autoactivated) mutant AtADR1s and analysed their self-
126 association by co-immunoprecipitation. We observed that transient over-expression of AtADR1,
127 AtADR1^{DV} and AtADR1-L1^{DV} induced a strong hypersensitive response-like cell death (HR), while the
128 over-expression of ADR1-L2^{DV}, although expressed, only resulted in a weak HR that was not reliably
129 reproducible (only 11 of 20 leaves showed HR symptoms; Fig. 1 g and h). We also found that the
130 AtADR1-induced HR occurred earlier in comparison to the AtADR1-L1^{DV} and AtADR1-L2^{DV} triggered
131 HR. Wildtype AtADR1-L1 and At-ADR1-L2 did not trigger a cell death response under our conditions
132 (Fig. 1 g-h). These data are consistent with the hypothesis that wildtype AtADR1 is already highly active
133 under steady-state conditions, whereas AtADR1-L1 and AtADR1-L2 are inactive under steady-state
134 conditions. However, introduction of the D to V mutation in the QHD motif renders AtADR1-L1^{DV} and
135 AtADR1-L2^{DV} into active proteins.

136

137 Our co-immunoprecipitation experiments revealed that the proteins inducing a strong cell death
138 response (AtADR1, AtADR1^{DV} and AtADR1-L1^{DV}) also strongly self-associated (Fig. 1 i). We also
139 observed self-association of wildtype AtADR1-L2 and the autoactivated AtADR1-L2^{DV}, however this
140 interaction was much weaker than for the highly active AtADR1, AtADR1^{DV} and AtADR1-L1^{DV} (Fig. 1 i).
141 These results indicate a correlation between self-association and cell death induction, suggesting that
142 the AtADR1s might activate HR via a similar mechanism as canonical CNLs – by the formation of an
143 oligomeric complex at the PM.

144

145 **Arabidopsis RNL and CNL PM localization and protein stability requires PM PI4P.** The PM
146 localization of AtADR1, AtADR1-L1 and AtADR1-L2 also suggests that they execute their immune

147 function at this cellular compartment as observed for other NLRs, such as AtRPM1¹⁷. Interestingly, for
148 both RNL families and many PM-localized CNLs, including AtRPM1, no transmembrane region or N- or
149 C-terminal protein lipidation motif could be identified and thus, they are most likely peripheral membrane
150 proteins (Supplementary Table 1)³⁸. Given the predicted structural homology of RNL CCR domains with
151 the phosphatidylinositol phosphate binding HeLo domain of mammalian MLKL²¹, we investigated
152 whether the presence of specific phosphoinositide species might be important for the PM localization
153 of Arabidopsis RNLs and CNLs. Since PI4P is one of the major phospholipids of the plant PM³⁰, we
154 tested whether RNL and CNL PM localization depends on PI4P. Transient expression of catalytic
155 domain of the PM-localized PI4P-specific yeast phosphatase SAC1p can be used to specifically
156 decrease the PI4P pool at the PM and therefore to determine the requirement of PI4P for the localization
157 and function of proteins of interest^{30,31,39}. We co-expressed the three AtADR1s (RNLs) and AtRPM1
158 (CNL) with SAC1 and determined their subcellular localization and protein abundance by confocal
159 microscopy and western blot analysis, respectively. The N-terminally myristoylated and PM localized
160 Arabidopsis CNL AtRPS5 was included as a control NLR as AtRPS5 PM localization (and function) was
161 not expected to be affected by PM PI4P reduction^{19,40}. Co-expression with the catalytically active
162 wildtype SAC1 (SAC1^{WT}) affected the PM localization of all tested NLRs except AtRPS5 (Fig. 2a,c,e,g;
163 Supplementary Fig. S2 a). By contrast, co-expression with the phosphatase dead mutant SAC1
164 (SAC1^{dead}) protein had no influence on the PM localization of AtADR1, AtADR1-L1, AtADR1-L2,
165 AtRPM1 and AtRPS5 (Fig. 2 a,c,e,g; Supplementary Fig. S2 a). Thus, the effect of SAC1 activity on the
166 PM localization of AtRNLs and AtRPM1 is specific. Co-localization of AtADR1 with SAC1^{WT} at the PM
167 was rarely detectable and the majority of AtADR1 was localized inside the cell, likely at the ER and/or
168 cytosol (Fig. 2a). We also observed less AtADR1 accumulation in western blot analysis upon co-
169 expression with SAC1^{WT} (Fig. 2b), indicating that most likely only the PM-localized pool of AtADR1 and
170 not the ER-localized pool is affected by SAC1^{WT} co-expression. No fluorescence was observed for either
171 AtADR1-L1 or AtRPM1, and only a very weak fluorescence for AtADR1-L2, after co-expression with
172 SAC1^{WT} (Fig. 2c,e,g). This indicates that depleting the PM PI4P pool severely affects protein
173 accumulation of AtADR1-L1, AtADR1-L2 and AtRPM1. Western blot analysis of AtADR1-L1, AtADR1-
174 L2 and AtRPM1 upon co-expression with SAC1^{WT} confirmed the lack of NLR protein accumulation (Fig.
175 2d,f,h). A similar observation was previously reported for a phosphatidylserine specific binding protein,
176 which is unstable in the Arabidopsis *pss1* mutant that lacks phosphatidylserine production⁴¹.

177

178 In order to test whether loss of NLR protein accumulation upon SAC1^{WT} co-expression was due to
179 degradation of the mis-localized proteins we analysed protein levels by western blot in presence of
180 protease and proteasome inhibitors. The specific inhibition of proteasomal degradation by Bortezomib
181 (BTZ) had an observable effect on the accumulation of AtADR1-L2 (compare lane 2 with lane 6 in
182 Supplementary Fig. S3c) and a weak effect on AtADR1 and AtADR1-L1 accumulation (Supplementary
183 Fig. 3a,b). This indicates that proteasomal degradation is, at least partially, responsible for the
184 degradation of mis-localized AtADR1s. In contrast, mis-localized AtRPM1 could not be stabilized in the
185 presence of BTZ (Supplementary Fig. S3d), suggesting the proteasome plays no role in AtRPM1
186 degradation. This is consistent with previously published data¹⁷.

187 Together these results clearly demonstrate that all three AtADR1s and AtRPM1 require PI4P or a high
188 electronegativity driven by PI4P at the PM for their proper localization and that loss of PM localization
189 severely affects protein stability. Degradation of the mis-localized NLRs is, at least for the RNLs,
190 partially mediated by the proteasome.

191

192 **Cell death function of Arabidopsis PM-localized RNLs and CNLs is PI4P dependent.** PM
193 localization of several NLRs, including AtRPM1, was shown to be important for their immune and cell
194 death function^{10,17-20}. The severe effect on the localization of the AtADR1s and AtRPM1 by depleting
195 PI4P from the PM, prompted us to analyse whether their cell death function was also affected. Transient
196 over-expression of the CC_R domains of the Arabidopsis RNLs ADR1, ADR1-L2 and NRG1.1 is sufficient
197 to induce a cell death response in *N. benthamiana* (Fig. 3a-c)¹⁶. CC_R domain induced cell death activity
198 was dramatically diminished by SAC1^{WT}, but not SAC1^{dead} co-expression (Fig. 3a-c). These results
199 suggest that the RNL CC_R domains induce cell death at the PM in a PI4P-dependent manner.
200 Expression of the AtADR1-L1 CC_R domain did not induce a visible cell death response in transient
201 expression assays under our growth conditions and hence, could not be tested for PI4P dependency
202 (Supplementary Fig. S4a). Interestingly, in contrast to the measurable negative effect of SAC1^{WT} activity
203 on the accumulation of the full-length NLR proteins (Fig. 2b, d, f, h) we did not observe a similar effect
204 on the CC_R domains (Fig. 3a-c). Altogether, PI4P depletion does not affect CC_R domain stability, but
205 substantially affects CC_R domain induced cell death.

206

207 Since the AtADR1 full-length protein induces a fast and strong cell death response in *N. benthamiana*,
208 we further tested whether SAC1^{WT} co-expression can suppress cell death induced by full-length
209 AtADR1 (Fig. 3 d). Similar to suppression of AtADR1 CC_R induced cell death by PI4P depletion, SAC1^{WT}
210 expression also suppressed cell death activity of full-length AtADR1 (Fig. 3d).

211

212 To examine if suppression of cell death activity by SAC1^{WT} is not restricted to the CC_R domains and
213 full-length wildtype AtADR1 we wanted to include the QHD motif mutants of the three AtADR1s in our
214 cell death suppression experiments. However, only the AtADR1^{DV} mutant induced a strong, fast and
215 reliable cell death response under our growth conditions (Fig. 1g). Consistent with the suppression of
216 wildtype AtADR1 induced cell death by SAC1^{WT} co-expression, SAC1^{WT} co-expression also suppressed
217 AtADR1^{DV} cell death activity (Fig. 3e). We conclude that PI4P depletion severely affects RNL and CC_R
218 domain cell death activity, most likely due to loss of PM localization.

219

220 AtRPM1 guards the immune regulatory protein RIN4 (RPM1 INTERACTING PROTEIN 4) and is
221 activated by an effector-triggered phosphorylation of RIN4 threonine 166^{42,43}. AtRPM1 activation can
222 be reconstituted in *N. benthamiana* by co-expression of AtRPM1 and a phosphomimic mutant of AtRIN4
223 (AtRIN4^{T166D})^{17,44}. The strong cell death response upon AtRPM1 activation by AtRIN4^{T166D} was
224 completely inhibited by SAC1^{WT} co-expression, but not by SAC1^{dead} (Fig. 3f). Cell death activity of
225 effector-activated AtRPM1 was also severely affected by SAC1^{WT} co-expression (Supplementary Fig.
226 S4b), demonstrating that AtRPM1 mediated cell death activity at the PM also depends on PI4P.

227 To demonstrate that the effect of decreasing the PM PI4P pool on cell death activity of the AtRNLs and
228 AtRPM1 is specific and not a general effect on cell death induced by NLRs, we analysed whether
229 SAC1^{WT} activity had any effect on cell death mediated by the myristoylated and “constitutively” PM
230 localized AtRPS5. Similar to the AtRPM1 mediated cell death response, the AtRPS5 mediated and
231 effector-triggered cell death can be reconstituted in transient expressions in *N. benthamiana*⁴⁵. Neither
232 the expression of SAC1^{WT} nor SAC1^{dead} suppressed effector-triggered and AtRPS5 mediated cell death
233 (Supplementary Fig. S2e). These results suggest that the effect of SAC1 activity on cell death induction
234 by the AtRNLs and AtRPM1 is specific.

235

236 Taken together, our results demonstrate that AtRNL and AtRPM1 cell death activity is significantly
237 affected by PI4P depletion from the PM and further suggest that cell death activity of all AtRNLs,
238 including the presumably ER-localized AtNRG1s^{6,7}, takes place at the PM.

239

240 **PI4P depletion affects PM localization of AtADR1, AtADR1-L1 and AtADR1-L2 CC_R domains.** Cell
241 death activity of the AtADR1 and AtADR1-L2 CC_R domain was notably diminished by SAC1^{WT} co-
242 expression (Fig. 3a,b). However, unlike the full length AtADR1s, the stability of the AtADR1 CC_R-
243 domains was not affected (Fig. 3a,b; Supplementary Fig. S4a). To test whether PI4P depletion affects
244 CC_R localization and hence function, we co-expressed the CC_R domains of all three AtADR1s with
245 SAC1^{WT} or SAC1^{dead} and analysed their localization by confocal microscopy. All three CC_R domains
246 localized to the PM in the presence of SAC1^{dead} (Fig. 4; Supplementary Fig. S5). We also observed that
247 the AtADR1 CC_R domains localized to dot-like structures and to ER membranes (Fig. 4a and
248 Supplementary Fig. S5a). However, the PM localization of all three CC_R domains was affected by
249 SAC1^{WT} co-expression. Fluorescence of the CC_R domains was detected at intracellular puncta and also
250 at ER membranes and/or the cytosol (Fig. 4; Supplementary Fig. S5). SAC1^{WT} triggered re-localization
251 was more visible for AtADR1 and AtADR1-L2 CC_R domains than for the AtADR1-L1 CC_R domain
252 (Supplementary Fig. S5).

253

254 Thus, PI4P depletion from the PM leads to a reduced PM-localization (and loss of cell death function)
255 of the CC_R domains and potentially a (mis-)localization to endosomal compartments and the ER or
256 cytosol. Proteins that are normally interacting with the PM in a PI4P- or electronegativity-dependent
257 manner have been shown to ‘adopt’ endosomal localization once the PM PI4P pool is depleted^{30,41}.

258

259 **AtADR1-, AtADR1-L1-, AtADR1-L2 CC_R and AtRPM1 CC domains specifically interact with**
260 **anionic lipids *in vitro*.** Reducing the abundance of PM PI4P levels negatively influenced the function,
261 localization and stability of the tested Arabidopsis RNLs and RPM1. Thus, it is very likely that a direct
262 interaction of AtADR1s and AtRPM1 with PM PI4P or other anionic lipids in general is required for their
263 cell death activity. Given the structural homology of the CC_R domain with the N-terminal HeLo domain
264 of MLKL²¹ and the importance of the CC domain for cell death function of many CNLs²² we investigated
265 whether the AtADR1s CC_R and the AtRPM1 CC domains bind to specific phospholipids. We generated
266 C-terminally haemagglutinin (HA)-tagged CC domain proteins *in vitro* and incubated the proteins on a

267 lipid array (PIP strip). All three AtADR1 CC_R domains and the AtRPM1 CC domain directly interacted
268 with PIPs, but not with other phospholipids or non-phosphorylated phosphoinositides (Fig. 5). A very
269 weak interaction was also observed with the anionic and low abundant phosphatidylserine (PS) and
270 phosphatidic acid (PA) (Fig. 5)⁴⁶. These results suggest a strong binding of the AtRNLs and AtRPM1
271 CC_R/CC domain with negatively charged PIPs most likely via an electrostatic interaction.

272

273 **PI(4,5)P₂ depletion has no impact on PM localization and cell death function of AtRNL and**
274 **AtRPM1.** The strong effect of PI4P depletion at the PM on the function and localization of AtRNLs and
275 AtRPM1 and the specific interaction of their CC_R/CC domain with anionic lipids (including PI4P) *in vitro*,
276 suggest that PI4P plays a major role for their interaction with and function at the PM. However,
277 phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) fulfils similar important cellular functions, is
278 specifically found at the plant PM, and is also required for the interaction of many proteins with the PM⁴⁷,
279 like the mammalian MLKL proteins^{26,27}. Although, PI(4,5)P₂ is most likely not required for plant PM
280 electronegativity³⁰. Our observation of the additional direct binding of the AtADR1s CC_R and AtRPM1
281 CC domains to PI(4,5)P₂ (Fig. 5), prompted us to test whether PI(4,5)P₂ is also required for AtRNL and
282 AtCNL PM localization and cell death function. We co-expressed the PM-anchored wildtype PI(4,5)P₂
283 5-phosphatase domain from the Drosophila OCRL protein (dOCRL^{WT}) that specifically depletes the
284 PI(4,5)P₂ pool at the plant PM⁴⁷ with AtADR1, AtADR1-L1, AtADR1-L2, AtRPM1 and AtRPS5. As a
285 control we co-expressed a phosphatase dead mutant version of dOCRL (dOCRL^{dead}) that is catalytically
286 inactive⁴⁷. Co-expression of neither dOCRL^{WT} nor dOCRL^{dead} had a visible effect on the (PM-)
287 localization or protein expression of the tested AtRNLs and AtCNLs (Supplementary Figure S2c,d and
288 S6). Co-expression of dOCRL^{WT} with the cell death inducing CC_R domains of AtADR1, AtADR1-L2 and
289 AtNRG1.1 did not inhibit their activity and a strong cell death induction was visible for all three CC_R
290 domains (Supplementary Fig. S7 a-c). Likewise, dOCRL^{dead} co-expression did not negatively affect the
291 activity of the tested CC_R domains. Similarly, depleting the PI(4,5)P₂ pool did not affect AtADR1 and
292 AtADR1^{DV}-induced cell death responses (Supplementary Fig. 7d,e). Further, we found no inhibition of
293 the cell death activity of AtRPM1 or AtRPS5 in either the presence of dOCRL^{WT} or dOCRL^{dead}
294 (Supplementary Fig. S4c, S2f and S7f). Consistent with the fact that PI(4,5)P₂ depletion does not affect
295 AtRNL and AtRPM1-mediated cell death, we also did not observe a negative effect on protein
296 accumulation by PM PI(4,5)P₂ depletion (Supplementary Fig. S6 and S7f). This suggests Arabidopsis
297 RNLs, RPM1 and RPS5 cell death activity at the PM is independent of PI(4,5)P₂.

298

299 Collectively, this demonstrates that PI(4,5)P₂ is likely not a major contributor for AtADR1s (RNLs),
300 AtRPM1 and AtRPS5 (CNL) localization and function at the PM.

301

302 Discussion

303 The Arabidopsis CNL ZAR1 oligomerizes upon effector-induced activation, followed by a potential
304 translocation to the PM where it is potentially forming a pore-like structure via the alpha 1 helix of its
305 CC domain^{10,48}. PM or endomembrane localization was shown to be necessary for the cell death and
306 immune function of many CNLs^{17,49}. Some CNLs localize to membranes via N-terminal myristoylation

307 and/or palmitoylation, and the residues required for this post-translational modification were
308 demonstrated to be important for CNL function¹⁹. However, the molecular mechanism underlying the
309 localization of non-acylated PM/membrane-localized NLRs remains elusive. We present data that
310 suggests a model in which AtRNLs and the CNL AtRPM1 require PI4P at the PM for proper localization,
311 protein stability and cell death function upon (auto-) activation (Fig. 6). The localization is most likely
312 regulated by direct binding of their CC/CC_R domain to anionic lipids (including the very abundant PI4P),
313 possibly via positive charges in this domain. We cannot rule out the possibility that other mechanisms
314 are also required, like the interaction with other (structural) lipids or proteins, e.g. integral membrane or
315 transmembrane proteins. However, the strong effect of PI4P depletion from the PM on NLR function
316 and stability, suggests that PI4P contributes significantly to RNL and CNL PM localization.

317
318 Interestingly, recent studies demonstrated that there is a reduction in PI4P and a specific enrichment
319 of PI(4,5)P₂ on interfacial membranes during successful infections, like the extra-haustorial membrane
320 (EHM) in *Arabidopsis* powdery mildew infections, the extra-invasive hyphal membrane (EIHM) in
321 *Arabidopsis Colletotrichum* infections or at the potato (*solanum tuberosum*) *Phytophthora infestans*
322 infection sites⁵⁰⁻⁵². The PI(4,5)P₂ enrichment at the EHM and EIHM is an essential susceptibility factor,
323 which is most likely pathogen-induced and requires the function of the host phosphatidylinositol 4-
324 phosphate 5-kinases (PIP5K)^{50,51}. It is possible that the depletion of PI4P and the simultaneous
325 enrichment of PI(4,5)P₂ at these host-pathogen interfaces result in a reduced accumulation of immune-
326 regulatory proteins, for example NLRs, by removing possible binding sites and/or enhancing
327 endocytosis of immune signaling components⁵⁰. Plants however have evolved means to counteract this
328 potentially pathogen/effector-induced enrichment of PI(4,5)P₂ by downregulating the activity of PIP5Ks
329 or upregulating the activity of phosphoinositide 5-phosphatases upon pathogen perception by cell-
330 surface localized immune receptors^{53,54}. Thus, actively changing or adjusting the lipid composition and
331 homeostasis of the plant PM is part of the evolutionary arms race between the host and the pathogen.
332 This indicates the importance of the regulation/manipulation of lipid homeostasis and the associated
333 changes in protein localization/stability in this battle.

334
335 Likewise, a correlation between the lipid composition of the PM and immunity, as well as NLR (CNL)
336 function and stability and an important function for phospholipase-dependent signalling in immunity was
337 previously reported⁵⁵⁻⁵⁹. Plant phospholipase families C (PLCs) and D (PLDs) are involved in many
338 aspects of abiotic and biotic stress responses⁶⁰. However, the exact mechanisms of how these enzymes
339 and their product(s) influence immunity are not well understood⁶¹. Perception of pathogen-derived
340 danger signals by NLRs and cell-surface localized pathogen-recognition receptors (PRRs) lead to rapid
341 recruitment and specific activation of PLDs and PLCs and to their recruitment to pathogen entry sites
342 at the PM, as well as a biphasic transient Ca²⁺ influx^{56,57,62}. PLDs and PLCs induce the production of
343 inositol polyphosphates, phosphatidic acid (PA) and diacylglycerol (DAG), all of which can function as
344 second messengers during immunity as well as other stress responses⁶¹. The PLC and PLD mediated
345 generation of PA is required for NLR-triggered ROS production and HR, and external application of PA
346 is sufficient to induce a cell death response and the transcriptional activation of the pathogen-responsive

347 *PR1* promoter⁵⁸. The hypothetical pore or ion (Ca²⁺)-channel forming capability of some CNLs at
348 membranes is presumably required for their cell death activity and downstream immune signalling^{21,63}.
349 It is very likely that RNLs, having a RPW8-like/HeLo-like CC domain, use a similar mechanism for cell
350 death induction and immunity. In light of our results it is tempting to hypothesize that (i) RNL (and most
351 CNLs) activation leads to oligomerization and (enhanced or induced) interaction with PM/membrane
352 anionic lipids, like PI4P, (ii) the formation of a transient Ca²⁺ channel/pore and the (iii) subsequent
353 activation of calcium dependent and probably NLR-interacting phospholipases that in turn produce lipid
354 messengers, such as PA and DAG, which (iv) might activate downstream signalling components
355 required for cell death and resistance (Supplementary Figure 8)^{10,21,55,58}.

356

357 **Methods**

358 **Plasmid construction**

359 The CDS from *ADR1* and *RPM1* were cloned into pENTR/D-TOPO (Thermo Fisher Scientific; Waltham,
360 USA), while the CDS from *ADR1-L1*, *ADR1-L2*, *ADR1 CC_R* (1-146aa), *ADR1-L1 CC_R* (1-155aa) and
361 *ADR1-L2 CC_R* (1-153aa) were cloned into pDONR221 (Invitrogen; Carlsbad, USA) generating pEntry
362 clones by gateway cloning (Life Technologies; Carlsbad, USA). The corresponding point mutations for
363 the QHV mutants *ADR1^{D461V}*, *ADR1-L1^{D489V}* and *ADR1-L2^{D484V}* were introduced by site-directed
364 mutagenesis PCR using primers listed in table S2. The PCR products were digested with *DpnI* (NEB;
365 Ipswich, USA) overnight and subsequently transformed into *Escherichia coli* DH5 α . The CDS of RPS5
366 was cloned into pDONR207 (Invitrogen; Carlsbad, USA). The *NRG1.1 CC_R* (1-180aa) CDS was
367 synthesized with 3' and 5' gateway attachment sites into the pUC57Kan vector (Genescript, Piscataway
368 NJ, USA). LR reactions (Gateway Cloning Technology, Life Technologies; Carlsbad, USA) were
369 performed to introduce specific CDS into a modified Estradiol-inducible destination vector pMDC7-
370 Citrine-HA⁶⁴, the Estradiol-inducible destination vector pABindmCherry⁶⁵ or the 35s-driven destination
371 vector pGWB641⁶⁶ as indicated. The 36 first amino acid of AtGPA1 (i.e. MAP sequence) were added
372 to mCHERRYnoSTOP in pDONR207⁶⁷ to generate MAP-mCHERRYnoSTOP in pDONR207.
373 2x35Sprom/pDONRP4-P1R⁶⁸, MAP-mCHERRYnoSTOP in pDONR207 and SAC1in pDONR-P2R-P3
374 (or SAC1dead in pDONR-P2RP3)³⁰ were recombined using LR reaction into pH7m34GW⁶⁹ to generate
375 2x35Sprom::MAP-mCHERRY-SAC1 in pH7m34GW (or 2x35Sprom::MAP-mCHERRY-SAC1dead in
376 pH7m34GW). 2x35Sprom in pDONRP4-P1R⁶⁸, MAP-mCHERRYnoSTOP in pDONR207 and dOCRL
377 in pDONR-P2R-P3 (or dOCRLdead in pDONR-P2RP3)⁴⁷ were recombined using LR reaction into
378 pH7m34GW⁶⁹ to generate 2x35Sprom::MAP-mCHERRY-dOCRL in pH7m34GW (or 2x35Sprom::MAP-
379 mCHERRY-dOCRLdead in pH7m34GW). Constructs were verified by sequencing and transformed into
380 *Agrobacterium tumefaciens* strain GV3101 and used for transient expression in *Nicotiana benthamiana*.

381

382 **Transient expression in *N. benthamiana***

383 *Agrobacterium tumefaciens* strains were grown overnight at 28°C in LB media containing the
384 appropriate antibiotics. The overnight cultures were centrifuged for 8 min at 8,500 rpm and the pellets
385 were resuspended in induction buffer (10 mM MgCl₂, 10 mM MES pH 5.6, 150 μ M acetosyringone).
386 The OD₆₀₀ was adjusted to 0.05 (35S::P19) and 0.3 (35s::RPS5-EYFP, Dex::PBS1-3x-HA,

387 Dex::AvrPphB-5x-myc, 35S::RPM1-EYFP, pRIN4::T7-RIN4^{T166D}, Dex::AvrRpm1-HA, 35s::MAP-mCh-
388 SAC1^{WT/dead}, 35s::MAP-mCh-dOCRL^{WT/dead}, 35s::ADR1/L1/L2-EYFP, Est::ADR1/L1/L2-Cit-HA,
389 Est::ADR1^{DV}/L1^{DV}/L2^{DV}-Cit-HA, Est::ADR1/L1/L2-mCherry, Est::ADR1^{DV}/L1^{DV}/L2^{DV}-mCherry,
390 35s::ADR1/L1/L2 CC, Est::ADR1/L1/L2 CC-Cit-HA) and samples were mixed as indicated.
391 Agrobacteria mixtures were infiltrated into young leaves of 4-6 week old *N. benthamiana* WT plants
392 using a 1-ml needleless syringe. The *N. benthamiana* plants were grown on soil under 12h light / 12h
393 dark cycles (24°C/22°C, 70% humidity). Induction of protein expression was done 24 hours post
394 infiltration using either 30 µM Dexamethasone (Sigma-Aldrich; St. Louis, USA) and 0.001% [v/v] Silwet
395 L-77 or 20 µM Estradiol (Sigma-Aldrich; St. Louis, USA) and 0.001% [v/v] Silwet L-77 by spraying.
396 Leaves were imaged for cell death or for protein localization at indicated timepoints.

397

398 **Chemical treatments**

399 For PIC and BTZ treatments, *N. benthamiana* leaves were infiltrated with the indicated constructs using
400 a 1 ml needleless syringe. At 23 hours post infiltration (hpi), leaves were infiltrated with induction buffer
401 (10 mM MgCl₂, 10 mM MES pH 5.6, 150 µM acetosyringone) only as Mock control or with induction
402 buffer containing 2.5 µM BTZ (Santa Cruz Biotechnology; Dallas, USA) or 1x HaltTM Protease Inhibitor
403 Cocktail (Thermo Fisher Scientific; Waltham, USA). For ADR1, 20 µM Estradiol and 0.001% Silwet was
404 infiltrated together with the Mock solution or the inhibitors to induce ADR1 expression. Leaf material
405 was harvested 4 hours (ADR1) or 5 hours (ADR1-L1, ADR1-L2, RPM1) post inhibitor/mock treatment.

406

407 **HR/Cell Death Assay**

408 Indicated constructs were transiently expressed in *N. benthamiana* leaves and leaves were imaged for
409 cell death at the indicated time points. Cell death images were taken under UV light using the Amersham
410 ImageQuant 800 western blot imaging system and an integrated Cy5 filter (GE Healthcare; Chalfont St.
411 Giles, UK). Images were processed with Adobe Photoshop CS2 for adjustment of brightness and
412 contrast. Note, since 35s::MAP-mCh-SAC1^{WT} often induces tissue collapse at around 52 hours post
413 infiltration, cell death imaging has to be done at earlier timepoints.

414

415 **Confocal imaging**

416 Protein localization was analysed at the indicated time points with the confocal laser scanning
417 microscope LSM880 from Zeiss (Oberkochen, Germany), using a 40x or 63x water-immersion objective
418 and the ZENblack software. EYFP and Citrine were excited using a 514 nm laser collecting emission
419 between 516-556 nm; RFP and mCherry were excited using a 561 nm laser with an emission spectrum
420 of 597-634 nm, Chlorophyll A was excited with a 561 nm laser and the emission spectrum was 661-682
421 nm. Focal plane images were processed with the ZENblue software (Zeiss) for adjustment of brightness
422 and contrast. Maximum Z-projection images were processed with ImageJ.

423

424 **Western blot analysis of transiently expressed proteins**

425 For protein extraction, 4 leaf discs (5mm diameter) were collected and frozen in liquid nitrogen,
426 homogenized using a tissue homogenizer (Retsch GmbH) and resuspended in 190 µl grinding buffer

427 (20 mM Tris-HCl pH 7, 150 mM NaCl, 1 mM EDTA pH 8, 1% [v/v] Triton X-100, 0.1% [w/v] SDS, 5 mM
428 DTT, 1x Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific; Waltham, USA)). Samples were
429 incubated on ice for 5-10 min and then centrifuged for 15 min at 13,000 rpm and 4°C. 30 µl 5x SDS
430 loading buffer (250 mM Tris-HCl pH 6.8, 50% [v/v] glycerol, 500 mM DTT, 10% [w/v] SDS, 0.005% [w/v]
431 bromphenol blue) was added to 120 µl of supernatant. Proteins were denatured by incubation at 95°C
432 for 5 min. Protein samples were resolved by electrophoresis on 8-10% SDS/PAGE gels, transferred to
433 nitrocellulose membranes (GE Healthcare; Chalfont St Giles, UK) using semi-dry transfer (Bio-Rad
434 Laboratories; Hercules, USA). Membranes were blocked in 5% [w/v] milk powder solved in 1x TBS with
435 1% [v/v] Tween-20 (TBS-T). Primary antibody incubations were done overnight at 4°C or for 1.5 hours
436 at RT in 5% [w/v] milk powder diluted in TBS-T. Primary and secondary antibody dilutions were as
437 follows: α-GFP 1:1500 (Roche Diagnostics; Basel, Switzerland), α-RFP 1:1000 (ChromoTek; Planegg-
438 Martinsried, Germany), α-Myc 1:1000 (ChromoTek; Planegg-Martinsried, Germany), α-T7 Tag HRP
439 conjugate 1:10.000 (Merck, Darmstadt, Germany), α-mouse HRP-conjugated 1:10.000 (Sigma-Aldrich;
440 St. Louis, USA), α-rat HRP-conjugated 1:10.000 (Thermo Fisher Scientific; Waltham, USA).
441 Chemiluminescence was detected using an Amersham Imager 600 or ImageQuant 800 (GE
442 Healthcare; Chalfont St Giles, UK). Images were processed with Adobe Photoshop CS2 for adjustment
443 of brightness and contrast.

444

445 **Co-immunoprecipitation**

446 Frozen *N. benthamiana* leaf tissue (~200 mg) was collected and ground with a pre-cooled mortar and
447 pestle with liquid nitrogen and then resuspended in 2.5 mL of extraction buffer (50mM HEPES pH 7.5,
448 50 mM NaCl, 10 mM EDTA pH 8.0, 0.5% [v/v] Triton X-100, 5 mM DTT, 1x Halt™ Protease Inhibitor
449 Cocktail (Thermo Fisher Scientific; Waltham, USA)). Samples were kept for 10-30 min on ice and then
450 cleared by centrifugation at 14,000 rpm for 5 min and 14,000 rpm for 15 min at 4°C. Protein extracts
451 were incubated for 1 h with 25 µl GFP trap (ChromoTek; Planegg-Martinsried, Germany) on a rotating
452 wheel at 4°C. Samples were captured by centrifugation at 2,400g at 4°C and washed two times with 1
453 ml of wash buffer (50 mM HEPES buffer pH 7.5, 150 mM NaCl, 10 mM EDTA pH 8.0, 0.2% [v/v] Triton
454 X-100, 5 mM DTT, 1x Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific; Waltham, USA)) by
455 incubating the extracts for 5 min on a rotating wheel at 4°C and two additional times by inverting the
456 tube six times. Bound proteins were eluted in 120 µl 2x SDS loading buffer (100 mM Tris-HCl pH 6.8,
457 20% [v/v] glycerol, 200 mM DTT, 4% [w/v] SDS, 0.002% [w/v] bromphenol blue) and denatured by
458 boiling the proteins at 95°C for 5 min.

459

460 ***In vitro* transcription and translation and PIP strip assay**

461 ADR1 CC_R (1-146aa), ADR1-L1 CC_R (1-155aa), ADR1-L2 CC_R-HA (1-153aa) and RPM1 CC-HA (1-
462 156aa) were expressed *in vitro* using the TnT® SP6 High-Yield Wheat Germ Protein Expression System
463 (Promega; Madison, USA) according to the manufacturer's instructions. A PCR-generated DNA
464 fragment was used as template for the transcription and translation reaction. Primers are listed in Table
465 S2. Protein synthesis was confirmed on western blot using an HA-specific antibody. PIP strips (Echelon
466 Biosciences; Salt Lake City, USA) were blocked overnight at 4°C in blocking buffer (PBS-T (0.1% [v/v]

467 Tween-20), 4% [w/v] fatty acid-free BSA). 18 µl of the TnT reaction was added to 3 mL fresh blocking
468 buffer and PIP strips were incubated for 1 h at RT with the protein. PIP strips were washed three times
469 for 10 min with PBS-T. Binding of the proteins to the lipids was analysed by immunodetection using an
470 HA-specific antibody. Primary antibody (α-HA 1:2000, Roche; Basel, Switzerland) incubation was done
471 for 1 hour and 20 min at RT in blocking buffer, secondary antibody (α-rat HRP-conjugated 1:10.000;
472 Thermo Fisher Scientific; Waltham, USA) incubation was done for 1 hour at RT in blocking buffer.
473 Chemiluminescence was detected using the Amersham ImageQuant 800 (GE Healthcare; Chalfont St
474 Giles, UK).

475

476 **Transmembrane and lipidation predictions**

477 Full length protein sequences of Arabidopsis RPM1, RPS5, ADR1, ADR1-L1, ADR1-L2, NRG1.1 and
478 NRG1.2 were used for prediction of potential transmembrane domains (TMDs) with three online tools:
479 TMHMM2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>)⁷⁰, CCTOP (<http://cctop.enzim.ttk.mta.hu/?>)
480 and PredictProtein (<https://predictprotein.org/>)⁷¹, and for lipidation with the online tools: NBA-Palm
481 (<http://nbapalm.biocuckoo.org/>)⁷², GPS-Palm (<http://gpspalm.biocuckoo.cn/>)⁷³ and ExpASY
482 Myristoylator (<https://web.expasy.org/myristoylator/>).

483

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680

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695

696 **Author contributions**

697 S.C.S. created RNL entry and destination constructs, performed confocal and cell death analysis for all
698 RNLs, the in vitro transcription and translation assay, the PIP strip analysis and all western blot analysis
699 for the RNL experiments and the BTZ treatments; F.M.A. performed confocal and cell death analysis
700 for all RPM1 experiments; S.S. created RPS5 entry and destination constructs, performed cell death
701 and western blot analysis for RPM1 and RPS5, and confocal analysis for RPS5.; A.B. did some cell
702 death analysis for RNLs.; E.S., V.B. and L.W. assisted in creating RNL and CNL entry and destination
703 constructs; Y.J. and M.C.C. provided unpublished SAC1 and dOCRL constructs; M.D. generated and

704 characterized SAC1 and dOCRL constructs; S.C.S., F.M.A., M.T.N. and F.E.K conceived the study and
705 designed the experiments; F.E.K. wrote the manuscript with help of S.C.S. and F.M.A.; S.S., A.B., Y.J.,
706 M.C.C., J.L.D. and M.T.N. reviewed and edited the manuscript.

707

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710

711 **Additional information**

712 Supplementary Table S1. Transmembrane domain and lipidation prediction summary for *Arabidopsis*
713 *thaliana* RNLs and the CNL RPM1 and RPS5.

714 Supplementary Table S2. Primer list

715

716 **Competing interests**

717 The authors declare no competing interests.

718

719 **Figure legends**

720 **Fig.1 AtADR1 proteins mainly localize to the PM and do self-associate.**

721 **a-f**, Single plane secant views showing AtADR1 proteins (ADR1, ADR1-L1, ADR1-L2) localize at the
722 plasma membrane (PM). The indicated ADR1 proteins fused to Citrine-HA or EYFP were transiently
723 co-expressed with the PM-resident protein BRI1-mRFP in *N. benthamiana* leaves and confocal imaging
724 was done at 4 (a, b, d) or 5 hours (f) post Estradiol induction or 2 days post infiltration (c, e). Localization
725 of ADR1s is shown with the first column (Citrine/YFP, in yellow) and the co-localized PM-localized BRI1
726 is shown in the second column (RFP, in magenta). Chloroplasts are shown in the third column
727 (Chlorophyll A, in cyan) and the merged images are shown in the fourth column (merge). Fluorescence
728 intensities were measured along the dotted line depicted in the merge images. Scale bars, 20 μ m. **g-i**,
729 ADR1s cell death function coincides with strong protein self-association. **g**, Transient expression of
730 steady-state (WT) or mutant auto-activated (DV) ADR1s-Citrine-HA fusion proteins in *N. benthamiana*.
731 Photos were taken under UV light at 23 hours post Estradiol induction and 47 hours post infiltration.
732 White areas correspond to dead leaf tissue. Numbers represent the number of leaves showing cell
733 death out of the number of leaves analysed. Asterisk indicates weak cell death. **h**, Immunoblot analysis
734 of the proteins infiltrated in (g) using anti-GFP antibody. Equal loading of the proteins is indicated by
735 the Rubisco band from the Ponceau staining (PS). Proteins were extracted 4 hours post Estradiol
736 induction. **i**, Auto-activated DV mutant ADR1 proteins self-associate. The indicated proteins were
737 transiently co-expressed in *N. benthamiana* and samples were harvested 4 hours post Estradiol
738 induction. Total proteins were immunoprecipitated with anti-GFP beads and immunoblotted with anti-
739 GFP and anti-RFP antibody. Immunoblots using total proteins prior to immunoprecipitation are shown
740 as input (upper panel) and immunoprecipitated proteins are shown in lower panel. Co-
741 immunoprecipitation was repeated three times with similar results. ADR1^{DV}: ADR1^{D461V}, ADR1-L1^{DV}:
742 ADR1-L1^{D489V}, ADR1-L2^{DV}: ADR1-L2^{D484V}.

743

744 **Fig. 2 PI4P depletion reduces PM localization and stability of AtADR1s and AtRPM1.**

745 Effects on the localization and stability of AtADR1s (ADR1, ADR1-L1, ADR1-L2) and AtRPM1 after
746 transient co-expression with SAC1^{dead} (upper panel) or SAC1^{WT} (lower panel). **a**, MAP-mCherry-
747 SAC1^{WT} co-expression affects ADR1-Citrine-HA PM localization but not its endoplasmic reticulum
748 localization. **c,e,g**, ADR1-L1-, ADR1-L2 and RPM1-EYFP fluorescence is not (c and g) or only weakly
749 (e) detectable when co-expressed with MAP-mCherry-SAC1^{WT}. Fusion proteins were transiently
750 expressed in *N. benthamiana* leaves and confocal imaging was done at 4 hours after Estradiol induction
751 (a), 2 days post infiltration (c, e) or 24 hours post infiltration (g). Localization of ADR1-Citrine-HA and
752 ADR1-L1-, ADR1-L2 and RPM1-EYFP proteins is shown in the first column (Citrine/YFP, in yellow) and
753 MAP-mCherry-SAC1^{WT} or MAP-mCherry-SAC1^{dead} is shown in the second column (mCherry, in
754 magenta). Chloroplasts are shown in the third column (Chlorophyll A, in cyan) and the merged images
755 are shown in the fourth column. Images are single plane secant views. Scale bars, 20 μ m. **b**,
756 Immunoblot analysis indicates a slightly reduced accumulation of ADR1-Citrine-HA after co-expression
757 with MAP-mCherry-SAC1^{WT} compared to co-expression with MAP-mCherry-SAC1^{dead}. **d,f,h**, Co-
758 expression of ADR1-L1 (d), ADR1-L2 (f) and RPM1 (h) -EYFP with MAP-mCherry-SAC1^{WT} severely
759 affects their stability. Immunoblot analysis of proteins infiltrated in (a, c, e, g) using anti-GFP and anti-
760 RFP antibody are shown. Equal loading of the proteins is indicated by the Rubisco band from the
761 Ponceau staining (PS). Protein samples were collected at 4 hours after Estradiol induction (b), 2 days
762 post infiltration (d, f) or 24 hours post infiltration (h).

763

764 **Fig. 3 MAP-mCherry-SAC1 strongly affects AtADR1s and AtRPM1 cell death activity.**

765 Cell death activity of autoactive AtADR1s CC_R domains, full-length AtADR1 and the AtADR1^{DV} mutant
766 as well as the phospho-mimic T7-RIN4^{T166D} activated RPM1 is suppressed by SAC1^{WT} co-expression.
767 **a-f** (upper panels), Transient expression of ADR1 CC_R (a), ADR1-L2 CC_R (b), NRG1.1 CC_R (c), ADR1
768 (d), ADR1^{D461V} (e) and phospho-mimic T7-RIN4^{T166D} (RIN4^{TD})-activated RPM1 Citrine-HA- or EYFP-
769 fusion proteins in *N. benthamiana* co-expressed with MAP-mCherry-SAC1^{WT} or MAP-mCherry-
770 SAC1^{dead}. Images of leaves were taken under UV light at 23 hours post infiltration (hpi) (a), 26 hpi (b),
771 28 hpi (c), 8 hp Estradiol induction (d), 30 hpi (e) and 24 hpi (f). Phospho-mimic T7-RIN4^{TD} was co-
772 expressed to activate RPM1. White areas on the leaves indicate dead tissue. Numbers represent the
773 number of leaves showing cell death out of the number of leaves analysed. Asterisk in (a and c)
774 indicates weak cell death. **a-f** (lower panels), Immunoblot analysis of the proteins infiltrated in the upper
775 panels using anti-GFP and anti-RFP (a-f) antibody. Membranes were horizontally cut into two pieces
776 and probed with anti-GFP or anti-RFP antibody (a-c). Equal loading of the proteins is indicated by the
777 Rubisco band from the Ponceau staining (PS). Protein samples were collected at 20 hpi (a-c), 24 hpi
778 (d), 4 hp Estradiol induction (e) or 22 hpi (f).

779

780 **Fig. 4 PI4P depletion affects PM localization of AtADR1 CC_R domains**

781 Co-expression of SAC1^{WT} noticeably affects ADR1 CC_R (a), ADR1-L1 CC_R (b) and ADR1-L2 CC_R (c)
782 localization. **a-c**, Citrine-HA tagged AtADR1 (ADR1, ADR1-L1, ADR1-L2) CC_R domains, MAP-mCherry-
783 SAC1^{dead} (upper panels) or MAP-mCherry-SAC1^{WT} (lower panels) were transiently co-expressed in *N.*

784 *benthamiana* leaves and confocal imaging was done at 3 hours (a) or 4 hours (b,c) post Estradiol
785 induction. MAP-mCherry-SAC1^{WT} induces ADR1 CCR, ADR1-L1 CCR and ADR1-L2 CCR re-localization
786 to intracellular puncta, most likely endosomes (white arrowheads in lower panels of a-c). Localization
787 of ADR1 CCR-Cit-HA domains is shown with the first column (Citrine, in yellow) and MAP-mCherry-
788 SAC1^{dead} or MAP-mCherry-SAC1^{WT} is shown in the second column (mCherry, in magenta).
789 Chloroplasts are shown in the third column (Chlorophyll A, in cyan) and the merged images are shown
790 in the fourth column (merge). Images are single plane secant views. Scale bars, 20 μ m.

791

792 **Fig. 5 AtADR1s CCR and AtRPM1 CC interact *in vitro* with anionic lipids**

793 Arabidopsis ADR1s CCR and RPM1 CC domains can directly bind to anionic lipids *in vitro*. **a-d**, *In vitro*
794 transcribed and translated AtADR1 (a), AtADR1-L1 (b) and AtADR1-L2 CCR (c) and AtRPM1 CC (d)
795 domains fused with a C-terminal single HA tag were incubated with a commercial PIP strip. Binding
796 was analysed by immunoblotting with anti-HA antibody. The analysed CC domains bind strongly to
797 PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3. A weak interaction was also
798 detected with PA and PS.

799

800 **Fig. 6 Proposed model of RNL and CNL localization and cell death/resistance function at the** 801 **plasma membrane.**

802 Localization of RNLs and non-acylated CNLs, for example AtRPM1, to the plasma membrane (PM) is
803 mediated by a direct interaction of their CCR or CC domains with anionic lipids, of which PI4P is the
804 most abundant at the plant plasma membrane. **a**, Expression of catalytical inactive and forced PM-
805 localized MAP-SAC1^{DEAD} does not affect RNL (ADR1s), myristoylated (RPS5) or non-acylated CNL
806 (RPM1) PM localization, and consequently also not their cell death activity upon (auto-)activation. **b**,
807 MAP-SAC1^{WT}-mediated PI4P depletion from the PM severely affects RNL and non-acylated CNL, but
808 not myristoylated CNL, localization. The decreased PI4P levels strongly affect PM electronegativity and
809 this leads to a loss of binding to the PM and rapid degradation of RNLs and non-acylated CNLs. The
810 reduced accumulation of RNLs and CNLs in the cell consequently leads to loss of RNL- and CNL-
811 mediated cell death induction. **c**, The localization of RNL CCR domains is not affected by MAP-
812 SAC1^{DEAD} expression, similar to full-length RNLs. Thus, there is no observable effect on CCR domain
813 autoactivity (cell death induction). **d**, PI4P depletion by MAP-SAC1^{WT} expression causes a re-
814 localization of the CCR domains to intracellular puncta, probably endosomal compartments as their
815 membranes might contain the highest electronegativity when MAP-SAC1^{WT} is expressed. This mis- or
816 re-localization of CCR domains does not lead to their degradation. However, CCR cell death activity is
817 severely reduced.

818

819 **Supplementary Figure legends**

820

821 **S1 Fig. AtADR1 proteins mainly localize to the PM.**

822 **a-h**, Maximum projection of Z-stack images clearly demonstrate AtADR1 proteins localization to the
823 plasma membrane in transient expressions in *N. benthamiana* leaves. **a,b,e-h**, AtADR1 proteins

824 (ADR1, ADR1-L1, ADR1-L2) localize mainly to the plasma membrane. The indicated ADR1 proteins
825 fused to EYFP or Citrine-HA were transiently co-expressed with PM-resident BRI1-mRFP fusion-protein
826 and confocal imaging was done at 4 hours (a, b, f) or 5 hours (h) post Estradiol induction or 2 days post
827 infiltration (e, g). **c,d.** ADR1 also localizes to the endoplasmic reticulum (ER). Wildtype ADR1 or ADR1^{DV}
828 fused to Citrine-HA was transiently co-expressed with the ER-localized VMA12-RFP fusion-protein and
829 confocal imaging was done at 3 hours (c) and 4 hours (d) post Estradiol induction. Localization of EYFP
830 and Citrine-HA tagged ADR1 proteins is shown with the first column (Citrine/YFP, in yellow) and the co-
831 localized PM-localized BRI1-mRFP or ER-localized VMA12-RFP is shown in the second column (RFP,
832 in magenta). Chloroplasts are shown in the third column (Chlorophyll A, in cyan) and the merged images
833 are shown in the fourth column. Images shown here are a maximum projection of Z-stack images. Scale
834 bars, 20 μ m.

835

836 **S2 Fig. RPS5 localization and cell death activity at the plasma membrane is not affected by MAP-**
837 **SAC1 or MAP-dOCRL co-expression.**

838 **a,** Plasma membrane localization of RPS5-EYFP is not affected by co-expression of MAP-mCherry-
839 SAC1^{dead} (upper panel) or MAP-mCherry-SAC1^{WT} (lower panel). **c,** Co-expression of MAP-mCherry-
840 dOCRL^{dead} (upper panel) or MAP-mCherry-dOCRL^{WT} (lower panel) does not affect RPS5-EYFP PM
841 localization. Indicated proteins were transiently expressed in *N. benthamiana* leaves and confocal
842 imaging was done at 24 hours post infiltration. Localization of RPS5-EYFP proteins is shown with the
843 first column (YFP, in yellow) and MAP-mCherry-SAC1^{WT}, MAP-mCherry-SAC1^{dead}, MAP-mCherry-
844 dOCRL^{dead} and MAP-mCherry-dOCRL^{WT} are shown in the second column (mCherry, in magenta).
845 Chloroplasts are shown in the third column (Chlorophyll A, in cyan) and the merged images are shown
846 in the fourth column (merge). Images are single plane secant views. Scale bars, 20 μ m. **b,d,**
847 Immunoblot analysis of the proteins infiltrated in (a) and (c) using anti-GFP and anti-RFP antibody.
848 Equal loading of the proteins is indicated by the Rubisco band from the Ponceau staining (PS). Samples
849 were collected 24 hours post infiltration. **e,f,** Effector (AvrPphB)-triggered and RPS5-EYFP mediated
850 cell death is not suppressed by MAP-mCherry-SAC1^{WT} (e) or MAP-mCherry-dOCRL^{WT} (f) co-
851 expression in *N. benthamiana*. (e) and (f) upper panels, leaf images showing cell death induction of
852 activated RPS5-EYFP. Transient expression of Dexamethasone-inducible AvrPphB-MYC and PBS1-
853 HA with constitutively expressed RPS5-EYFP, MAP-mCherry-SAC1^{WT} or MAP-mCherry-SAC1^{dead} (e)
854 or MAP-mCherry-dOCRL^{dead} and MAP-mCherry-dOCRL^{WT} (f). Leaf images were taken under UV light
855 at 24 hours post Dexamethasone induction, which corresponds to 2 days post infiltration (e,f). AvrPphB-
856 MYC and PBS1-HA expression was induced with 30 μ M Dexamethasone to activate RPS5-EYFP.
857 White areas indicate dead leaf tissue. Numbers represent the number of leaves showing cell death out
858 of the number of leaves analysed. (e) and (f) lower panels, Immunoblot analysis of the proteins infiltrated
859 in the upper panels using anti-GFP and anti-RFP antibody. Equal loading of the proteins is indicated by
860 the Rubisco band from the Ponceau staining (PS). Protein samples were collected at 6 hours post
861 Dexamethasone induction, which corresponds to 28 hours post infiltration.

862

863 **S3 Fig. Degradation of mis-localized AtRPM1 and AtADR1 proteins is not or only partially**
864 **blocked by proteasome inhibitors.**

865 **a-c**, Bortezomib (BTZ) treatment partially inhibits degradation of mis-localized ADR1 proteins. **d**, Mis-
866 localized RPM1 protein degradation can neither be blocked by a protease inhibitor cocktail (PIC) nor
867 BTZ. Shown are immunoblot analysis of ADR1 (a), ADR1-L1 (b), ADR1-L2 (c) and RPM1 (d) Citrine-
868 HA or EYFP fusion proteins that were transiently co-expressed with MAP-mCherry-SAC1^{WT} or MAP-
869 mCherry-SAC1^{dead} in *N. benthamiana* using anti-GFP antibody. Equal loading of the proteins is
870 indicated by the Rubisco band from the Ponceau staining (PS). Samples were collected 4 hours (a) or
871 5 hours (b-d) post inhibitor and Estradiol (a) treatments, which corresponds to 27 (a) and 25 hours (b-
872 d) post infiltration.

873

874 **S4 Fig. Effector-triggered AtRPM1-mediated cell death is reduced by PI4P depletion**

875 **a** upper panel, CC_R domain of AtADR1-L1 induces no visible cell death symptoms and thus no effect of
876 MAP-mCherry-SAC1^{WT} co-expression on AtADR1-L1 CC_R activity is observable. Transient co-
877 expression of ADR1-L1 CC_R-EYFP, MAP-mCherry-SAC1^{WT} or MAP-mCherry-SAC1^{DEAD} in *N.*
878 *benthamiana* leaves. **b** upper panel, mCherry-SAC1^{WT} co-expression noticeably reduced cell death
879 activity of AvrRpm1-HA activated RPM1-EYFP. Transient expression of RPM1-EYFP, AvrRPM1-HA
880 and MAP-mCherry-SAC1^{WT} or MAP-mCherry-SAC1^{DEAD} in *N. benthamiana* leaves. **c** upper panel, Cell
881 death activity of AvrRpm1-HA activated RPM1-EYFP was not blocked by co-expression of MAP-
882 mCherry-dOCRL^{dead} or MAP-mCherry-dOCRL^{WT}. Transient expression of RPM1-EYFP, AvrRPM1-HA
883 and MAP-mCherry-dOCRL^{dead} or MAP-mCherry-dOCRL^{WT} in *N. benthamiana* leaves. AvrRPM1-HA
884 expression was induced with 30 μM Dexamethasone 20 hours post infiltration. Leaf images were taken
885 under UV light at 24 hours post infiltration (a) or 24 hours post Dexamethasone induction (b and c).
886 White areas indicate dead leaf tissue. Numbers represent the number of leaves showing cell death
887 out of the number of leaves analysed. Asterisk in (b) indicates weak cell death. **a-c** lower panels,
888 Immunoblot analysis of the proteins infiltrated in leaves shown in upper panels using anti-GFP and anti-
889 RFP antibody. Equal loading of the proteins is indicated by the Rubisco band from the Ponceau staining
890 (PS). Protein samples were collected at 20 hours post infiltration (a) and 6 hours post Dexamethasone
891 induction, which corresponds to 26 hours post infiltration (b and c).

892

893 **S5 Fig. PI4P depletion affects the PM localization of Arabidopsis ADR1 CC_R domains**

894 **a-c**, Co-expression SAC1^{WT} affects ADR1 CC_R (a), ADR1-L1 CC_R (b) and ADR1-L2 CC_R (c) localization.
895 Citrine-HA tagged AtADR1 (ADR1, ADR1-L1, ADR1-L2) CC_R domains were transiently co-expressed
896 with MAP-mCherry-SAC1^{dead} (a-c upper panel) or MAP-mCherry-SAC1^{WT} (a-c lower panel) in *N.*
897 *benthamiana* leaves. Confocal imaging was done at 3 hours (a) or 4 hours after Estradiol induction
898 (b,c). ADR1 CC_R, ADR1-L1 CC_R and ADR1-L2 CC_R domains re-localization to intracellular puncta,
899 potentially endosomes, is indicated (white arrow heads). Localization of ADR1 CC_R-Citrine-HA domains
900 is shown with the first column (Citrine, in yellow) and MAP-mCherry-SAC1^{WT} or MAP-mCherry-SAC1^{dead}
901 is shown in the second column (mCherry, in magenta). Chloroplasts are shown in the third column

902 (Chlorophyll A, in cyan) and the merged images are shown in the fourth column (merge). Images shown
903 here are a maximum projection of Z-stack images. Scale bars, 20 μm .

904

905 **S6 Fig. PI(4,5)P₂ is not required for the PM localization and stability of AtADR1s and AtRPM1.**

906 **a, c, e, g**, Plasmamembrane localization of AtADR1s (ADR1-Citrine-HA, ADR1-L1-EYFP, ADR1-L2-
907 EYFP) and AtRPM1-EYFP is not altered when dOCRL^{dead} (upper panel) or dOCRL^{WT} (lower panel) is
908 co-expressed. Proteins were transiently expressed in *N. benthamiana* leaves and confocal imaging was
909 done 3 hours post Estradiol induction (a), 2 days post infiltration (c, e) or 24 hours post infiltration (g).
910 Localization of Citrine-HA/EYFP tagged ADR1s and RPM1-EYFP is shown with the first column
911 (Citrine/YFP, in yellow) and MAP-mCherry-dOCRL^{WT} or MAP-mCherry-dOCRL^{dead} is shown in the
912 second column (mCherry, in magenta). Chloroplasts are shown in the third column (Chlorophyll A, in
913 cyan) and the merged images are shown in the fourth column (merge). Images are single plane secant
914 views. Scale bars, 20 μm . **b, d, f, h**, Immunoblot analysis of proteins infiltrated in (a, c, e, g) using anti-
915 GFP and anti-RFP antibody show no effect on NLR stability by dOCRL^{WT} or dOCRL^{dead} co-expression.
916 Equal loading of proteins is indicated by the Rubisco band from the Ponceau staining (PS). Samples
917 were collected at 4 hours post Estradiol induction (b), 2 days post infiltration (d, f) or 24 hours post
918 infiltration (h).

919

920 **S7 Fig. AtADR1s and AtRPM1 cell death activity is not affected by depletion of PI(4,5)P₂ from the
921 plasma membrane via MAP-dOCRL co-expression.**

922 **a-f**, dOCRL^{WT} co-expression does not affect the cell death induced by ADR1 CCR (a), ADR1-L2 CCR
923 (b), NRG1.1 CCR (c) domains, full-length ADR1 (d), mutant ADR1^{DV} (e) or RPM1 (f). **a-f upper panels**,
924 Transient expression of Citrine-HA or EYFP tagged autoactive, ADR1 (d), ADR1^{D461V} mutant (e) and
925 phospho-mimic T7-RIN4^{T166D} activated RPM1 (f) co-expressed with MAP-mCherry-dOCRL^{WT} or MAP-
926 mCherry-dOCRL^{dead} in *N. benthamiana*. Leaf images were taken under UV light at 24 hours post
927 infiltration (hpi) (a), 26 hpi (b), 28 hpi (c), 9 hours post Estradiol induction (d), 30 hpi (e) and 24 hpi (f).
928 Phospho-mimic T7-RIN4^{T166D} (RIN4^{TD}) was co-expressed to activate RPM1. White areas in leaves
929 indicate dead tissue. Numbers represent the number of leaves showing cell death out of the number of
930 leaves analysed. Asterisk in (c) indicates weak HR. **a-f lower panels**, Immunoblot analysis of proteins
931 infiltrated in the upper panels using anti-GFP and anti-RFP antibody. Equal loading of proteins is
932 indicated by the Rubisco band from the Ponceau staining (PS). Samples were collected at 20 hpi (a-c),
933 24 hpi (d), 4 hours post Estradiol induction (e) or 22 hpi (f).

934

935 **S8 Fig. Proposed model of AtRNL localization, oligomerization and function during immunity.**

936 Arabidopsis RNLs constitutively localize at the plasma membrane through the interaction of their CCR
937 domains with anionic lipids, including PI4P. (1) RNL activation, either by pathogen infection or
938 autoactivating mutations, leads to conformational changes inducing oligomerization, (2) the formation
939 of a transient Ca²⁺ channel/pore and the (3) subsequent recruitment or activation of calcium dependent
940 and probably NLR-interacting phospholipases that (4) in turn produce lipid messengers, such as PA
941 and DAG, which (5) might activate downstream signalling components required for NLR-mediated (6)

942 cell death and resistance outputs. The lipase-like protein EDS1 (ENHANCED DISEASE SUSCEPTIBLE
 943 1) and its sequence-related direct partners SAG101 (SENESCENCE ASSOCIATED GENE 101) and
 944 PAD4 (PHYTOALEXIN DEFICIENT 4) are key immune regulators of NLR-mediated immunity, but also
 945 of basal resistance.

946 **Supplementary Table 1.** Transmembrane domain and lipidation prediction summary for *Arabidopsis*
 947 *thaliana* RNLs and the CNL RPM1 and RPS5.

948

NLR (type)	TMD prediction		Lipidation prediction		
	Tool	result	Tool	# of sites	position
AtRPM1 (CNL) At3g07040	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	3	438,567,704
	PredictProtein	no	ExPASy Myristoylator	0	
AtRPS5 (CNL) At1g12220	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	4	4,103,106,463
	PredictProtein	no	ExPASy Myristoylator	1	N-terminus
AtADR1 (RNL) At1g33560	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	1	661
	PredictProtein	no	ExPASy Myristoylator	0	
AtADR1-L1 (RNL) At4g33300	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	4	301,347,638,690
	PredictProtein	no	ExPASy Myristoylator	0	
AtADR1-L2 (RNL) At5g04720	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	2	73,685
	PredictProtein	no	ExPASy Myristoylator	0	
AtNRG1.1 (RNL) At5g66900	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	5	198,592,683,705,731
	PredictProtein	no	ExPASy Myristoylator	0	
AtNRG1.2 (RNL) At5g66910	TMHMM2.0	no	NBA-Palm	0	
	CCTOP	no	GPS-Palm	6	200,598,689,711,737,764
	PredictProtein	no	ExPASy Myristoylator	0	

949

950

951 **Supplementary Table S2.** Primer list.

Primer	sequence	purpose
FEK_101_4	GACGCAACACGtGTtTTTGAGAGACCTAG	ADR1 D461V (site-directed mutagenesis)
FEK_101_5	CTAGGTCTCTCAAACAaCGTgTTGCGTC	ADR1 D461V (site-directed mutagenesis)
FEK_101_2	GTGACACAGCATGtGTtTCTGCGAGAC	ADR1-L1 D489V (site-directed mutagenesis)
FEK_101_3	GTCTCGCAGAACAaCATGCTGTGTAC	ADR1-L1 D489V (site-directed mutagenesis)
FEK_948	GTCACGCAGCATGtGTtTCTAAGAGATG	ADR1-L2 D484V (site-directed mutagenesis)
FEK_949	CATCTCTTAGAACAaCATGCTGCGTGAC	ADR1-L2 D484V (site-directed mutagenesis)
FEK_131_1	cgtagatctatttagtgacactatagaacagaccaccATGGCTTCGGCTACTGTTGATTTT	SP6 RPM1 CC 1-156 (TnT)
FEK_131_2	cgtagatcCTAAGCGTAATCTGGAACGTCATATGGGATACTTTCATCGCCATCA TCAAT	1xHA rev for RPM1 CC 1-156 (TnT)
FEK_132_9	cgtagatctatttagtgacactatagaacagaccaccATGGCTTCGTTTCATAGATC	SP6 ADR1 CC 1-146 (TnT)
FEK_133_0	cgtagatcCTAAGCGTAATCTGGAACGTCATATGGGATAATCATTCCGCTCAGTC AAC	1xHA rev for ADR1 CC 1-146 (TnT)
FEK_133_1	cgtagatctatttagtgacactatagaacagaccaccATGGCCATCACCGATTTTTTCG	SP6 ADR1-L1 CC 1-155 (TnT)
FEK_133_2	cgtagatcCTAAGCGTAATCTGGAACGTCATATGGGATATCCCCCAATTTTCATG GAAC	1xHA rev for ADR1-L1 CC 1-155 (TnT)
FEK_131_3	cgtagatctatttagtgacactatagaacagaccaccATGGCAGATATAATCGGCG	SP6 ADR1-L2 CC 1-153 (TnT)
FEK_131_4	cgtagatcCTAAGCGTAATCTGGAACGTCATATGGGATATCCCTGAGTTTCATA GAAC	1xHA rev for ADR1-L2 CC 1-153 (TnT)

5phos	catcatactccttgctgctgccgctgccgctatggtgagcaagggcgaggagg	5' phosphorylated primers for MAP-mCherrynoSTOP/pDONR207 cloning
5phos_R	TCGACTTCTACTGCAGAGTAAGCCCATGGTAGCCTGCTTTTTGTACAAACT TGGC	5' phosphorylated primers for MAP-mCherrynoSTOP/pDONR207 cloning

952











