

1 *Short title:* PLC2 in plant immunity

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9 Role for PLC2 in MAMP-Triggered Immunity by Modulating ROS Production
10 in Arabidopsis

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29 *One sentence summary:* Arabidopsis Phospholipase C 2 (PLC2) participates
30 in a branch of microbe-associated molecular patterns-triggered immunity that
31 involves reactive oxygen species regulated processes.

32

33 *List of author contribution:* AML conceived the original research plans; AML,
34 MXA, MEA and CZ designed and supervised the experiments and analyzed
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39

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52 *Key words:* *Arabidopsis thaliana*, phospholipase C, *AtPLC2*, reactive oxygen
53 species, NADPH oxidase, RBOHD, flagellin, flg22, *Pseudomonas syringae*
54 pv. *tomato*, *Erysiphe pisi*

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57

58 **ABSTRACT**

59 The activation of phosphoinositide-specific phospholipase C (PI-PLC) is one
60 of the earliest responses triggered by the recognition of several microbe-
61 associated molecular patterns (MAMPs) in plants. The Arabidopsis PI-PLC
62 gene family is composed of nine members. Previous studies suggested a role
63 for PLC2 in MAMP-triggered immunity (MTI) as it is rapidly phosphorylated *in*
64 *vivo* upon treatment with the bacterial MAMP flg22. Here we analyzed the role
65 of PLC2 in plant immunity using an artificial microRNA to silence *PLC2*
66 expression in Arabidopsis. We found that *PLC2*-silenced plants were more
67 susceptible to the type III secretion system-deficient bacterial strain
68 *Pseudomonas syringae* pv. *tomato* (*Pst* DC3000 *hrcC*⁻ (*Pst* DC3000 *hrcC*⁻)
69 and to the non-adapted pea powdery mildew *Erysiphe pisi*. However, *PLC2*-
70 silenced plants display normal susceptibility to virulent (*Pst* DC3000) and
71 avirulent *P. syringae* strains (*Pst* DC3000 AvrRPM1), conserving typical HR
72 features. In response to flg22, the *PLC2*-silenced plants maintain wild type
73 MAPKs activation and *PHI1*, *WRKY33* and *FRK1* gene expression, but
74 reduced reactive oxygen species (ROS)-dependent responses such as
75 callose deposition and stomatal closure. Accordingly, the generation of ROS
76 upon flg22 treatment was compromised in the *PLC2*-deficient plants
77 suggesting an effect of PLC2 in a branch of MTI and non-host resistance that
78 involves early ROS-regulated processes. Consistently, PLC2 associates with
79 RBOHD, evidencing a potential regulation of the Arabidopsis NADPH oxidase
80 by PLC2.

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84 INTRODUCTION

85 Plants are constantly challenged by microbial pathogens and to resist
86 them, they exhibit various defense mechanisms. A first line of inducible
87 defenses is triggered by the recognition of microbe-associated molecular
88 patterns (MAMP) by cell-surface pattern recognition receptors (PRRs)
89 (Antolin-Llovera et al., 2014). This recognition induces MAMP-triggered
90 immunity (MTI), which confers resistance to multiple microbes (Couto and
91 Zipfel, 2016). Adapted plant pathogens use secreted effector proteins to,
92 among other things, interfere with MTI, resulting in the so-called effector-
93 triggered susceptibility (ETS). Eventually, microbial effectors can become
94 detected by intracellular nucleotide-binding leucine-rich repeat (NLR) proteins
95 triggering a second line of defense called effector-triggered immunity (ETI)
96 (Jones and Dangl, 2006).

97 After recognition of MAMPs, a series of rapid responses are initiated,
98 including an increase in cytosolic Ca^{2+} , generation of apoplastic reactive
99 oxygen species (ROS), activation of mitogen-activated protein kinases
100 (MAPKs) and Ca^{2+} -dependent protein kinases (CDPKs), callose deposition
101 and stomatal closure (Boller and Felix, 2009; Segonzac and Zipfel, 2011).
102 Among the best-studied responses to MAMPs are those triggered following
103 recognition of bacterial flagellin by the *Arabidopsis thaliana* (hereafter
104 *Arabidopsis*) leucine-rich repeat receptor kinase (LRR-RK) FLAGELLIN
105 SENSING 2 (FLS2) (Felix et al., 1999; Gomez-Gomez and Boller, 2000; Sun
106 et al., 2013). Upon ligand recognition, FLS2 forms a complex with the LRR-
107 RK BRASSINOSTEROID RECEPTOR 1-ASSOCIATED KINASE 1 (BAK1),
108 also known as SOMATIC EMBRYOGENESIS-RELATED KINASE 3 (SERK3)
109 (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Sun et al.,
110 2013). This complex interacts with and phosphorylates the receptor-like
111 cytoplasmic kinase BOTRYTIS INDUCED KINASE 1 (BIK1) (Veronese et al.,
112 2006; Lu et al., 2010; Zhang et al., 2010). Upon activation, BIK1
113 phosphorylates the plasma membrane NADPH oxidase RBOHD thus priming
114 apoplastic ROS production (Kadota et al., 2014; Li et al., 2014).

115 Several lipids and lipid-derived metabolites have been shown to
116 function in signal transduction pathways leading to the activation of plant
117 defense responses (Laxalt and Munnik, 2002; Munnik and Vermeer, 2010;

118 Hung et al., 2014; Hong et al., 2016). Specifically, phosphoinositide-specific
119 phospholipase C (PI-PLC) is rapidly activated in plant cells after recognition of
120 different MAMPs, such as xylanase, flg22 and chitosan (van der Luit et al.,
121 2000; Laxalt et al., 2007; Raho et al., 2011), or of pathogen effector proteins
122 (de Jong et al., 2004; Andersson et al., 2006). PI-PLC catalyzes the
123 hydrolysis of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol
124 (4,5) bisphosphate PI(4,5)P₂ to generate water-soluble IP₂ and IP₃
125 respectively, and diacylglycerol (DAG), which remains in the membrane. In
126 plants, DAG produced by PI-PLC activity is phosphorylated by DAG kinase
127 (DGK) to produce phosphatidic acid (PA), which regulates several protein
128 targets (Arisz et al., 2009; Testerink and Munnik, 2011; Munnik, 2014). PA
129 has been specifically implicated in the modulation of immune signaling
130 components, such as MAPKs and PHOSPHOINOSITIDE-DEPENDENT
131 PROTEIN KINASE 1 (PDK1) (Farmer and Choi, 1999; Lee et al., 2001;
132 Szczegielniak et al., 2005; Anthony et al., 2006). In particular, PA binds to the
133 NADPH oxidase isoforms RBOHD and RBOHF to induce ROS during ABA-
134 mediated stomatal closure (Zhang et al., 2009). Additionally, it has been
135 shown that PLC activity is required for ROS production during ETI responses
136 (de Jong et al., 2004; Andersson et al., 2006).

137 In animals, IP₃ triggers release of Ca²⁺ from intracellular stores by
138 activating a ligand-gated calcium channel at the endoplasmic reticulum. In
139 plants, no clear homologue of the IP₃-activated Ca²⁺ channel has been
140 identified (Munnik and Testerink, 2009). Instead IP₂ and IP₃ are further
141 phosphorylated by inositolpolyphosphate kinase (Williams et al., 2015) to: i)
142 IP₆ which, stimulates the release of Ca²⁺ from intracellular stores in guard
143 cells (Lemtiri-Chlieh et al., 2000), affects gene transcription, mRNA export and
144 regulates the auxin receptor TIR1 (Zonia and Munnik, 2006; Lee et al., 2015);
145 ii) IP₅ which is part of the jasmonate receptor COI1 (Munnik and Nielsen
146 2011); and iii) IP₇ and IP₈, involved in plant defense (Laha et al., 2015). In
147 addition, PIP and PIP₂, originally characterized as PLC substrates, do have
148 signaling properties themselves, since many proteins involved in membrane
149 trafficking and signal transduction have domains that bind to these lipids
150 (Munnik and Nielsen, 2011; Delage et al., 2013; Heilmann, 2016).

151 The Arabidopsis genome contains nine genes encoding PI-
152 PLCs, (*AtPLC1* to *AtPLC9*) (Mueller-Roeber and Pical, 2002). *AtPLC2*
153 (hereafter PLC2) is the most abundant PLC isoform, which expresses highly
154 and constitutively, and localizes to the plasma membrane (Pokotylo et al.,
155 2014). PLC2 is also rapidly phosphorylated following flg22 recognition (Nuhse
156 et al., 2007). In this work, we analyzed the role of PLC2 in resistance to *P.*
157 *syringae* and *Erysiphe pisi* and in defenses triggered upon flg22 perception.
158 We found that PLC2 plays an important role in MTI and non-host resistance
159 and that it associates with RBOHD, evidencing a putative regulation of the
160 Arabidopsis NADPH oxidase and consequently of ROS-dependent processes
161 by PLC2.
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163

164 **RESULTS**

165 ***PLC2* Silencing by Artificial microRNA**

166 To study the role of *PLC2* in plant defense, we developed *PLC2*-
167 silenced *Arabidopsis* plants by constitutively expressing a specific artificial
168 microRNA (*amiR*). Expression analysis using qPCR of *PLC2* in leaves of T4
169 *amiR PLC2* homozygous plants showed that *PLC2* was stably silenced (Fig.
170 1A). Expression of *PLC7* (closest homologue to *PLC2*), *PLC4* (co-expressed
171 with *PLC2*) and *PLC1* (the second most abundant PLC) (Pokotylo et al., 2014)
172 were not altered in *PLC2*-silenced plants (Supplemental Fig. S1). Western
173 blot analysis using a specific anti-*PLC2* antibody (Otterhag et al., 2001)
174 showed highly reduced levels of *PLC2* protein in *amiR* silenced lines (Fig.
175 1B).

176

177 ***PLC2*-silenced Plants are More Susceptible to *Pseudomonas syringae***
178 **DC3000 *hrcC* Strain and to the Non-adapted Pathogen *Erysiphe pisi***

179 To investigate the role of *PLC2* in plant innate immunity we tested the
180 *PLC2* silenced plants interactions with two different pathogens. First, we
181 selected *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) as a
182 hemibiotrophic pathogen that infects *Arabidopsis* (Xin and He, 2013). The
183 virulence of *Pst* DC3000 on *Arabidopsis* depends on the type III secretion
184 system (TTSS) which allows MTI suppression (Block and Alfano, 2011). Thus,
185 proliferation of the *Pst* DC3000 mutant strain *hrcC* lacking a functional TTSS
186 is restricted in this plant (Hauck et al., 2003). We used *Pst* DC3000 *hrcC* to
187 evaluate MTI in *PLC2*-silenced plants. After spraying adult plants with this
188 bacterium, pathogen proliferation was assessed at day one and three post-
189 inoculation. *PLC2*-silenced plants were more susceptible to *Pst* DC3000 *hrcC*
190 than wild type (Fig. 2A) indicating that *PLC2* is likely involved in MTI.

191 We further studied the growth of the virulent wild type *Pst* DC3000,
192 whose TTSS effectors interfere with MTI (Block and Alfano, 2011). No
193 significant difference in proliferation of this adapted pathogen was detected
194 between both plants (Fig. 2B top) indicating that *PLC2* silencing does not
195 further increase ETS.

196 In order to study if *PLC2* also played a role during ETI, we infiltrated
197 *Arabidopsis* leaves with an avirulent strain of *Pst* DC3000 expressing the type

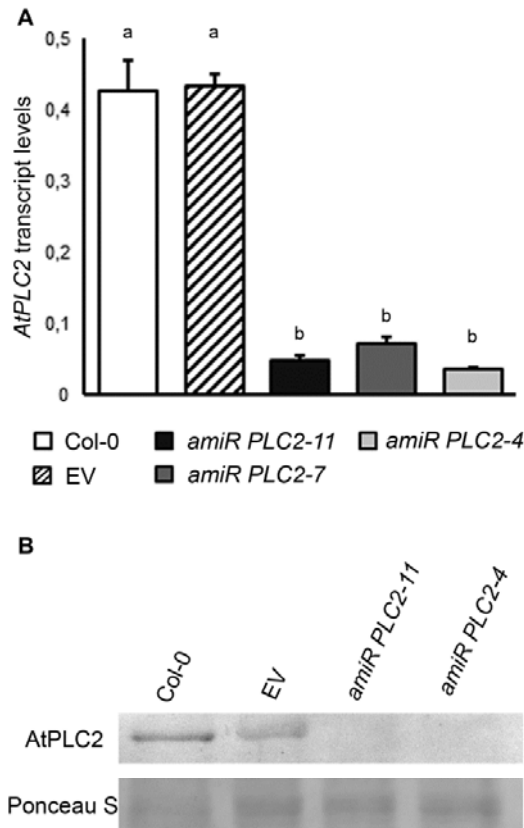


Figure 1. PLC2 silencing by artificial micro RNAs in Arabidopsis.
 A, Total RNA was isolated from leaves of 4-5 weeks old Col-0 or PLC2 silenced plants (T4 homozygous lines amiR PLC2-4, -7 and -11). Relative transcript levels of PLC2 were determined by RT-qPCR. Transcript levels were normalized to ACT2. Error bars represent standard deviations of 3-9 individual plants. Different letters indicate significant difference at $P < 0.001$ (multiple comparison using one-way ANOVA, Tukey's test).
 B, PLC2 protein levels were analyzed by western blot using anti-AtPLC2 antibody in leaves of 4-5 weeks old Col-0; empty vector (EV); amiR PLC2-11 and amiR PLC2-4 independent silenced lines. Ponceau S staining (PS) of Rubisco Subunit L is included as a loading control.

198 III-secreted effector AvrRpm1, which is recognized by the NLR RPM1 (Block
 199 and Alfano, 2011). *PLC2*-silenced plants showed the same ability of wild type
 200 in constraining growth of this strain (Fig. 2B bottom) indicating that the lack of
 201 *PLC2* does not affect AvrRpm1-recognition-triggered growth restriction.

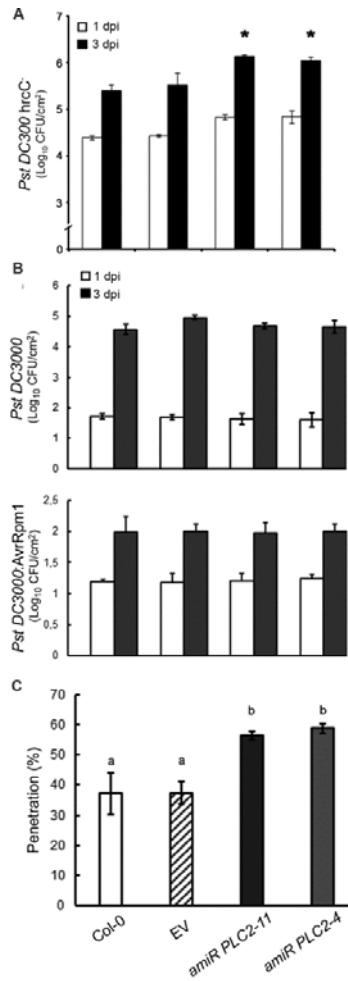


Figure 2. Growth of *Pseudomonas syringae* and *Erysiphe pisi* (*E. pisi*) in Arabidopsis PLC2-silenced plants. Wild type (Col-0), empty vector (EV) and PLC2-silenced lines (amiR PLC2-11 and 4) were used. **A**, PLC2-silenced plants are more susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 hrcC- mutant. Bacteria were inoculated by spray at OD600=0.1 and the number of colony forming units (CFU) per cm² of leaf extracts was determined. A representative experiment of 4 biological replicates is depicted, where asterisks (*) indicate significant differences regarding EV control according to t-test (P<0.01). **B**, PLC2-silenced lines showed no susceptibility differences during virulent and avirulent *Pseudomonas syringae* pv. *tomato* DC3000 infection. *Pseudomonas syringae* pv. *tomato* DC3000 (virulent) and *Pseudomonas syringae* pv. *tomato* DC3000:AvrRpm1 (avirulent) were inoculated by infiltration at OD600=0.0002 and CFU per cm² of leaf was calculated. A representative experiment of 4 biological replicates is depicted. **C**, PLC2-silenced plants are more susceptible to the non-adapted pea powdery mildew *Erysiphe pisi* (*E. pisi*). The penetration rate at 3 days after inoculation was calculated as % of successful penetration of at least 50 germinated spores on three independent leaves. Error bars represents standard error of the mean. Different letters indicate significant difference at P<0.05 (multiple comparison using one-way ANOVA, Tukey's test). A representative experiment of 4 biological independent replicates is depicted.

202 Moreover, the HR type cell death induced by *Pst* DC3000 AvrRpm1 was
 203 identical in wild type and in *PLC2*-silenced plants (Supplemental Fig. S2A).
 204 The effect of *PLC2* silencing on HR was also tested by ion leakage
 205 experiments using Arabidopsis plants expressing AvrRpm1 under the control

206 of a dexamethasone-inducible promoter (*DEX::AvrRpm1*) (Andersson et al.,
207 2006). As a negative control, *AvrRpm1* was expressed in a *RPM1* knockout
208 background (*rpm1-3*) (*DEX::AvrRpm1/rpm1-3*) (Mackey et al., 2002; Mackey
209 et al., 2003). We stably silenced *PLC2* by transforming both backgrounds with
210 *ubi::amiR-PLC2* (Supplemental Fig. S2C). Leaf discs from
211 *DEX::AvrRpm1/Col-0* or *DEX::AvrRpm1/rpm1-3* silenced and non-silenced
212 plants were induced with dexamethasone, and ion leakage was measured at
213 different time points. This experiment demonstrated no significant difference
214 between the *PLC2*-silenced plants and wild type (Supplemental Fig. S2B)
215 confirming that *PLC2* is not required for *AvrRpm1*-induced HR.

216 Finally, we tested the ability of *PLC2*-silenced plants to restrict entry of
217 the non-adapted pathogen *Erysiphe pisi* (*E. pisi*) the causal agent of pea
218 (*Pisum sativum*) powdery mildew. Arabidopsis displays non-host resistance
219 (NHR) towards *E. pisi* (Kuhn et al., 2016), whose spores are restricted from
220 penetrating the epidermal cell wall. This resistance relies on basal defenses
221 and MAMP recognition that function also against powdery mildews adapted to
222 Arabidopsis (Kuhn et al., 2016). We assayed epidermal penetration of the
223 pathogen on wild type and *PLC2*-silenced plants (Fig. 2C). We observed a
224 significantly increased success in penetration of the epidermis by *E. pisi*
225 spores on *PLC2*-silenced plants compared to wild type, indicating that *PLC2*
226 is involved in non-host resistance. Altogether, the above-presented results
227 suggest a role for *PLC2* in MAMP-triggered immunity.

228

229 **PLC2 is Required for MTI ROS-regulated Processes**

230 In order to study the role of *PLC2* in MTI we used the MAMP flg22, a
231 22-amino acid sequence of the conserved N-terminal part of flagellin that is
232 recognized by the FLS2 receptor (Gomez-Gomez and Boller, 2000), and
233 studied two different MTI branches, MAPK- and ROS-dependent plant
234 defense cascades (Bigeard et al., 2015).

235 Flg22-induced activation of a particular MAPKs cascade is an early
236 event that regulates transcriptional reprogramming which finally results in
237 resistance (Bethke et al., 2012). Western blot analysis of Arabidopsis wild
238 type seedlings treated with flg22 using an antibody directed against the
239 conserved phosphorylation motif on the activation loop of MAPKs, recognized

240 three immunoreactive bands (i.e. MPK6, MPK3 and MPK4/11, (Bethke et al.,
241 2012) 15 min after treatment (Supplemental Fig. S3). *PLC2*-silenced lines
242 showed a similar MAPKs activation as wild type plants (Supplemental Fig.
243 S3). Similarly, the flg22-induced expression of *FRK1*, *PHI1* and *WRKY33*,
244 which are MAPK-, and CDPK-dependent MAMP-activated immune marker
245 genes (Boudsocq et al., 2010) showed no significant differences between wild
246 type and *PLC2*-silenced seedlings (Supplemental Fig. S4). These results
247 suggest that *PLC2* is not required for this particular branch of MTI signaling.

248 Since oxidative burst is a MAPK-independent signaling event occurring
249 after flg22 recognition in plant immunity (Zhang et al., 2007; Xu et al., 2014)
250 we further studied the role of *PLC2* in ROS-dependent processes. First of all,
251 we analyzed flg22-induced callose deposition (Luna et al., 2011). To this end,
252 leaves were infiltrated with flg22 and 18 hours later stained with aniline-blue
253 for callose visualization. *PLC2*-silenced lines showed significantly less callose
254 deposition upon flg22 treatment, compared to leaves of control plants, which
255 were either transformed with the empty vector or non-transformed wild type
256 (Fig. 3).

257 An earlier response of active immunity at the pre-invasive level is the
258 closure of the stomata upon MAMP perception, which is also a ROS-
259 dependent defense response (Mersmann et al., 2010; Kadota et al., 2014; Li
260 et al., 2014). In order to evaluate if stomatal closure was affected in *PLC2*-
261 silenced plants, epidermal peels were treated with flg22. As shown in Figure
262 4, flg22-mediated induction of stomatal closure was impaired in epidermal
263 peels of *PLC2*-silenced plants, whereas ABA-induced stomatal closure was
264 unaffected. Together, these results imply that *PLC2* is required for ROS-
265 dependent immune responses.

266

267 ***PLC2* is Required for flg22-induced ROS Burst**

268 Flg22 perception triggers a fast and transient increase of apoplastic
269 ROS (Chinchilla et al., 2007). Using a luminol/peroxidase-based method,
270 apoplastic ROS levels were quantified in flg22-treated leaf discs. A
271 representative experiment is shown in Figure 5a, indicating that in *PLC2*-
272 silenced line 11 (*amiR PLC2-11*) ROS accumulation had similar kinetics but
273 significantly lower levels than in control plants. To estimate such reduction, we

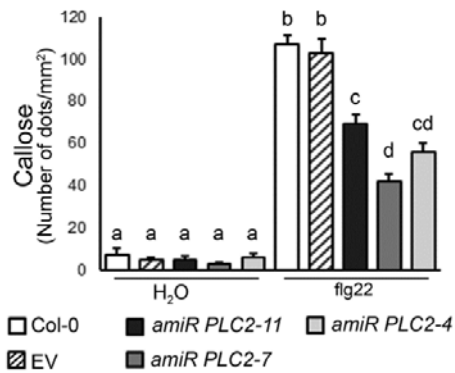


Figure 3. PLC2-silenced plants exhibit impaired flg22-induced callose deposition. Leaves from 4- to 5-week-old Col-0 or amiR PLC2 plants were infiltrated with 1 μ M flg22 or H₂O as a control and incubated for 18 h and callose deposition was measured as dots per area. Error bars represent standard error of the mean. Different letters indicate significant difference at $P < 0.001$ (one-way ANOVA, Tukey's test) $n = 3$.

274 quantified apoplastic ROS in additional independent experiments including
 275 three different silenced lines, as well as a control line carrying an empty vector
 276 (EV). All *PLC2* silenced lines reduced ROS accumulation in response to flg22
 277 (40 to 75% regarding control plants) (Fig. 5B). Therefore, our results
 278 demonstrated that *PLC2* is required for full ROS accumulation following flg22
 279 recognition in Arabidopsis leaf discs.

280

281 **PLC2 Associates with RBOHD**

282 Flg22-induced ROS burst is generated via activation of the plasma
 283 membrane NADPH oxidase RBOHD (Nuhse et al., 2007; Zhang et al., 2007).
 284 Our results show that *PLC2* is required for the flg22-mediated ROS burst that
 285 is generated via RBOHD activation (Fig. 5). As mentioned earlier, *PLC2*
 286 localizes at the plasma membrane, where RBOHD exists in a complex with
 287 FLS2 and BIK1 (Kadota et al., 2014; Li et al., 2014). To investigate whether
 288 *PLC2* associates with RBOHD, we immunoprecipitated N-terminally FLAG-
 289 tagged RBOHD (stably expressed in Arabidopsis under its own promoter) by
 290 using anti-FLAG affinity beads. In three independent biological experiments

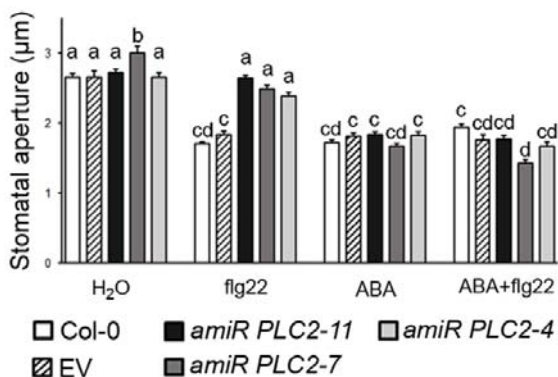


Figure 4. PLC2-silenced plants exhibit impaired flg22-induced stomatal closure. Epidermal peels from Col-0 and PLC2-silenced plants were incubated in opening buffer under light for 3 h. The peels were treated with H₂O, 1 µM flg22, 50 µM ABA or 50 µM ABA + 1 µM flg22 for 1 h. The results show the mean of 90-120 stomata measured from three independent experiments. Error bars represent SE of the means. Different letters denote statistical difference (one-way analysis of variance, Dunn's Method $P < 0.05$).

291 PLC2 co-immunoprecipitated with RBOHD *in planta* (Fig. 6). PLC2 could not
 292 be immunoprecipitated in wild type plants that did not express FLAG-RBOHD.
 293 Notably, the brassinosteroid receptor BRI1 (used here as an unrelated plasma
 294 membrane located protein control) was not detected in anti-FLAG
 295 immunoprecipitates (Fig. 6). In addition, experiments in the presence of flg22
 296 revealed that the association was independent of the ligand binding to FLS2,
 297 since the same amount of PLC2 was immunoprecipitated in treated and non-
 298 treated plants (Fig. 6).

299

300 DISCUSSION

301 The participation of PI-PLCs activity in signaling after recognition of
 302 different MAMPs such as xylanase, flg22 and chitosan (van der Luit et al.,
 303 2000; Laxalt et al., 2007; Raho et al., 2011), or pathogen effector proteins (de
 304 Jong et al., 2004; Andersson et al., 2006) has been previously described
 305 (Laxalt and Munnik, 2002; Munnik, 2014). Here, we show genetic evidence
 306 that PLC2 is particularly involved in MTI signaling. The molecular details of PI-
 307 PLC signaling in plants are still unclear but there is evidence that *i*) PI4P is

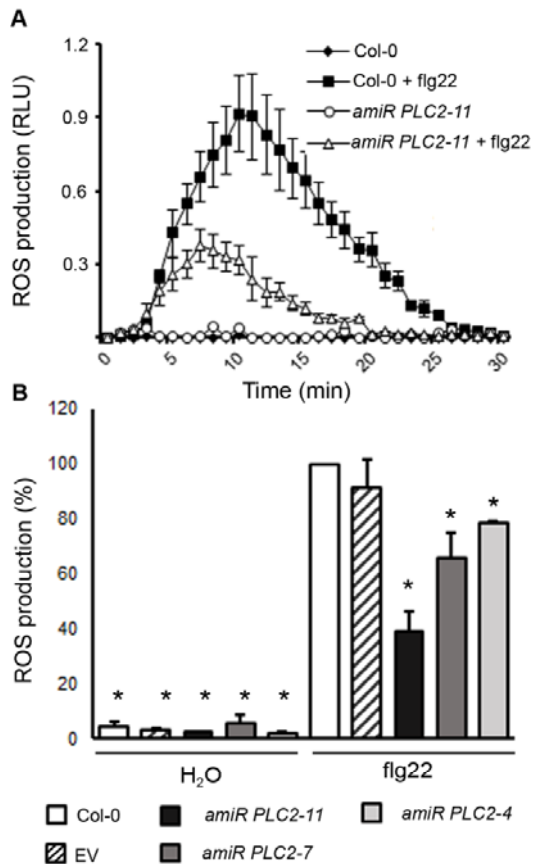


Figure 5. PLC2-silenced plants exhibit impaired flg22-induced oxidative burst.

Production of reactive oxygen species (ROS) was measured with a luminol-based assay in Col-0 or amiR PLC2 plants. A, Leaf disks from 4- to 5-week-old plants were incubated with 100 nM flg22 and the luminescence was measured every 1 min for 30 min and expressed as relative light units (RLU). A representative experiment is shown using wild type (Col-0) and a PLC2-silenced line (amiR PLC2-11) plants. B, Total ROS production was calculated integrating the areas under the curves and referring to Col-0 wild type treated with flg22 as 100%. Average of 4 independent experiments is shown. Error bars represent SE of the means. The asterisk indicates statistically significant differences compared to flg22-treated Col-0 plant (ANOVA, Multiple Comparisons versus Control Group Dunnett's Method $P < 0,001$).

308 most likely the substrate, and *ii*) the phosphorylated products of IP₂, and DAG,
 309 including various inositol polyphosphates (IPPs), PA and diacylglycerol
 310 pyrophosphate have a role as secondary messengers (Munnik, 2014). PA is
 311 involved in the modulation of immune signaling components, such as MAPKs,

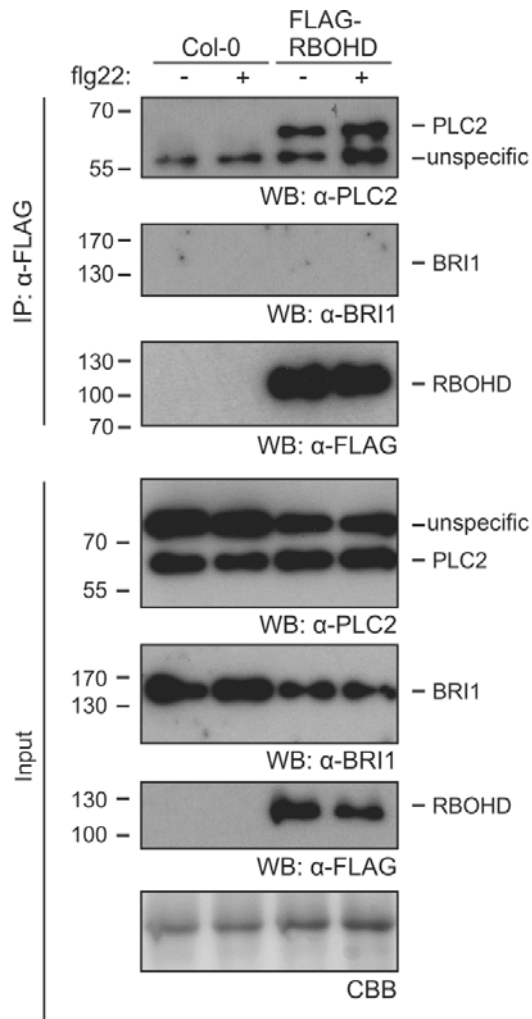


Figure 6. PLC2 associates with RBOHD. Co-immunoprecipitation of PLC2 and RBOHD in stable transgenic Arabidopsis seedlings (T3) expressing FLAG-RBOHD- (pRBOHD:FLAG-RBOHD) treated (+) or not (-) with 1 μ M flg22 for 15 min. Total protein extracts (input) were subjected to immunoprecipitation with anti-FLAG beads followed by immunoblot analysis with anti-PLC2 (α -PLC2) and anti-FLAG (α -FLAG) antibodies as indicated. Protein extracts of Col-0 plants were used as negative controls. Anti-BRI1 (α -BRI1) antibodies were used as plasma membrane protein not associated with RBOHD. Coomassie brilliant blue (CBB). These experiments were performed three times with similar results.

312 PDK1 and RBOHD (Farmer and Choi, 1999; Lee et al., 2001; Szczegieliński et
 313 al., 2005; Anthony et al., 2006; Zhang et al., 2009). However, we cannot
 314 exclude that IP₂ can be very rapidly phosphorylated to IP₆ and thus probably
 315 increase Ca²⁺ in the cytosol, or also participate in auxin signaling via TIR1 and

316 COI1-JA signalling among others (Xue et al., 2009; Munnik, 2014; Williams et
317 al., 2015). Indeed, mutants with altered IPP levels showed altered defense
318 responses (Murphy et al., 2008; Donahue et al., 2010; Mosblech et al., 2011;
319 Hung et al., 2014; Laha et al., 2015). Whether these compounds are
320 generated downstream of PLC2 is likely but still remains to be shown.

321

322 **PLC2 is Required for Plant Immunity**

323 Recently, using tomato and virus induced-gene silencing (VIGS) of different
324 PLCs, SIPLC4 was found to be specifically involved in the HR upon AVR4
325 perception, while SIPLC6 is required for multiple R protein-mediated
326 responses (Vossen et al., 2010), suggesting that in tomato both PLCs
327 participate on ETI responses. Similarly, over-expression of *SIPLC3* enhanced
328 the Cf-4/Avr4-triggered HR (Abd-El-Haliem et al., 2016). Further studies on
329 tomato showed that SIPLC2 is required for xylanase induced-gene
330 expression, ROS production, and plant susceptibility against *Botrytis cinerea*
331 (Gonorazky et al., 2014; Gonorazky et al., 2016).

332 Here, we assayed three different strains of the hemibiotrophic
333 pathogen *Pst* DC3000: the virulent wild type strain to study the role of PLC2 in
334 effector-triggered susceptibility (ETS), the avirulent strain expressing
335 AvrRpm1 to determine if PLC2 played a role during ETI, and the non-virulent
336 *hrcC*⁻ strain mutated in the type III secretion system to investigate if PLC2 was
337 required for MTI. *PLC2*-silenced plants showed increased susceptibility to *Pst*
338 DC3000 *hrcC*⁻ but not to the virulent or avirulent strains, suggesting that this
339 protein is mostly involved in MTI. Further studies showed that basal
340 resistance against the non-adapted pathogen pea powdery mildew, *Erysiphe*
341 *pisi* was also impaired in *PLC2*-silenced plants. In this non-host interaction
342 with *Arabidopsis*, the first line of defense is the recognition of MAMPs, such
343 as chitin by the receptor CERK1, triggering a series of immune responses
344 including MAPKs activation and ROS burst mediated by NADPH oxidases
345 (Kuhn et al., 2016).

346

347 **PLC2 Participates in RBOHD-dependent Plant Defense Responses**

348 Callose accumulation is an MTI response that requires RBOHD (Luna
349 et al., 2011), and flg22-induced callose deposition is reduced on *PLC2*-

350 silenced plants. Another RBOHD-dependent response is the flg22-induced
351 stomatal closure (Mersmann et al., 2010; Kadota et al., 2014; Li et al., 2014).
352 The restriction of microbial entry by stomatal closure is one of the first MTI
353 responses (Melotto et al., 2006). *fls2* mutant plants are impaired in stomatal
354 closure in response to flg22 and show increased susceptibility to *Pst* DC3000
355 when sprayed onto the leaf surface but not when infiltrated into leaves
356 (Gomez-Gomez et al., 2001; Zipfel et al., 2004; Chinchilla et al., 2006; Zeng
357 and He, 2010). Importantly, the action of ABA on stomatal immunity seems to
358 occur downstream or independently of the PRR complex because *fls2*, *bik1*,
359 and *rbohD* mutants exhibit wild type stomatal closure in response to
360 exogenous ABA (Macho et al., 2012; Kadota et al., 2014). Accordingly, we
361 demonstrate that PLC2 is required for flg22-induced stomatal closure,
362 whereas ABA-dependent stomatal closure is unaffected. These results show
363 that PLC2 is required for callose deposition and stomatal closure following
364 flg22 perception in Arabidopsis plants.

365

366 **PLC2 Acts Upstream of RBOHD Activation**

367 We have demonstrated that PLC2 is required for flg22-induced ROS
368 production. ROS production upon flg22 perception in Arabidopsis is
369 dependent on the NADPH oxidase RBOHD (Kadota et al., 2014; Li et al.,
370 2014). Post-translational regulation of RBOHD activation involves Ca^{2+} via
371 direct binding to EF hand motifs, phosphorylation by Ca^{2+} -dependent (i.e.
372 CPKs) and -independent protein kinases (i.e. BIK1) (Logan et al., 1997;
373 Boudsocq et al., 2010; Kadota et al., 2014; Li et al., 2014; Kadota et al.,
374 2015). By using PLC inhibitors, PLC activation has been suggested to be
375 required for ROS production upon xylanase, chitosan and the race specific
376 elicitor Avr4 (de Jong et al., 2004; Laxalt et al., 2007; Raho et al., 2011). PA
377 has also been shown to interact directly with RBOHD and enhance ROS
378 production (Zhang et al., 2009). Upon cryptogein treatments of tobacco BY2
379 cells, PLC and DGK inhibitors or silencing of the cluster III of the tobacco
380 DGK family resulted in reduced PA and ROS production (Cacas et al., 2016).
381 Therefore, it could be speculated that the second messengers derived from
382 PLC2 activation, PA and/or increase cytosolic Ca^{2+} via i.e. IP_6 , could positively
383 regulate the NADPH oxidase activity, since *PLC2*-silenced plants showed

384 reduced ROS production in response to flg22.

385 Flg22 activates MAPK signaling pathways leading to the induction of
386 immune gene expression. MPK3, MPK4/11 and MPK6 activation act
387 independently of the RBOHD-mediated ROS burst (Zhang et al., 2012; Xu et
388 al., 2014). Flg22-treated *PLC2*-silenced plants showed similar levels of MAPK
389 activation and immune gene expression as the wild type, suggesting that
390 MAPK signalling is independent from PLC2 presence.

391 RBOHD exists in a complex with the receptor kinase FLS2, interacting
392 directly with BIK1 (Kadota et al., 2014; Li et al., 2014). Our results show that
393 PLC2 is associated with RBOHD, and this association is ligand-independent.
394 In *Arabidopsis*, the receptor complex FLS2-BAK1 perceives flg22 and
395 activates by phosphorylation the downstream kinases BIK1 and PBL1, which
396 induce an influx of extracellular Ca^{2+} in the cytosol (Li et al., 2014; Ranf et al.,
397 2014). PLC2 contains a Ca^{2+} -dependent phospholipid-binding domain (C2)
398 and EF-hand domains (Otterhag et al., 2001). In addition, it is localized at the
399 plasma membrane and is rapidly phosphorylated upon flg22 treatment
400 (Niittyala et al., 2007; Nuhse et al., 2007). One can envisage that PLC2 is part
401 of the FLS2, BIK1, RBOHD complex, and that BIK1 or another component of
402 the receptor complex phosphorylates PLC2 leading to the generation of
403 second messengers like PA or IP_6 , which in turn, positively regulate or are
404 required to sustain/reinforce the activity of RBOHD.

405

406 **Other Roles of PLC2**

407 Seeking for knock-out mutant plants for *PLC2* we could not recover
408 homozygous mutants, and therefore decided to silence *PLC2*. Nevertheless,
409 further characterization showed that this gene is expressed during early
410 megagametogenesis and in the embryo after fertilization being required for
411 both reproductive- and embryo development, presumably by controlling
412 mitosis and/or the formation of cell-division planes (Li et al., 2015; Di Fino et
413 al., 2016). The fact that we were able to obtain *PLC2*-silenced lines could be
414 related with *i*) low expression levels of the 35S::*amiR-PLC2* in the
415 reproductive organs and embryos or *ii*) the silencing not being fully effective,
416 with low levels of PLC2 in the gametophyte and/or embryos being sufficient
417 for correct development. These findings suggest that the mechanisms for

418 PLC2 activation and/or its downstream targets, such as RBOHD, could be
419 similar in both the sporophyte during flg22 perception and the gametophyte
420 during development. Arabidopsis has five somatic embryogenesis receptor
421 kinases (SERKs) proteins. SERK3/BAK1 and SERK4/BKK1 associate with
422 FLS2 and BIK1 (Chinchilla et al., 2007; Lu et al., 2010; Zhang et al., 2010;
423 Roux et al., 2011). SERK1 and SERK2 are crucial in regulating male fertility
424 and are expressed in the ovule, female gametophyte, early embryos, and
425 vascular cells (Hecht et al., 2001; Kwaaitaal et al., 2005; Albrecht et al., 2005;
426 Colcombet et al., 2005). We speculate that PLC2 has a role in
427 gametogenesis and embryo development, probably by signaling
428 downstream of LRR-RLKs like kinases like SERKs. Nonetheless, whether
429 PLC2 is specific for FLS2-BAK1-BIK1 receptor-complex or participates in the
430 signaling of other receptor-complexes, like CERK1, as suggested by the
431 results obtained with *E. pisi*, remains to be elucidated.

432

433 **CONCLUSION**

434 The activity of PI-PLC in signaling after recognition of different MAMPs has
435 been described earlier. The Arabidopsis genome contains nine PI-PLC genes,
436 however, until the present work, it was not known which one was specifically
437 linked to plant defense response. We here present genetic evidence that
438 PLC2 participates in MAMP-triggered immunity. PLC2 is required for ROS
439 production and ROS-dependent responses elicited by the MAMP flg22. PLC2
440 associates with RBOHD, suggesting a positive regulation of the Arabidopsis
441 NADPH oxidase activity by PLC2.

442

443 **MATERIALS AND METHODS**

444 **Plant Material and Growth Conditions**

445 Seeds from Arabidopsis (Col-0) transformed with an artificial microRNA
446 (amiR) targeting specifically *PLC2* (*amiR PLC2*) under the control of the
447 CaMV 35S promoter or with the empty vector were germinated in soil
448 (soil:vermiculite:perlite (3:1:1)) and kept at 4°C for 2 days. Then, they were
449 grown at 25°C using a 16h light/ 8h dark photoperiod. In case of infections
450 (bacterial and fungus) plants were grown at 22°C in 8h light/ 16h dark
451 photoperiod.

452 For ion leakage experiments, Col-0 or *rpm1.3* mutant plants
 453 transformed with the coding sequence for the *P. syringae* pv. *tomato*
 454 *AvrRpm1* under the control of a dexamethasone inducible promoter (Aoyama
 455 and Chua, 1997) were grown as described at 22°C in 8h light/ 16h dark cycle.
 456 Both backgrounds were transformed with *amiR-PLC2* under the control of the
 457 Ubiquitin 10 promoter (pUBQ10).

458

459 **amiR PLC2 Silencing Constructs**

460 *AtPLC2* (At3g08510) silencing was performed using a specific artificial
 461 microRNA (*amiR*) designed with WMD3 Web microRNA designer
 462 (<http://wmd3.weigelworld.org>). Arabidopsis *miR319* was used as a template
 463 and the cloning strategy was according to Ossowski et al., 2009.

464 Primers for artificial micro RNA cloning.

I PLC2 miR-s	gaTTAAACACTCAGTAATTGCGCtctctctttgtattcc
II PLC2 miR-a	gaGCGCAATTACTGAGTGTTTAAtcaaagagaatcaatga
III PLC2 miR*s	gaGCACAATTACTGACTGTTTATtcacaggctgtagatg
IV PLC2 miR*a	gaATAAACAGTCAGTAATTGTGCtctacatatattcct

465 Capital letters denote *AtPLC2* targeted site.

466 The *amiR PLC2* was cloned into pCHF3 vector (kanamycine resistance
 467 in plants) driven by the CaMV 35S promoter or into pUBQ10 destination
 468 vector driven by the Ubiquitin 10 promoter (Basta resistance in plants).

469

470 **Arabidopsis Transformation**

471 Arabidopsis plants were transformed using floral dip method (Zhang,
 472 Henriques, Lin, Niu, & Chua, 2006). T1 plants were sown in MS-Agar
 473 (Murashige and Skoog medium with Gamborg's Vitamin, Agar 1%) plates with
 474 Kanamycin (50 µg/ml for pCHF3:amiRPLC2) or BASTA (10 µg/ml for
 475 pUBQ10:amiRPLC2). After two weeks, resistant plants were transferred to
 476 soil. T3 or T4 homozygous plants on which silencing levels were checked by
 477 qPCR were used for experiments.

478

479 **Expression Analysis by RT-qPCR**

480 Total RNA was extracted from ten-day-old seedlings or leaves from 4-5
481 week old plants using the Trizol method according to the manufacturer
482 instructions (Invitrogen, NY, USA). Complementary DNA (cDNA) was
483 synthesized on 1 µg of total RNA by MMLV reverse transcriptase (RT) from
484 Promega (Madison, USA) using oligo-dT primer in a final volume of 20 µl. The
485 cDNA was diluted to a final volume of 100 µl and 2.5 µl were used for
486 quantitative PCR (qPCR). The Fast Universal SYBR Green Master mix from
487 Roche (Mannheim, Germany) was employed, using a Step-one Real-time
488 PCR machine from Applied Biosystems (California, USA). The standard
489 amplification program was used. The expression levels of the gene of interest
490 were normalized to that of the constitutive *ACT2* (At3g18780) gene by
491 subtracting the cycle threshold value of *ACT2* from the CT value of the gene
492 (Δ CT). The nucleotide sequences of the specific primers for qPCR analysis
493 are listed in supplemental table S1. The annealing temperature for each
494 primer was 60 °C. LinRegPCR was the program employed for the analysis of
495 real time qPCR data (Ruijter et al., 2009).

496

497 **Western blot Analysis**

498 Polyclonal antibodies were prepared as described in (Otterhag et al.,
499 2001). A peptide KDLGDEEVWGREVPSFIQR corresponding to residues
500 266-284 of AtPLC2 was synthesized. One rabbit was immunized at 2-weeks
501 interval and serum was collected after the second boost. Protein extraction
502 buffer [100 mM NaPi pH 7.5, 150 mM NaCl, 1 mM EDTA and SIGMA
503 proteinase inhibitor cocktail] was added to an equal volume of 4-5 week old
504 grounded leaves tissue, mixed and centrifuged for 10 min at 10.000 g. Protein
505 concentration in the supernatant was determined. Samples were loaded onto
506 a 10% SDS–polyacrylamide gel, blotted on to nitrocellulose membranes, and
507 stained with Ponceau S for loading control. Membranes were incubated
508 overnight in PBS-T containing polyclonal anti-PLC2 antibody (1:2000). The
509 blot was washed three times with PBST and revealed using a secondary anti-
510 rabbit IgG antibody coupled to alkaline phosphatase according to the
511 manufacturer instructions (SIGMA).

512

513 **Bacterial Infection Assays**

514 6-8 week-old plants were used for bacterial inoculations. Strains
515 *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (virulent), *Pst* DC3000
516 *AvrRpm1* (avirulent) and *Pst* DC3000 *hrcC*⁻ mutant were maintained on solid
517 *Pseudomonas* agar F (King's B medium, Biolife, Italy) supplemented with 50
518 mg L⁻¹ rifampicin and 50 mg L⁻¹ kanamycin. Virulent and avirulent strains
519 were inoculated into the abaxial side of leaves by needleless syringe
520 infiltration with bacterial suspensions (10 mM MgCl₂; OD₆₀₀=0.00002). The
521 bacteria were extracted at 1 or 3 days post-infiltration and the number of
522 colony forming units (CFU) was determined after serial dilution and plating as
523 described (Johansson et al., 2014). The strain *Pst* DC3000 *hrcC*⁻ was
524 inoculated by spraying (MgCl₂ 10mM; OD₆₀₀=0.1; Silwet 0.02 %). Plants were
525 kept covered with a transparent lid for 6 hours. Samples were taken at day 1
526 and 3 post-inoculation with a cork borer N° 1. Bacterial growth was evaluated
527 as previously described (Katagiri et al., 2002). Data shown are from one
528 experiment representative of four independent biological assays. Each assay
529 contained 3 pools of 4 leaf-discs, collected from 4 independent plants.

530

531 **Ion Leakage**

532 Ion leakage was measured in leaf discs after infiltration of *Pst*
533 DC3000 *AvrRpm1* as well in leaf discs of Col-0 plants expressing the coding
534 sequence of *P. syringae AvrRpm1*, under the control of a Dex-inducible
535 promoter (Andersson et al., 2006) as described in (Johansson et al., 2014).
536 Leaf discs from 4- to 5-week-old empty vector or *PLC2*-silenced plants in wild
537 type or *rpm1-3* background were placed in deionized water during 1-2 hours,
538 then washed and transferred to six well cultivation plates containing 10 mL
539 water (four discs per well). For the Dex inducible *AvrRpm1* plants, leaf discs
540 were treated with 20 µM dexamethasone. The release of electrolytes from the
541 leaf discs was determined every 30 min for 5 hrs using a conductivity meter
542 (Orion, Thermo scientific) as described in (Johansson et al., 2014). The
543 experiment was repeated twice.

544

545 **Fungal Inoculation and Scoring of Fungal Penetration**

546 The non-host powdery mildew fungi *Erysiphe pisi* (isolate CO-01) was
547 propagated on pea (*Pisum sativum* L. cv. *Kelvedon wonder*) plants.
548 Inoculations were carried out powdering spores on leaves of 4-week-old
549 Arabidopsis wild type and *PLC2* silenced plants. After 3 days post-inoculation
550 leaves were stained with trypan blue as described (Koch & Slusarenko, 1990).
551 The penetration rate after inoculation was calculated as percentage of
552 successful penetration attempt (penetration ending in plant cell death) as
553 described (Pinosa et al., 2013) on at least 50 germinated spores on three
554 independent leaves per genotype. The experiment was repeated 4 times.

555

556 **MAPK Activation**

557 MAPK assays were performed on six 2-week-old seedlings grown in
558 liquid Murashige-Skoog (MS) medium (including vitamins; Duchefa) and 1%
559 sucrose. Seedlings were elicited with 1 mM flg22 for 5, 15 or 30 min and
560 frozen in liquid nitrogen. MAPK activation was monitored by western blot with
561 antibodies that recognize the dual phosphorylation of the activation loop of
562 MAPK (pTEpY). Phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204) rabbit
563 monoclonal antibodies from Cell Signaling were used according to the
564 manufacturer's protocol (1:5000). Blots were stained with Coomassie Brilliant
565 Blue to verify equal loading.

566

567 **Callose Deposition**

568 Leaves from 4- to 5-week-old plants were fully infiltrated with 1 μ M
569 flg22 or water for 18 h. Leaves were then incubated in 96% EtOH until all
570 tissue was transparent, washed in 0.07 M phosphate buffer (pH =9), and
571 incubated for 2 hours in 0.07 M phosphate buffer containing 0.01% aniline-
572 blue. Observations were performed with an epifluorescence microscope with
573 UV filter (Excitation 365/10 nm, emission 460/50 nm). Number of callose dots
574 was calculated using ImageJ software (Schneider et al., 2012). Two leaves
575 from three independent plants were analyzed per line (six microscopic fields
576 of 1 mm² for each leaf) in three independent experiment.

577

578 **Epidermal Peel Preparation and Stomatal Aperture Measurement**

579 Epidermal peels were obtained from the abaxial surface of fully

580 expanded leaves. The peels were pre-incubated in opening buffer [10 mM
581 MES, pH 6.1 (MES titrated to its pKa with KOH), 10 mM KCl] under white light
582 at 25 °C, to promote stomatal opening. After 3 h pre-incubation, flg22 (1 µM)
583 or ABA (50 µM) (Sigma, St Louis, MO, USA), were added to the opening
584 buffer and incubated for 1 h. Stomatal apertures were measured from digital
585 pictures taken with a Nikon Coolpix 990 (Nikon, Tokyo, Japan) camera
586 coupled to an optical microscope (Nikon Eclipse 2000). Then, the stomatal
587 pore width was digitally determined using the image analysis software Image
588 J. Aperture values are the mean of 90-120 stomata measured from at least
589 three independent experiments.

590

591 **ROS Detection**

592 Leaf discs from 4-5 week-old plants were placed in 96 wells black
593 plates floating in 200 µl of deionized water over night. ROS production was
594 triggered with 100 nM flg22 ("N"- QRLSTGSRINSAKDDAAGLQIA-"C",
595 Genbiotech S.R.L.) applied together with 20 mM luminol (SIGMA, cat# A8511)
596 and 0.02 mg/ml of horseradish peroxidase (SIGMA, cat # P6782).
597 Luminescence was measured with a luminometer (Thermo Scientific^(R)
598 Luminoskan Ascent Microplate). Each plate contained 36 leaf discs for flg22
599 treatment and 12 leaf discs for mock treatments of the same Arabidopsis line.
600 Every plate was measured over a period of 30 min with an interval of 1 min,
601 and repeated in four independent experiments.

602

603 **Seedlings Protein Extraction and Immunoprecipitation**

604 For immunoprecipitation studies in seedlings, Arabidopsis
605 *rbohdpRBOHD::FLAG-RBOHD* (Kadota et al. 2014) seeds were surface-
606 sterilized with chlorine gas and germinated on plates containing Murashige-
607 Skoog (MS) medium (with Gamborg's vitamins; Duchefa) and 1% sucrose
608 and 0.8% agar for the first 7 days at 22°C and with a 16-h light period.
609 Seedlings were transferred to liquid MS medium supplemented with 1%
610 sucrose and grown under the same conditions for additional 7 days.

611 Two-week-old seedlings were treated with flg22 (1 µM) or water and
612 ground to a fine powder in liquid nitrogen with sand (Sigma-Aldrich). Proteins

613 were isolated in extraction buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM
614 NaCl, 10% glycerol, 10 mM DTT, 1 mM NaF, 1 mM Na₂MoO₄·2H₂O, 1%
615 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich), 1% (v/v) P9599
616 Protease Inhibitor Cocktail (Sigma-Aldrich), 100 μM phenylmethylsulphonyl
617 fluoride and 1% (v/v) IGEPAL CA-630 (Sigma-Aldrich). Extracts were
618 incubated 30 min at 4°C and centrifuged for 20 min at 16,000 g at 4°C.
619 Supernatants were incubated for 1-2 h at 4°C with ANTI-FLAG M2 Affinity Gel
620 (Sigma-Aldrich), and washed 5 times with extraction buffer. Beads were
621 heated at 55°C in SDS loading buffer for 20 min to release proteins. For
622 immunoblotting, antibodies were used at the following dilutions: α-PLC2
623 (1:5000), α-FLAG-HRP (Sigma-Aldrich, 1:5000), α-Rabbit-HRP (Sigma-
624 Aldrich, 1:10000) and anti-BRI1 (1:5000).

625

626 **Accession Numbers**

627 *AtPLC2* (At3g08510).

628

629 **Supplemental Data**

630 Table S1. Primer sequences

631 Figure S1. PLC2 silencing specificity.

632 Figure S2. PLC2 is not involved in the programmed cell death during the
633 effector triggered immunity upon recognition of AvrRpm1 from *Pseudomonas*
634 *syringae*.

635 Figure S3. PLC2 is not required for flg22-induced MAPK activation.

636 Figure S4. MAMPs-activated gene expression is not deregulated in PLC2-
637 silenced seedlings

638

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640 We thank Miss Alexandra Leschnin and Dr Oskar Johansson for helping with
641 ion leakage measurement, bacterial and fungal infections.

642

643 **FIGURE LEGENDS**

644

645 **Figure 1.** *PLC2* silencing by artificial micro RNAs in Arabidopsis.

646 A, Total RNA was isolated from leaves of 4-5 weeks old Col-0 or *PLC2*
647 silenced plants (T4 homozygous lines *amiR PLC2-4*, *-7* and *-11*). Relative
648 transcript levels of *PLC2* were determined by RT-qPCR. Transcript levels
649 were normalized to *ACT2*. Error bars represent standard deviations of 3-9
650 individual plants. Different letters indicate significant difference at $P < 0.001$
651 (multiple comparison using one-way ANOVA, Tukey's test).

652 B, PLC2 protein levels were analyzed by western blot using anti-AtPLC2
653 antibody in leaves of 4-5 weeks old Col-0; empty vector (EV); *amiR PLC2-11*
654 and *amiR PLC2-4* independent silenced lines. Ponceau S staining (PS) of
655 Rubisco Subunit L is included as a loading control.

656
657 **Figure 2.** Growth of *Pseudomonas syringae* and *Erysiphe pisi* (*E. pisi*) in
658 Arabidopsis *PLC2*-silenced plants.

659 Wild type (Col-0), empty vector (EV) and *PLC2*-silenced lines (*amiR PLC2-11*
660 and 4) were used.

661 A, *PLC2*-silenced plants are more susceptible to *Pseudomonas syringae* pv.
662 *tomato* DC3000 *hcrC* mutant. Bacteria were inoculated by spray at $OD_{600}=0.1$
663 and the number of colony forming units (CFU) per cm^2 of leaf extracts was
664 determined. A representative experiment of 4 biological replicates is depicted,
665 where asterisks (*) indicate significant differences regarding EV control
666 according to t-test ($P<0.01$).

667 B, *PLC2*-silenced lines showed no susceptibility differences during virulent
668 and avirulent *Pseudomonas syringae* pv. *tomato* DC3000 infection.
669 *Pseudomonas syringae* pv. *tomato* DC3000 (virulent) and *Pseudomonas*
670 *syringae* pv. *tomato* DC3000:AvrRpm1 (avirulent) were inoculated by
671 infiltration at $OD_{600}=0.0002$ and CFU per cm^2 of leaf was calculated. A
672 representative experiment of 4 biological replicates is depicted.

673 C, *PLC2*-silenced plants are more susceptible to the non-adapted pea
674 powdery mildew *Erysiphe pisi* (*E. pisi*). The penetration rate at 3 days after
675 inoculation was calculated as % of successful penetration of at least 50
676 germinated spores on three independent leaves. Error bars represents
677 standard error of the mean. Different letters indicate significant difference at
678 $P<0.05$ (multiple comparison using one-way ANOVA, Tukey's test). A
679 representative experiment of 4 biological independent replicates is depicted.

680
681 **Figure 3.** *PLC2*-silenced plants exhibit impaired flg22-induced callose
682 deposition.

683 Leaves from 4- to 5-week-old Col-0 or *amiR PLC2* plants were infiltrated with
684 1 μ M flg22 or H_2O as a control and incubated for 18 h and callose deposition
685 was measured as dots per area. Error bars represent standard error of the
686 mean. Different letters indicate significant difference at $P<0.001$ (one-way
687 ANOVA, Tukey's test) $n=3$.

688
689 **Figure 4.** *PLC2*-silenced plants exhibit impaired flg22-induced stomatal
690 closure.

691 Epidermal peels from Col-0 and *PLC2*-silenced plants were incubated in
692 opening buffer under light for 3 h. The peels were treated with H_2O , 1 μ M
693 flg22, 50 μ M ABA or 50 μ M ABA + 1 μ M flg22 for 1 h. The results show the
694 mean of 90-120 stomata measured from three independent experiments.
695 Error bars represent SE of the means. Different letters denote statistical
696 difference (one-way analysis of variance, Dunn's Method $P<0.05$).

697
698 **Figure 5.** *PLC2*-silenced plants exhibit impaired flg22-induced oxidative burst.
699 Production of reactive oxygen species (ROS) was measured with a luminol-
700 based assay in Col-0 or *amiR PLC2* plants.

701 A, Leaf disks from 4- to 5-week-old plants were incubated with 100 nM flg22
702 and the luminescence was measured every 1 min for 30 min and expressed
703 as relative light units (RLU). A representative experiment is shown using wild
704 type (Col-0) and a *PLC2*-silenced line (*amiR PLC2-11*) plants.

705 B, Total ROS production was calculated integrating the areas under the
706 curves and referring to Col-0 wild type treated with flg22 as 100%. Average of
707 4 independent experiments is shown. Error bars represent SE of the means.
708 The asterisk indicates statistically significant differences compared to flg22-
709 treated Col-0 plant (ANOVA, Multiple Comparisons versus Control Group
710 Dunnett's Method $P < 0,001$).

711

712 **Figure 6.** *PLC2* associates with RBOHD.

713 Co-Immunoprecipitation of *PLC2* and RBOHD in stable transgenic
714 Arabidopsis seedlings (T3) expressing FLAG-RBOHD- (pRBOHD:FLAG-
715 RBOHD) treated (+) or not (-) with 1 μ M flg22 for 15 min. Total protein
716 extracts (input) were subjected to immunoprecipitation with anti-FLAG beads
717 followed by immunoblot analysis with anti-*PLC2* (α -*PLC2*) and anti-FLAG (α -
718 FLAG) antibodies as indicated. Protein extracts of Col-0 plants were used as
719 negative controls. Anti-BRI1 (α -BRI1) antibodies were used as plasma
720 membrane protein not associated with RBOHD. Coomassie brilliant blue
721 (CBB). These experiments were performed three times with similar results.

722

723

724 **Figure S1.** *PLC2* silencing specificity.

725 Relative transcript levels of *PLC1* (the second most abundant *PLC*), *PLC4*
726 (similar expression pattern than *PLC2*) and *PLC7* (high sequence similarity to
727 *PLC2*) were determined by RT-qPCR. Total RNA was isolated from leaves of
728 4- to 5-week-old Col-0 or *amiR PLC2-11* silenced plants (T4 homozygous
729 lines). Transcript levels were normalized to *ACT2*. Error bars represent
730 standard deviations of 3 individual plants.

731

732 **Figure S2.** *PLC2* is not involved in the programmed cell death during the
733 effector triggered immunity upon recognition of AvrRpm1 from *Pseudomonas*
734 *syringae*.

735 A, Electrolyte leakage in leaf discs from wild type (Col-0), Empty vector (EV)
736 and *PLC2* silenced lines (*amiR PLC2 11* and *4*) inoculated by vacuum
737 infiltration with *Pseudomonas syringae* DC3000 carrying AvrRpm1.

738 B, Electrolyte leakage in leaf discs from DEX::AvrRpm1/Col-0 (solid symbols)
739 and DEX::AvrRpm1/*rpm1-3* (open symbols), *PLC2*-silenced lines (indicated
740 as *amiR*) or non-silenced plants, were incubated with dexamethasone. The
741 conductivity of the solution was measured at the times indicated. Mean and
742 standard deviation are shown (n=6). The experiment was replicated 2 times
743 with similar results.

744 C, Silencing of *PLC2* by artificial micro RNA in AvrRpm1/Col-0 or
745 AvrRpm1/*rpm1* plants. Total RNA was isolated from leaves of 4-5 weeks old
746 AvrRpm1/Col-0 or AvrRpm1/*rpm1-3* or *PLC2* silenced plants (T3 homozygous
747 lines *amiR*). Relative transcript levels of *PLC2* were determined by RT-qPCR.
748 Transcript levels were normalized to *ACT2*. Error bars represent standard
749 deviations of 3 individual plants.

750

751 **Figure S3.** *PLC2* is not required for flg22-induced MAPK activation.
752 MAPK activation assay in wild type (Col-0), empty vector (EV), and *PLC2*
753 silenced lines. Fourteen-day-old seedlings were treated with 1 μ M flg22 for 0
754 (-) or 15 (+) min. Total protein extracts were subjected to immunoblot analysis
755 with anti-phospho MAPK; anti-*PLC2* (α -*PLC2*) and anti-*FLS2* antibodies as
756 indicated. CBB, Coomassie Brilliant Blue (loading control). The experiment
757 was performed at least 3 times with similar results.

758
759 **Figure S4.** *PLC2* is not required for flg22-induced MAPK dependent-gene
760 expression
761 Ten-day-old Col-0 and *PLC2* silenced (*amiR PLC2-11*) seedlings were treated
762 with 1 μ M flg22 for 0 min, 30 min or 60 min as indicated. Total RNA was
763 extracted for transcript analysis. Transcript levels of *PHI1*, *WRKY33* and
764 *FRK1* and were determined by qPCR. The data were normalized using *ACT2*
765 as a reference gene. Error bars show SE from three independent
766 experiments.
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