Short title: PLC2 in plant immunity

* *Corresponding author:* Ana Maria Laxalt. Instituto de Investigaciones Biológicas IIB-Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar del Plata, 7600 Mar del Plata, Argentina. Tel+54-223-4753030. amlaxalt@mdp.edu.ar

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

Juan Martín D'Ambrosio[1], Daniel Couto[2], Georgina Fabro[3], Denise Scuffi[1], Lorenzo Lamattina[1], Teun Munnik[4], Mats X. Andersson[5], María E. Álvarez[3], Cyril Zipfel[2], Ana M. Laxalt[1*]

[1] Instituto de Investigaciones Biológicas IIB-Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar del Plata, 7600 Mar del Plata, Argentina

[2] The Sainsbury Laboratory, Norwich Research Park, Norwich, England, United Kingdom.

[3] Centro de Investigaciones en Química Biológica de Córdoba CIQUIBIC, UNC-CONICET, Universidad Nacional de Córdoba, X5000HUA Córdoba, Argentina

[4] Swammerdam Institute for Life Sciences, Section Plant Cell Biology, University of Amsterdam, 1098 XH Amsterdam, The Netherlands.

[5] Department of Biological and Environmental Sciences, University of Gothenburg, SE–405 30 Gothenburg, Sweden

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

List of author contribution: AML conceived the original research plans; AML, MXA, MEA and CZ designed and supervised the experiments and analyzed the data; JMD performed most of the experiments and analyzed the data; DC, GF and DS performed some of the experiments. AML conceived the project and wrote the article with contributions of all the authors; LL and TM supervised and complemented the writing.

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Corresponding author email: amlaxalt@mdp.edu.ar

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ABSTRACT

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

INTRODUCTION

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

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

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

Hung et al., 2014; Hong et al., 2016). Specifically, phosphoinositide-specific phospholipase C (PI-PLC) is rapidly activated in plant cells after recognition of different MAMPs, such as xylanase, flg22 and chitosan (van der Luit et al., 2000; Laxalt et al., 2007; Raho et al., 2011), or of pathogen effector proteins (de Jong et al., 2004; Andersson et al., 2006). PI-PLC catalyzes the hydrolysis of phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol (4,5) bisphosphate $PI(4,5)P_2$ to generate water-soluble IP_2 and IP_3 respectively, and diacylglycerol (DAG), which remains in the membrane. In plants, DAG produced by PI-PLC activity is phosphorylated by DAG kinase (DGK) to produce phosphatidic acid (PA), which regulates several protein targets (Arisz et al., 2009; Testerink and Munnik, 2011; Munnik, 2014). PA has been specifically implicated in the modulation of immune signaling components, such as MAPKs and PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1 (PDK1) (Farmer and Choi, 1999; Lee et al., 2001; Szczegielniak et al., 2005; Anthony et al., 2006). In particular, PA binds to the NADPH oxidase isoforms RBOHD and RBOHF to induce ROS during ABA-mediated stomatal closure (Zhang et al., 2009). Additionally, it has been shown that PLC activity is required for ROS production during ETI responses (de Jong et al., 2004; Andersson et al., 2006).

In animals, IP_3 triggers release of Ca^{2+} from intracellular stores by activating a ligand-gated calcium channel at the endoplasmic reticulum. In plants, no clear homologue of the IP_3-activated Ca^{2+} channel has been identified (Munnik and Testerink, 2009). Instead IP_2 and IP_3 are further phosphorylated by inositolpolyphosphate kinase (Williams et al., 2015) to: i) IP_6 which, stimulates the release of Ca^{2+} from intracellular stores in guard cells (Lemtiri-Chlieh et al., 2000), affects gene transcription, mRNA export and regulates the auxin receptor TIR1 (Zonia and Munnik, 2006; Lee et al., 2015); ii) IP_5 which is part of the jasmonate receptor COI1 (Munnik and Nielsen 2011); and iii) IP_7 and IP_8, involved in plant defense (Laha et al., 2015). In addition, PIP and PIP_2, originally characterized as PLC substrates, do have signaling properties themselves, since many proteins involved in membrane trafficking and signal transduction have domains that bind to these lipids (Munnik and Nielsen, 2011; Delage et al., 2013; Heilmann, 2016).

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

RESULTS

PLC2 Silencing by Artificial microRNA

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

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

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

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

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

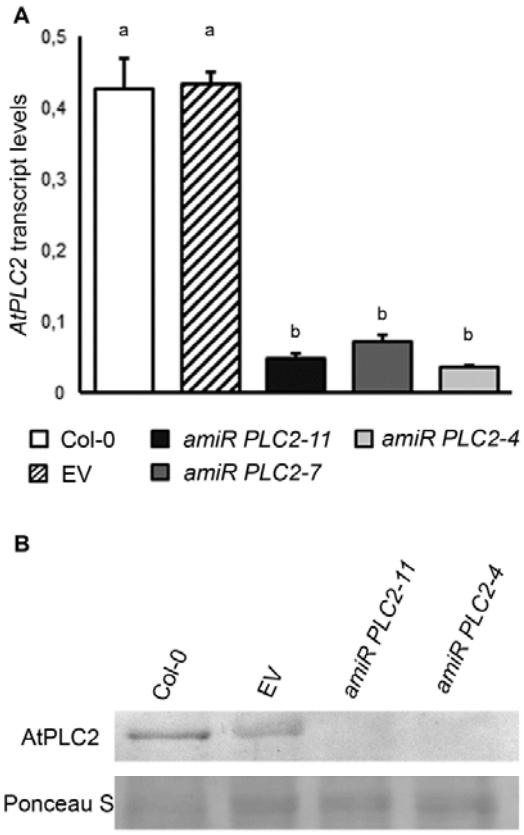

Figure 1. PLC2 silencing by artificial micro RNAs in Arabidopsis.
A, Total RNA was isolated from leaves of 4-5 weeks old Col-0 or PLC2 silenced plants (T4 homozygous lines amiR PLC2-4, -7 and -11). Relative transcript levels of PLC2 were determined by RT-qPCR. Transcript levels were normalized to ACT2. 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 affects AvrRpm1-recognition-triggered growth restriction.

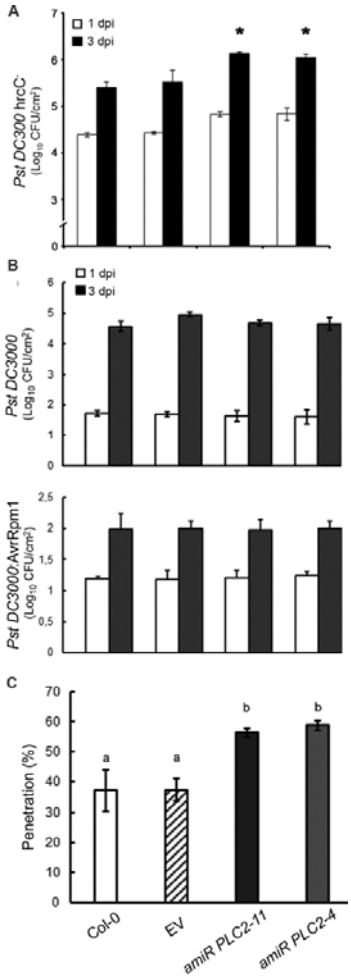

Figure 2. Growth of *Pseudomonas syringae* and *Erysiphe pisi* (*E. pisi*) in Arabidopsis PLC2-silenced plants.
Wild type (Col-0), empty vector (EV) and PLC2-silenced lines (amiR PLC2-11 and 4) were used.
A, PLC2-silenced plants are more susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 hrcC- 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.

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

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

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

PLC2 is Required for MTI ROS-regulated Processes

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

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

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

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

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

PLC2 is Required for flg22-induced ROS Burst

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

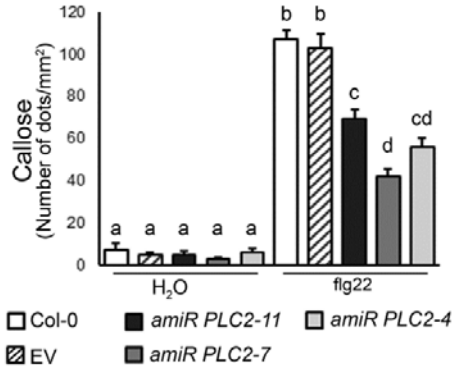

Figure 3. PLC2-silenced plants exhibit impaired flg22-induced callose deposition.
Leaves from 4- to 5-week-old Col-0 or amiR PLC2 plants were infiltrated with 1 µM flg22 or H2O as a control and incubated for 18 h and callose deposition was measured as dots per area. Error bars represent standard error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

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

PLC2 Associates with RBOHD

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

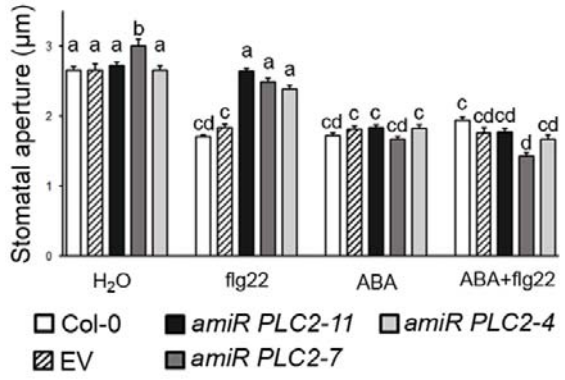

Figure 4. PLC2-silenced plants exhibit impaired flg22-induced stomatal closure.
Epidermal peels from Col-0 and PLC2-silenced plants were incubated in opening buffer under light for 3 h. The peels were treated with H2O, 1 µM flg22, 50 µM ABA or 50 µM ABA + 1 µM flg22 for 1 h. The results show the mean 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 $P<0.05$).

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

DISCUSION

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

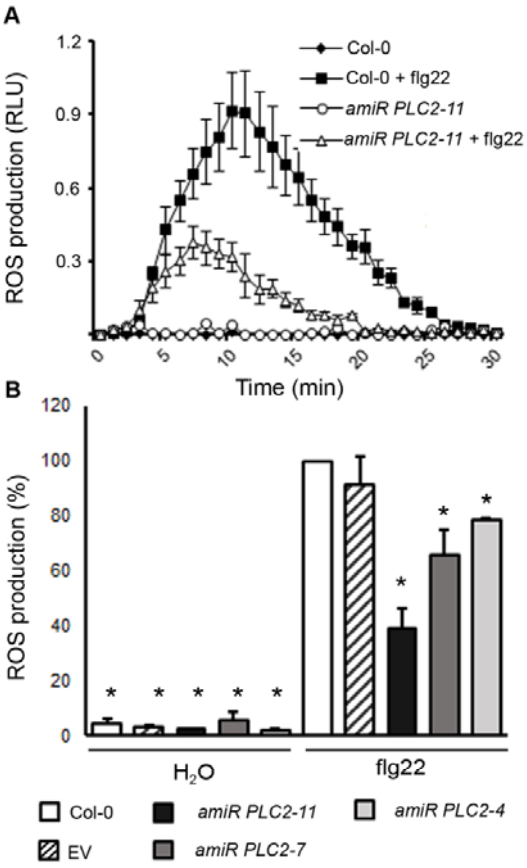

Figure 5. PLC2-silenced plants exhibit impaired flg22-induced oxidative burst.
Production of reactive oxygen species (ROS) was measured with a luminol-based assay in Col-0 or amiR PLC2 plants.
A, Leaf disks from 4- to 5-week-old plants were incubated with 100 nM flg22 and the luminescence was measured 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).

most likely the substrate, and *ii)* the phosphorylated products of IP_2, and DAG, including various inositol polyphosphates (IPPs), PA and diacylglycerol pyrophosphate have a role as secondary messengers (Munnik, 2014). PA is involved in the modulation of immune signaling components, such as MAPKs,

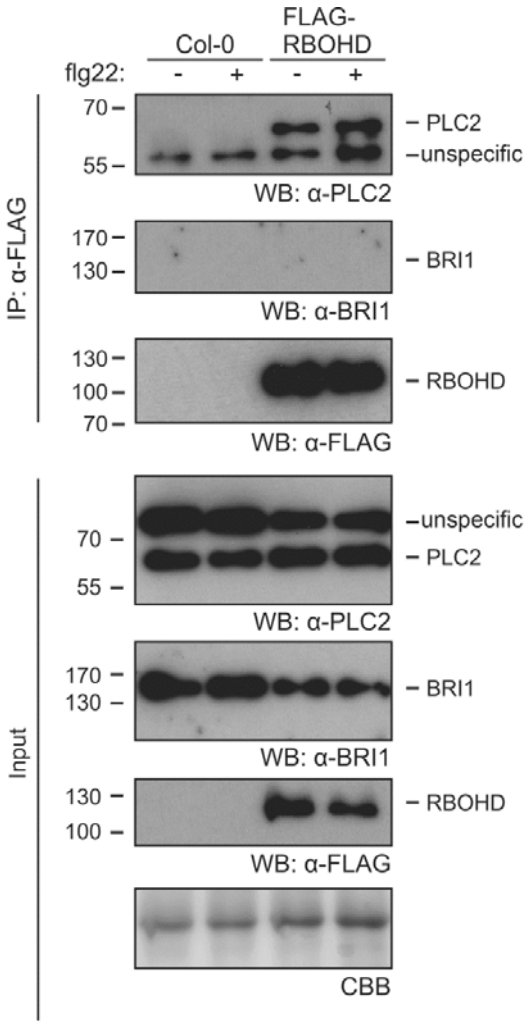

Figure 6. PLC2 associates with RBOHD.
Co-Immunoprecipitation of PLC2 and ROBHD in stable transgenic Arabidopsis seedlings (T3) expressing FLAG-RBOHD- (pRBOHD:FLAG-RBOHD) treated (+) or not (-) with 1 µM flg22 for 15 min. Total protein extracts (input) were subjected to immunoprecipitation with anti-FLAG 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. Anit-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; Szczegielniak et
313 al., 2005; Anthony et al., 2006; Zhang et al., 2009). However, we cannot
314 exclude that IP_2 can be very rapidly phosphorylated to IP_6 and thus probably
315 increase Ca^{2+} in the cytosol, or also participate in auxin signaling via TIR1 and

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

PLC2 is Required for Plant Immunity

Recently, using tomato and virus induced-gene silencing (VIGS) of different PLCs, SlPLC4 was found to be specifically involved in the HR upon AVR4 perception, while SlPLC6 is required for multiple R protein-mediated responses (Vossen et al., 2010), suggesting that in tomato both PLCs participate on ETI responses. Similarly, over-expression of *SlPLC3* enhanced the Cf-4/Avr4-triggered HR (Abd-El-Haliem et al., 2016). Further studies on tomato showed that SlPLC2 is required for xylanase induced-gene expression, ROS production, and plant susceptibility against *Botrytis cinerea* (Gonorazky et al., 2014; Gonorazky et al., 2016).

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

PLC2 Participates in RBOHD-dependent Plant Defense Responses

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

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

PLC2 Acts Upstream of RBOHD Activation

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

reduced ROS production in response to flg22.

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

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

Other Roles of PLC2

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

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

CONCLUSION

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

MATERIALS AND METHODS

Plant Material and Growth Conditions

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

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

amiR PLC2 Silencing Constructs

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

Primers for artificial micro RNA cloning.

I PLC2 miR-s	gaTTAAACACTCAGTAATTGCGCtctctcttttgtattcc
II PLC2 miR-a	gaGCGCAATTACTGAGTGTTTAAtcaaagagaatcaatga
III PLC2 miR*s	gaGCACAATTACTGACTGTTTATtcacaggtcgtgatatg
IV PLC2 miR*a	gaATAAACAGTCAGTAATTGTGCtctacatatatattcct

Capital letters denote *AtPLC2* targeted site.

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

Arabidopsis Transformation

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

Expression Analysis by RT-qPCR

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

Western blot Analysis

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

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 $MgCl_{2;}$ OD_{600}=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 ($MgCl_2$ 10mM; OD_{600}=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 µM
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 ηm, emission 460/50 ηm). Number of callose dots
574 was calculated using ImageJ software (Schneider et al., 2012). Two leafs
575 from three independent plants were analyzed per line (six microscopic fields
576 of 1 mm^2 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

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

ROS Detection

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

Seedlings Protein Extraction and Immunoprecipitation

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

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

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

Accession Numbers

AtPLC2 (At3g08510).

Supplemental Data

Table S1. Primer sequences
Figure S1. PLC2 silencing specificity.
Figure S2. PLC2 is not involved in the programmed cell death during the effector triggered immunity upon recognition of AvrRpm1 from Pseudomonas syringae.
Figure S3. PLC2 is not required for flg22-induced MAPK activation.
Figure S4. MAMPs-activated gene expression is not deregulated in PLC2-silenced seedlings

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FIGURE LEGENDS

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.

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 OD_{600}=0.1 and the number of colony forming units (CFU) per cm^2 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 OD_{600}=0.0002 and CFU per cm^2 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.

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 µM flg22 or H_2O 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.

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 H_2O, 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).

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).

Figure 6. PLC2 associates with RBOHD.
Co-Immunoprecipitation of PLC2 and ROBHD in stable transgenic Arabidopsis seedlings (T3) expressing FLAG-RBOHD- (pRBOHD:FLAG-RBOHD) treated (+) or not (-) with 1 µM 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. Anit-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.

Figure S1. *PLC2* silencing specificity.
Relative transcript levels of *PLC1* (the second most abundant *PLC*), *PLC4* (similar expression pattern than *PLC2*) and *PLC7* (high sequence similarity to *PLC2*) were determined by RT-qPCR. Total RNA was isolated from leaves of 4- to 5-week-old Col-0 or *amiR PLC2-11* silenced plants (T4 homozygous lines). Transcript levels were normalized to *ACT2*. Error bars represent standard deviations of 3 individual plants.

Figure S2. *PLC2* is not involved in the programmed cell death during the effector triggered immunity upon recognition of AvrRpm1 from *Pseudomonas syringae*.
A, Electrolyte leakage in leaf discs from wild type (Col-0), Empty vector (EV) and *PLC2* silenced lines (*amiR PLC2 11* and *4*) inoculated by vacuum infiltration with *Pseudomonas syringae* DC3000 carrying AvrRpm1.
B, Electrolyte leakage in leaf discs from DEX::AvrRpm1/Col-0 (solid symbols) and DEX::AvrRpm1/*rpm1-3* (open symbols), PLC2-silenced lines (indicated as *amiR*) or non-silenced plants, were incubated with dexamethasone. The conductivity of the solution was measured at the times indicated. Mean and standard deviation are shown (n=6). The experiment was replicated 2 times with similar results.
C, Silencing of *PLC2* by artificial micro RNA in AvrRpm1/Col-0 or AvrRpm1/*rpm1* plants. Total RNA was isolated from leaves of 4-5 weeks old AvrRpm1/Col-0 or AvrRpm1/*rpm1-3* or *PLC2* silenced plants (T3 homozygous lines *amiR*). Relative transcript levels of *PLC2* were determined by RT-qPCR. Transcript levels were normalized to *ACT2*. Error bars represent standard deviations of 3 individual plants.

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

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

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