Short title: PLC2 in plant immunity

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Phospholipase C 2 affects MAMP-Triggered Immunity by Modulating ROS Production

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One sentence summary: Arabidopsis Phospholipase C 2 (PLC2) participates in a branch of microbe-associated molecular pattern-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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Abbreviations

BAK1, BRASSINOSTEROID RECEPTOR 1-ASSOCIATED KINASE 1
BIK1, BOTRYTIS INDUCED KINASE 1
CDPK, Ca^{2+}-dependent protein kinases
DAG, diacylglycerol
ETI, effector-triggered immunity
ETS, effector-triggered susceptibility
FLS2, Flagellin sensing 2
IP2, inositol bisphosphate
IP3, inositol trisphosphate
MAMPS, microbe-associated molecular patterns
MAPK, mitogen-activated protein kinases
MTI, MAMP-triggered immunity
NLR, nucleotide-binding leucine-rich repeat
PA, phosphatidic acid
PDK1, Phosphoinositide-dependent protein kinase 1
PI4P, phosphatidylinositol 4-phosphate
PIP2, phosphatidylinositol (4,5) bisphosphate
PI-PLC, phosphoinositide-specific phospholipase C
PRRs, pattern recognition receptors
RBOHD, respiratory burst oxidase homolog D
ROS, reactive oxygen species
SERK, Somatic embryogenesis-related kinase

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 are 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 hypersensitive response (HR) features. In response to flg22, *PLC2*-silenced plants maintain wild-type MAPK activation and *PHI1*, *WRKY33* and *FRK1* immune marker gene expression, but have reduced reactive oxygen species (ROS)-dependent responses such as callose deposition and stomatal closure. Accordingly, the generation of ROS upon flg22 treatment is 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 the NADPH oxidase RBOHD, suggesting its potential regulation 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 (or the derived peptide flg22) 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 PIP_2 to generate water-soluble inositol bisphosphate (IP_2) or IP_3, 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 generate: 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 (Tan et al., 2007; Lee et al., 2015); ii) IP_5, which is part of the jasmonate receptor COI1 (Sheard et al., 2010); and iii) IP_7 and IP_8, which are 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 is strongly and constitutively expressed, 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 responses triggered upon flg22 perception. We found that PLC2 plays an important role in stomatal pre-invasion immunity and non-host resistance, and that it associates with RBOHD, suggesting a potential 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. S1A). Western blot analysis using a specific anti-PLC2 antibody (Otterhag et al., 2001) showed strongly reduced levels of PLC2 protein in *amiR* silenced lines (Fig. 1B). The *PLC2*-silenced plants show similar morphological features than wild type plants under standard growth conditions (Supplemental Fig. S1B).

PLC2-silenced Plants are More Susceptible to the Bacterial *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*$^-$ Strain and to the Non-adapted Fungus *Erysiphe pisi*

To investigate the role of PLC2 in plant innate immunity we tested the interaction of *PLC2*-silenced plants 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 one and three days post-inoculation. *PLC2*-silenced plants were more susceptible to *Pst* DC3000 *hrcC*$^-$ than wild type plants (Fig. 2A). Under natural conditions, *Pst* enters host plants, through wounds or natural openings such as stomata, and then spreads and multiplies in intercellular spaces (Beattie and Lindow, 1995). The infiltration of bacteria with a syringe bypasses the first steps of the natural infection process. When *Pst* DC3000 *hrcC*$^-$ was infiltrated, no significant difference was detected between *PLC2*-silenced and non-silenced plants (Fig.

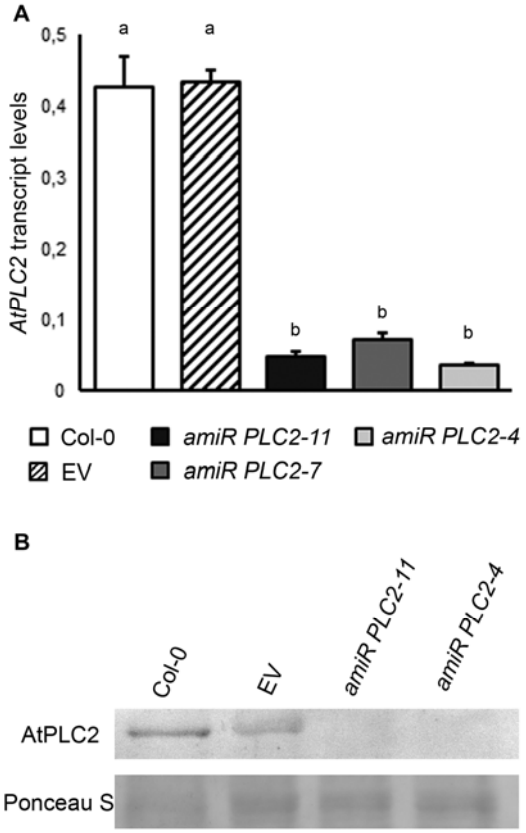

Figure 1. *PLC2* silencing by artificial micro RNAs in Arabidopsis.
A, Total RNA was isolated from leaves of 4-5 weeks old Col-0 or *PLC2* silenced plants (T4 homozygous lines amiR *PLC2-4*, *-7* and *-11*). Relative transcript levels of PLC2 were determined by RT-qPCR. Transcript levels were normalized to *ACT2*. Error bars represent standard deviations of 3-9 individual plants. Different letters indicate significant difference (ANOVA for unbalanced samples, post-hoc Tukey-Krumer test at $P<0.001$).
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.

2B). These experiments indicate that PLC2 is likely involved in stomatal related MTI responses.

We further studied the growth of the virulent wild type *Pst* DC3000, whose TTSS effectors do interfere with MTI (Block and Alfano, 2011). No

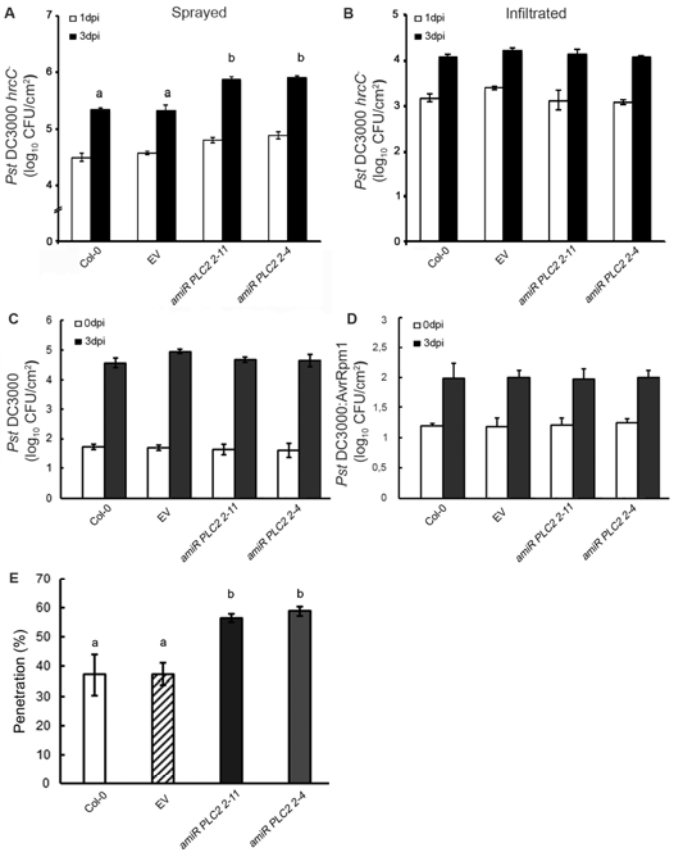

Figure 2. Growth of *Pseudomonas syringae* and *Erysiphe pisi* (E. pisi) in Arabidopsis *PLC2*-silenced plants.
Wild type (Col-0), empty vector (EV) and *PLC2*-silenced lines (*amiR PLC2-11* and *4*) were used.
A, *PLC2*-silenced plants are more susceptible to *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*⁻ mutant. Bacteria were inoculated by spraying at OD_{600}=0.1 and the number of colony forming units (CFU) per cm^2 of leaf extracts were determined. Data from three biological replicates each with three technical replicates were averaged (n=9) and ANOVA performed considering each replicate as a factor. Error bars represents standard error of the mean. Different letters indicate significant difference between genotypes (ANOVA P<0.001, post-hoc Tukey test).
B, *PLC2*-silenced plants showed no differences in susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC*⁻ mutant when the bacteria is syringe-inoculated into the leaf apoplast. Bacterial suspension was inoculated at OD_{600}=0.0001 and the number of colony forming units (CFU) per cm^2 of leaf extracts were determined. Data from three biological replicates each with three technical replicates were averaged (n=9) and ANOVA performed considering each replicate as a factor. No significant differences were observed between genotypes. Error bars represent standard error.
C and D, *PLC2*-silenced lines showed no differences in susceptibility to virulent (C) and avirulent (D) *Pseudomonas syringae* pv. *tomato* DC3000 infections. *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. No significant differences were observed regarding EV control according to t-test (*P<0.05*).
E, *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 SE of the mean. Different letters indicate significant difference (multiple comparison using one-way ANOVA, post-hoc Tukey's test at P<0.05). A representative experiment of 4 biological independent replicates is depicted.

227 significant difference in proliferation of this adapted pathogen was detected
228 between both plant genotypes (Fig. 2C) indicating that *PLC2* silencing does
229 not have an effect when the virulent pathogen is infiltrated.

230 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 III-secreted effector AvrRpm1, which is recognized by the NLR RPM1 (Block and Alfano, 2011). *PLC2*-silenced plants showed the same ability as wild type in constraining growth of this strain (Fig. 2D) indicating that the lack of *PLC2* does not affects AvrRpm1-recognition-triggered growth restriction. Moreover, the HR cell death measured by ion leakage 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 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 plants (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. 2E). We observed a significantly increased success in penetration of the epidermis by *E. pisi* spores on *PLC2*-silenced plants compared to wild type plants, indicating that PLC2 is involved in non-host resistance. Altogether, the above-presented results suggest that PLC2 might play a role in MTI establishment.

PLC2 is Involved in ROS-regulated Processes During MTI

In order to study the function 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 the two distinct MAPK- and ROS-dependent branches of MTI signaling (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 phosphorylated motif on the activation loop of MAPKs, recognized three immunoreactive bands 15 min after treatment corresponding to at least MPK6, MPK3 and MPK4/11 (Bethke et al., 2012) (Supplemental Fig. S3). *PLC2*-silenced lines showed a similar MAPKs activation as wild type plants (Supplemental Fig. S3). Similarly, 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; Segonzac and Zipfel, 2011; 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

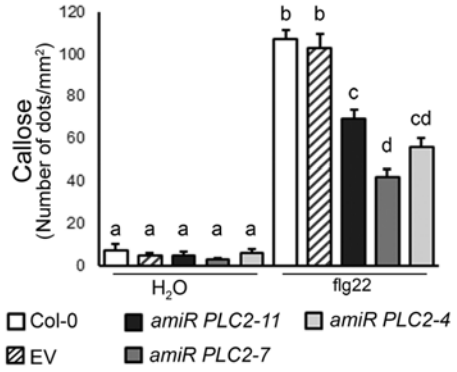

Figure 3. *PLC2*-silenced plants exhibit impaired flg22-induced callose deposition.
Leaves from 4- to 5-week-old Col-0 or *amiR PLC2* plants were infiltrated with 1 µM flg22 or H_2O as a control and incubated for 18 h and callose deposition was measured as dots per area. Six different microscopic areas (1 mm^2) were taken per leaf. Two different leafs per individual was analyzed. Three independent plants were analyzed per line per experiment. Three independent experiments were performed. Error bars represent standard error of the mean. Different letters indicate significant difference (ANOVA for unbalanced samples, post-hoc Tukey-Kramer test at $P<0.001$).

unaffected. Together, these results imply that PLC2 is required for full activation of stomatal ROS-dependent immune responses.

PLC2 is Involved in flg22-induced ROS Burst

Flg22 perception triggers a fast and transient increase of apoplastic ROS (Felix et al., 1999). 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 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 showed a reduction ROS levels in response to flg22 (40 to 75% compared to control plants) (Fig. 5B). Thus, our results demonstrated that PLC2 is required for the full ROS accumulation following flg22 recognition in Arabidopsis leaf discs.

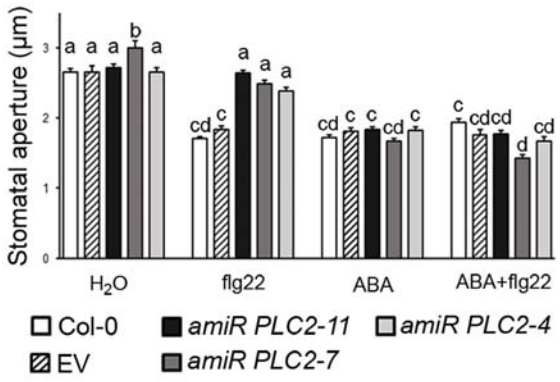

Figure 4. *PLC2*-silenced plants exhibit impaired flg22-induced stomatal closure.
Epidermal peels from Col-0 and *PLC2*-silenced plants were incubated in opening buffer under light for 3 h. The peels were treated with H_2O, 1 µM flg22, 50 µM ABA or 50 µM ABA + 1 µM flg22 for 1 h. The results show the mean of 90-120 stomata measured from three independent experiments. Error bars represent SE of the means. Different letters denote statistical difference (ANOVA for unbalanced samples, post-hoc Tukey-Kramer test at $P<0.05$).

316 **PLC2 Associates with RBOHD**

317 Flg22-induced ROS burst is generated via activation of the plasma
318 membrane NADPH oxidase RBOHD (Nuhse et al., 2007; Zhang et al., 2007).
319 Our results show that PLC2 is required for the flg22-mediated ROS burst that
320 is generated via RBOHD activation (Fig. 5). As mentioned earlier, PLC2 is
321 localized at the plasma membrane, where also RBOHD exists in a complex
322 with FLS2 and BIK1 (Kadota et al., 2014; Li et al., 2014). To investigate
323 whether PLC2 associates with RBOHD we immunoprecipitated N-terminally
324 FLAG-tagged RBOHD (stably expressed in Arabidopsis under its own
325 promoter) using anti-FLAG affinity beads. In three independent biological
326 experiments PLC2 co-immunoprecipitated with RBOHD *in planta* (Fig. 6 and
327 Supplemental Fig. S5). PLC2 could not be immunoprecipitated in wild type
328 plants that did not express FLAG-RBOHD. Notably, the brassinosteroid
329 receptor BRI1 (used here as an unrelated plasma membrane located protein
330 control) was not detected in anti-FLAG immunoprecipitates (Fig. 6). In
331 addition, experiments in the presence of flg22 revealed that the association
332 was independent of the ligand binding to FLS2, since the same amount of

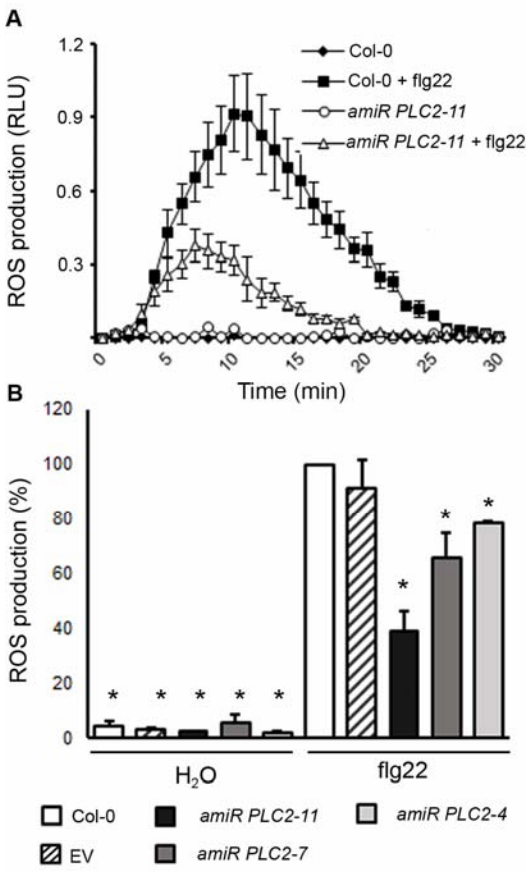

Figure 5. *PLC2*-silenced plants exhibit impaired flg22-induced oxidative burst.
Production of reactive oxygen species (ROS) was measured with a luminol-based assay in Col-0 or *amiR PLC2* plants.
A, Leaf disks from 4- to 5-week-old plants were incubated with 100 nM flg22 and the luminescence was measured 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 for unbalanced samples, Multiple Comparisons versus Control Group post-hoc Dunnett's Method at $P<0.05$).

PLC2 was immunoprecipitated in treated as in non-treated plants (Fig. 6).

DISCUSION

The participation of PI-PLCs activity in signaling after recognition of

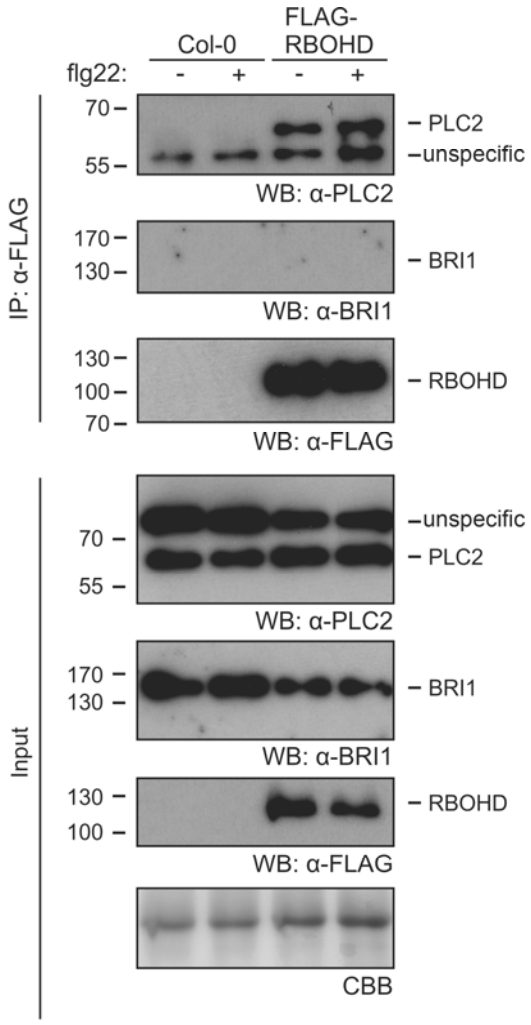

Figure 6. PLC2 associates with RBOHD.
Co-Immunoprecipitation of PLC2 and ROBHD in stable transgenic Arabidopsis seedlings (T3) expressing FLAG-RBOHD- (pRBOHD:FLAG-RBOHD) treated (+) or not (-) with 1 µM flg22 for 15 min. Total protein extracts (input) were subjected to immunoprecipitation with anti-FLAG 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.

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 and PIP_2 are most likely the substrates, and *ii)* the phosphorylated products of IP_2, IP_3 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, PDK1 and RBOHD (Farmer and Choi, 1999; Lee et al., 2001; Szczegielniak et al., 2005; Anthony et al., 2006; Zhang et al., 2009). Unfortunately, in Arabidopsis we were not able to detect *in vivo* flg22-induced PA increase by radiolabelling with ^{32}Pi on seedlings, cotyledons or leaf discs (Supplemental Fig. S6). We can envisage different plausible explanations: i) flg22 triggers a very local increase of PA in specialized cells or tissue, so when we measure overall *in vivo* production in organs with different tissues, the signal gets diluted; ii) PA may be rapidly produced and metabolized, and thus difficult to detect or iii) ^{32}Pi-labeling conditions are not sensitive enough to distinguish small PA differences in Arabidopsis. However those undetectable changes in the levels of signaling lipids can have strong effects on plant responses. We cannot exclude that IP_2 and IP_3 can be very rapidly phosphorylated to IP_6 and thus probably increase Ca^{2+} in the cytosol, or also participate in auxin signaling via TIR1 and COI1-JA signaling 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 remains to be demonstrated.

PLC2 is Required for Full Activation of Plant Immunity

In tomato, using 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 NLR-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

375 production, and plant susceptibility against *Botrytis cinerea* (Gonorazky et al.,
376 2014; Gonorazky et al., 2016).
377 Here, we assayed three different strains of the hemibiotrophic
378 pathogen *Pst* DC3000: the virulent wild type strain to study the role of PLC2 in
379 effector-triggered susceptibility (ETS), the avirulent strain expressing
380 AvrRpm1 to determine if PLC2 played a role during ETI, and *hrcC*$^{-}$ strain
381 mutated in the type III secretion system to investigate if PLC2 was required for
382 MTI. *PLC2*-silenced plants showed increased susceptibility to *Pst* DC3000
383 *hrcC*$^{-}$ but not to the virulent or avirulent strains, suggesting that this protein is
384 mostly involved in MTI. When the *hrcC*$^{-}$ strain was infiltrated, no differences in
385 susceptibility were found between *PLC2*-silenced and wild type plants,
386 indicating that the differences found when the strain was sprayed could be
387 explain by the role of the stomata closure during infection. In order to further
388 corroborate that PLC2 does not affect MTI final output when the bacteria are
389 syringe-infiltrated into the apoplast, we studied whether flg22 leads to
390 induced-plant resistance in *PLC2*-silenced plants. Supplemental figure S7
391 shows that treatment of plants with flg22 triggers resistance to syringe-
392 infiltrated *Pst* DC3000 in wild type as well as in *PLC2*-silenced plants. These
393 results suggests that PLC2 controls stomatal pre-invasive but not post-
394 invasive immunity. Accordingly, *PLC2*-silenced plants are impaired in stomatal
395 closure upon flg22 treatment. These results suggest a role of PLC2 in
396 stomatal immunity.
397 Further studies indicated that also basal resistance against the non-
398 adapted pathogen pea powdery mildew, *Erysiphe pisi* was impaired in *PLC2*-
399 silenced plants. In this non-host interaction with Arabidopsis, the first line of
400 defense is the recognition of MAMPs, such as chitin by the LYK5-CERK1
401 receptor complex, triggering a series of immune responses including MAPKs
402 activation and ROS burst mediated by NADPH oxidases (Kuhn et al., 2016).
403
404 **PLC2 Participates in RBOHD-dependent Plant Defense Responses**
405 Callose accumulation is an MTI response that requires RBOHD (Luna
406 et al., 2011), and flg22-induced callose deposition is reduced on *PLC2*-
407 silenced plants. Another RBOHD-dependent response is flg22-induced
408 stomatal closure (Mersmann et al., 2010; Kadota et al., 2014; Li et al., 2014).

409 The restriction of microbial entry by stomatal closure is one of the first MTI
410 responses (Melotto et al., 2006). *fls2* mutant plants are impaired in stomatal
411 closure in response to flg22 and show increased susceptibility to *Pst* DC3000
412 when sprayed onto the leaf surface but not when infiltrated into leaves
413 (Gomez-Gomez et al., 2001; Zipfel et al., 2004; Chinchilla et al., 2006; Zeng
414 and He, 2010). Importantly, the action of ABA on stomatal immunity seems to
415 occur downstream or independently of the PRR complex because *fls2*, *bik1*,
416 and *rbohD* mutants exhibit wild type stomatal closure in response to
417 exogenous ABA (Macho et al., 2012; Kadota et al., 2014). Accordingly, we
418 demonstrate that PLC2 is involved in flg22-induced stomatal closure, whereas
419 ABA-dependent stomatal closure is unaffected. These results show that PLC2
420 is involved in callose deposition and stomatal closure following flg22
421 perception in Arabidopsis plants.
422
423 **PLC2 Acts Upstream of RBOHD Activation**
424 We have demonstrated that PLC2 is required for full activation of flg22-
425 induced ROS production. ROS production upon flg22 perception in
426 Arabidopsis is dependent on the NADPH oxidase RBOHD (Kadota et al.,
427 2014; Li et al., 2014). Post-translational regulation of RBOHD activation
428 involves Ca^{2+} via direct binding to EF hand motifs, phosphorylation by Ca^{2+}-
429 dependent (i.e. CPKs) and -independent protein kinases (i.e. BIK1) (Logan et
430 al., 1997; Boudsocq et al., 2010; Kadota et al., 2014; Li et al., 2014; Kadota et
431 al., 2015). By using PLC inhibitors, PLC activation has been suggested to be
432 required for ROS production upon xylanase, chitosan and Avr4 (de Jong et
433 al., 2004; Laxalt et al., 2007; Raho et al., 2011). PA has also been shown to
434 interact directly with RBOHD and enhance ROS production (Zhang et al.,
435 2009). Upon cryptogein treatments of tobacco BY2 cells, PLC and DGK
436 inhibitors or silencing of the cluster III of the tobacco DGK family resulted in
437 reduced PA and ROS production (Cacas et al., 2016). Therefore, it could be
438 speculated that the second messengers derived from PLC2 activation, PA
439 and/or increase cytosolic Ca^{2+} via i.e. IP_6, could positively regulate the
440 NADPH oxidase activity, since *PLC2*-silenced plants showed reduced ROS
441 production in response to flg22.
442 Flg22 activates MAPK signaling pathways leading to the induction of

443 immune gene expression. MPK3, MPK4/11 and MPK6 activation act
444 independently of the RBOHD-mediated ROS burst (Zhang et al., 2012; Xu et
445 al., 2014). Flg22-treated *PLC2*-silenced plants showed similar levels of MAPK
446 activation and immune gene expression as the wild type, suggesting that
447 MAPK signaling is independent of PLC2.
448 RBOHD exists in a complex with the receptor kinase FLS2, interacting
449 directly with BIK1 (Kadota et al., 2014; Li et al., 2014). Our results show that
450 PLC2 is associated with RBOHD, and this association is ligand-independent.
451 In Arabidopsis, the receptor complex FLS2-BAK1 perceives flg22 and
452 activates the downstream kinases BIK1 and PBL1 by phosphorylation, which
453 induce an influx of extracellular Ca^{2+} in the cytosol (Li et al., 2014; Ranf et al.,
454 2014). PLC2 contains a Ca^{2+}-dependent phospholipid-binding domain (C2)
455 and EF-hand domains (Otterhag et al., 2001). In addition, PLC2 is localized to
456 the plasma membrane and is rapidly phosphorylated upon flg22 treatment
457 (Niittyla et al., 2007; Nuhse et al., 2007). One can envisage that PLC2 is part
458 of the FLS2, BIK1, RBOHD complex, and that BIK1 or another component of
459 the receptor complex phosphorylates PLC2 leading to the generation of
460 second messengers like PA or IP_6, which in turn, positively regulate or are
461 required to sustain/reinforce the activity of RBOHD.
462
463 **Other Roles of PLC2**
464 Seeking for knock-out mutant plants for *PLC2* we could not recover
465 homozygous mutants, and therefore decided to silence *PLC2*. Nevertheless,
466 further characterization showed that this gene is expressed during early
467 megagametogenesis and in the embryo after fertilization being required for
468 both reproductive- and embryo development, presumably by controlling
469 mitosis and/or the formation of cell-division planes (Li et al., 2015; Di Fino et
470 al., 2017). The fact that we were able to obtain *PLC2*-silenced lines could be
471 related with *i)* low expression levels of the 35S::*amiR-PLC2* in the
472 reproductive organs and embryos or *ii)* the silencing not being fully effective,
473 with low levels of PLC2 in the gametophyte and/or embryos being sufficient
474 for correct development. The requirement of PLC2 during development
475 suggests that the mechanisms for PLC2 activation and/or its downstream
476 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 SERKs. Nonetheless, whether PLC2 is specific for FLS2-BAK1-BIK1 receptor-complex or participates in the signaling of other receptor-complexes, like LYK5-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 full activation of 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).

Bacterial Infection Assays

6-8 week-old plants were used for bacterial inoculations. Strains Pseudomonas *syringae* pv. *tomato* (*Pst*) DC3000 (virulent), *Pst* DC3000

AvrRpm1 (avirulent) and *Pst* DC3000 *hrcC*$^-$ mutant were maintained on solid Pseudomonas agar F (King's B medium, Biolife, Italy) supplemented with 50 mg L^{-1} rifampicin (for *Pst* DC3000 *hrcC*$^-$) or plus 50 mg L^{-1} kanamycin (for virulent and avirulent *Pst* strains). Bacterial suspensions of virulent and avirulent strains were inoculated into the abaxial side of leaves with a needleless syringe (10 mM $MgCl_2$; OD_{600}=0.00002). The bacteria were extracted at 1 or 3 days post-infiltration and the number of colony forming units (CFU) was determined after serial dilution and plating as described (Johansson et al., 2014). The strain *Pst* DC3000 *hrcC*$^-$ was inoculated either by spraying ($MgCl_2$ 10mM; OD_{600}=0.1; Silwet 0.02 %) or by infiltration with a needless syringe ($MgCl_2$ 10mM; OD_{600}=0.0001). Following spray-inoculations, plants were kept covered with a transparent lid for 6 hours. For spray- and syringe- inoculated plants samples were taken at day 0, 1 or 3 post-inoculation with a cork borer N° 1. Bacterial growth was evaluated as previously described (Katagiri et al., 2002). Data shown in Figure 2A, C, D and E corresponds to one experiment representative of four independent biological assays performed. Again, in Figure 2B, one of three biologically replicates that showed similar results is depicted. For all cases, each value in the graphs represents the average ± SD of three technical replicates (3 pools of 4 leaf-discs collected from 4 independent plants at each time-point) that were grinded, diluted and plated separately.

Ion Leakage

Ion leakage was measured in leaf discs after infiltration of *Pst* DC3000 AvrRpm1 (OD_{600}=0.1) as well in leaf discs of Col-0 plants expressing the coding sequence of *P. syringae AvrRpm1*, under the control of a Dex-inducible promoter (Andersson et al., 2006) as described in (Johansson et al., 2014). Leaf discs from 4- to 5-week-old empty vector or *PLC2*-silenced plants in wild type or *rpm1-3* background were placed in deionized water during 1-2 hours, then washed and transferred to six well cultivation plates containing 10 mL water (four discs per well). For the Dex inducible *AvrRpm1* plants, leaf discs were treated with 20 µM dexamethasone. The release of electrolytes from the leaf discs was determined every 30 min for 5 hrs using a conductivity

meter (Orion, Thermo scientific) as described in (Johansson et al., 2014). The experiment was repeated twice.

Fungal Inoculation and Scoring of Fungal Penetration

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

MAPK Activation

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

Callose Deposition

Leaves from 4- to 5-week-old plants were fully infiltrated with 1 µM flg22 or water for 18 h. Leaves were then incubated in 96% EtOH until all tissue was transparent, washed in 0.07 M phosphate buffer (pH =9), and incubated for 2 hours in 0.07 M phosphate buffer containing 0.01% aniline-blue. Observations were performed with an epifluorescence microscope with UV filter (Excitation 365/10 ηm, emission 460/50 ηm). Number of callose dots was calculated using ImageJ software (Schneider et al., 2012). Six different microscopic areas (1 mm^2) were taken per leaf. Two different leaves per

individual were analyzed. Three independent plants were analyzed per line per experiment. Three independent experiments were performed.

Epidermal Peel Preparation and Stomatal Aperture Measurement

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-well 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, 5 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).

AtRBOHD (At5G47910)

AtBRI1 (At4g39400)

Supplemental Data

Table S1. Primer sequences
Figure S1. PLC2 silencing specificity and phenotype of silenced plants.
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.
Figure S5. PLC2 associates with RBOHD.
Figure S6. Effect of flg22 on the formation of PA
Figure S7. Flg22-induced resistance.

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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- to 5-week-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 (ANOVA for unbalanced samples, post-hoc Tukey-Krumer test at $P<0.001$).
B, PLC2 protein levels were analyzed by western blot using anti-AtPLC2 antibody in leaves of 4- to 5-week-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 cm2 of leaf extracts was determined. Data from three biological replicates each with three technical replicates were averaged (n=9) and ANOVA performed considering each replicate as a factor. Error bars represents standard error of the mean. Different letters indicate significant difference between genotypes (ANOVA $P<0.001$, post-hoc Tukey test).
B, *PLC2*-silenced plants do not show increased susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 *hrcC-* mutant when the bacteria is syringe-inoculated into the leaf apoplast. Bacterial suspension was inoculated at $OD_{600}=0.0001$ and the number of colony forming units (CFU) per cm2 of leaf extracts was determined. Data from three biological replicates each with three technical replicates were averaged (n=9) and ANOVA performed considering each replicate as a factor. No significant differences were observed between genotypes. Error bars represent standard error.
C and D, PLC2-silenced lines showed no differences in susceptibility to virulent (C) and avirulent (D) *Pseudomonas syringae* pv. *tomato* DC3000 infections. *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. No significant differences were observed regarding EV control according to t-test ($P<0.05$).
E, *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 SE of the mean. Different letters indicate significant difference (multiple comparison using one-way ANOVA, post-hoc Tukey's test at $P<0.05$). 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. Six different microscopic areas (1 mm^2) were taken per leaf. Two different leafs per individual was analyzed. Three independent plants were analyzed per line per experiment. Three independent experiments were performed. Error bars represent standard error of the mean. Different letters indicate significant difference (ANOVA for unbalanced samples, post-hoc Tukey-Kramer test at $P<0.001$).

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 (ANOVA for unbalanced samples, post-hoc Tukey-Kramer test at $P<0.05$).

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 for unbalanced samples, Multiple Comparisons versus Control Group post-hoc Dunnett's Method at $P<0.05$).

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
A, *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 where asterisks (*) indicate significant differences regarding Col-0 according to t-test (P<0.01).
B, Growth phenotype of 5-week-old *amiR PLC2* silenced 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 OD_{600}=0.1.
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 microRNA in AvrRpm1/Col-0 or AvrRpm1/*rpm1* plants. Total RNA was isolated from leaves of 4- to 5-week-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. Different letters indicate significant difference (multiple comparison using one-way ANOVA, post-hoc Tukey's test at P<0.05).

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.

Figure S5. PLC2 associates with RBOHD

A and B Independent experiments of 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. Coomassie brilliant blue (CBB).

Figure S6. Effect of flg22 on the formation of PA in $^{32}P_i$-prelabeled Arabidopsis seedling (A), cotyledon (B) or leaf disc (C). After treatment, lipids were extracted, separated by EtAc to resolve PA from the rest of the phospholipids, and quantified by Phosphoimaging. Quantification of PA levels as percentage of total ^{32}P-incorporated into all phospholipids according to Munnik and Zarza, 2013.

A. Five-days-old seedlings were prelabelled O/N (~16 hrs) in $^{32}P_i$ and the next day re-incubated with another fresh dose of $^{32}P_i$ to boost the ATP pools for 30 min after which seedlings were treated with different concentrations of flg22 for 30 min. Each sample is composed of three seedlings with the experiment performed in duplo. The experiment is representative for at least four independent experiments.

B. Seven-days-old cotyledon were prelabelled O/N (~16 hrs) in $^{32}P_i$ and the next day treated with 500 nM flg22 for the time indicated. Each sample is composed of six cotyledon with the experiment performed in triplo. The experiment is representative of three independent experiments.

C. Leaf discs were excised from fully expanded leaves and prelabelled O/N (~16 hrs) in $^{32}P_i$ and the next day treated with 500 nM flg22 for the time indicated. Each sample is composed of one disc and the experiment was performed in triplo. The experiment is representative for at least three independent experiments.

Figure S7. *PLC2* silenced lines shows no differences in flg22-induced resistance. 5 weeks old Arabidopsis plants wild type (Col-0) empty vector (EV) and PLC2 silenced lines (amiR) were pre-infiltrated (24 h) with H_2O or flg22 (1 µM) and then inoculated by needleless-syringe with a bacterial suspension of *Pseudomona syringae* pv. *tomato* DC3000 (OD_{600}=0.0002). The number of colony forming units (CFU) per cm^2 of leaf extracts was determined after 2 days post infection according to Zipfel et al. (2004). A representative experiment of 4 biological replicated is depicted.

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