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Phospholipase C2 Affects MAMP-Triggered Immunity by Modulating ROS Production

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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 (Arabidopsis thaliana) PI-PLC gene family is composed of nine members. Previous studies suggested a role for PLC2 in MAMP-triggered immunity, 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) and to the nonadapted pea (Pisum sativum) powdery mildew Erysiphe pisi. However, PLC2-silenced plants display normal susceptibility to virulent (Pst DC3000) and avirulent (Pst DC3000 AvrRPM1) P. syringae strains, conserving typical hypersensitive response features. In response to flg22, PLC2-silenced plants maintain wild-type mitogen-activated protein kinase activation and PHII, 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-deficient plants, suggesting an effect of PLC2 in a branch of MAMP-triggered immunity and nonhost resistance that involves early ROS-regulated processes. Consistently, PLC2 associates with the NADPH oxidase RBOHD, suggesting its potential regulation by PLC2.

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 (MAMPs) by cell surface pattern recognition receptors (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. Eventually, microbial effectors can become detected by intracellular nucleotide-binding Leu-rich repeat (NLR) proteins, triggering a second line of defense called effector-triggered immunity (ETI; Jones and Dangl, 2006). After the recognition of MAMPs, a series of rapid responses are initiated, including an increase in cytosolic Ca2+, generation of apoplastic reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs) and Ca2+-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 the recognition of bacterial flagellin (or the derived peptide flg22) by the Arabidopsis (Arabidopsis thaliana) Leu-rich repeat receptor kinase (LRR-RK) FLAGELLIN SENSING2 (FLS2). By FLS2, the flagellin elicitor is perceived and cleaved to generate flagellin peptides. These peptides activate FLS2 and other NLRs, which are then recruited to the plasma membrane to initiate ETI (Wang et al., 2014). ROS are generated in response to MAMP perception and play a central role in activating the immune response (Stam et al., 1999; Yoo et al., 2007). Upon activation, ROS mediate multiple signaling events, including the activation of mitogen-activated protein kinases (MAPKs), which are key components of the plant immune response (Van der Hoorn et al., 2000).

The Arabidopsis plasmid DNA (PDD)-sensitive (PDS) gene family is composed of 11 members. Several PDS members are rapidly phosphorylated upon treatment with the bacterial MAMPs flg22. PDS phosphorylation is dependent on the activation of protein kinase A (PKA) (Stam et al., 1999). The Arabidopsis PKA (PKA) family is composed of 11 members, and they have been shown to be involved in different cellular processes, including growth and development (Tanaka et al., 2000). Ongoing projects in our laboratory are focused on characterizing the role of PKA in plant immunity and on identifying the regulatory mechanisms that control PKA phosphorylation in response to MAMPs.

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(FLS2; Felix et al., 1999; Gómez-Gómez and Boller, 2000; Sun et al., 2013). Upon ligand recognition, FLS2 forms a complex with the LRR–RR BRASSINOSTEROID RECEPTOR1–ASSOCIATED KINASE1 (BAK1), also known as SOMATIC EMBRYOGENESIS–RELATED KINASE3 (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 KINASE1 (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 the 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 and phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate water-soluble inositol bisphosphate (IP₂) and phosphatidylinositol (4,5) bisphosphate (PI(4,5)P₂). PI-PLC catalyzes the hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate water-soluble inositol bisphosphate (IP₂) and phosphatidylinositol (4,5) bisphosphate (PI(4,5)P₂). Plant Physiol. Vol. 175, 2017 971

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 quantitative PCR (qPCR) of PLC2 in leaves of T4 amiR-PLC2 homozygous plants showed that PLC2 was stably silenced (Fig. 1A). The expression of PLC7 (the closest homolog to PLC2), PLC4 (coexpressed with PLC2), and PLC1 (the second most abundant PLC; Pokotylo et al., 2014) was 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 to wild-type plants under standard growth conditions (Supplemental Fig. S1B).

PLC2-Silenced Plants Are More Susceptible to the Bacterial P. syringae pv tomato DC3000 hrcC Strain and to the Nonadapted Fungus E. 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 (Pst) strain 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 (Pst 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 1 and 3 d postinoculation. PLC2-silenced plants were more susceptible to Pst DC3000 hrcC than wild-type plants (Fig. 2A). Under natural conditions, Pst enters host plants,

The Arabidopsis genome contains nine genes encoding PI-PLCs (AtPLC1–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 also is rapidly phosphorylated following flg22 recognition (Nühse et al., 2007). In this work, we analyzed the role of PLC2 in resistance to Pseudomonas syringae and Erysiphe pisi and in responses triggered upon flg22 perception. We found that PLC2 plays an important role in stomatal preinvasion immunity and nonhost resistance and that it associates with RBOHD, suggesting a potential regulation of the Arabidopsis NADPH oxidase and, consequently, of ROS-dependent processes by PLC2.
In the proliferation of this adapted pathogen was detected between both plant genotypes (Fig. 2C), indicating that PLC2 silencing does not have an effect when the virulent pathogen is infiltrated.

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 the wild type in constraining the growth of this strain (Fig. 2D), indicating that the lack of PLC2 does not affect AvrRpm1 recognition-triggered growth restriction. Moreover, the hypersensitive response (HR) cell death measured by ion leakage induced by *Pst* DC3000 AvrRpm1 was identical in wild-type and PLC2-silenced plants (Supplemental Fig. S2A). The effect of PLC2 silencing on 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 an rpm1-3 knockout background (rpm1-3; DEX::AvrRpm1/rpm1-3; Mackey et al., 2002, 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 nonsilenced 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 the entry of the nonadapted pathogen *E. pisi*, the causal agent of pea (*Pisum sativum*) powdery mildew. Arabidopsis displays nonhost resistance toward *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 the 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 with wild-type plants, indicating that PLC2 is involved in nonhost 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 (Gómez-Gómez and Boller, 2000), and studied the two distinct MAPK- and ROS-dependent branches of MTI signaling (Bigeard et al., 2015).
Figure 2. Growth of *P. syringae* and *E. pisi* in Arabidopsis PLC2-silenced plants. Wild-type (Col-0), empty vector (EV), and PLC2-silenced lines (amiR-PLC2-11 and amiR-PLC2-4) were used. A, PLC2-silenced plants are more susceptible to the *Pst DC3000*.
Flg22-induced activation of a particular MAPK 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 MAPK activation to wild-type plants (Supplemental Fig. S3). Similarly, flg22-induced expression of FRK1, PHII, 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, we analyzed flg22-induced callose deposition (Luna et al., 2011). To this end, leaves were infiltrated with flg22 and, 18 h later, stained with Aniline Blue for callose visualization. PLC2-silenced lines showed significantly less callose deposition upon flg22 treatment compared with leaves of control plants, which were either transformed with the empty vector or nontransformed wild-type plants (Fig. 3).

An earlier response of active immunity at the preinvasive 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 the full activation of stomatal ROS-dependent immune responses.

**PLC2 Is Involved in the 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. All PLC2-silenced lines showed a reduction in ROS levels in response to flg22 (40%–75% compared with control plants; Fig. 5B). Thus, our results demonstrated that PLC2 is required for the full ROS accumulation following flg22 recognition in Arabidopsis leaf discs.

**PLC2 Associates with RBOHD**

The flg22-induced ROS burst is generated via activation of the plasma membrane NADPH oxidase RBOHD (Nühse 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 is localized at the plasma membrane, where RBOHD also 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 the control of its own promoter) using anti-FLAG affinity beads. In three independent biological experiments, PLC2 coimmunoprecipitated with RBOHD in planta (Fig. 6; Supplemental Fig. S5). PLC2 could not be immunoprecipitated in wild-type

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**Figure 2. (Continued.)**

**hrC** mutant. Bacteria were inoculated by spray at OD<sub>600</sub> = 0.1, and the number of colony-forming units (CFU) per cm<sup>2</sup> of leaf extracts was determined. Data from three biological replicates each with three technical replicates were averaged (n = 9), and ANOVA was performed considering each replicate as a factor. Error bars represent se. Different letters indicate significant differences between genotypes (ANOVA, P < 0.001, posthoc Tukey’s test). dpi, Days postinoculation. B, PLC2-silenced plants do not show increased susceptibility to the *Pst DC3000 hrC* mutant when the bacteria are syringe inoculated into the leaf apoplast. Bacterial suspension was inoculated at OD<sub>600</sub> = 0.0001, and the number of CFU per cm<sup>2</sup> of leaf extracts was determined. Data from three biological replicates each with three technical replicates were averaged (n = 9), and ANOVA was performed considering each replicate as a factor. No significant differences were observed between genotypes. Error bars represent se. C and D, PLC2-silenced lines showed no differences in susceptibility to virulent (C) and avirulent (D) *Pst DC3000* infections. *Pst DC3000* (virulent) and *Pst DC3000*:AvrRpm1 (avirulent) were inoculated by infiltration at OD<sub>600</sub> = 0.0002, and CFU per cm<sup>2</sup> of leaf was calculated. A representative experiment of four biological replicates is depicted. No significant differences were observed regarding the EV control according to Student’s test (P > 0.05). E, PLC2-silenced plants are more susceptible to the nonadapted pea powdery mildew *E. pisi*. The penetration rate at 3 d after inoculation was calculated as the percentage of successful penetration of at least 50 germinated spores on three independent leaves. Error bars represent se. Different letters indicate significant differences (multiple comparison using one-way ANOVA, posthoc Tukey’s test at P < 0.05). One representative experiment of four biologically independent replicates is depicted.
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 as in nontreated plants (Fig. 6).

DISCUSSION

The participation of PI-PLC activity in signaling after the 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 described previously (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 (1) phosphatidylinositol 4-phosphate and PIP$_2$ are most likely the substrates and (2) the phosphorylated products of IP$_2$, IP$_3$, and DAG, including various inositol polyphosphates, PA, and DAG pyrophosphate, have roles 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 radiolabeling with $^{32}$P$_i$ on seedlings, cotyledons, or leaf discs (Supplemental Fig. S6). We can envisage different plausible explanations: (1) flg22 triggers a very local increase of PA in specialized cells or tissues, so when we measure overall in vivo production in organs with different tissues, the signal gets diluted; (2) PA may be rapidly produced and metabolized and, thus, difficult to detect; or (3) $^{32}$P$_i$ 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$_3$ and IP$_2$ can be very rapidly phosphorylated to IP$_6$ and, thus, probably increase Ca$^{2+}$ in the cytosol or also participate in immune signaling via TIR1 and CO1-JA signaling, among others (Xue et al., 2009; Munnik, 2014; Williams et al., 2015). Indeed, mutants with altered inositol polyphosphate 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 (Solanum lycopersicum), using virus-induced-gene silencing of different PLCs, SlPLC4 was found to be involved specifically 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 in ETI responses. Similarly, overexpression of SlPLC3 enhanced the Cf-4/Avr4-triggered HR (Abd-El-Haliem et al., 2016). Further studies in tomato showed that SlPLC2 is required for...
xylanase-induced gene expression, ROS production, and plant susceptibility against *Botrytis cinerea* (Gonorazky et al., 2014, 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, the avirulent strain expressing AvrRpm1 to determine if PLC2 played a role during ETI, and the *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 strain, suggesting that this protein is mostly involved in MTI. When the *hrcC* strain was infiltrated, no differences in susceptibility were found between PLC2-silenced and wild-type plants, indicating that the differences found

![Figure 5](image.png)

**Figure 5.** PLC2-silenced plants exhibit impaired flg22-induced oxidative burst. The production of ROS was measured with a luminol-based assay in Col-0 or amiR-PLC2 plants. A, Leaf discs 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 referred to the Col-0 wild type treated with flg22 as 100%. Averages of four independent experiments are shown. Error bars represent S.E. Asterisks indicate statistically significant differences compared with the flg22-treated Col-0 plant (ANOVA for unbalanced samples, multiple comparisons versus control group post hoc Dunnett's method at *P* < 0.05). EV, Empty vector.

![Figure 6](image.png)

**Figure 6.** PLC2 associates with RBOHD. Coimmunoprecipitation of PLC2 and RBOHD was performed 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 (IP) with anti-FLAG beads followed by immunoblot analysis with anti-FLAG (α-FLAG) and anti-PLC2 (α-PLC2) antibodies as indicated. Protein extracts of Col-0 plants were used as negative controls. Anti-BRI1 (α-BRI1) antibodies were used as plasma membrane protein not associated with RBOHD. CBB, Coomassie Brilliant Blue. These experiments were performed three times with similar results.
when the strain was sprayed could be explained by the role of stomata closure during infection. In order to further corroborate that PLC2 does not affect MTI final output when the bacteria are syringe infiltrated into the apoplast, we studied whether flg22 leads to induced plant resistance in PLC2-silenced plants. Supplemental Figure S7 shows that treatment of plants with flg22 triggers resistance to syringe-infiltrated *Pst* DC3000 in wild-type as well as PLC2-silenced plants. These results suggest that PLC2 controls stomatal preinvasive but not postinvasive immunity. Accordingly, PLC2-silenced plants are impaired in stomatal closure upon flg22 treatment. These results suggest a role of PLC2 in stomatal immunity.

Further studies also indicated that basal resistance against the nonadapted pathogen pea powdery mildew, *E. pisi*, was impaired in PLC2-silenced plants. In this nonhost interaction with Arabidopsis, the first line of defense is the recognition of MAMPs, such as chitin by the LYK5-CERK1 receptor complex, triggering a series of immune responses including MAPK 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 in PLC2-silenced plants. Another RBOHD-dependent response is 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 (Gómez-Gómez 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 of the pattern recognition receptor 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 involved in flg22-induced stomatal closure, whereas ABA-dependent stomatal closure is unaffected. These results show that PLC2 is involved in 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 the full activation of 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). Posttranslational regulation of RBOHD activation involves Ca\(^{2+}\) via direct binding to ER hand motifs and phosphorylation by Ca\(^{2+}\)-dependent (i.e. CPKs) and Ca\(^{2+}\)-independent (i.e. BIK1) protein kinases (Logan et al., 1997; Boudsocq et al., 2010; Kadota et al., 2014, 2015; Li et al., 2014). By using PLC inhibitors, PLC activation has been suggested to be required for ROS production upon xylanase, chitosan, and Avr4 (de Jong et al., 2004; Laxalt et al., 2007; Rahoo et al., 2011). PA also has been shown to interact directly with RBOHD and enhance ROS production (Zhang et al., 2009). Upon cryptogein treatment of tobacco (*Nicotiana tabacum*) 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., 2017). Therefore, it could be speculated that the second messengers derived from PLC2 activation, PA and/or increased cytosolic Ca\(^{2+}\) via IP\(_6\), for example, 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 signaling is independent of PLC2.

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 that this association is ligand independent. In Arabidopsis, the receptor complex FLS2-BAK1 perceives flg22 and activates the downstream kinases BIK1 and PBL1 by phosphorylation, 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, PLC2 is localized to the plasma membrane and is rapidly phosphorylated upon flg22 treatment (Niittylä et al., 2007; Nöhse et al., 2007). One can envisage that PLC2 is part of the FLS2, BIK1, and RBOHD complex and that BIK1 or another component of the receptor complex phosphatases 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 knockout 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., 2017). The fact that we were able to obtain PLC2-silenced lines could be related to (1) low expression levels of the 35S::amiR-PLC2 in the reproductive organs and embryos or (2) the silencing not being fully effective, with low levels of PLC2 in the gametophyte and/or embryos being sufficient for correct development. The requirement for PLC2 during development
suggests that the mechanisms for PLC2 activation and/or its downstream targets, such as RBOHD, could be similar in both the sporophyte during fig22 perception and the gametophyte during development. Arabidopsis has five SERK 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; Albrecht et al., 2005; Colcombet et al., 2005; Kwaaitaal et al., 2005). We speculate that PLC2 plays a role in gametogenesis and embryo development, probably by signaling downstream of LRR-RKs like SERKs. Nonetheless, whether PLC2 is specific for the 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 the recognition of different MAMPs was described earlier. The Arabidopsis genome contains nine PI-PLC genes; however, until this work, it was not known which one was specifically linked to the plant defense response. We here present genetic evidence that PLC2 participates in MTI. PLC2 is required for the full activation of ROS production and ROS-dependent responses elicited by the MAMP fig22. 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 (Arabidopsis thaliana Col-0) transformed with an artificial microRNA targeting specifically PLC2 (amiR-PLC2) under the control of the cauliflower mosaic virus 35S promoter or with the empty vector were germinated in soil (soil:vermiculite:perlite, 3:1:1) and kept at 4°C for 2 d. Then, they were grown at 25°C using a 16-h-light/8-h-dark photoperiod. In the case of infections (bacterial or viral), plants were inoculated either by spraying with Pseudomonas syringae pv tomato AvrRpm1 or with the empty vector were germinated in soil (soil:vermiculite:perlite, 3:1:1) and kept at 4°C for 2 d. Then, they were grown at 25°C using a 16-h-light/8-h-dark photoperiod. In the case of infections (bacterial and fungal), plants were grown at 22°C in an 8-h-light/16-h-dark photoperiod.

For ion leakage experiments, Col-0 or rpm1-3 mutant plants transformed with the coding sequence for Pseudomonas syringae pv tomato AvrRpm1 under the control of a dexamethasone-inducible promoter (Aoyama and Chua, 1997) were grown as described at 22°C in an 8-h-light/16-h-dark cycle. Both backgrounds were transformed with amiR-PLC2 under the control of the Ubiquitin10 promoter (pUBQ10).

amiR-PLC2-Silencing Constructs

AtPLC2 (At3g08510) silencing was performed using a specific artificial microRNA designed with WMD3 Web microRNA designer (http://wmd3.weigelworld.org). Arabidopsis mirR319 was used as a template, and the cloning strategy was according to Ossowski et al. (2008).

Primers for Artificial MicroRNA Cloning

The following primers were used: PLC2 miR-s, 5′-gaTTAAAACACTCAG-TAATTCGCCATTGGTTATTt-3′; PLC2 miR-a, 5′-gcGCGAATTCAGTCTGTTGTAATcagagaaactaaggta-3′; PLC2 miR-s, 5′-gaCACAAATTGAGATCTGTTTAGTCAAgtctattcatgctatctctc-3′; and PLC2 miR-a, 5′-gaATAAACACTCAG-TAATTCGCCATTGGTTATTt-3′. Uppercase letters denote APLC2-targeted sites. The amiR-PLC2 was cloned into pCHF3 vector (kanamycin resistance in plants) driven by the cauliflower mosaic virus 35S promoter or into pUBQ10 designation vector driven by the Ubiquitin10 promoter (Basta resistance in plants).

Arabidopsis Transformation

Arabidopsis plants were transformed using the floral dip method (Zhang et al., 2006). T1 plants were sown on Murashige and Skoog (MS) agar (MS medium with Gamborg’s vitamins and 1% agar) plates with kanamycin (50 μg·mL⁻¹ for pCHF3:amiR-PLC2) or BASTA (10 μg·mL⁻¹ for pUBQ10:amiR-PLC2). After 2 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 10- to 14-d-old seedlings or leaves from 4- to 5-week-old plants using the Trizol method according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized on 1 μg of total RNA by MMLV reverse transcriptase from Promega 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 was used for qPCR. The Fast Universal SYBR Green Master Mix from Roche was employed, using a Step-One Real-Time PCR machine from Applied Biosystems. The standard amplification program was used. The expression levels of the gene of interest were normalized to those of the constitutive AC2 (At3g18780) gene by subtracting the cycle threshold value of AC2 from the cycle threshold 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

Polypeptide antibodies were prepared as described (Otterhag et al., 2001). A peptide, KDLGDEEVWGREVPSFIQR, corresponding to residues 266 to 284 of AtPLC2 was synthesized. One rabbit was immunized at 2-week intervals, 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-Aldrich protease inhibitor cocktail) was added to an equal volume of 4- to 5-week-old ground leaf tissue, mixed, and centrifuged for 10 min at 10,000g. Protein concentration in the supernatant was determined. Samples were loaded onto a 10% SDS-polyacrylamide gel, blotted onto nitrocellulose membranes, and stained with Ponceau S for a loading control. Membranes were incubated overnight in phosphate-buffered saline plus Tween 20 containing polyclonal anti-PLC2 antibody (1:2,000). The blot was washed three times with phosphate-buffered saline plus Tween 20 and revealed using a secondary anti-rabbit IgG antibody coupled to alkaline phosphatase according to the manufacturer’s instructions (Sigma-Aldrich).

Bacterial Infection Assays

Six- to 8-week-old plants were used for bacterial inoculations. Strains Pst DC3000 (virulent), Pst DC3000 AvrRpm1 (avirulent), and Pst DC3000 hrcC⁻ mutant were maintained on solid Pseudomonas agar F (King’s B medium; Bioline) 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 MgCl₂, OD₆₀₀ = 0.00002). The bacteria were extracted at 1 or 3 d postinoculation with a needleless syringe (10 mM MgCl₂, OD₆₀₀ = 0.1; 0.02% Silwet). Bacterial growth was evaluated as described previously (D’Ambrosio et al., 2017). Bacterial inoculations were performed in Gamborg’s B5 medium containing 5% Suc and 2% agar at 60°C. Bacterial suspensions were adjusted to OD₆₀₀ = 0.0002. 60°C. Bacterial suspensions were adjusted to OD₆₀₀ = 0.0002. The bacteria were extracted at 1 or 3 d postinoculation with a needleless syringe (10 mM MgCl₂, OD₆₀₀ = 0.1; 0.02% Silwet) or by infiltration with a needleless syringe (10 mM MgCl₂, OD₆₀₀ = 0.0002). Following spray inoculations, plants were kept under a transparent lid for 6 h. For spray- and syringe-inoculated plants, samples were taken at day 0, 1, or 3 postinoculation with a number 1 cork borer. Bacterial growth was evaluated as described previously (Katagiri et al., 2002). Data shown in Figure 2, A, C, D, and E, correspond to one experiment representative of four independent biological assays performed. As a result, in Figure 2B, one of three biological replicates that showed similar results is depicted. For all cases, each value in the graphs represents the average ± SD of three technical replicates (three pools of four leaf discs collected from four independent plants at each time point) that were ground, diluted, and plated separately.
Ion Leakage

Ion leakage was measured in leaf discs after infiltration of Pst DC3000 AvrRpm1 (OD600 = 0.1), as well as in leaf discs of Col-0 plants expressing the coding sequence of P. syringae AvrRpm1, under the control of a dexamethasone-inducible promoter (Anderson et al., 2006) as described (Johansson et al., 2014). Leaf discs from 4- to 5-week-old empty vector or PLC2-silenced plants in the wild-type or rpm1-3 background were placed in deionized water during 1 to 2 h and then washed and transferred to six-well cultivation plates containing 10 mL of water (four discs per well). For the dexamethasone-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 h using a conductivity meter (Orion; Thermo Scientific) as described (Johansson et al., 2014). The experiment was repeated twice.

Fungal Inoculation and Scoring of Fungal Penetration

The nonhost powdery mildew fungus *Erysiphe pisi* (isolate CO-01) was propagated on pea (*Pisum sativum* ‘Kelvedon Wonder’) plants. Inoculations were carried out by powdering spores on leaves of 4-week-old Arabidopsis wild-type and PLC2-silenced plants. At 3 d postinoculation, leaves were stained with Trypan Blue as described (Koch and Slusarenko, 1990). The penetration rate after inoculation was calculated as the percentage of successful penetration attempts (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 four times.

MAPK Activation

MAPK assays were performed on six 2-week-old seedlings grown in liquid MS medium (including vitamins; Duchefa) and 1% Suc. Seedlings were elicited by powdering spores on leaves of 4-week-old Arabidopsis wild-type and PLC2-silenced plants. At 3 d postinoculation, leaves were stained with Trypan Blue as described (Koch and Slusarenko, 1990). The penetration rate after inoculation was calculated as the percentage of successful penetration attempts (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 four times.

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% ethanol until all tissue was transparent, washed in 0.07 M phosphate buffer (pH 9), and incubated for 2 h in 0.07 M phosphate buffer containing 0.01% Aniline Blue. Observations were performed with an epifluorescence microscope with UV filter (excitation, 365/10 nm; emission, 460/50 nm). The number of callose dots was calculated using ImageJ software (Schneider et al., 2012). Six different microscopic areas (1 mm²) 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 preincubated in opening buffer (10 mM MES, pH 6.1; MES titrated to its pKa with KOH, and 10 mM KCl) under white light at 25°C to promote stomatal opening. After 3 h of preincubation, Flg22 (1 μM) or ABA (50 μM; Sigma-Aldrich) was added to the opening buffer and incubated for 1 h. Stomatal apertures were measured from digital images taken with a Nikon Coolpix 990 camera coupled to an optical microscope (Nikon Eclipse 2000). Then, the stomatal pore width was digitally determined using the image-analysis software ImageJ. Aperture values are means of 90 to 120 stomata measured from at least three independent experiments.

ROS Detection

Leaf discs from 4- to 5-week-old plants were placed on 96-well black plates floating in 200 μL of deionized water overnight. ROS production was triggered with 100 nM Flg22 (Q-QLRTSGLRNSAKDQADGLQAA-C; Genbiotech) and its pKa with KOH, and 10 mM KCl) under white light at 25°C to promote stomatal opening. After 3 h of preincubation, Flg22 (1 μM) or ABA (50 μM; Sigma-Aldrich) was added to the opening buffer and incubated for 1 h. Stomatal apertures were measured from digital images taken with a Nikon Coolpix 990 camera coupled to an optical microscope (Nikon Eclipse 2000). Then, the stomatal pore width was digitally determined using the image-analysis software ImageJ. Aperture values are means of 90 to 120 stomata measured from at least three independent experiments.

discs for mock treatment 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 independent experiments.

Seedling Protein Extraction and Immunoprecipitation

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

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 Na3MoO4·H2O, 1% phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich), 1% (v/v) P9599 protease inhibitor cocktail (Sigma-Aldrich), 100 μM phenylmethylsulfonyl fluoride, and 1% (v/v) IGEPA CA-630 (Sigma-Aldrich). Extracts were incubated 30 min at 4°C and centrifuged for 20 min at 16,000g at 4°C. Supernatants were incubated for 1 to 2 h at 4°C with ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) and washed five 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: an-PLC2 (1:5,000), α-FLAG-HRP (Sigma-Aldrich; 1:5,000), α-rabbit-HRP (Sigma-Aldrich; 1:10,000), and anti-BRI1 (1:5,000).

Accession Numbers

Accession numbers are as follows: AHP122 (A15g08510), A1RBOHD (At1g47910), and A1BR1 (At4g94040).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. PLC2 silencing specificity and phenotypes of silenced plants.

Supplemental Figure S2. PLC2 is not involved in programmed cell death during ETI upon recognition of AvrRpm1 from *P. syringae*.

Supplemental Figure S3. PLC2 is not required for Flg22-induced MAPK activation.

Supplemental Figure S4. MAMP-activated gene expression is not deregulated in PLC2-silenced seedlings.

Supplemental Figure S5. PLC2 associates with RBOHD.

Supplemental Figure S6. Effect of Flg22 on the formation of PA.

Supplemental Figure S7. Flg22-induced resistance.

Supplemental Table S1. Primer sequences.

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Literature Cited


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