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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 (Antolín-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; 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 (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol 4-phosphate and phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) to generate water-soluble inositol bisphosphate (IP2) and phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2), 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 implicated specifically in the modulation of immune signaling components, such as MAPks and PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE1 (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 abscisic acid (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, IP3 triggers the release of Ca2+ from intracellular stores by activating a ligand-gated calcium channel at the endoplasmic reticulum. In plants, no clear homolog of the IP3-activated Ca2+ channel has been identified (Munnik and Testerink, 2009). Instead, IP2 and IP3 are further phosphorylated by inositol polyphosphate kinase (Williams et al., 2015) to generate (1) IP6, which stimulates the release of Ca2+ from intracellular stores in guard cells (Lemtiri-Chlieh et al., 2000), affects gene transcription and mRNA export, and regulates the auxin receptor TIR1 (Tan et al., 2007; Lee et al., 2015); (2) IP7, which is part of the jasmonate receptor COI1 (Sheard et al., 2010); and (3) IP7 and IP8, which are involved in plant defense (Laha et al., 2015). In addition, PIP and PIP2, 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–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 response 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.

**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*–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,
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 nonsilenced plants (Fig. 2B). These experiments indicate that PLC2 is likely involved in stomata-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 significant difference 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 HR also was 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 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...
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 PIP2 are most likely the substrates and (2) the phosphorylated products of IP2, IP3, 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 radionubelirng with 32Pi 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) 32Pi 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 IP2 and IP3 can be very rapidly phosphorylated to IP6, and, thus, probably increase Ca2+ 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 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 *SIPC3* enhanced the Cf-4/Avr4-triggered HR (Abd-El-Haliem et al., 2016). Further studies in tomato showed that SlIPC2 is required for

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**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 water as a control and incubated for 18 h, and callose deposition was measured as dots per area. 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. Error bars represent st. Different letters indicate significant differences (ANOVA for unbalanced samples, posthoc Tukey-Kramer test at P < 0.001). EV, Empty vector.

**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 water, 1 μM flg22, 50 μM ABA, or 50 μM ABA + 1 μM flg22 for 1 h. The results show means of 90 to 120 stomata measured from three independent experiments. Error bars represent st. Different letters denote statistical differences (ANOVA for unbalanced samples, posthoc Tukey-Kramer test at P < 0.05). EV, Empty vector.
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*₂ 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...
when the strain was sprayed could be explained by the role of stomatal 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 tested whether pgl22 leads to induced plant resistance in PLC2-silenced plants. Supplemental Figure S7 shows that treatment of plants with pgl22 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 pgl22 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-CKER1 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 pgl22-induced callose deposition is reduced in PLC2-silenced plants. Another RBOHD-dependent response is pgl22-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 pgl22 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 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 pgl22-induced stomatal closure, whereas ABA-dependent stomatal closure is unaffected. These results show that PLC2 is involved in callose deposition and stomatal closure following pgl22 perception in Arabidopsis plants.

PLC2 Acts Upstream of RBOHD Activation

We have demonstrated that PLC2 is required for the full activation of pgl22-induced ROS production. ROS production upon pgl22 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 EF 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; Raho 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_{3} for example, could positively regulate the NADPH oxidase activity, since PLC2-silenced plants showed reduced ROS production in response to pgl22.

Pgl22 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). Pgl22-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 pgl22 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 pgl22 treatment (Niittylä et al., 2007; Nühse 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 phosphorylates PLC2, leading to the generation of second messengers like PA or IP_{3} 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 meigametogenesis 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 has a role in gametogenesis and embryonic development, probably by signaling downstream of LRR-RKs like SERKs. Nonetheless, whether PLC2 is specifically expressed in the ovule, female gametophyte, early embryos, and vascular cells remains to be elucidated.

**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-day-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 ACT2 (At3g18780) gene by subtracting the cycle threshold value of ACT2 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**

Polyclonal antibodies were prepared as described (Ottherg et al., 2001). A peptide, KLDGEDEWVGRVPSFQKR, 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; Biolife) 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, O D₆₀₀ = 0.00002). The bacteria were extracted at 1 or 3 d postinfiltration, and the number of colony-forming units was determined after serial dilution and plating as described (Johansson et al., 2014). The strain Pst DC3000 hrcC⁻ was inoculated either by spraying (10 mM MgCl₂, OD₆₀₀ = 0.1; 0.02% Silwet) or by infiltration with a needleless syringe (10 mM MgCl₂, OD₆₀₀ = 0.0001). Following spray inoculations, plants were kept covered with 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.

**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 on Gamborg B medium (King's B medium; Biolife) supplemented with 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, O D₆₀₀ = 0.00002). The bacteria were extracted at 1 or 3 d postinfiltration, and the number of colony-forming units was determined after serial dilution and plating as described (Johansson et al., 2014). The strain Pst DC3000 hrcC⁻ was inoculated either by spraying (10 mM MgCl₂, OD₆₀₀ = 0.1; 0.02% Silwet) or by infiltration with a needleless syringe (10 mM MgCl₂, OD₆₀₀ = 0.0001). Following spray inoculations, plants were kept covered with 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.

**Primers for Artificial MicroRNA Cloning**

The following primers were used: PLC2 miR-r, 5'-gaTTAAACACTCAG-TAATTTTGGCAGTTTATGATC-3'; PLC2 miR-r, 5'-gaGGCAATACCTACTGATTTGTTAATcCagagaattc-3'; PLC2 miR-r, 5'-gaATCAGAACATTTGACTGTTTAAACTGagCggATggCTGTTA-3' and PLC2 miR-r, 5'-gaATAGACAGTACTACGATTGTTGTTTTGCTGTT-3'. Uppercase letters denote AtPLC2-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 destination vector driven by the Ubiquitin10 promoter (Basta resistance in plants).
Ion Leakage

Ion leakage was measured in leaf discs after infiltration of Pe DC3000 AvrRpm1 (OD₅₉₀ = 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 (Andersson 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 suspending 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 growing in liquid MS medium (including vitamins; Duchefa) and 1% Suc. 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 (1:5,000). 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 90% ethanol until all tissue was transparent, washed in 0.07M 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 epi-fluorescence 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 0.9], and 10 mM KCl) under white light at 25°C to promote stomatal opening. After 3 h of preincubation, flg22 (1 μM) or flg22 (1 μM) 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 200). 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 μM Flg22 (N-QRLSTGSRINSAKDO/AGLQIA-C; Gembiochem) applied together with 20 μM luminol (Sigma-Aldrich; catalog no. A8511) and 0.02 mg mL⁻¹ horseradish peroxidase (Sigma-Aldrich; catalog no. P6782). Luminescence was measured with a luminometer (Thermo Scientific Luminoskan Ascent Microplate). Each plate contained 36 leaf discs for flg22 treatment and 12 leaf 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 roboh/pRBOHD::FLAG-pRBOHD (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) IGEPL 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: α-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: AIP2LC2 (At5g08510), AIPRBOHD (At5g47910), and AABRI1 (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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