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Planta
An International Journal of Plant Biology

ISSN 0032-0935
Volume 247
Number 4

Planta (2018) 247:1001-1009
DOI 10.1007/s00425-018-2843-8

Springer

Planta (2018) 247:1001–1009
https://doi.org/10.1007/s00425-018-2843-8

ORIGINAL ARTICLE

The sesquiterpene botrydial from *Botrytis cinerea* induces phosphatidic acid production in tomato cell suspensions

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Received: 14 September 2017 / Accepted: 30 December 2017 / Published online: 16 January 2018

Abstract

Main conclusion **The phytotoxin botrydial triggers PA production in tomato cell suspensions via PLD and PLC/DGK activation. PLC/DGK-derived PA is partially required for botrydial-induced ROS generation.**

Phosphatidic acid (PA) is a phospholipid second messenger involved in the induction of plant defense responses. It is generated via two distinct enzymatic pathways, either via phospholipase D (PLD) or by the sequential action of phospholipase C and diacylglycerol kinase (PLC/DGK). Botrydial is a phytotoxic sesquiterpene generated by the necrotrophic fungus *Botrytis cinerea* that induces diverse plant defense responses, such as the production of reactive oxygen species (ROS). Here, we analyzed PA and ROS production and their interplay upon botrydial treatments, employing tomato (*Solanum lycopersicum*) cell suspensions as a model system. Botrydial induces PA production within minutes via PLD and PLC/DGK. Either inhibition of PLC or DGK diminishes ROS generation triggered by botrydial. This indicates that PLC/DGK is upstream of ROS production. In tomato, PLC is encoded by a multigene family constituted by *SlPLC1–SlPLC6* and the pseudogene *SlPLC7*. We have shown that *SlPLC2*-silenced plants have reduced susceptibility to *B. cinerea*. In this work, we studied the role of SlPLC2 on botrydial-induced PA production by silencing the expression of *SlPLC2* via a specific artificial microRNA. Upon botrydial treatments, *SlPLC2*-silenced-cell suspensions produce PA levels similar to wild-type cells. It can be concluded that PA is a novel component of the plant responses triggered by botrydial.

Keywords Defense · Necrotroph · Phospholipase · Phospholipid · Phytotoxin · Plant

Abbreviations

DGK	Diacylglycerol kinase
DPI	Diphenyleneiodonium chloride
EtAc	Ethyl acetate
MAMP	Microbe-associated molecular patterns
PA	Phosphatidic acid
PIP	Phosphatidylinositol 4-phosphosphate
PIP_2	Phosphatidylinositol 45-bisphosphate
PLC	Phospholipase C
PLD	Phopholipase D

Juan Martin D'Ambrosio and Gabriela Gonorazky contributed equally.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00425-018-2843-8) contains supplementary material, which is available to authorized users.

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Introduction

Plants are exposed to a great diversity of pathogenic microorganisms that include virus, bacteria, and fungus. According to their lifestyles, plant pathogens are divided into biotrophs, necrotrophs, and hemibiotrophs (Glazebrook 2005). Biotrophs obtain nutrients from living host tissues. Necrotrophs induce death of host cells to obtain nutrients to grow and colonize the plant. Hemibiotrophs display both biotrophic and necrotrophic style during

distinct stages of their lifecycles (Glazebrook 2005). Plants can detect the presence of pathogenic microorganisms by sensing pathogen-derived molecules. These include microbe-associated molecular patterns (MAMPs), protein effectors, and phytotoxins (Thomma et al. 2011; Mengiste 2012). MAMPs are molecules that have a conserved chemical structure that activate a first line of defense, which is termed MAMP-triggered immunity (Thomma et al. 2011). Protein effectors are pathogen-derived molecules that are specifically recognized by the corresponding plant resistance protein and induce effector-triggered immunity (Thomma et al. 2011). Phytotoxins are secreted by necrotrophic pathogens to induce cell necrosis and leakage of nutrients (Mengiste 2012). These include alkaloids, polyketides, terpenes, non-ribosomal peptides, or metabolites of mixed biosynthetic origin (Mengiste 2012). MAMPs, protein effectors, and phytotoxins activate enzymes that produce reactive oxygen species (ROS) in plants. These enzymes include NADPH oxidase and peroxidases, which generate O_2^- and H_2O_2, respectively. ROS production is one of the earliest defense events induced in the host. Downstream of ROS, other responses such as altered expression of defense-related genes, and cell death are triggered (Chisholm et al. 2006; Mengiste 2012; Wrzaczek et al. 2013).

Botrytis cinerea is a broad-host range fungal necrotroph that infects almost all vegetables and fruit crops, including solanaceous plants, and causes vast economic damage pre- and post-harvesting (Dean et al. 2012). *B. cinerea* produces ROS, phytotoxins, and cell-wall-degrading enzymes to induce necrosis of plant tissues (van Kan 2006; van Baarlen et al. 2007), and also triggers ROS production and hypersensitive response (HR) in the host, a form of programmed cell death that favors the infection process (van Kan 2006). Recently, several *B. cinerea* molecules that activate plant responses have been identified (Mengiste 2012; Wang et al. 2014b). One of them is botrydial, which is the primary phytotoxic metabolite of *B. cinerea* (Collado and Viaud 2016). Botrydial is a sesquiterpene that is produced during infection of several plant species (Deighton et al. 2001). It has been implicated in virulence, since its addition facilitates fungal penetration and plant colonization (Colmenares et al. 2002). Botrydial treatment induces chlorosis and cell collapse in a similar way to *B. cinerea* infection (Colmenares et al. 2002). Rossi et al. (2011) demonstrated that botrydial triggers ROS production, expression of defense genes, and HR in Arabidopsis plants. It was observed as well that botrydial-induced HR is modulated by host-signaling pathways mediated by the phytohormones salicylic acid and jasmonic acid. Based on these results, the authors concluded that botrydial acts not only as a phytotoxin, but also as an elicitor of plant defense responses (Rossi et al. 2011). Until now, the direct cellular targets of botrydial are not known.

Phosphatidic acid (PA) is a phospholipid molecule that plays a role as a second messenger in plant cells during the response to microbial attack. Upon pathogen perception, PA is produced within minutes by enzymatic activation of phospholipase C in concerted action with diacylglycerol kinase (PLC/DGK) or by activation of phospholipase D (PLD) (Testerink and Munnik 2011). Phosholipase C hydrolyses phosphatidylinositol 4-phosphosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4-bisphosphate (IP_2) or inositol 1,4,5-trisphosphate (IP_3), respectively. DAG is rapidly phosphorylated by DGK to generate PA (Testerink and Munnik 2011). IP_3 has been described in animals as a signal for Ca^{2+} release from internal stores. In plants, IP_2 and IP_3 can be further phosphorylated to IP_6, which might be the signal that induces Ca^{2+} release (Munnik 2014). PLD hydrolyses structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine to produce PA (Wang et al. 2014a). The accumulation of PA is one of the earliest plant responses as evidenced upon treatment with several elicitors. The MAMPs xylanase, chitotetraose, chitosan, *N*-acetylchitooligosaccharides, produced by fungi, cryptogein, from the oomycete *Phyotophtora cryptogea*, and the bacterial flagellin-derived peptide flg22, induce PA production via PLC/DGK and/or PLD (van der Luit et al. 2000; den Hartog et al. 2003; Yamaguchi et al. 2003, 2005; Bargmann et al. 2006; Laxalt et al. 2007; Raho et al. 2011; Cacas et al. 2017). The protein effector Avr4 from the fungus *Cladosporium fulvum* triggers PA generation in tobacco cell suspensions that express the resistance protein Cf4 (de Jong et al. 2004). Similar results have been obtained in Arabidopsis plants that express the resistance proteins RPM1/RPS2 treated with the protein effectors AvrRpm1 and AvrRpt2 from *Pseudomonas syringae* (Andersson et al. 2006). It has been well documented that ROS production, expression of defense-related genes, and cell death triggered upon elicitor perception are induced downstream of PLC/DGK and PLD activation (Testerink and Munnik 2011).

Plant PLCs and PLDs are encoded by gene families (Munnik 2014; Wang et al. 2014a). In the *Arabidopsis thaliana* genome, there are seven known functional *PLC* genes (*AtPLC1–AtPLC7*) (Mueller-Roeber and Pical 2002) and two pseudogenes (*AtPLC8–AtPLC9*). We have recently demonstrated that silencing of *AtPLC2* results in increased susceptibility to *P. syringae* pv. *tomato* (*Pst*) DC3000 *hrcC*$^-$ (*Pst* DC3000 *hrcC*$^-$) and to the non-adapted pea powdery mildew *Erysiphe pisi* and that AtPLC2 plays a role in flg22-induced defense responses (D'Ambrosio et al. 2017). In tomato (*Solanum lycopersicum*), there are six PLC genes (*SlPLC1–SlPLC6*) and one pseudogene (*SlPLC7*) (Abd-El-Haliem et al. 2016). It has been demonstrated that SlPLC4 is specifically involved in the induction of HR upon AVR4 perception in tomato plants (Vossen et al. 2010). Instead,

SlPLC6 is a more general component of defense signaling (Vossen et al. 2010). We have reported that silencing of *SlPLC2* resulted in reduced susceptibility of tomato plants to *B. cinerea* infection (Gonorazky et al. 2016). In the *Arabidopsis thaliana* genome, there are 12 known functional *PLD* genes (Wang et al. 2014a). The PLD isoform of *Arabidopsis thaliana*, AtPLDß1, plays a role in resistance to *B. cinerea* infection, since *AtPLDß1* knock out plants are more susceptible to this fungus (Zhao et al. 2013). Altogether, this evidence indicates that there is a differential requirement of PLC and PLD isoforms for plant defense. Nevertheless, the nature of the *B. cinerea* molecules that would trigger PLC/DGK and PLD activation has not been established.

The aim of this work was to determine whether the sesquiterpene botrydial, one of the best known phytotoxins produced by *B. cinerea*, is able to induce PA production as a required second messenger involved in ROS generation. We studied PLD and PLC/DGK activation in tomato cell suspensions upon botrydial treatment. In addition, we studied the role of SlPLC2 on botrydial-induced PA production by silencing the expression of *SlPLC2* via a specific artificial microRNA.

Materials and methods

Isolation of botrydial

Botrydial was isolated and purified as described previously (Moraga et al. 2016), dried with gaseous N_2 and storage at − 20 °C. Botrydial powder was dissolved in acetone/water (4:6, v/v) to obtain a 20 mM concentration and subsequently diluted with water for treatment of tomato cell suspensions. Control experiments were performed in the presence of the corresponding dilution of the acetone/water vehicle.

Tomato cell suspensions and treatments

Suspension cultured tomato cells (*Solanum lycopersicum* cv. Money Maker; line Msk8) were grown as described earlier (Laxalt et al. 2007). Suspension cells of 4–5 days were used for the indicated treatments.

^{32}Pi phospholipid labeling and analyses

Aliquots of 80 µL of tomato cell suspensions were employed. For long labeling experiments, tomato cells were labeled for 3 h with 185,000 Bq carrier-free orthophosphate (^{32}Pi) (CNEA, The National Atomic Energy Commission, Buenos Aires, Argentina) prior to botrydial or vehicle (control) treatments. For short-labeling experiments, tomato cells were labeled for 2 min prior to the treatments. Lipids were extracted and separated by thin layer chromatography (TLC) silica-60 plates (Merck) employing ethyl acetate (EtAc/isooctane/formic acid/H_2O (13:2:3:10, by vol.), or alkaline solvent [$CHCl_3$:MeOH:(25%, w/v) NH_4OH:H2O (90:70:4:16, by vol.)] as a mobile phase according to Laxalt et al. (2007). Radiolabelled phospholipids were visualized by phosphoimaging and quantified with the Storm software (Storm; Amersham).

Quantification of ROS production by fluorometry

For this assay, 80 µL of tomato cells were placed into Greiner 96-well plate and pre-incubated for 30 min with 4 µM of the fluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA, Molecular Probes) in the absence or presence of the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), the PLC inhibitor U73122, and the DGK inhibitor R59022 (Sigma). Cells were subsequently treated with 100 µM botrydial or its vehicle. Fluorometric measurements were performed in a Fluoroskan Ascent microwell fluorometer using Chroma filters D480-40 and D525-30 for excitation and emission, respectively. Fluorescence was measured every 2 min over 60 min at room temperature. Each experiment was performed with three technical replicates. Total ROS production was calculated by subtracting to the fluorescence value of each timepoint the fluorescence value of time 0. Then, we integrate the areas under the curves and the values were expressed as fold increase relative to control cells.

amiR-SlPLC2 silencing constructs

Silencing of *SlPLC2* (Solyc06g051620) 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. (2008). Primers for artificial microRNA cloning.

I PLC2 miR-s	ga**TAAATAGGCTCTTAATGT CTG**tctctcttttgtattcc
II PLC2 miR-a	ga**CAGACATTAAGAGCCTAT TTA**tcaaagagaatcaatga
III PLC2 miR*s	ga**CAAACATTAAGAGGCTAT TTT**tcacaggtcgtgatatg
IV PLC2 miR*a	ga**AAAATAGCCTCTTAATGT TTG**tctacatatatattcct

Capital letters denote *SlPLC2* targeted site

The *amiR-SlPLC2* was cloned into pCHF3 vector (kanamycine resistance in plants) driven by the CaMV 35S promoter.

Tomato cell transformation

Tomato cell transformation was performed according to Bargmann et al. (2006). Briefly, 10 mL of cell suspensions were inoculated with 200 µL of an over night culture of *Agrobacterium tumefaciens* GC 3101 carrying the vector pCHF3:*amiR-SlPLC2*. After 3 days of incubation at 25 °C without shaking, cells were placed in MS medium (1% agar, 3% sucrose 5.4 µM IAA, 1 µM BAP) in the presence of 50 µg mL^{-1} kanamycin. After 2 weeks, transgenic callus were selected and silencing was evaluated by qPCR. Callus from silenced lines were grown in liquid medium and weekly subcultured during 2 months. Transcript levels of all six *SlPLCs* were evaluated in the silenced-cell suspensions by qPCR as previously described (Vossen et al. 2010; Gonorazky et al. 2014, 2016).

Results

To investigate whether botrydial induces PA formation in tomato cell suspensions, time-course experiments were performed. Tomato cells were pre-labeled with $^{32}P_i$ for 3 h and treated with 100 µM botrydial or its vehicle (control) for 0 to 30 min. In leaves of *Capsicum annumm*, *Phaseolus vulgaris* and *Arabidopsis thaliana* infected with *B. cinerea,* the concentration of botrydial determined upon 4 and 6 dpi was between 0.44 and 260 nmoles g^{-1} fresh weight (Deighton et al. 2001). Rossi et al. (2011) analyzed the induction of plant defense responses in Arabidopsis leaves employing 161–3220 µM of botrydial. Botrydial triggered a gradual increase in PA levels in cell suspensions incubated with botrydial (Fig. 1a). Control cells showed no variation in PA content throughout the experiment (Fig. 1a). ^{32}P-phospholipid spots were quantified and PA levels were calculated as a percentage in relation with structural phospholipids. Botrydial induced a significant increase of PA (Fig. 1b).

PA can be generated via two enzymatic pathways, PLD and/or PLC/DGK. We first tested whether PLD contributes to the botrydial-induced PA formation. PLD activity is measured in vivo, which consists in the quantification of the transfer of the phosphatidyl group from its substrate to a primary alcohol, such as 1-butanol. The level of the product, phosphatidylbutanol (PBut), is a relative measure of PLD activity (Munnik 2001). Cells were pre-labeled with $^{32}P_i$ for 3 h and subsequently treated with different doses of botrydial during 15 or 30 min in the presence of 0.5% (v/v) 1-butanol. Botrydial induced a significant increase of PBut and PA levels (Fig. 2, Fig. S1). Thus, botrydial induces early PLD activation in tomato cells. Increasing doses of botrydial trigger higher production of PA in tomato cells regardless the presence of 1-butanol (Fig. 2c, d).

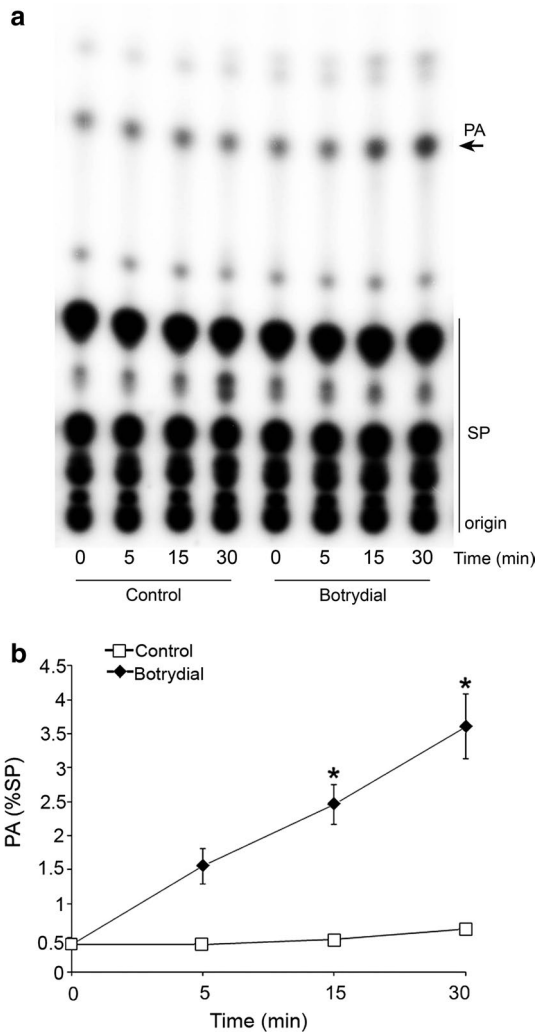

Fig. 1 Botrydial induces PA production in tomato cell suspensions. Tomato cell suspensions were labeled with ^{32}Pi for 3 h and then treated with 100 µM botrydial or its vehicle (control) for the indicated times (min). Total lipids were extracted and separated by EtAc TLC system. **a** Representative phosphoimage is shown ($n = 3$). *SP* structural phospholipids, *PA* phosphatidic acid. **b** Quantification of PA levels expressed as % of SP. Error bars represent the standard error of means ($n = 3$). Asterisks indicate significant difference from $t = 0$ control samples according to one-way ANOVA, post hoc Dunnet's test ($P < 0.05$)

To elucidate whether botrydial also elicits PA formation via PLC/DGK, a short-labeling strategy was performed (Munnik 2001). The strategy is based on the fact that $^{32}P_i$ is slowly incorporated into structural phospholipids, but rapidly into the ATP pool. Thus, in a short-labeling protocol, ^{32}P-ATP is used as a substrate by the DGK to phosphorylate the PLC-derived diacylglycerol (DAG) to ^{32}PA. In contrast, to detect ^{32}PA derived from PLD, a long time $^{32}P_i$ labeling protocol is required. Accordingly, tomato cells were labeled with ^{32}Pi for 2 min and treated with 100 µM botrydial or its vehicle for 0–30 min. PA levels significantly increased

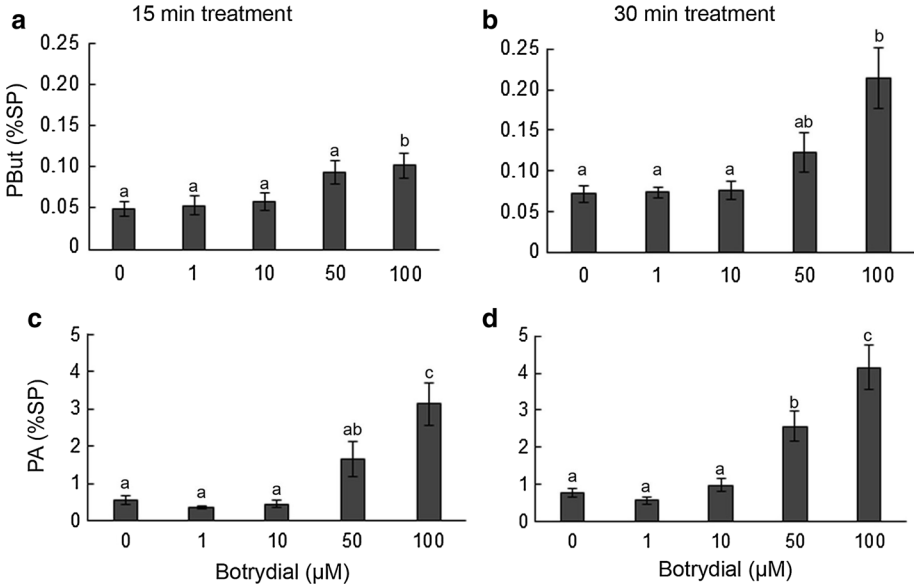

Fig. 2 Botrydial induces PLD activity in a dose dependent manner. Tomato cell suspensions were labeled with ^{32}Pi for 3 h and then treated with different doses of botrydial for 15 min (**a**, **c**) or 30 min (**b**, **d**) in the presence of 0.5% 1-butanol. Lipids were extracted and separated by EtAc TLC system. Quantification of PBut (**a**, **b**) and PA levels (**c**, **d**) are expressed as a % of SP. Error bars represent the standard error of means ($n = 3$). Different letters indicate significant difference between treatments according to one-way ANOVA, multiple comparison, post hoc Tukey's test ($P < 0.05$)

upon treatment with botrydial, while these remained constant throughout the experiment in control cells (Fig. 3). Concomitantly with PLC activation, the PLC substrates PIP and PIP_2 levels were lower in botrydial-treated cells than in control cells upon 30 min of treatment (Fig. 3). This result indicates that PIP and PIP_2 hydrolysis is linked to botrydial-induced PA increase and that botrydial triggers PLC/DGK activation in tomato cells.

Induction of plant oxidative burst is required by *B. cinerea* to infect the host (van Kan 2006). Govrin and Levine (2000) reported that inhibition of NADPH oxidase with DPI significantly diminishes ROS production and reduces fungal colonization. Therefore, the authors concluded that plant oxidative burst induced by *B. cinerea* infection is partially dependent on NADPH oxidase activity (Govrin and Levine 2000). It has been demonstrated that botrydial induces ROS production in Arabidopsis plants (Rossi et al. 2011). We studied whether botrydial requires NADPH oxidase for ROS production. For this, we employed DPI, as an NADPH oxidase inhibitor that has been used in cell suspensions and entire plant systems (Piedras et al. 1998; Govrin and Levine 2000; Orozco-Cardenas et al. 2001; de Jong et al. 2004). Since PLC is required for NADPH oxidase activation (de Jong et al. 2004; Gonorazky et al. 2010; Raho et al. 2011; D'Ambrosio et al. 2017), we also tested U73122 as a PLC inhibitor. In addition, we used R59022 as inhibitor of DGK. U73122 and R59022 have been used as inhibitors of PA production and downstream responses such as ROS and nitric oxide production, gene expression, cell death, and FLS2 internalization (de Jong et al. 2004; Gonorazky et al. 2010; Sueldo et al. 2010; Raho et al. 2011; Abd-El-Haliem et al. 2016). Botrydial elicits oxidative burst in tomato cell suspensions (Fig. 4a, b). Incubation of tomato cells with DPI, U73122, or R59022 partially inhibited botrydial-induced ROS production (Fig. 4a, b). These results indicate that NADPH oxidase is involved in botrydial-induced ROS and that activation of PLC/DGK is required for a full ROS response in botrydial-treated tomato cell suspensions.

Previously, we have shown that SlPLC2 participates in plant susceptibility to *B. cinerea* (Gonorazky et al. 2016). To determine if SlPLC2 is required for PA production induced by botrydial, we stably silenced *SlPLC2* in tomato cell suspensions by employing the Arabidopsis artificial microRNA technique (Ossowski et al. 2008). A significant reduction of *SlPLC2* transcript levels occurred in *amiR-SlPLC2.9* and *amiR-SlPLC2.10* independent lines compared to wild type (Fig. 5a). Transcript levels of the other *SlPLC* genes were evaluated showing no significant changes in expression levels of *SlPLC1, 3, 4,* and *5,* but a partial increase in *SlPLC6* transcripts (Fig. S2), which is low expressed in wild-type cells compared to *SlPLC2* transcript levels, as previously reported (Gonorazky et al. 2014). We analyzed the role of SlPLC2 in botrydial-induced PA production by performing short-labeling experiments in the silenced lines. PA levels in both *amiR-SlPLC2.9* and *amiR-SlPLC2.10* cell suspensions lines treated with botrydial were not significantly different from wild type (Fig. 5b). This suggests that SlPLC2 is not involved in botrydial-induced PA production.

Discussion

In this report, we show that the phytotoxin botrydial triggers PA production in tomato cell suspensions via PLD and PLC/DGK activation and that PLC/DGK-derived PA is partially required for botrydial-induced ROS generation. We also report that *SlPLC2*-silenced-cell suspensions produce PA levels similar to wild type. It can be concluded

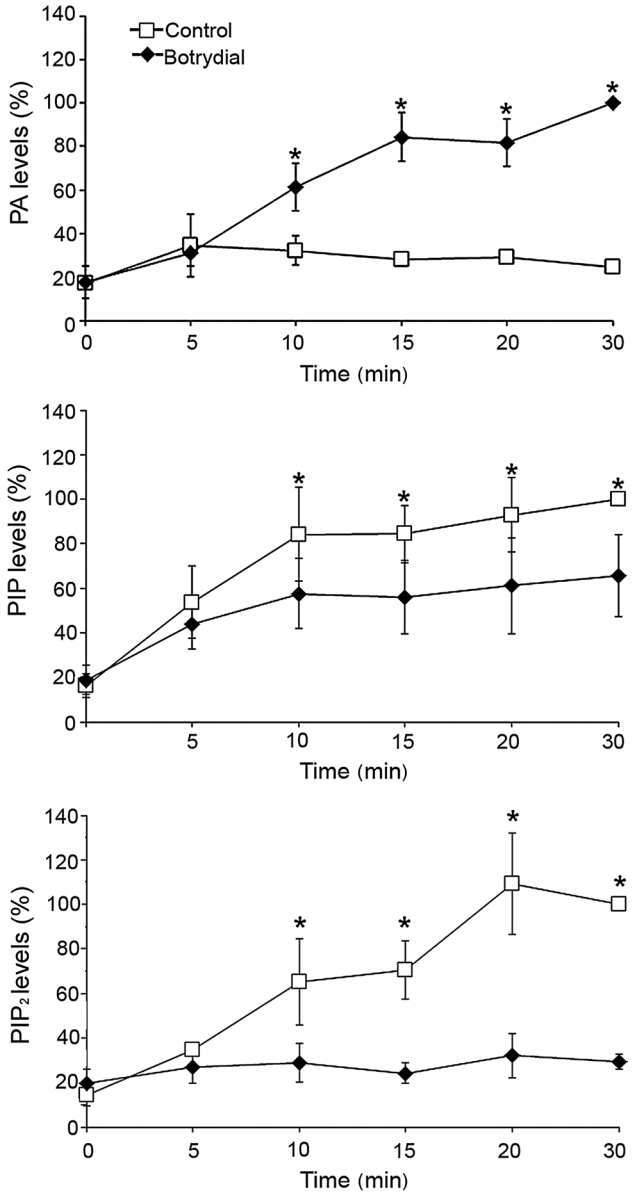

Fig. 3 Botrydial triggers PA production via PLC/DGK. Tomato cell suspensions were labeled with ^{32}Pi for 2 min and then treated with 100 µM botrydial or its vehicle (control) for the indicated times. Total lipids were extracted and separated by alkaline TLC system. Quantification of PA levels is expressed as %, setting the levels of cells treated with botrydial for 30 min as 100%. Quantification of PIP and PIP_2 production is expressed as %, taking the levels of cells treated with the vehicle for 30 min as 100%. Error bars represent the standard error of means ($n = 3$). Asterisks indicate significant difference from $t = 0$ according to one-way ANOVA, post hoc Dunnet's test ($P < 0.05$)

that PA is a novel component of the plant responses triggered by botrydial and that an SlPLC isoform different from SlPLC2 might be involved in the activation of the signaling pathway induced by botrydial.

In plants, MAMPs and effector proteins are recognized by distinct receptors, but in both cases, this leads to the activation of very similar signaling pathways that elicit plant defense responses (Boller and Felix 2009). Less is known about the mechanisms by which plants sense phytotoxins, but interestingly, it has been reported that several of their molecular targets are common to MAMPs and effector proteins (Mengiste 2012). Here, we demonstrate that the phytotoxin botrydial triggers PA production in tomato cell suspensions. This response occurs within minutes and it is dose dependent. Similarly, it has been shown that PA early accumulated in response to well-described MAMPs such as xylanase, flg22, chitotetraose, *N*-acetylchito-oligosacharides, chitosan and cryptogein, and the effector proteins Avr4 from *C. fulvum* and AvrRpm1 and AvrRpt2 from *P. syringae* (van der Luit et al. 2000; den Hartog et al. 2003; de Jong et al. 2004; Yamaguchi et al. 2005; Andersson et al. 2006; Bargmann et al. 2006; Raho et al. 2011; Cacas et al. 2017).

The phospholipases PLD and PLC/DGK are activated during plant defense to generate PA (Testerink and Munnik 2011). We determined that PLD and PLC/DGK are early activated by botrydial treatment. When we analyzed the PLC/DGK enzymatic pathway, we detected a significant increase of PA levels upon botrydial treatment of tomato cells and a decrease of the PLC substrates, PIP and PIP_2. Since PIP is also a PIP_2 precursor (Munnik 2014), we cannot discard that part of the PIP pool is being phosphorylated by PIP kinases to replace PIP_2 levels. It has been reported that xylanase, chitosan, and *N*-acetylchitooligosaccharides induce both PLD and PLC/DGK activity within minutes (van der Luit et al. 2000; Yamaguchi et al. 2003, 2005; Raho et al. 2011). Other MAMPs like chitotetraose, flg22, cryptogein, and the protein effector Avr4 mainly activate PLC/DGK (van der Luit et al. 2000; de Jong et al. 2004; Cacas et al. 2017). Altogether, this evidence suggests that PA production is a general response to biotic stress. Rossi et al. (2011) reported that tissue necrosis starts to be visualized in Arabidopsis leaves upon 3 h of inoculation with botrydial. We observed that botrydial induces cell death in tomato cell suspensions upon 18 h of treatment, but it does not induce cell death upon 1 h of incubation (data not shown). Activation of PLD also occurs upon a significant increase of $[Ca^{2+}]$ when cellular compartmentalization is lost (Bargmann et al. 2009). Since botrydial has been described as a toxin that also plays a role as an elicitor molecule (Rossi et al. 2011), PA production derived from PLD could be triggered by both activation of defense signaling pathways and cellular damage.

Induction of the plant oxidative burst is required by *B. cinerea* to infect the host (van Kan 2006). One of the phytotoxic effects of botrydial is the early induction of ROS accumulation in Arabidopsis leaves (Rossi et al. 2011). Accordingly, we show that botrydial triggers oxidative burst

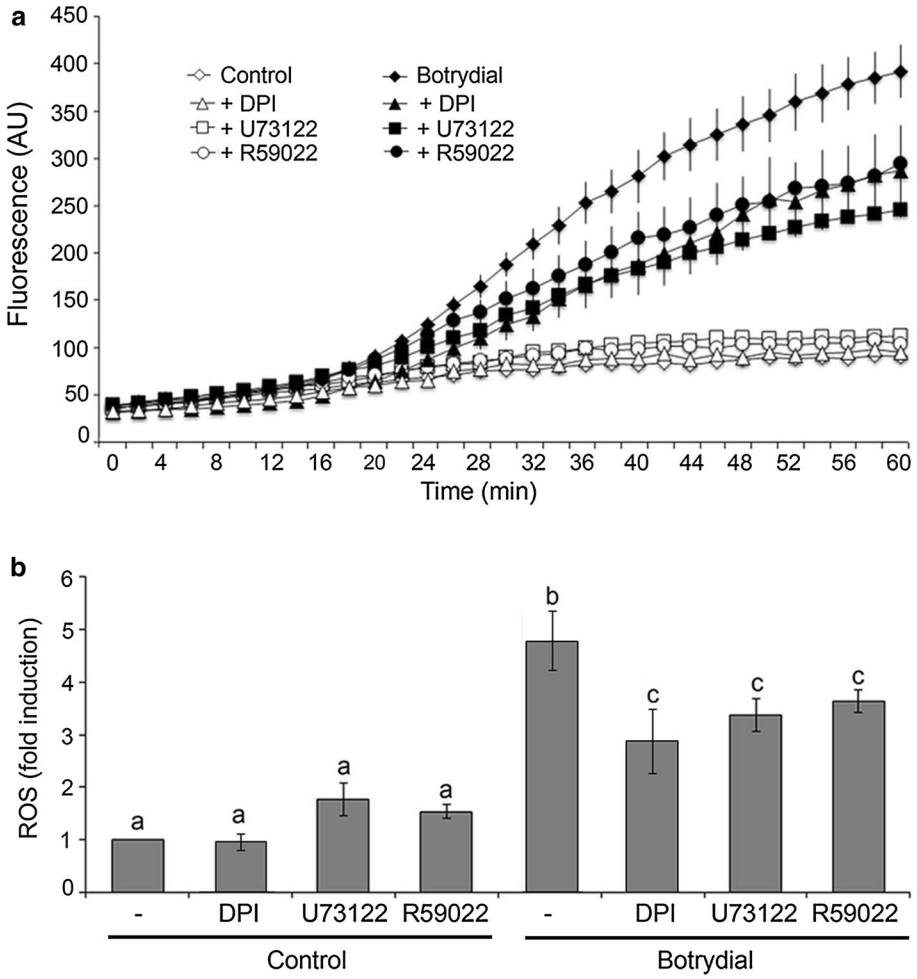

Fig. 4 PLC/DGK inhibitors diminish botrydial-induced ROS production. Tomato cell suspensions were treated with 100 µM botrydial or its vehicle (control) in the absence or presence of 10 µM DPI, 10 µM U73122 or 10 µM R59022. ROS levels were determined in a microwell fluorometer using H_2DCF-DA. **a** Results of a representative experiment are shown ($n \geq 3$). **b** Total ROS production was calculated by subtracting the fluorescence value of time 0 to the fluorescence value of each timepoint and then integrating the areas under the curves. These are expressed as fold increase relative to control cells. Error bars represent the standard error of means ($n \geq 3$). Different letters indicate significant difference between treatments according to one-way ANOVA, multiple comparison, post hoc Tukey's test ($P < 0.01$)

in tomato cell suspensions. Inhibition of NADPH oxidase reduces botrydial-induced ROS production. Therefore, botrydial partially triggers ROS generation via NADPH oxidase in tomato cell suspensions. This is consistent with results published by Govrin and Levine (2000), who reported that inhibition of NADPH oxidase diminishes the necrosis produced by *B. cinerea*. NADPH oxidase is a target protein of PA. It was demonstrated that PA triggers NADPH oxidase activity, while specific mutations in PA binding sites of RBOHD and RBOHF lessen ROS production (Zhang et al. 2009). We show that inhibition of PLC and DGK activity reduces oxidative burst triggered by botrydial in tomato cell suspensions. Therefore, botrydial-induced PLC/DGK activation is upstream of ROS production. It has been demonstrated that activation of PLC/DGK is required for ROS production induced by xylanase, chitosan, *N*-acetychitooligosaccharide, cryptogein and Avr4 (Yamaguchi et al. 2003; de Jong et al. 2004; Laxalt et al. 2007; Raho et al. 2011; Cacas et al. 2017). We reported that silencing of *SlPLC2* in tomato plants diminishes oxidative burst upon *B. cinerea* (Gonorazky et al. 2016). Similarly, Arabidopsis *AtPLC2* silenced plants produce less ROS upon flagelline treatment (D'Ambrosio et al. 2017). Consistently, AtPLC2 associates with RBOHD (D'Ambrosio et al. 2017). Altogether, this evidence indicates that PLC regulates ROS production upon distinct elicitor treatments.

Tomato *SlPLC* gene family is composed by six genes (*SlPLC1-SlPLC6*) and one pseudogene (Abd-El-Haliem et al. 2016). Previously, we reported that silencing of *SlPLC2* reduces plant susceptibility to *B. cinerea* (Gonorazky et al. 2016). Here, we observed that *SlPLC2*-silenced-cell suspensions produced PA levels similar to wild type upon botrydial treatment. *SlPLC2*-silenced tomato cells have a significant reduction of *SlPLC2* transcript levels in *amiR-SlPLC2.9* and *amiR-SlPLC2.10* lines compared to wild type, concomitantly with a partial increase of *SlPLC6* levels. Nevertheless, in wild-type cells, *SlPLC2* transcript levels are tenfold higher than *SlPLC6* levels, as previously shown (Gonorazky et al. 2014). On the other hand, augmentation of *SlPLC2* transcript levels has been reported in *SlPLC4*- and *SlPLC6*-silenced tomato plants (Vossen et al. 2010). In spite of this, the authors observed that *SlPLC4*- and *SlPLC6*-silenced plants had different phenotypes from wild-type plants. *SlPLC4*-silenced plants developed less

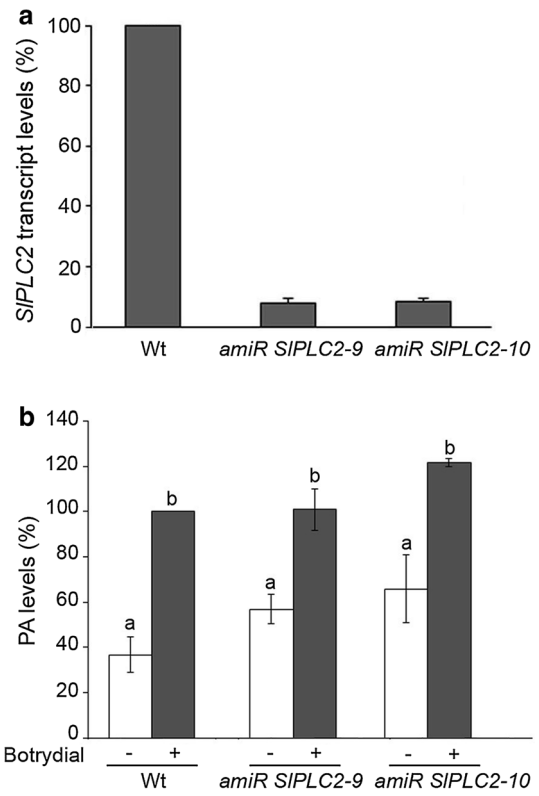

Fig. 5 SlPLC2 is not involved in botrydial-induced PA production. **a** SlPLC2 was silenced in tomato cells suspensions using a specific artificial microRNA expressed under the constitutive promoter CaMV 35S. Two independent lines were selected named *amiR-SlPLC2.9* and *amiR-SlPLC2.10*. Total RNA was isolated and *SlPLC2* transcript levels were determined by RT-qPCR. Transcript levels were normalized to *SlACT* and *SlPLC2* transcript levels of wild-type cells were taken as 100%. Error bars represent the standard errors of means ($n = 2$). **b** Tomato cell suspensions were labeled with ^{32}Pi for 2 min and then treated with 100 µM botrydial or its vehicle (control) for 15 min. Total lipids were extracted and separated in an EtAc TLC system. Quantification of PA levels is expressed as % of maximal production, taking the levels of wild-type cells treated with botrydial as 100%. Error bars represent the standard error of means ($n = 3$). Different letters indicate significant difference between treatments according to one-way ANOVA, multiple comparison, post hoc Tukey's test ($P < 0.05$)

HR upon treatment with the effector protein AVR4 of *C. fulvum*, while *SlPLC6*-silenced plants were less resistant to *C. fulvum*, *P. syringae,* and *Verticillium dahliae* infection. This indicates that there is no redundancy between SlPLC isoforms (Vossen et al. 2010). Therefore, our results suggest that SlPLC2 is not involved in botrydial-induced PA production and that an SlPLC isoform different from SlPLC2 may be activated by botrydial.

In summary, we demonstrate that botrydial triggers PA production via PLD and PLC/DGK and that PA positively regulates ROS production. A follow-up of our work will be to identify the SlPLC and SlPLD isoforms involved in the signaling pathway triggered by botrydial.

Author contribution statement AML and GG conceived the original research plans, designed and supervised the experiments and analyzed the data; JMD performed most of the experiments and analyzed the data; DJS and AADP performed some of the experiments. JM and IGC provided the botrydial and discuss the project, AML and GG conceived the project and wrote the article with contributions of all the authors; LL supervised and complemented the writing.

Funding This work was financially supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Universidad Nacional de Mar del Plata (UNMdP) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). J.M.D'Ambrosio is a postdoctoral researcher of the research career of CONICET. G. Gonorazky, A.M. Laxalt, and L. Lamattina are members of the research career of CONICET.

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