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Silencing of the tomato phosphatidylinositol-phospholipase C2 (SlPLC2) reduces plant susceptibility to *Botrytis cinerea*

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SUMMARY

The tomato [*Solanum lycopersicum* (*Sl*)] *phosphatidylinositol-phospholipase C* (*PI-PLC*) gene family is composed of six members, named *SlPLC1* to *SlPLC6*, differentially regulated on pathogen attack. We have previously shown that the fungal elicitor xylanase induces a raise of *SlPLC2* and *SlPLC5* transcripts and that SlPLC2, but not SlPLC5, is required for xylanase-induced expression of defense-related genes. In this work we studied the role of SlPLC2 in the interaction between tomato and the necrotrophic fungus *Botrytis cinerea*. Inoculation of tomato leaves with *B. cinerea* increases *SlPLC2* transcript levels. We knocked-down the expression of *SlPLC2* by virus-induced gene silencing and plant defense responses were analyzed upon *B. cinerea* inoculation. *SlPLC2* silenced plants developed smaller necrotic lesions concomitantly with less proliferation of the fungus. Silencing of *SlPLC2* resulted as well in a reduced production of reactive oxygen species. Upon *B. cinerea* inoculation, transcript levels of the salicylic acid (SA)-defense pathway marker gene *SlPR1a* were diminished in *SlPLC2* silenced plants compared to non-silenced infected plants, while transcripts of the jasmonic acid (JA)-defense gene markers *Proteinase Inhibitor I* and *II* (*SlPI-I* and *SlPI-II*) were increased. This implies that SlPLC2 participates in plant susceptibility to *B. cinerea*.

Keywords: cell death, defense gene, jasmonic acid, necrotrophic fungus, phospholipid signalling, reactive oxygen species, salicylic acid.

INTRODUCTION

Phosphatidylinositol-phospholipase C (PI-PLC) catalyzes the hydrolysis of the signal molecules phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol (4,5) bisphosphate [PI(4,5)P_2] to produce inositol 2-phosphate (IP_2) or inositol 3-phosphate (IP_3) and diacylglycerol (DAG). In plants, IP_2 and IP_3 can be further phosphorylated to IP_6, which acts as a second messenger inducing the release of calcium (Ca^{+2}) from intracellular stores (Meijer and Munnik, 2003). The other PI-PLC product, DAG, is phosphorylated by DAG kinase (DGK) to produce phosphatidic acid (PA) (Meijer and Munnik, 2003). The activation of PLC is one of the earliest host responses upon treatment of plant cells with pathogen-associated molecular patterns (PAMPs) (Laxalt and Munnik, 2002). These are conserved compounds of pathogenic microbes that are perceived by immune receptors present in resistant plants. For example, the fungal PAMPs xylanase, chitosan and *N*-acetyloligosaccharides, as well as the bacterial flagellin-derived peptide flg22, induce PA production via PLC/DGK in tomato, alfalfa and rice cells (Bargmann *et al.*, 2006; den Hartog *et al.*, 2003; Laxalt *et al.*, 2007; van der Luit *et al.*, 2000; Raho *et al.*, 2011; Yamaguchi *et al.*, 2003, 2005). Moreover, the race-specific pathogen effector Avr4, from the fungus *Cladosporium fulvum*, induces PLC activity in *Cf4*-expressing tobacco cells (de Jong *et al.*, 2004). Production of PA via PLC/DGK has also been reported in *RPM1*/*RPS2*- Arabidopsis plants upon the perception of the specific effectors AvrRpm1 and AvrRpt2 from *Pseudomonas syringae* (Andersson *et al.*, 2006). It has been well documented that PLC/DGK activation triggers downstream plant defense responses like reactive oxygen species (ROS) production, induction of defense genes and cell death (Testerink and Munnik, 2011). There are seven known functional *PLC* genes and two pseudogenes in the *Arabidopsis thaliana* genome (Mueller-Roeber and Pical, 2002). Multiple *PLC* genes have been found in several plant species such as rice, potato and tomato (Kopka *et al.*, 1998; Song and Goodman, 2002; Vossen *et al.*, 2010). It has been demonstrated that PLCs are regulated at transcriptional level upon biotic stress. *Oryza sativa* (Os) *PLC1* transcript levels increase upon treatment of rice with *P. syringae* or the salicylic acid (SA) analogue, benzothiadiazol (Chen *et al.*, 2007; Song and Goodman, 2002). In tomato [*Solanum lycopersicum* (Sl)], Vossen *et al.* (2010) characterized a *PLC* gene family composed of six members named *SlPLC1* to *SlPLC6*. These authors found that the expression levels of *SlPLCs* are distinctly increased in tomato plants inoculated with *C. fulvum* (Vossen *et al.*, 2010). By performing virus-induced gene

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[Correction added on 22 September 2016, after first online publication: Two authors, Ahmed M. Abd-El-Haliem and Matthieu H.A.J. Joosten, were not listed in the original version. This was an error and they have now been added as authors.]

silencing (VIGS) assays, it was demonstrated that *SlPLC4* is specifically involved in the induction of the plant hypersensitive response (HR) upon AVR4 perception (Vossen *et al.*, 2010). Instead, *SlPLC6* is a more general component of defense signalling, since it is required for resistance against *C. fulvum*, *Verticillium dahliae* and *P. syringae* (Vossen *et al.*, 2010). Based on this evidence, the authors concluded that there is a differential requirement of PLC isoforms for plant defense. We have recently demonstrated that xylanase induces an increase of *SlPLC2* and *SlPLC5* transcript levels both in tomato cell suspensions and tomato plants (Gonorazky *et al.*, 2014). We found by VIGS assays that SlPLC2, but not SlPLC5, is required for xylanase-induced expression of the SA-defense gene marker *Pathogenesis Related1* (*SlPR1*) and the HR tomato gene marker *Hypersensitive Response 203J* (*SlHSR203J*) (Gonorazky *et al.*, 2014). Since xylanase is a PAMP, the aim of this work was to investigate whether *SlPLC2* plays a role in plant-pathogen interactions.

Botrytis cinerea is a necrotrophic fungus that infects over 200 plant species including several crops such as grape, strawberry and solanaceous plants (Dean *et al.*, 2012). *B. cinerea* produces ROS, phytotoxins and enzymes, such as xylanase, that are required to induce necrosis of plant tissues (van Baarlen *et al.*, 2007; van Kan, 2006). This pathogen also triggers programmed cell death pathways in the host, activating ROS production and HR, which favors the infection process (van Kan, 2006). It was demonstrated that the growth of *B. cinerea* is accompanied by H_2O_2 production and expression of *HSR203J* in the plant (Govrin and Levine, 2000). Infection of plants with *B. cinerea* also activates SA- and jasmonic acid (JA)-signalling pathways (Glazebrook, 2005). The expression of *PR1a* and the transcription factor that regulates its expression, *Non-expressed PR1* (*NPR1*), is induced upon *B. cinerea* inoculation (Abuqamar *et al.*, 2009; Diaz *et al.*, 2002; El Oirdi *et al.*, 2011; Flors *et al.*, 2007). Transcript levels of the JA-gene markers *Proteinase Inhibitor I* (*PI-I*) and *Proteinase Inhibitor II* (*PI-II*) are also augmented by *B. cinerea* inoculation (Abuqamar *et al.*, 2009; Diaz *et al.*, 2002; El Oirdi *et al.*, 2011).

Previously, we demonstrated that SlPLC2 is required for xylanase-induced expression of defense genes. The goal of this work was to study whether SlPLC2 regulates defense responses to the necrotrophic pathogen *B. cinerea* by transient silencing of *SlPLC2* in tomato plants.

RESULTS

To investigate whether SlPLC2 plays a role in tomato-*B. cinerea* interaction, first we studied the expression of *SlPLC2* in tomato leaflets during *B. cinerea* infection. Figure 1A shows that *SlPLC2* transcripts significantly increased in tomato leaflets inoculated with *B. cinerea* at 48 and 72 h post-inoculation (hpi). In addition, transcript levels of the other *SlPLCs* were studied for later analysis of *SlPLC2* silencing specificity. At 0 hpi, *SlPLC1-SlPLC6* transcripts were comparable to those reported by Vossen *et al.* (2010; Fig. 1A). *SlPLC1* was the only *SlPLC* gene whose transcript levels were significantly reduced upon inoculation (Fig. 1A). *SlPLC3, SLPLC4* and *SlPLC5* were increased in tomato leaflets inoculated with *B. cinerea* at different time points (Fig. 1A). Transcript levels of *SlPLC6* did not significantly change throughout the experiment (Fig. 1A).

Expression of the SA-defense gene markers, *SlPR1a* and *SlPR2b*, and the JA-defense gene markers, *SlPI-I* and *SlPI-II*, were used as a read-out for transcriptional defense-related gene activation upon *B. cinerea* infection (Abuqamar *et al.*, 2009; Diaz *et al.*, 2002; El Oirdi *et al.*, 2011; Flors *et al.*, 2007; Uppalapati *et al.*, 2007).Transcript levels of *SlPR1a* and *SlPR2b* were significantly increased at 48 hpi, while *SlPI-I* and *SlPI-II* were induced at 6 and 24 hpi, respectively (Fig. 1B). The transcript levels of the *B. cinerea ACTINE* (*BcACT*) gene were quantified by qRT-PCR at different time points after inoculation, as a quantitative measure for the fungal biomass (Benito *et al.*, 1998). Figure 1C shows that *BcACT* increased in a time dependent manner from 0 to 72 hpi.

As mentioned previously, it was demonstrated that SlPLC2 is involved in xylanase-induced defense gene expression (Gonorazky *et al.*, 2014). To investigate whether SlPLC2 plays a role in tomato-*B. cinerea* interaction, *SlPLC2* expression was knocked down by VIGS employing a tobacco-rattle virus (TRV) construct (Gonorazky *et al.*, 2014). As a negative control we used a TRV with the β-*glucuronidase* (*GUS*) gene (TRV:*GUS*), which has no homologues in plants. Under normal growth conditions, the TRV:*SlPLC2* plants displayed no apparent morphological alterations, as reported earlier (Gonorazky *et al.*, 2014). Silencing specificity of *SlPLC2* was studied by measuring transcript levels of the six *SlPLCs* in TRV:*GUS* and TRV:*SlPLC2* leaflets inoculated with *B. cinerea*. For this, we analyzed only the time points at which the expression of each *SlPLC* changed significantly upon *B. cinerea* inoculation of wild type plants (Fig. 1A). For *SlPLC6*, which expression did not change upon infection, we chose 24 hpi as an intermediate point. Figure 2 shows that there was a 40% reduction of *SlPLC2* transcript levels in TRV:*SlPLC2* leaflets upon 72 hpi. This result demonstrates that *SlPLC2* was knocked down. Differences in *SlPLC2* transcript levels could not be detected between TRV:*GUS* and TRV:*SlPLC2* plants at 0 hpi, since the *SlPLC2* transcript levels are very low (0,01 in relation to *SlACT*). Transcript levels of the other five *SlPLC* genes were not reduced in TRV:*SlPLC2* leaflets (Fig. 2). It was observed as well that transcripts of *SlPLC1* were significantly higher in TRV:*SlPLC2* than in TRV:*GUS* leaflets (Fig. 2). This indicates that transient silencing of *SlPLC2* was specific. Analysis of the expression of all six *SlPLC* genes in TRV:*GUS* and TRV:*SlPLC2* plants at additional time points was included as Supporting Information (Fig. S1).

Once established that *SlPLC2* was silenced, we analyzed the role of SlPLC2 in the tomato - *B. cinerea* interaction. Figure 3A shows that necrotic lesions produced in tomato leaflets by *B.*

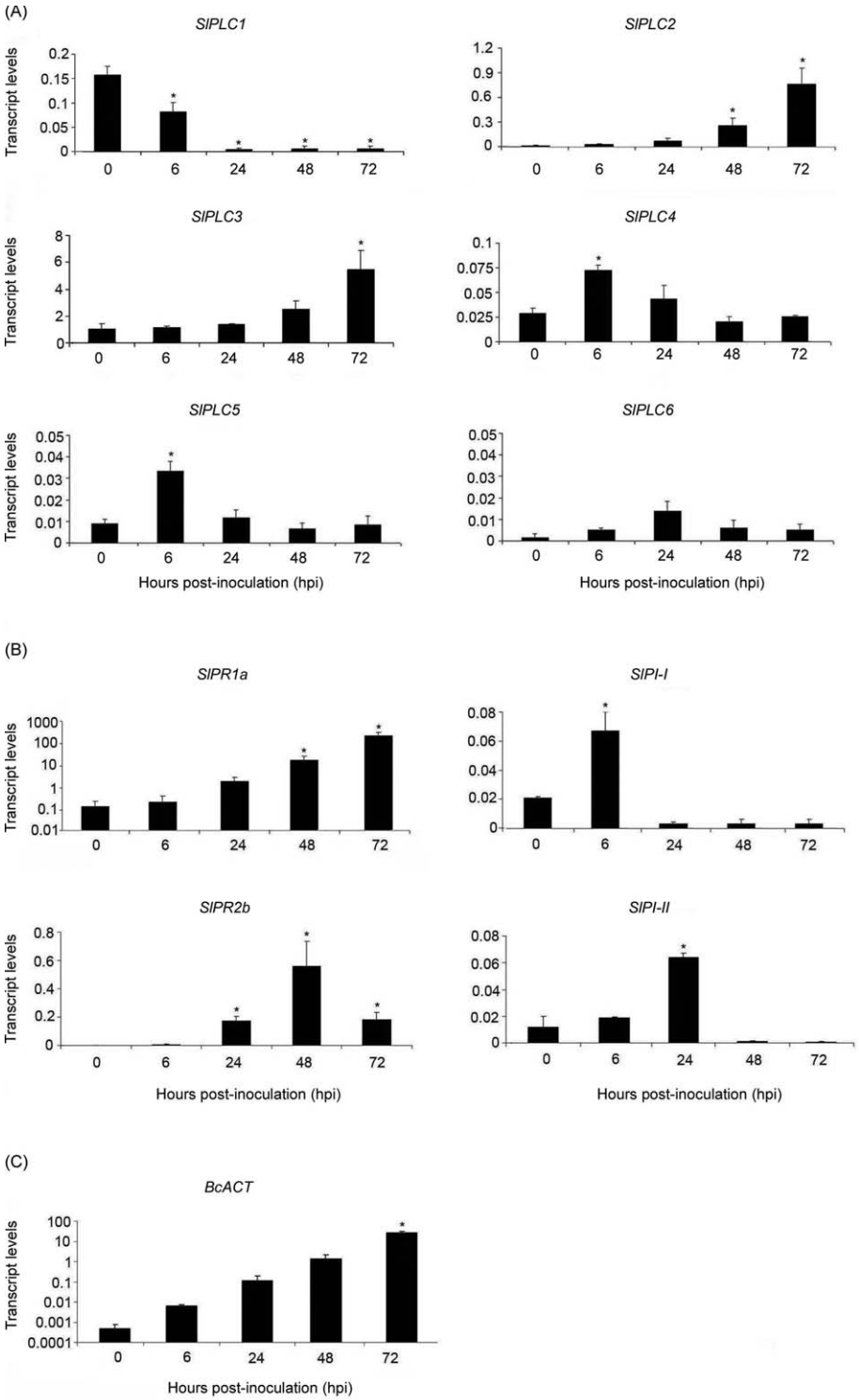

Fig. 1 Transcript levels of *SlPLC* gene family, SA- and JA- defense gene markers and *BcACT* during the interaction between tomato and *B. cinerea*. Detached leaves from 6 weeks old tomato plants were droplet inoculated with *B. cinerea* isolate B05.10 spore suspension (10^6 spores.mL^{-1}). Leaflets were harvested at the indicated hours post-inoculation (hpi). Total RNA was isolated and transcript levels of *SlPLC1 – SlPLC6* genes (A), SA-defense gene markers *SlPR1a* and *SlPR2b* and JA-defense gene markers *SlPI-I* and *SlPI-II* (B), and *BcACT* (C), were determined by RT-qPCR. Transcript levels were normalized to *SlACT*. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different from 0 hpi samples according to a t-test ($P < 0.05$).

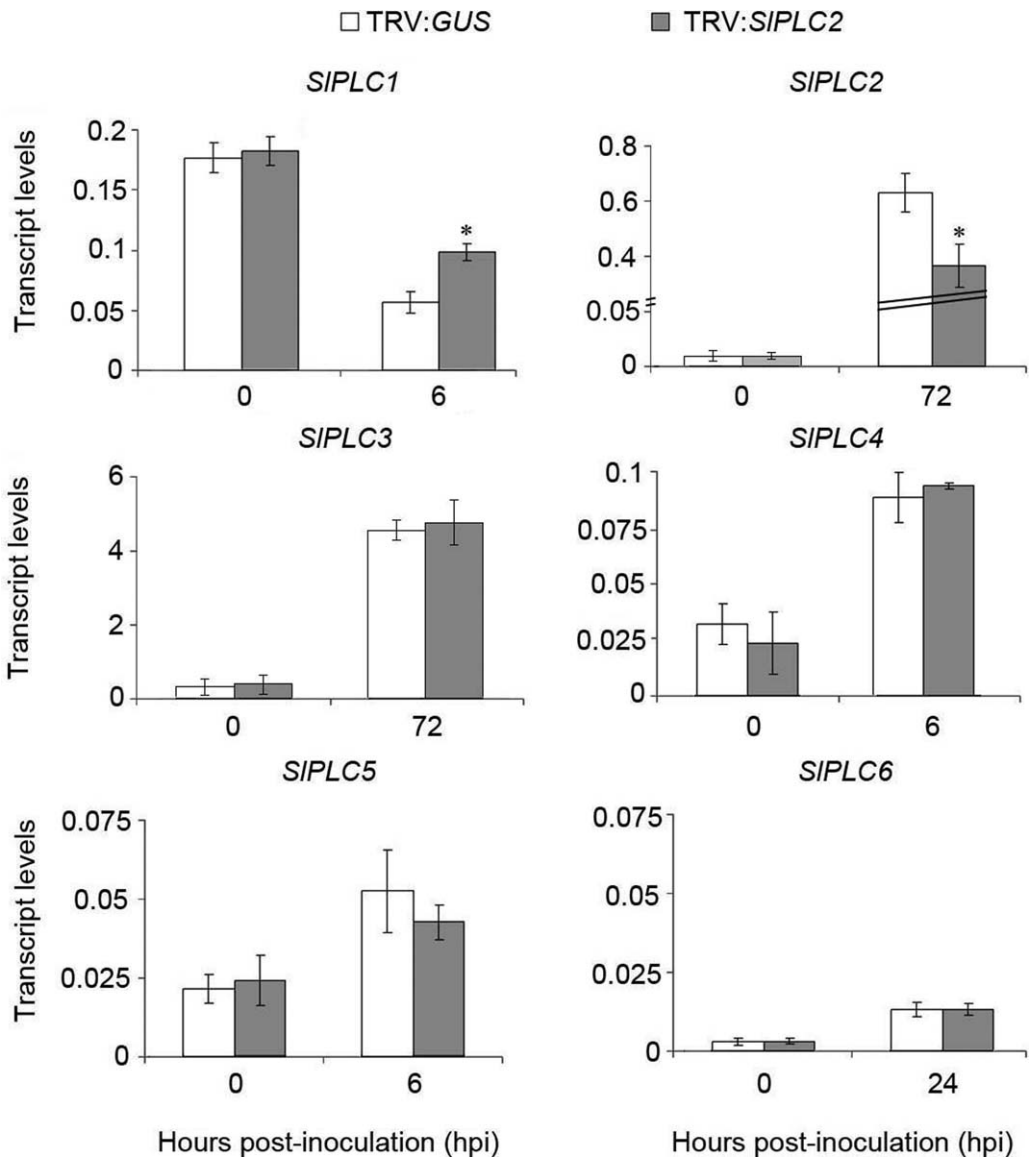

Fig. 2 Specificity of virus-induced gene silencing of *SlPLC2* on tomato. Fourteen days old tomato seedlings were agroinfiltrated with the tobacco rattle virus (TRV) silencing constructs TRV:*GUS* (control) or TRV:*SlPLC2*. After 4 weeks, detached leaves were droplet inoculated with *B. cinerea* isolate B05.10 spore suspension (10^6 spores.mL^{-1}). Leaflets were harvested at the indicated hours post-inoculation (hpi). Total RNA was isolated and transcript levels of the six *SlPLC* genes were determined by RT-qPCR. Transcript levels were normalized to *SlACT*. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different from inoculated TRV:*GUS* samples according to a t-test ($P < 0.05$).

cinerea inoculation were smaller in TRV:*SlPLC2* plants than in TRV:*GUS* plants. To quantitatively confirm this phenotype, the lesion expansion rate produced by *B. cinerea* between 48 and 72 hpi was measured. As shown in Fig. 3B, the average lesion expansion rate was 21% lower in TRV:*SlPLC2* than in TRV:*GUS* plants. To determine whether *B. cinerea* growth was affected in TRV:*SlPLC2* infected plants, the transcript levels of *BcACT* were quantified in TRV:*GUS* and TRV:*SlPLC2* leaflets inoculated with *B. cinerea* at 72 hpi. These experiments showed that *BcACT* transcripts were 70% lower in TRV:*SlPLC2* than in TRV:*GUS* leaflets (Fig. 3C). This indicates that *B. cinerea* proliferation was significantly lower in TRV:*SlPLC2* plants. Altogether, it can be concluded that *SlPLC2* silenced plants were less susceptible to *B. cinerea* infection.

B. cinerea actively triggers an oxidative burst during plant cuticle penetration and primary lesion formation to favor its growth (van Kan, 2006). It was examined whether there was a difference between TRV:*GUS* and TRV:*SlPLC2* plants in H_2O_2 accumulation during the tomato – *B. cinerea* interaction. H_2O_2 production was detected by DAB staining. As shown in Fig. 4A, TRV:*SlPLC2*

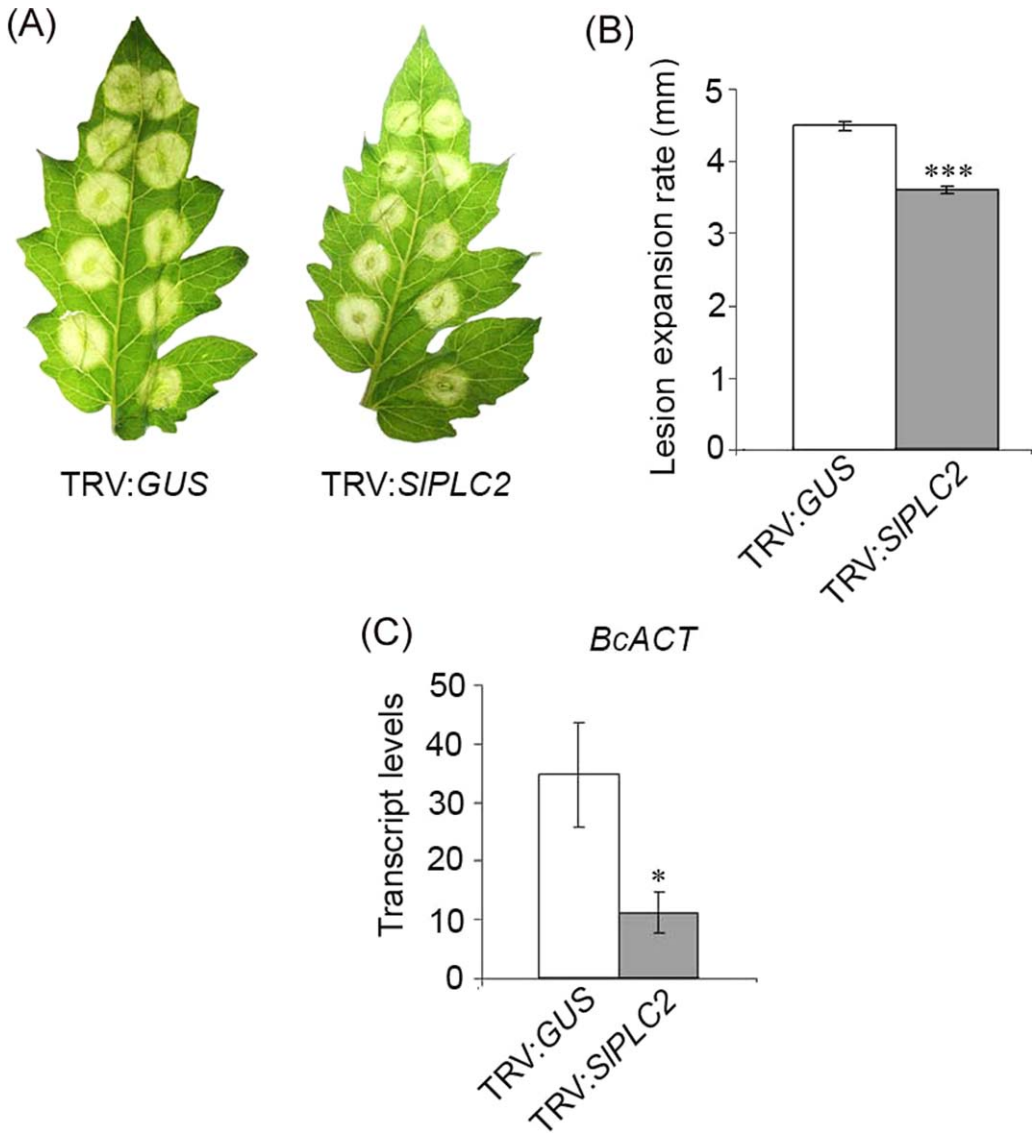

Fig. 3 Susceptibility of *SlPLC2* silenced tomato plants to *B. cinerea* infection. Fourteen days old tomato seedlings were agroinfiltrated with the constructs TRV:*GUS* (control) or TRV:*SlPLC2*. After 4 weeks, detached leaves were droplet inoculated with *B. cinerea* isolate B05.10 spore suspension (10^6 spores.mL^{-1}). (A) Pictures were taken from representative leaflets at 72 h post-inoculation (hpi). (B) Lesion diameter of 300-400 inoculation sites was measured at 48 and 72 hpi and the average lesion expansion rate was calculated. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different from inoculated TRV:*GUS* samples according to a t-test ($P < 0.0001$). (C) Leaflets were harvested at 72 hpi. Total RNA was isolated and transcript levels of *BcACT* were determined by RT-qPCR. Transcript levels were normalized to *SlACT*. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different from inoculated TRV:*GUS* samples according to a t-test ($P < 0.05$).

leaflets inoculated with *B. cinerea* displayed less DAB stained area than TRV:*GUS* leaflets. DAB precipitation was 40% lower in TRV:*SlPLC2* than in TRV:*GUS* leaflets (Fig. 4B). This result indicates that H_2O_2 production was reduced in *SlPLC2* silenced tomato plants inoculated with *B. cinerea*.

The expression of SA- and JA-defense gene markers was quantified in TRV:*GUS* and TRV:*SlPLC2* leaflets inoculated with *B. cinerea*. Transcript levels of *SlPR1a* were 30% lower in TRV:*SlPLC2* than in TRV:*GUS*, while no significant differences were detected in *SlPR2b* transcripts (Fig. 5). In contrast, transcript levels of *SlPI-I* and *SlPI-II* were over 3-fold higher in TRV:*SlPLC2* than in TRV:*GUS* leaflets (Fig. 5).

DISCUSSION

In this report we show that transient silencing of *SlPLC2* resulted in a reduction of the lesion expansion rate, together with a diminished *B. cinerea* growth, less H_2O_2 production and differential expression

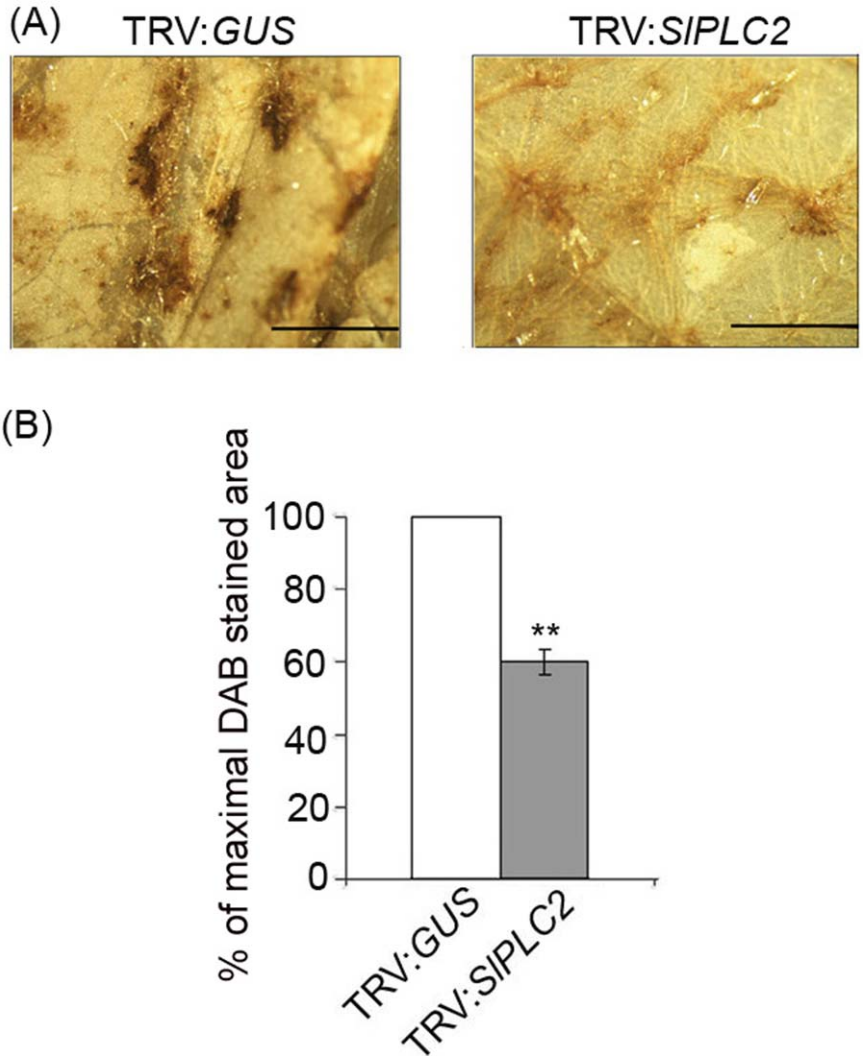

Fig. 4 Production of H_2O_2 on *SlPLC2* silenced tomato plants inoculated with *B. cinerea*. Fourteen days old tomato seedlings were agroinfiltrated with the constructs TRV:*GUS* (control) or TRV:*SlPLC2*. After 4 weeks, detached leaves were spray inoculated with *B. cinerea* isolate B05.10 spore suspension (10^6 spores.mL^{-1}). Leaflets were harvested at 24 hpi and H_2O_2 production was detected by 3,3-diaminobenzidine (DAB) staining immediately after harvesting. (A) DAB stained tissue was macroscopically observed. Pictures were taken from representative leaflets (scale bars = 1.5 mm). (B) Quantification of DAB stained area was performed using ImageJ 1.3 software and expressed as a percentage of inoculated TRV:*GUS* samples which was set to 100%. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different according to a t-test ($P < 0.001$).

pattern of defense genes upon *B. cinerea* inoculation. This implies that SlPLC2 participates in plant susceptibility to *B. cinerea*.

A property of signalling enzymes in general is that treatments that activate them often rapidly enhance expression of their genes. The response could be a positive feedback mechanism to prime the cell for further stimulation (Hirt, 1999; Yamamoto, 1998). Accordingly, distinct evidence indicates that PI-PLCs are also modulated at a transcriptional level in response to biotic stress (Chen *et al.*, 2007; Gonorazky *et al.*, 2014; Vossen *et al.*, 2010). Particularly in tomato, xylanase treatment increases *SlPLC2* and *SlPLC5* transcript levels (Gonorazky *et al.*, 2014). From these two *SlPLC* genes, *SlPLC2* showed the highest induction (Gonorazky *et al.*, 2014). Here we demonstrate that *SlPLC2* expression was induced in tomato plants inoculated with *B. cinerea*, together with *SlPLC3, SlPLC4* and *SlPLC5*. Induction of *SlPLC2* occurred simultaneously with an enhanced expansion of *B. cinerea* lesions between 48 and 72 hpi. Vossen *et al.* (2010) showed that expression of all six *SlPLCs* is differentially induced during interaction between tomato and *C. fulvum*. *SlPLC2* maximum expression coincided with the time point at which *C. fulvum* biomass starts to increase significantly (Vossen *et al.*, 2010). Altogether, these results point out that *SlPLC2* is induced upon perception of distinct fungal pathogens.

In order to study the involvement of SlPLC2 in the tomato – *B. cinerea* interaction, *SlPLC2* was transiently silenced in tomato plants by VIGS. As a necrotrophic pathogen, *B. cinerea* requires induction of plant cell death to grow and infect the host (van Kan, 2006). It has been demonstrated that *accelerated cell death* (*acd*) mutants are more susceptible to *B. cinerea* (van Baarlen *et al.*, 2007). Inversely, mutation of type 2 metacaspases, which have been associated with induction of plant cell death, resulted in plants significantly less susceptible to this fungus (van Baarlen *et al.*, 2007). Virus-induced gene silencing of *SlPLC2* resulted in a significant reduction of the lesion expansion rate in tomato leaflets inoculated with *B. cinerea*. In addition, there was a drastically reduced growth of *B. cinerea* in

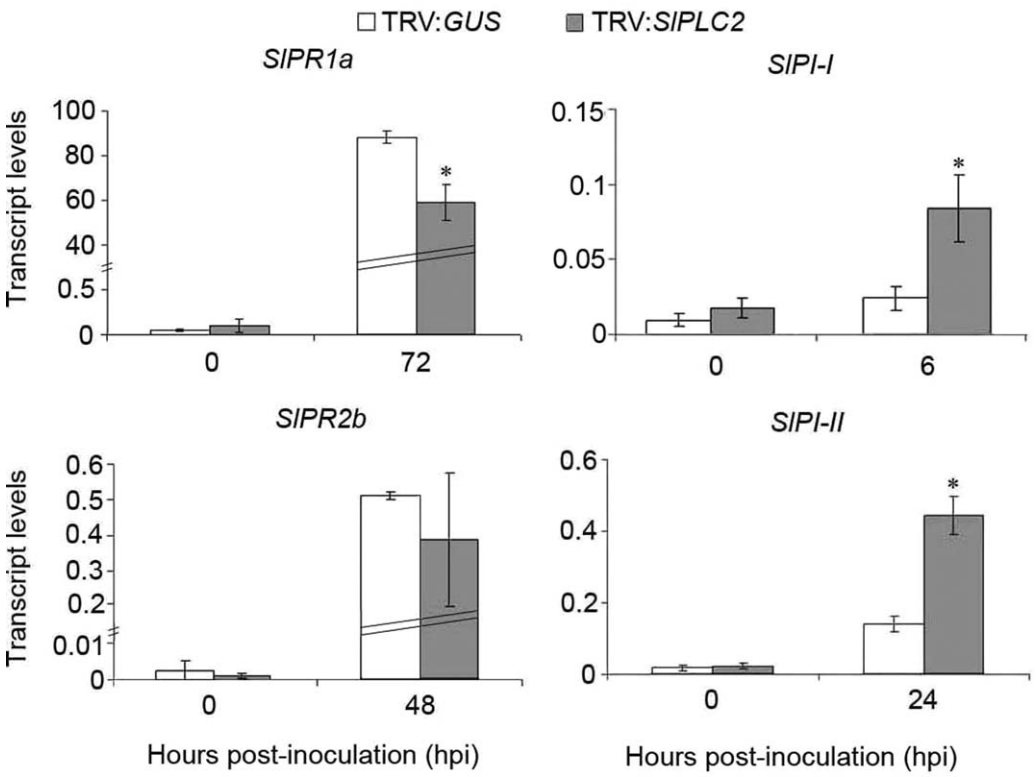

Fig. 5 Transcript levels of SA- and JA-defense gene markers on *SlPLC2* silenced tomato plants inoculated with *B. cinerea*. Fourteen days old tomato seedlings were agroinfiltrated with the constructs TRV:*GUS* (control) or TRV:*SlPLC2*. After 4 weeks, detached leaves were droplet inoculated with *B. cinerea* isolate B05.10 spore suspension (10^6 spores.mL^{-1}). Leaflets were harvested at the indicated hours post-inoculation (hpi). Total RNA was isolated and transcript levels of the SA-defense gene markers *SlPR1a* and *SlPR2b*, and the JA-defense gene markers *SlPI-I* and *SlPI-II* were determined by RT-qPCR. Transcript levels were normalized to *SlACT*. Error bars represent standard errors of three independent experiments. Asterisks denote that means are significantly different from inoculated TRV:*GUS* samples according to a t-test ($P < 0.05$).

SlPLC2 silenced plants. Therefore, it can be concluded that SlPLC2 is required for plant susceptibility to *B. cinerea*. We have previously reported that xylanase-induced cell death requires PLC activation (Laxalt *et al.*, 2007). Interestingly, it has been demonstrated that xylanase is required by *B. cinerea* to be fully virulent on tomato plants (Brito *et al.*, 2006). It remains to be elucidated whether xylanase and/or other molecules produced by *B. cinerea* induce SlPLC2 activity.

Induction of the plant oxidative burst is required by *B. cinerea* to infect the host (van Kan, 2006). This is partially dependent on NADPH oxidase activity, since inhibition of this enzyme significantly diminishes ROS production and reduces fungal colonization (Govrin and Levine, 2000). *SlPLC2* silenced tomato plants showed less H_2O_2 production than non-silenced plants inoculated with *B. cinerea*. This is consistent with the less susceptible phenotype of *SlPLC2* silenced plants to this fungus. Host cell death requires the active participation of both, the pathogen and the host (van Kan, 2006). Therefore, the reduced H_2O_2 production, required to induce cell death, leads to smaller *B. cinerea* lesions. At the same time, less *B. cinerea* proliferation induces less H_2O_2 production. It has been demonstrated that activation of PLC is required for ROS production induced by xylanase, chitosan, *N*-acetychitooligosaccharide and the race specific elicitor Avr4 (de Jong *et al.*, 2004; Laxalt *et al.*, 2007; Raho *et al.*, 2011; Yamaguchi *et al.*, 2003). In addition, PLC activation correlates in time with early oxidative burst upon pathogen recognition (Andersson *et al.*, 2006). It has been reported that PA and Ca^{2+} positively regulate NADPH oxidase activity with the consequent increase in O_2^- generation, which is a precursor of H_2O_2 (Zhang *et al.*, 2009). Therefore, it could be speculated that the second messengers derived from SlPLC2 activation positively regulate the NADPH oxidase activity in tomato-*B. cinerea* interaction.

It has been postulated that, in general terms, SA-regulated defense responses favor plant infection by necrotrophs like *B. cinerea*, while JA-regulated defense responses are involved in restricting the disease produced by this kind of pathogens. The inverse model is proposed for (hemi)biotrophs (Glazebrook, 2005; Pieterse *et al.*, 2009). The final balance between SA- and JA-signalling pathways would determine the establishment of the infection. Therefore, a reduction of plant susceptibility to necrotrophic pathogens results in an increase of susceptibility to

(hemi)biotrophs, and vice versa (Glazebrook, 2005; Pieterse *et al.*, 2009). Partial silencing of *SlPLC2* in tomato plants infected with *B. cinerea* resulted in lower transcript levels of *SlPR1a* and higher transcripts of *SlPI-I* and *SlPI-II*. This indicates that silencing of *SlPLC2* increases the basal resistance of tomato to *B. cinerea*, and slower disease development results in a lower expression of *SlPR1a* and higher expression of *SlPI-I* and *SlPI-II*. These results are in accordance to El Oirdi *et al.* (2011), who demonstrated that transient silencing of the transcription factor that induces *SlPR1a* gene expression, *SlNPR1*, diminishes susceptibility of tomato plants to *B. cinerea*. Tomato *SlNPR1* silenced plants presented higher transcript levels of *SlPI-I* and *SlPI-II*, indicating that *SlNPR1* negatively regulates *SlPI-I* and *SlPI-II* (El Oirdi *et al.*, 2011). Inversely, silencing of *SlPI-I* and *SlPI-II* by VIGS increased susceptibility to *B. cinerea* (El Oirdi *et al.*, 2011). It has been previously reported that NPR1 modulates both SA- and JA-signalling pathways (Pieterse *et al.*, 2009). A follow up of our work will be to determine the connection between NPR1 and SlPLC2.

Activation of PI-PLCs modulate levels of their substrates, PI4P and PI(4,5)P_2, and generate IP_2, IP_3 and DAG (Meijer and Munnik, 2003). PI4P and PI(4,5)P_2 act as molecular signals that can bind to proteins, thus modifying their localization and/or their activity (Munnik and Nielsen, 2011). Arabidopsis mutants expressing a mammalian type I inositol polyphosphate 5-phosphatase, characterized by low levels of IP_3, had a reduced cytosolic Ca^{+2} increase in response to flagellin, delayed induction of defense gene expression such as *PR1* and compromised plant defense to *P. syringae* (Ma *et al.*, 2012; Hung *et al.*, 2014). Ca^{+2} activates diverse proteins involved in plant defense such as phospholipase D (PLD), which produces PA from structural phospholipids, NADPH oxidase and Ca^{+2}-dependent protein kinases (Kadota *et al.*, 2015; Meijer and Munnik, 2003; Romeis and Herde, 2014). In plants, DAG produced by PI-PLCs is subsequently phosphorylated to the signal molecule PA, which is involved in the induction of distinct defense responses such as ROS production, expression of defense genes and cell death (Testerink and Munnik, 2011). The biochemical mechanisms by which SlPLC2 regulates downstream signalling during tomato - *B. cinerea* interaction remain to be elucidated.

In summary, we demonstrated that SlPLC2 contributes to plant susceptibility to *B. cinerea*. Future work will be carried out to determine whether other SlPLCs are involved in the tomato-*B. cinerea* interaction. Also, a future challenge will be to study the role of SlPLC2 in the interaction between tomato and (hemi)biotrophs pathogens.

EXPERIMENTAL PROCEDURES

Plant and fungal material

MM-Cf0 tomato plants were grown in soil under a 16 h light/8 h dark regime, at 21°C and 70% relative humidity. *B. cinerea* strain B05.10 was maintained and conidia was isolated as described (Benito *et al.*, 1998).

Virus-induced gene silencing (VIGS) assays and inoculation of tomato leaves

SlPLC2 gene of 10-day-old tomato seedlings was silenced employing a tobacco rattle virus (TRV) as previously described (Gonorazky *et al.*, 2014). As a negative control a TRV conteining part of the sequence of the *β-glucuronidase* (*GUS*) gene was used (Gonorazky *et al.*, 2014). Compound leaves of 6-week-old TRV:*GUS* and TRV:*SlPLC2* plants were detached for *B. cinerea* inoculation. Harvest and pre-incubation of conidia and tomato leaf handling were performed as described (Benito *et al.*, 1998). Leaflets were droplet inoculated (for lesion diameter measurements and RNA isolation) or spray inoculated (for detection of H_2O_2 production) with *B. cinerea* spore suspension (10^6 spores.mL^{-1}). For droplet inoculations, 8-10 of 4 μL droplets were applied to each leaflet of detached compound leaves, except the apical. Incubations of droplet or sprayed inoculated leaves were performed in humid chambers at 20°C in the dark (Benito *et al.*, 1998). Lesion diameters of 300-400 inoculation sites were measured with a caliper at 48 and 72 h post-inoculation (hpi). The average lesion expansion rate was calculated by subtracting to each 72 hpi lesion diameter the corresponding measure made at 48 hpi.

Detection of H_2O_2 production

Sprayed inoculated leaflets were harvested at 24 hpi and incubated with 20 mg.mL^{-1} 3,3-diaminobenzidine (DAB) in 50 mM sodium acetate at room temperature in the dark for 5 h, immediately after harvesting. DAB locally polymerizes as soon as it comes into contact with H_2O_2 in the presence of peroxidase, and it is visualized as a brown precipitate (Thordal-Christensen *et al.*, 1997). Leaflets were bleached with 100% ethanol. Stained areas were quantified using the program ImageJ after generating an extension for the plugin *Phenotype Quant* (Abd-El-Haliem, 2012). The new extension (called 'DAB Quant') was generated by training the program to recognize and measure the surfaces of DAB stained areas in scanned images of DAB stained leaves. [Correction added on 22 September 2016, after first online publication: the wording has been amended to include more details on the *Phenotype Quant* plugin.]

cDNA synthesis and quantitative PCR analysis

Total RNA was extracted using Trizol as described by the manufacturer (Invitrogen, NY, USA). Complementary DNA (cDNA) was synthesized using MMLV reverse transcriptase (RT) from Promega (Madison, USA) and an oligo-dT primer on 1 μg of total RNA as a template. The cDNA was diluted to a final volume of 200 μL and 2.5 μL was used for quantitative PCR (qPCR). The Fast Universal SYBR Green Master mix from Roche (Mannheim, Germany) was employed, using a Step-one Real-time PCR machine from Applied Biosystems (California, USA). The standard amplification program was used. The nucleotide sequences of the specific primers for qPCR analysis of *SlPLC1* to *SlPLC6*, *SlPR1a*, *SlPR2b*, *SlPI-II*, *SlACT* and *BcACT* were previously reported (ten Have *et al.*, 2010; Lopez-Raez *et al.*, 2010; Uppalapati *et al.*, 2007; Vossen *et al.*, 2010). The primers used for *SlPI-I* were 5′-GACTCTAACTTGATGTGCGAAGG-3′ (forward primer) and 5′-TCAAAAAGACGAACTCGATCAC-3′ (reverse primer). Stepone Software

v2.1 (Applied Biosystems) was used to analyze the transcript amounts of all genes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Silencing specificity of S1PLC2 on tomato.