Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family members and the role of PLC4 and PLC6 in HR and disease resistance

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SUMMARY
The perception of pathogen-derived elicitors by plants has been suggested to involve phosphatidylinositol-specific phospholipase-C (PI-PLC) signalling. Here we show that PLC isoforms are required for the hypersensitive response (HR) and disease resistance. We characterised the tomato [Solanum lycopersicum (Sl) PLC gene family. Six SlPLC-encoding cDNAs were isolated and their expression in response to infection with the pathogenic fungus Cladosporium fulvum was studied. We found significant regulation at the transcriptional level of the various SlPLCs, and SlPLC4 and SlPLC6 showed distinct expression patterns in C. fulvum-resistant Cf-4 tomato. We produced the encoded proteins in Escherichia coli and found that both genes encode catalytically active PI-PLCs. To test the requirement of these SlPLCs for full Cf-4-mediated recognition of the effector Avr4, we knocked down the expression of the encoding genes by virus-induced gene silencing. Silencing of SlPLC4 impaired the Avr4/Cf-4-induced HR and resulted in increased colonisation of Cf-4 plants by C. fulvum expressing Avr4. Furthermore, expression of the gene in Nicotiana benthamiana enhanced the Avr4/Cf-4-induced HR. Silencing of SlPLC6 did not affect HR, whereas it caused increased colonisation of Cf-4 plants by the fungus. Interestingly, SlPLC6, but not SlPLC4, was also required for resistance to Verticillium dahliae, mediated by the transmembrane Ve1 resistance protein, and to Pseudomonas syringae, mediated by the intracellular Pto/Prf resistance protein couple. We conclude that there is a differential requirement of PLC isoforms for the plant immune response and that SlPLC4 is specifically required for Cf-4 function, while SlPLC6 may be a more general component of resistance protein signalling.

Keywords: disease resistance, innate immunity receptors, nucleotide-binding leucine-rich repeat, phospholipid signalling, receptor-like protein, virus-induced gene silencing.

INTRODUCTION
In their interactions with pathogenic organisms, plants must be able to perceive adverse external stimuli. Perception seems to rely largely on innate immunity receptors that specifically recognize pathogen-derived ligands. The Arabidopsis thaliana genome encodes hundreds of potential innate immunity receptors that are predicted to be localized at the plasma membrane [receptor-like proteins (RLPs) and receptor-like kinases (RLKs)] or intracellularly [nucleotide-binding leucine-rich repeat proteins (NB-LRRs)] (Shiu et al., 2004; Fritz-Laylin et al., 2005). Using such a wide repertoire of receptors, plants are able to recognise a broad spectrum of extracellular and intracellular elicitors. Recognition
results in the activation of a complex set of defence responses and can result in microscopically or macroscopically visible cell death, the so-called hypersensitive response (HR), that contributes to resistance to pathogens (Jones and Dangl, 2006). The mechanism by which recognition subsequently results in a comprehensive cellular response is the subject of our research.

In animal cells, phospholipid-based signal transduction is a common mechanism for relaying extracellular signals perceived by transmembrane receptors (reviewed by Berridge and Irvine, 1989). Upon stimulation, these receptors either directly or indirectly activate phospholipid-hydrolysing enzymes, thereby producing second-messenger molecules that diffuse laterally through the membrane or into the cytoplasm, often resulting in increased fluxes of calcium ions (Ca^{2+}). For example, activation of phosphatidylinositol-specific phospholipase C (PI-PLC), the enzyme that is subject of this paper, can result in the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). Both the reduced levels of substrate and the increased levels of the reaction products have a signalling function in animal cells.

Phosphatidylinositol (4,5)-bisphosphate provides a docking site for various proteins and is a key regulator of actin organisation and membrane traffic. Diacylglycerol remains in the intracellular leaflet of the plasma membrane, where it can activate protein kinase C (PKC). Inositol trisphosphate is released into the cytoplasm and binds ligand-gated Ca^{2+} channels (IP_3 receptors) in intracellular membranes, resulting in the release of Ca^{2+} from intracellular stores. In plants, the role of PIP_2 in cytoskeleton organisation and membrane traffic appears to be quite similar to that in animal cells (Kost et al., 1999; Helling et al., 2006; König et al., 2008). However, the function of the PLC reaction products DAG and IP_3 appears to be quite different since plants lack the equivalents of their respective targets (i.e. PKC and IP_3 receptors). It is therefore postulated that in plants the phosphorylated products of DAG (phosphatidic acid (PA) and diacylglycerol pyrophosphate) and of IP_3 (inositol hexakisphosphate (IP_6)) function as second messengers (Laxalt and Munnik, 2002; Xia et al., 2003; van Schooten et al., 2006; Zonia and Munnik, 2006; van Leeuwen et al., 2007; Xue et al., 2007). Many plant genomes encode PI-PLCs (Kopka et al., 1998; Müller-Röber and Pical, 2002; Mikami et al., 2004; Das et al., 2005; Munnik and Testerink, 2009) and activation of the enzymes in response to a large variety of signals has been shown. For example, PLC activity is induced rapidly upon exposure to heat, cold, salt and osmotic stress but also in response to endogenous signals like altered abscisic acid levels (reviewed in Meijer and Munnik, 2003; Müller-Röber and Pical, 2002; Xue et al., 2007).

The induction of PI-PLC activity in response to biotic stress has also been reported. For example, treatment of perceptive plant cell cultures with elicitors that are produced by a broad range of pathogens, so-called pathogen-associated molecular patterns (PAMPs), such as xylanase, flagellin and chitin (van der Luit et al., 2000; Yamaguchi et al., 2005) rapidly results in the accumulation of PA. This increase in PA appears to originate, at least in part, from the PLC product DAG which is phosphorylated by diacylglycerol kinase (DGK). Later it was shown that besides PAMPs, the race-specific effector Avr4 from the pathogenic fungus Cladosporium fulvum also induces the accumulation of PA within minutes after addition to cell cultures expressing the cognate Cf-4 resistance (R) gene from tomato [Solanum lycopersicum (Sl)]. Here, PA was found to originate from the sequential activity of PLC and DGK (de Jong et al., 2004). Successively, it was shown that two effectors from Pseudomonas syringae, AvrRpm1 and AvrRpt2, which are perceived by the intracellular R proteins RPM1 and RPS2, respectively, also cause a rapid induction of PLC activity in Arabidopsis cells (Andersson et al., 2006). A role for PLC has been implicated not only in elicitor recognition processes but also in downstream disease resistance signalling. It has been shown, for example, that OsPLC1 transcript levels increase upon treatment of rice cell suspension cultures with benzo[ghi]dioxin (BTH) or Xanthomonas oryzae. In addition, the resulting oxidative burst could be partially suppressed by treatment with PLC inhibitors (Song and Goodman, 2002; Chen et al., 2007).

In several processes, such as ABA perception (Sanchez and Chua, 2001), pollen tube growth (Dowd et al., 2006; Helling et al., 2006), cytokinin signalling (Repp et al., 2004) and drought tolerance (Wang et al., 2008), the involvement of PLCs has been demonstrated genetically. To our knowledge, all evidence that PLCs are involved in plant immunity comes from inhibitor studies and no reports are available using molecular-genetic tools. Here, we describe the identification and characterisation of a set of PI-PLC-encoding cDNAs from tomato. We subsequently studied the transcriptional regulation of the six corresponding SIPLC genes in different organs and in response to pathogen infection. SIPLC4 and SIPLC6 showed distinct expression patterns in resistant tomato and these genes were therefore selected for further studies. The encoded proteins were produced in Escherichia coli and we could show that both SIPLC4 and SIPLC6 are catalytically active PI-PLCs. Using a combination of virus-induced gene silencing (VIGS) and ectopic expression experiments we show that these enzymes are required for efficient plant defence responses. In addition, the two PLCs are shown to have non-overlapping roles in disease resistance.

RESULTS

Characterisation of the PLC gene family of tomato

To identify PLCs of tomato, we searched publicly accessible tomato expressed sequence tag (EST) databases (TIGR,
SOL) using the tBLASTn protocol with the Arabidopsis AtPLC1 protein as a query. This resulted in 10 significant hits. Using this sequence information, primers were designed to obtain complete cDNA sequences. Sequence analysis of the amplified fragments revealed that the tomato genome expresses at least six different PLC genes and the corresponding cDNAs were designated SPLC1 to SPLC6. The encoded proteins all show the typical plant PLC-type of domain organisation (Munnik et al., 1998), consisting of a non-conserved N-terminal domain, followed by a conserved PI-PLC-X domain, a non-conserved spacer region, a conserved PI-PLC-Y and a conserved C2 or CaLB (calcium-dependent lipid-binding) domain at the C-terminus (Figure 1a and Figure S1 in Supporting Information). The PI-PLC-X and PI-PLC-Y domains together form a barrel-like structure containing the active site residues (Ellis et al., 1998). The C2 domain is expected to have a regulatory function in response to Ca$^{2+}$ and phospholipids (Cho and Stahelin, 2005). Using PSORT, a potential N-terminal mitochondrial import signal was found in the SPLC2 and SPLC3 proteins. No obvious subcellular localisation could be predicted for the other PLC proteins.

The amino acid sequences of the six tomato PLC proteins were aligned with 25 PLC sequences from other plant species and one human PLC sequence (Figure S1). The derived most parsimonious tree (Figure 1b) shows four major clades. One clade, containing SPLC2 and SPLC3, only contains sequences from Solanaceae, whereas SPLC1 clearly relates to potato [Solanum tuberosum (St)] PLC1. Dedicated nucleotide sequence alignments show over 95% identity between the potato and tomato PLC sequences. Therefore, the SPLC1, SPLC2 and SPLC3 genes were named after their potato relatives.

A second clade with sequences of mixed origin could be distinguished. The two tomato proteins in this clade were named SPLC4 and SPLC5 from top to bottom, as no clear orthologues could be identified. One remaining tomato PLC protein, which shows a slight relationship to AtPLC1 and AtPLC3, was named SPLC6, without any reference to homologous sequences from other species. Furthermore, we could distinguish a clade that seems to contain monocot PLC sequences exclusively, whereas another clade contains PLC sequences from Rosaceae exclusively.

**SPLC gene expression patterns**

In order to identify SPLC genes that are potentially involved in the resistance response of tomato to *C. fulvum* in the leaves, we first investigated basal SPLC gene expression. A set of gene-specific primers was designed and used for real-time PCR on cDNA from cotyledons, flowers, fruits, leaves, roots and stems of healthy tomato plants. The six PLC genes were expressed in all organs tested (Figure S2); however, clear differences are observed in the transcript abundance of the individual SPLC genes. SPLC3 is the most abundantly expressed PLC gene. Its average expression level corresponds to 20% of the tomato actin (SlACT) Ct value, whereas SPLC5 transcripts show the lowest abundance in each organ (about 0.1% of SlACT).

The instantaneous increase in PLC activity that was observed in Cf-4-expressing cell suspension cultures upon treatment with Avr4 is likely to be achieved at the post-transcriptional level (de Jong et al., 2004). To test whether PLCs are also regulated at the transcriptional level, Cf-4 and Cf-0 tomato plants were inoculated with an Avr4-expressing strain of *C. fulvum*, resulting in an incompatible and a
compatible interaction, respectively. Water-treated Cf-4 plants were included as a mock treatment. Leaflets were taken before inoculation and at 2–3-day intervals after inoculation. Subsequently, real-time PCR analysis was performed to determine the expression levels of the genes of interest relative to expression levels of SlACT. As an additional control for gene expression we tested the expression level of SIGAPDH. The transcript remained constant throughout the experiment (data not shown). As shown in Figure 2, the expression of C. fulvum Avr9 (van Kan et al., 1991) and Ecp6 (Bolton et al., 2008) showed that colonisation was not successful in resistant Cf-4 plants, as the transcript levels remained low. However, in susceptible Cf-0 plants an increased expression of over 1000-fold for Avr9 and 50-fold for Ecp6 was observed. In Cf-4 plants there was a rapidly enhanced expression of the plant defence marker PR-1a, whereas in Cf-0 these transcripts accumulated more slowly. These kinetics are typical for an incompatible and a compatible interaction, respectively (van Kan et al., 1992). In mock-treated plants, SlPLC2, SlPLC3, SlPLC4 and SlPLC6 expression levels were relatively stable throughout the experiment. Towards the end of the experiment, the expression of SlPLC1 was induced while SlPLC5 expression was repressed. These trends might be related to the age of the leaves and/or the conditions under which the plants were grown. In the incompatible interaction, the expression levels of SlPLC3 and SlPLC6 were not significantly affected as compared with their expression in the mock-treated plants, whereas the levels of SIPLC1, SIPLC2, SIPLC4 and SIPLC5 transcripts significantly increased. This increase was transient for SIPLC1 and SIPLC4, as their expression levels decreased again at day 10 to reach the same levels as in the mock-treated plants. Interestingly, SIPLC2 and SIPLC5 reached their maximum expression levels at day 7. The concise regulation of SIPLC transcript levels at day 7 coincides with the time point at which the fungal biomass starts to increase significantly in the compatible interaction as compared to the incompatible interaction. This suggests a role for the SIPLC genes in the resistance response. However, the induction of the SIPLC transcripts does not seem to be a direct response of the Cf-4 plants to the Avr4 effector, as in the compatible interaction SIPLC1, SIPLC4 and SIPLC5 transcript accumulation follows similar kinetics as in the incompatible interaction. SIPLC2, SIPLC3 and SIPLC6 transcript accumulation shows slightly different kinetics in the compatible as compared with the incompatible interaction.

SIPLC4 and SIPLC6 encode catalytically active enzymes that convert phosphatidylinositol into diacylglycerol

SIPLC4 and SIPLC6 show distinct expression patterns in resistant Cf-4 plants upon inoculation with C. fulvum. SIPLC4 is a representative of the group whose expression peaks at day 7, whereas SIPLC6 expression is not affected.

Figure 2. Expression patterns of Avr9, Ecp6, PR-1a and the SIPLC genes during the interaction between tomato and Cladosporium fulvum. The Cf-4 and Cf-0 tomato plants were inoculated with a strain of C. fulvum expressing Avr4 or mock-treated with water. Leaflets were taken at the indicated days post-inoculation from three different plants and pooled. In these samples the expression levels of the indicated genes were measured by quantitative PCR. Relative expression levels (RQ) are shown using SlACT as an endogenous control. The day 0 samples were used as calibrators and were set to 1. Note the exponential scale of the Y-axis of the plots for Avr9, Ecp6 and PR-1a. Avr9 and Ecp6 transcripts were not detected in the mock-treated plants. Error bars represent standard deviations of two quantitative PCR samples from the same cDNA archive. The experiment was performed three times independently, with similar results. The result of a representative experiment is shown.

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Therefore in our further studies we decided to focus on the role of these two genes in defence. First we determined whether both genes indeed encode catalytically active PI-PLCs. For this we expressed the genes in E. coli (strain BL21) as glutathione S-transferase (GST)-fusion constructs. We expressed N-terminal fusions of GST and the full-length sequence of SlPLC4 and SlPLC6, using the pGEX-KG plasmid (Guan and Dixon, 1991). To exclude interference of possible co-purifying endogenous PI-hydrolysing activity from E. coli itself in our enzyme activity assays, we also included an empty vector (GST-only)-transformed control. Induction of gene expression and subsequent purification steps resulted in the isolation of highly purified recombinant proteins with the expected molecular weights, which are 93.5 kDa for GST-SlPLC4 and 92 kDa for GST-SlPLC6. For the GST-only control the expected GST band of 27 kDa was observed (results not shown). Both GST-SlPLC4 and GST-SlPLC6 displayed phosphoinositide-specific lipase activity as they are both able to hydrolyse PI and produce DAG in a time-dependent manner. This is shown for GST-SlPLC4 in Figure 3a. Interestingly, the enzymatic activity of both enzymes increased when decreasing the pH of the reaction buffer (Figure 3b). For GST-SlPLC4 and GST-SlPLC6 the pH optimum appears to be around 5.0 and 6.0, respectively. Figure 3b also shows that there is no co-purification of possible endogenous PI-hydrolysing activity of E. coli itself, as there is no enzymatic activity present in the GST-only control.

Unexpectedly, neither GST-SlPLC4 nor GST-SlPLC6 hydrolysed PIP2 under the reaction conditions that we tested (results not shown). This may reflect a strict substrate specificity compared with the PLC1, PLC2 and PLC3 enzymes from S. tuberosum, which were all shown to hydrolyse both PI and PIP2 (Kopka et al., 1998). Furthermore, we tested the ability of GST-SlPLC4 and GST-SlPLC6 to hydrolyze additional phospholipids, such as phosphatidylcholine (PC; results not shown) or phosphatidylethanolamine (PE), which in addition to PA is present in the PI substrate preparation (Figure 3), but we did not observe any degradation of these phospholipids under the applied reaction conditions.

SIPLC4 is required for Avr4/Cf-4-induced HR

After having shown that both SIPLC4 and SIPLC6 are indeed catalytically active PI phospholipases, we set out to investigate the requirement for these PLCs in the Avr4/Cf-4-induced HR. For this we knocked down the expression of the encoding genes using tobacco rattle virus (TRV)-induced gene silencing. Conserved parts of the SIPLC4 and SIPLC6 cDNAs were cloned into RNA2 of TRV. Ten-day-old Cf-4 seedlings were infected with either the recombinant TRV strains (designated TRV:PLC4 and TRV:PLC6) or a TRV strain that did not contain an insert (TRV-only). After 3 weeks, samples were collected to confirm that the target genes were efficiently knocked down.

As shown in Figure 4, which presents the results of one out of three independent experiments, the targeted SIPLC4 (grey arrows) and SIPLC6 (black arrows) genes were indeed silenced. The expression levels of the targeted genes varied between 5 and 50% of the levels of the TRV-only control plants. Virus-induced gene silencing of SIPLC4 and SIPLC6 appeared to be remarkably specific, since the transcript levels of the other five PLC genes in the TRV:PLC4- and TRV:PLC6-inoculated plants were not significantly suppressed. Surprisingly, the transcript levels of SIPLC2 were slightly (two- to threefold) higher in some of the tested TRV:PLC4- and TRV:PLC6-inoculated plants, as compared with the TRV-only-inoculated plants.

Now we had established that the targeted PLC genes were effectively and specifically silenced, we set out to test the role of PLC gene expression in the Avr4/Cf-4-induced HR. Leaflets of Cf-4 plants were injected with Avr4 protein at a total of eight sites left and right of the mid-vein, 3 weeks after TRV inoculation. As shown in Figure 5a, leaflets from TRV-only- and TRV:PLC6-inoculated plants showed a HR in response to Avr4, which is visible as brown necrotic tissue. Interestingly, the plants inoculated with TRV:PLC4 did not show this HR, and only slight chlorosis was observed at most sites of Avr4 injection. A similar effect was observed in the TRV:Cf-4-inoculated plants. Since VIGS in tomato tends
to cause ‘patchy’ silencing (Liu et al., 2002a) and because the efficiency of silencing is different in individual leaflets, we quantitatively confirmed the loss of HR. A total of 400 spots were injected with Avr4 in three independent experiments, for each TRV construct. The sites mounting an HR were counted and the percentage of responsive spots was calculated. The response of the TRV-only-inoculated plants was set to 100% (Figure 5b). In the TRV:PLC4- and the TRV:Cf-4-inoculated plants the HR was reduced to approximately 50% of the response in the TRV-only-inoculated plants. In contrast, the TRV:PLC6-inoculated plants showed a response that was similar to the TRV-only-inoculated plants. These results allowed us to conclude that SlPLC4 is required for the Avr4/Cf-4-induced HR.

Ectopic expression of SlPLC4 in Nicotiana benthamiana

We next wanted to test whether over-expression of SlPLC4 affects the Avr4/Cf-4-induced HR. As tomato plants are not suitable for transient over-expression of genes through agroinfiltration we used Cf-4-transgenic Nicotiana benthamiana plants which are highly amenable to ectopic expression studies (Gonzalez-Lamothe et al., 2006; Gabriëls et al., 2007). These plants respond to injection of Avr4 protein with a similar sensitivity as Cf-4 tomato plants, resulting in a typical HR within 2 days (Gabriëls et al., 2006). The SlPLC4 open reading frame, driven by the 35S promoter, was expressed through agroinfiltration in the left half of a leaf. The right half of the same leaf was infiltrated with Agrobacterium tumefaciens carrying the beta-glucuronidase (GUS) gene in the same vector backbone. Three days post-agroinfiltration both halves of the leaf were challenged with two concentrations of Avr4 protein. The high Avr4 concentration (50 µg ml⁻¹, position 3) triggered a HR within 2 days in both leaf halves, while the low concentration (5 µg ml⁻¹, position 2) caused a HR only in the leaf half expressing SlPLC4 (Figure 6a, see arrow). Infiltration of Avr4 into leaves of N. benthamiana not expressing Cf-4, but expressing SlPLC4 in the left leaf half and GUS in the right leaf half, did not cause a HR (Figure 6b). Infiltration medium itself did not cause any response in either leaf half (Figure 6a,b; injections at position 1). These results show that the HR observed upon challenge with Avr4 is Cf-4-dependent and that SlPLC4 expression by itself does not cause a-specific cell death in response to Avr4. The results shown in Figure 6 were consistently observed in five independent experiments (Table S1). Accumulation of SlPLC4 protein was confirmed by western blot analysis of extracts of leaves infiltrated with a 4× cMyc-tagged version of SlPLC4 in the same vector backbone. The molecular weight of the tagged SlPLC4 protein is predicted to be 70.5 kDa, and we indeed observed a band of this size (Figure 6c). Thus, ectopic expression of SlPLC4 in Cf-4 N. benthamiana plants causes an increased sensitivity to Avr4.

Both SlPLC4 and SlPLC6 are involved in Cf-4-mediated resistance to C. fulvum

Having established that SlPLC4 is involved in the Avr4/Cf-4-induced HR, we tested whether VIGS of SlPLC4 or SlPLC6 affects the resistance of tomato to C. fulvum. Therefore, tomato Cf-4 plants were inoculated with either TRV:PLC4, TRV:PLC6, TRV:Cf-4 or TRV-only and 3 weeks later the plants were inoculated with a C. fulvum strain expressing Avr4, as well as the constitutively expressed transgenic marker GUS. Finally, 2 weeks later, the leaves were inspected for disease symptoms. Macroscopically, no obvious disease symptoms were observed, also not in the TRV:Cf-4-inoculated plants in which resistance is expected to be suppressed. To reveal whether C. fulvum had colonised the tomato leaflets, the transgenic GUS marker was used. Blue staining clearly
indicated colonisation of the intercellular spaces of the leaflets by fungal mycelial structures in the TRV-inoculated plants. (Figure 7a-b). The arrowheads indicate fungal stroma underneath the stomata in TRV:Cf-4 and TRV:PLC6-inoculated plants. At a later stage of infection, outgrowth of conidiophores was observed in TRV:Cf-4-inoculated plants but not in the TRV:PLC4- and TRV:PLC6-inoculated plants. In leaflets of the TRV-only-inoculated plants no significant blue staining was observed. These histological data strongly suggest that both SlPLC4 and SlPLC6 are required for full Cf-4-mediated resistance.

In order to obtain quantitative support for our observations, we studied the presence of C. fulvum-derived transcripts in the TRV-inoculated Cf-4 plants. Two weeks after inoculation with C. fulvum, three leaflets of the plants were picked in two independent experiments. Both experiments revealed similar results, and in Figure 7c the results of one experiment are shown. Avr9 and Ecp6 transcripts could be detected in TRV-only plants, albeit at very low levels. These are probably derived from the C. fulvum inoculum surviving on the surface of the leaf. In two out of three leaflets harvested from TRV:PLC4-inoculated plants we found a fivefold increase in Ecp6 mRNA as compared with the TRV-only-inoculated plants. The mRNA levels of Avr9 were also significantly higher, although to a lesser extent. Leaflets of the TRV:PLC6-inoculated plants showed an 8- to 25-fold induction of Ecp6 mRNA, whereas Avr9 mRNA levels had increased 4- to 15-fold. These quantitative data confirmed our histological data, and we conclude that both SlPLC4 and SlPLC6 are required for full Cf-4-mediated resistance.

**SlPLCs are required for Ve1- and Pto/Prf-mediated resistance**

So far, we have studied the requirement of the SlPLCs in responses mediated by the transmembrane R protein Cf-4, acting against the foliar pathogen C. fulvum. In tomato, resistance to the vascular fungal pathogen *Verticillium dahliae* is mediated by another transmembrane R protein, Ve1, which like the Cf proteins belongs to the class of receptor-like proteins (Fradin and Thomma, 2006; Fradin et al., 2009). To investigate whether Ve1-mediated resistance also requires PLCs, VIGS of SlPLC4 or SlPLC6 was applied to the tomato cultivar Motelle that contains the Ve1 gene. Two weeks after TRV inoculation the plants were root-inoculated with conidiospores of V. dahliae. While TRV-only- and TRV:PLC4-inoculated plants remained fully resistant upon *V. dahliae* inoculation, TRV:PLC6-inoculated plants were clearly compromised in Ve1-mediated resistance as the plants showed clear *V. dahliae*-induced stunting at 14 days post-inoculation (Figure 8a). Subsequent plating of stem sections from *V. dahliae*-inoculated plants revealed that explants of the TRV:PLC6-inoculated plants showed more fungal outgrowth, representative of increased fungal colonisation as compared with the TRV-only- and TRV:PLC4-inoculated plants (Figure 8b).

In order to determine whether in addition to transmembrane R proteins intracellular R proteins also require PLCs to function, we studied the interaction between tomato and the bacterium *Pseudomonas syringae* pv. *tomato* (Pst) expressing AvrPto. Here, resistance is established through the concerted action of Pto, which is a protein kinase, and Prf, an NB-LRR protein. TRV:PLC4 and TRV:PLC6 were

Figure 5. SlPLC4, but not SlPLC6, is required for the Avr4/Cf-4-induced hypersensitive response (HR).

(a) Leaflets of Cf-4 tomato plants, inoculated with the indicated tobacco rattle virus (TRV) strains, were injected with Avr4 at eight sites. Pictures were taken from representative leaflets 4 days after Avr4 injection.

(b) Quantification of the Avr4/Cf-4-induced HR in tomato. Injected sites that developed a HR were counted and the average response is expressed as a percentage of the maximum average response. Error bars represent the standard deviation of the average of three independent experiments.
inoculated onto Pto- and Prf-expressing tomato plants and 3 weeks later the plants were inoculated with Psst expressing AvrPto. TRV-only-inoculated plants remained free of symptoms, as expected for an incompatible interaction (Figure 8c). Plants inoculated with TRV:Prf rapidly developed typical speck symptoms, indicating significantly compromised resistance as a result of Prf silencing. Interestingly, bacterial speck symptoms were also observed on plants inoculated with TRV:PLC6, whereas TRV:PLC4-inoculated plants remained devoid of symptoms (Figure 8c). To quantify the extent of colonisation by the bacteria, leaf samples were taken directly after inoculation (day 0) and 4 days after inoculation. The number of bacteria in these samples was assessed in a colony count assay. As expected for an incompatible interaction, the number of bacteria did not increase in the case of inoculation with TRV-only (Figure 8d). Also, TRV:PLC4 inoculation did not result in increased bacterial growth. However, TRV:Prf-inoculated plants showed an approximately 2000-fold increase in colony-forming units, whereas the TRV:PLC6-inoculated plants showed an approximate 200-fold increase in colonisation by Psst after 4 days (Figure 8d). This is in agreement with the intensity of the speck symptoms observed (Figure 8c). We conclude that SIPLC6 is required for full function of both transmembrane and intracellular R proteins. Since no role for SIPLC4 was found in Ve1- and Pto/Prf-mediated resistance and because the role of SIPLC4 appeared to be most pronounced in the Avr4/Cf-4-induced HR (Figure 5a,b) we speculated that SIPLC4 could also be involved in the HR rather than in the resistance induced by other R proteins. To date, the effector that is perceived by the Ve1 protein has not been identified. Therefore, we only tested the effect of PLC gene silencing on the AvrPto/Prf-induced HR and compared this with the effect on the Avr4/Cf-4-induced HR. The TRV:PLC4 and TRV:PLC6 constructs were inoculated onto N. benthamiana containing either the Cf-4 or the Pto transgene and 3 weeks later the plants were agroinfiltrated with Avr4 and AvrPto, respectively. Similar to what was observed in tomato (Figure 5a,b), in N. benthamiana inoculation with TRV:PLC4, but not with TRV:PLC6, also compromised the Avr4/Cf-4-induced HR (Figure 8e). However, neither inoculation with TRV:PLC4 nor with TRV:PLC6 affected the AvrPto-induced HR, while TRV:Prf-inoculated plants showed a clearly suppressed HR. It is concluded that SIPLC4, in contrast to SIPLC6, is specifically required for Cf-4-mediated resistance responses.

**DISCUSSION**

**The PLC gene family**

We have identified and characterised six cDNAs from tomato encoding different PLC proteins (Figure 1). The encoded proteins show a domain organisation that is typical for plant PI-PLCs (Müller-Röber and Pical, 2002). Comparison of the sequences with PLCs from other plant species reveals that sequence differentiation of PLC proteins has occurred at several points during evolution, since monocot-, Rosaceae- and Solanaceae-specific clades could be identified in a phylogenetic tree (Figure 1b). Interestingly, in the N-termini of both SIPLC2 and SIPLC3 a potential mitochondrial localisation signal was found. This sequence precedes a series of α-helices upstream of the X-domain which was previously annotated as a single EF-hand motif (Otterhag et al., 2001). However, the primary structure of the tomato proteins does not fit the EF-hand consensus from Prosite (data not shown). A double EF-hand motif could be involved in binding of a Ca²⁺ ion. Although the function of the N-termini of PLC proteins remains unknown, it is clear that they have an important role because deletion abolishes the in vitro activity of the protein (Otterhag et al., 2001).
Transcriptional activation of **PLC** genes

We found that all six **PLC** genes have a basal expression level in all tested organs from tomato plants (Figure S2), suggesting that potentially all PLC proteins can be rapidly activated by an environmental trigger without **de novo** transcription. However, it has been reported that besides the PLC enzyme activity, the transcript levels of **PLC** genes are also regulated in response to several types of abiotic stress (Hirayama et al., 1995; Hunt et al., 2004; Kim et al., 2004; Lin et al., 2004; Tasma et al., 2008). Interestingly, a recent report shows that the transcript levels of **OsPLC1** in rice cell suspensions respond to BTH and **X. oryzae** (Chen et al., 2007).

Here we have shown the **in planta** responsiveness of the tomato **PLC** gene family to infection with **C. fulvum**. The expression levels of five **PLC** genes were transiently upregulated in an incompatible interaction with **C. fulvum**, as SIPLC1, SIPLC2, SIPLC3, SIPLC4 and SIPLC5 showed a peak in expression at day 7 (Figure 2). It can be concluded that this is a relatively late event, since **PR1a** transcript levels had already increased at day 5. Especially since the **PLC** transcripts were also upregulated in the compatible interaction, we conclude that transcriptional regulation is a response to fungal infection.

**PLC** isoforms have distinct functions in **Cf-4**-mediated disease resistance

We have shown that the SIPLC4 and SIPLC6 open reading frames encode enzymatically active PI-PLCs, as the heterologously expressed recombinant GST-SIPLC4 and GST-SIPLC6 proteins both efficiently hydrolyse PI, thereby generating DAG (Figure 3). Interestingly, the enzymes appeared to have a relatively low pH optimum, which might indicate that they are fully active when acidification of the cytosol occurs during initiation of the Cf-mediated defence response (de Jong et al., 2000). We could not show activity of the PLCs using substrates different from PI, which might indicate that the affinity for these substrates is lower, or even absent. Alternatively, we might not yet have found the optimal conditions and micellar preparations for these additional putative substrates.

Virus-induced gene silencing of SIPLC4 or SIPLC6 compromises **Cf-4**-mediated resistance.

We have shown that the SIPLC4 and SIPLC6 open reading frames encode enzymatically active PI-PLCs, as the heterologously expressed recombinant GST-SIPLC4 and GST-SIPLC6 proteins both efficiently hydrolyse PI, thereby generating DAG (Figure 3). Interestingly, the enzymes appeared to have a relatively low pH optimum, which might indicate that they are fully active when acidification of the cytosol occurs during initiation of the Cf-mediated defence response (de Jong et al., 2000). We could not show activity of the PLCs using substrates different from PI, which might indicate that the affinity for these substrates is lower, or even absent. Alternatively, we might not yet have found the optimal conditions and micellar preparations for these additional putative substrates.

Virus-induced gene silencing of SIPLC4 and SIPLC6 was shown to be effective as the expression of the target genes was knocked down to 5–50% of the levels in the control
The TRV:PLC4 and TRV:PLC6 inserts do have a few stretches of 21–25 nucleotides in common with other PLCs. However, silencing was remarkably specific since we did not observe a significant decrease in the expression levels of other PLC genes. Interestingly, the expression of SlPLC2 was slightly enhanced in some of the TRV:PLC4- and TRV:PLC6-inoculated plants (Figure 4). It can be speculated that in this way the plant compensates for the loss of expression of SlPLC4 and SlPLC6.

Virus-induced gene silencing of SlPLC4 resulted in a drastically reduced Avr4/Cf-4-induced HR (Figure 5). In addition, ectopic expression of SlPLC4 in Cf-4-transgenic N. benthamiana leaves resulted in an enhanced HR in response to Avr4 (Figure 6). These complementary experiments clearly demonstrate that SlPLC4 is involved in the Avr4/Cf-4-induced HR. Our finding that SlPLC4 is not involved in the Pto/Prf-mediated HR (Figure 8e) shows that SlPLC4 is not generally required for the HR. Virus-induced gene silencing of SlPLC6, however, did not affect the Avr4-induced HR in Cf-4 plants, suggesting that SlPLC6 has a function in the resistance response of the plant that differs from SlPLC4. Potentially, the distinct transcriptional
regulation of SIPlC4 and SIPlC6 accounts for these different functions. An increased expression of SIPlC4, as is observed at day 7 of the interaction with C. fulvum (Figure 2), might result in an enhanced sensitivity to Avr4, similar to what was observed upon ectopic expression of SIPlC4 (Figure 6).

We find that both SIPlC4 and SIPlC6 are required for full Avr4/Cf-4-induced resistance to C. fulvum (Figure 7). The fact that inoculation with the silencing constructs did not allow the fungus to proceed to later stages of infection (conidioaphore outgrowth and sporulation), suggests that the fungus is eventually recognised and (partial) defence responses are mounted. This could be caused by partial and patchy silencing of the SIPlC4 and SIPlC6 genes and/or functional redundancy with other PLC genes. SIPlC4 and SIPlC6 are possibly involved in different aspects of the resistance response. This is supported by our finding that SIPlC4 is more important for mounting the HR, while SIPlC6 is more important for the actual resistance to colonisation by the pathogen.

Besides a mechanistic difference, a temporal distinction between PLC functions can also be made. Rapid activation of PLC after recognition of an elicitor suggests that the first wave of PLC activation is based on post-translational modification and/or changed localisation of the enzyme. Since at a later stage after pathogen perception PLC genes are transcriptionally regulated (Figure 2), it is very likely that additional wave(s) of PLC activity are required for the actual resistance response. The idea that the first wave of PLC activation is a post-transcriptional event is supported by the finding that AtPlC2 is rapidly phosphorylated after the addition of flagellin to a cell suspension culture expressing the transmembrane receptor FLS2 (Nühse et al., 2007). Interestingly, a phosphorylated peptide of AtPlC2 that was identified localizes to the spacer between the X- and Y-domains. This spacer is the most variable region and is only conserved in a subset of the PLCs (Figure S1). Only in SIPlC4 is the serine residue that is phosphorylated in AtPlC2 conserved, while in SIPlC6, for example, this domain is absent. This also indicates that SIPlC4 and SIPlC6 can be subject to different types of regulation.

SIPlC6 is required for multiple R protein-mediated responses

In contrast to Cf-4-mediated resistance, Ve1- and Pto/Prf-mediated resistance appear not to require SIPlC4. However, knock down of SIPlC6 does inhibit Ve1 and Pto/Prf function (Figure 8). It is surprising that two transmembrane RLPs, Cf-4 and Ve1, require different PLC proteins to be functional. As Cf-4 and Ve1 function in different tissues (leaf mesophyll cells and the tissue surrounding the xylem vessels, respectively), there might be a different PLC requirement. The finding that besides Cf-4 and Ve1, the intracellular R protein couple Pto/Prf requires SIPlC6 as well is intriguing, as this suggests that PLC signalling is a common mechanism employed by both transmembrane and intracellular immune receptors. In the light of this it is interesting to note that RPM1 has been described to localise to the inner leaflet of the plasma membrane (Boyés et al., 1998) where PI(2,4)P, a potential PLC substrate, is present (Kost et al., 1999; van Leeuwen et al., 2007). Possibly, a particular PLC isoform is required at the plasma membrane to relay elicitor perception into an intracellular response. Another PLC isoform could then be required for a more general signalling response.

The PLC signalling pathway

As mentioned before, in animal cells, activation of PLC results in PI(3,4,5)P hydrolysis and the formation of the second messengers IP3 and DAG, which eventually evoke downstream signalling responses. In plants, however, the phosphorylated forms of IP3 and DAG, which are IP6 and additional derivatives and PA, respectively, seem to be important signalling molecules (Zonia and Munnik, 2006). Certain plant PI-PLCs can hydrolyse PI4P and PI(4,5)P equally well in vitro, but the in vivo substrate is unknown. Also, since plant PLCs mostly resemble the PLC type of isoenzymes (Tasma et al., 2008), the PI4P and DAG, are IP6 and PI(4,5)P2 also emerging as signalling molecules themselves, PLC might also function as an attenuator of their signalling capacity.

The phosphorylated products of IP3 may be involved in the release of Ca2+ from internal stores or from the apoplast, thereby inducing transient spikes in cytoplasmic Ca2+ concentration (Munnik and Testerink, 2009). Dependent on the subcellular location, lag time, amplitude and frequency, a specific calcium signature is generated that further specifies downstream signalling (Garcia-Brugger et al., 2006; Lecourieux et al., 2006; Ma and Berkowitz, 2007). Interestingly, the presence of a C2 domain in the C-terminus of plant PI-PLCs, which is predicted to be a calcium-dependent lipid-binding domain, provides additional clues for potential feedback mechanisms.

There are several reports dealing with the role of PA in disease resistance signalling. One report describes the identification of several PA-binding proteins, among which is Hsp90 (Testerink et al., 2004). Hsp90 plays an important role in pathogen perception since it is required for the activity of both intracellular and transmembrane R proteins (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Belkhadir et al., 2004; de la Fuente van Bentem et al., 2005; Gabriëls et al., 2006). A second target of PA is the phosphoinositide-dependent protein kinase Abl. Binding to PA activates Abl, which subsequently results in activation of the AGC kinase AbcA3 (Anthony et al., 2004). AbcA3 is also known as OX11 kinase, which was identified as an important mediator of oxidative burst signalling (Rentel et al., 2004). The kinase acts upstream of a MAP kinase
cascade involved in basal resistance against *Hyaloperonospora arabidopsis*. Recently, an AGC kinase from tomato, Adi3, was identified which inhibits a MAP kinase cascade involved in disease resistance-associated cell death (Devarenne et al., 2006). Despite these opposite functions, it is apparent that PDKs and AGC kinases form a link between phospholipid signalling and downstream MAP kinase cascades involved in disease resistance (Bögre et al., 2003). Our finding that multiple PLC-dependent events are involved in disease resistance could be related to the involvement of multiple independent MAP kinase cascades in disease resistance that work in parallel or sequentially (Asai et al., 2002; Ekengren et al., 2003; Menke et al., 2004; del Pozo et al., 2004; Brodersen et al., 2006; Stulemeijer et al., 2007). In line with the observations described above, Zhang et al. (2007) have reported that overexpression of a rice DGK and a poly-A tail primer on 1 μg of total RNA as a template. The cDNA was diluted to a final volume of 150 μl and the RNA present in the aqueous phase was prepared at 19°C (8 h). Truncated just after the potential transit peptides and prior to the predicted α-helices, thereby corresponding to the sequence of mature AtPLC1. Protein sequences were subjected to a first alignment by T-Coffee (Notredame et al., 2000). Phylogeny was performed using PHYLU P v.3.6.1.2 (Felsenstein, 1989). A single most parsimonious tree was constructed using the HsPLC3α as an outgroup and compared with a consensus tree that was constructed using 1000 bootstraps and maximum parsimony. The consensus tree was almost identical to the most parsimonious tree.

**Plant material, fungal and bacterial strains**

For the PLC gene expression studies we used Cf0 and Cf-4 plants, derived from the tomato cultivar Money Maker, that were inoculated with a strain of *C. fulvum* expressing Avr4 (race 5). For VIGS experiments we used transgenic Cf0 plants expressing only the Hcr9-4D homologue of the Cf-4 resistance locus (Thomas et al., 1997). Silenced plants were inoculated with transgenic *C. fulvum* race 5 pPMPGUS. Resistance to *Pst* isolate DC3000 was assayed in tomato RG-PtoR (Pto/Pto, Prf/Prf), while resistance against *V. dahliae* was assayed in tomato cultivar Motelle (Ve/Ve). For transient expression studies we used transgenic *N. benthamiana* expressing Hcr9-4D (Gabriëls et al., 2006). The plants were grown in the greenhouse at a relative humidity of 70%. The day temperature was 21°C (16 h) and night temperature was 19°C (8 h). For agroinfiltration we used *A. tumefaciens* strain GV3101.

**cDNA synthesis and Q-PCR analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen, http://www.invitrogen.com/). The RNA present in the aqueous phase was purified using the RNAeasy extraction kit (Qiagen, http://www.qiagen.com/) including an on-column RNase-Free DNase treatment. Complementary DNA was synthesized using Superscript III (Invitrogen) and a poly-A tail primer on 1 μg of total RNA as a template. The cDNA was diluted to a final volume of 150 μl and 3 μl was used for quantitative PCR. We used the Eurogentec SYBR-green detection kit (http://www.eurogentec.com/) on an ABI 7300 machine (Applied Biosystems, http://www3.appliedbiosystems.com/). The standard amplification program was used with the primers listed in Table S3. The PCR products were derived from cDNA and not from the remaining genomic DNA in the RNA preparation since omission of reverse transcriptase did not result in a PCR product within 40 cycles for each tested sample (data not shown). ABI-7300 SDS v.1.3.1 relative quantification software was used to calculate relative quantities (RQ) of cDNA. SiACT was used as endogenous control.

**Heterologous expression of recombinant SIPLC4 and SIPLC6 and phospholipase activity assays**

First, the full-length SIPLC6 cDNA was amplified from cDNA derived from Cf4-1 and Avr4-expressing tomato seedlings (Gabriëls et al., 2006). For this, RNA was isolated after induction of the HR in the seedlings, which results in elevated levels of SIPLC6 expression (data not shown). The complete SIPLC6 cDNA was obtained in two steps. First, by PCR using primer (5'-TCCCATATAAATGGAACATTAAACA-3') spanning the stop codon and the 3'-UTR. Second, by a nested PCR using the primers (5'-TTCTAGATATGTCTAATGGTAAGCAACATTTCCA-3') just upstream of the predicted start codon and a primer on the 3' end of the SIPLC6 cDNA (5'-TGAAGTCGAAACGCAT-3'). A PCR was performed on cDNA derived from Cf0, producing a 650-bp product. This PCR product could not be purified, because a potential start codon for the predicted PLC1 protein (AtPLC1). Primers were designed based on the sequence of multiple PLC-dependent events are involved in disease resistance that work in parallel or sequentially (Asai et al., 2002; Ekengren et al., 2003; Menke et al., 2004; del Pozo et al., 2004; Brodersen et al., 2006; Stulemeijer et al., 2007). In line with the observations described above, Zhang et al. (2008) have reported that overexpression of a rice DGK in tobacco enhances its resistance to *Phytophthora parasitica var. nicotianae*, suggesting that increased accumulation of PA stimulates disease resistance responses. Future research will be required to study the timing and interactions between the multitudes of PLC-mediated processes and their relationship with other defence signalling events.

**EXPERIMENTAL PROCEDURES**

Cloning and phylogenetic analysis of SIPLC cDNA sequences

Expressed sequence tags (ESTs) were selected from the SOL and TIGR EST databases using gBLASTn search with the Arabidopsis PLC1 protein (AtPLC1). Primers were designed based on the sequences of multiple independent MAP kinase cascades in disease resistance that work in parallel or sequentially (Asai et al., 2002; Ekengren et al., 2003; Menke et al., 2004; dell Pozo et al., 2004; Brodersen et al., 2006; Stulemeijer et al., 2007). In line with the observations described above, Zhang et al. (2008) have reported that overexpression of a rice DGK in tobacco enhances its resistance to *Phytophthora parasitica var. nicotianae*, suggesting that increased accumulation of PA stimulates disease resistance responses. Future research will be required to study the timing and interactions between the multitudes of PLC-mediated processes and their relationship with other defence signalling events.
fusion, SlPLC4 was amplified from a plasmid containing full-length SlPLC4 using the primers (5’-TTCTTAGATGGGGAATATGAGTTGCT-GAGTGTG-3’) and 5’-ACTCGAGTCATAAATACCGGCAGTAGG-3’), cloned into pGEMT and then isolated by digestion with XbaI and Xhol. The pGEX:SlPLC4 and pGEX:SLPCL6 constructs and an empty pGEX vector control were transformed to E. coli strain BL21. The bacteria were grown for 2 h at 37°C in 500 ml of standard liquid broth, while shaking at 225 rpm, after which synthesis of the fusion proteins was induced by the addition of 0.4 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen) and further incubation for 4 h at 27°C and shaking at 225 rpm. Cells were harvested by centrifugation (4000 g for 15 min) and the pellet was washed by resuspending it in cold PBS (pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). After centrifugation, pellets were resuspended in 1/16 of the initial culture volume using cold extraction buffer (50 mM 2-aminopropanediol (TRIS)-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA) supplemented with protease inhibitor cocktail (Complete, Roche, http://www.roche.com/), 0.2 mg ml−1 lysozyme (Sigma, http://www.sigmaaldrich.com/) and 6 mM diethiothreitol (DTT). Cells were lysed using a French press (SLM Instruments, http://www.sagescientific.com) and after centrifugation (10 000 g for 15 min) 0.1% (final concentration) Triton X-100 (Sigma) was added to the supernatant, followed by incubation for 60 min at 4°C on a roller mixer. Subsequently the recombinant proteins were affinity purified using glutathione Sepharose 4B beads according to the manufacturer’s instructions (GE Healthcare, http://www.gehealthcare.com/). The concentration of the purified fusion proteins was estimated by comparison with BSA standards on Coomassie brilliant blue-stained SDS-PAGE gels.

The PI-PLC activity assay was essentially performed as described by Melin et al. (1992), Drøbak et al. (1994) and Kopka et al. (1998). The assay was carried out in 50-μl reaction volumes, each containing 5 μg of GST-SPlC4, GST-SPlC6 or GST-only protein in 50 mM TRIS/maleate (pH 6.25), 10 mM MgCl2 and 10 mM CaCl2, when phosphatidylinositol (PI), phosphatidylcholine (PC) or phosphatidylethanolamine (PE) were used as the substrate. With PI-P2 as the substrate, 10 μM Ca2+ was used (Kopka et al., 1998). Substrates were added as a micellar-lipid solution, made of one of the following substances: 30 μg PI-mixture (l-α-phosphatidylinositol; also including PE and PAI) (Sigma, 10 μg PI-P2= 1.2-dipalmitoylphosphatidylcholine; Sigma) or 20 μg PC (l-α-phosphatidylcholine) (Sigma). As a standard, 12 μg diacetyl glycerol (1,2-dipalmitoyl-sn-glycerol, Cayman, http://www.caymanchem.com/) was used. The reaction mixtures were incubated at 25°C for up to 2 h.

Reactions products were purified according to Melin et al. (1992), dried under nitrogen and then dissolved in 10 μl chloroform and loaded onto silica gel plates (TLC silica gel 60, Merck, http://www.merck.com/). Thin layer chromatography was performed in one dimension using two solvents in which the plates were first run to half of their length in the first solvent [ethyl acetate/iso-octane/formic acid:H2O (12:2:3:10, v/v/v/v)], then plates were allowed to dry before a full run in the second solvent [hex-ane:diethyl ether:acetic acid (9:1:0.5, v/v/v)]. A TLC analysis using these two solvents ensured that all tested phospholipids were effectively separated. Finally, plates were dried and transferred to a sealed chamber containing iodine crystals (Sigma) to allow staining of reaction products.

**VIGS in tomato, HR and disease assays**

For VIGS we used the pTRV-RNA1 and pTRV-RNA2 vectors described by Liu et al. (2002b). The pTRV-RNA2-derived constructs TRV:Cl-4 and TRV:Prf have been described before (Eekenren et al., 2003; Gabriëls et al., 2006). The insert for TRV:PLC4 was amplified using primers 5’-TGTTAGCCGTGATCCTACCAAGGGTACTAG-3’ and primer 5’-TGTTACCTCTTCATAACCTCAGCAG-3’. For TRV:PLC6 primers 5’-CAGGATTCCTCGTCCTTTCCACTTG-3’ and 5’-CAGGGTCCTGAAAGCCTATAAGGGAGTG-3’ were used on MM-c0 cdNA as a template. The PCR products were ligated into the Asp718 and BamHI restriction sites in pYL159. The integrity of the inserts of the resulting clones was confirmed by DNA sequencing. The cotyledons of seedlings were agroinfiltrated (OD600 = 2) with a mixture of pTRV-RNA1 and the pTRV-RNA2-derived constructs (combined in a 1:1 ratio). Three weeks post-TRV inoculation, plants were either inoculated with C. fulvum race 5 (expressing Avr4, pGPD::GUS, V. dahliae, PetDC3000) injected with Avr4 protein or agroinfiltrated with Avr4 or AvrPrf.

The C. fulvum inoculations were performed as described by Stulemeijer et al. (2007). Colonisation of the leaflets by C. fulvum was assessed 2 weeks later by X-glucuronide (Biosynth AG, http://www.biosynth.com/) staining to reveal GUS activity or by quantitative PCR. For V. dahliae inoculations, plants were uprooted 2 weeks post-TRV inoculation and inoculated by dipping the roots for 3 min in a suspension of 106 conidia ml−1 water. Colonization of the stem tissue by V. dahliae was assessed 2 weeks after inoculation with the fungus by plate assays. Stem sections were made immediately above the cotyledons up to the third compound leaf and surface-sterilised. Five slices were plated onto potato dextrose agar (five slices per plate) and incubated for 2 weeks at 22°C. Inoculation and determination of colonisation with Pet DC3000 was performed as described by Eekenren et al. (2003).

For the HR assays using Avr4 protein, Avr4 was purified from the culture filtrate of Pichia pastoris expressing Avr4 using the 6His/FLAG (HF) affinity tag. The HF tag was removed by digestion of 1 mg ml−1 Avr4-HF with EKMax protease (Invitrogen) for 16 h at 37°C. The reaction mixture was 20- or 200-fold diluted in infiltration medium (0.01% TWEEN-80 in water) and injected into leaflets using a Hamilton syringe at various sites. Agroinfiltration of Avr4 and AvrPrf into transgenic Cf-4- and Pto-expressing N. benthamiana was done as described by Gabriëls et al. (2006).

**SlPLC4 expression in N. benthamiana**

The SlPLC4 expression construct was made using a forward primer overlapping the start codon (5’-CACTCGAGCATGGGGAATATGAGTTGCT-GAGTGTG-3’) and a reverse primer overlapping the stop codon (5’-TGTCGGTTGATTGTTACTTTCTAGGGCTACTTGTG-3’) with a GC clamp. The PIN1 transcriptional terminator sequence was fused downstream by overlap extension using forward primer 5’-CAGGATTCCTCGTCCTTTCCACTTG-3’ and reverse primer 5’-CAGGGTCCTGAAAGCCTATAAGGGAGTG-3’. The product was ligated into the Xhol and EcoRI restriction sites downstream of the 35S promoter of a PMG8000-based binary vector (van der Hoorn et al., 2001). The same insert was ligated into a derivative of this vector containing four repeats of the cMyC sequence resulting in an N-terminal, in-frame fusion. The integrity of the constructs was confirmed by sequence analysis. Prior to agroinfiltration the bacterial cultures were mixed in a 1:1 ratio with an A. tumefaciens culture containing a binary vector encoding the p19 silencing suppressor from tomato bushy stunt virus in order to prevent gene silencing (Voinnet et al., 2003).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:
Figure S1. Alignment of phosphatidylinositol-specific phospholipase-C (PI-PLC) protein sequences from various plant species and human PLC\(\alpha\)3.
Figure S2. Relative transcript abundance of PI-PLC genes in different organs of tomato plants.
Table S1. Quantification of the Avr4-induced hypersensitive response (HR) in N. benthamiana plants transiently expressing SIPLC4.

Table S2. Expressed sequence tag (EST) sequence data and primer sequences used for the cloning of tomato PLC cDNAs.
Table S3. Primers and probes used for quantitative PCR.

Please note: As a service to our authors and readers, this journal delivers Supporting Information supplied by the authors. Such sequences used for the cloning of tomato PLC genes in Arabidopsis thaliana.

REFERENCES


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Enhanced expression of phospholipase C 1 (ZmPLC1) improves drought tolerance in transgenic maize. *Planta*, 227, 1127–1140.


