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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 *Sl*PLC-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 *SlPLC*s, 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 *Sl*PLCs 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, *Sl*PLC6, but not *Sl*PLC4, 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 *Sl*PLC4 is specifically required for Cf-4 function, while *Sl*PLC6 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 benzothiadiazol (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 *SlPLC* genes in different organs and in response to pathogen infection. *SlPLC4* and *SlPLC6* 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 *Sl*PLC4 and *Sl*PLC6 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 *At*PLC1 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 *SlPLC1* to *SlPLC6*. 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 *Sl*PLC2 and *Sl*PLC3 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 *Sl*PLC2 and *Sl*PLC3, only contains sequences from Solanaceae, whereas *Sl*PLC1 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 *SlPLC1, SlPLC2* and *SlPLC3* 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 *Sl*PLC4 and *Sl*PLC5 from top to bottom, as no clear orthologues could be identified. One remaining tomato PLC protein, which shows a slight relationship to *At*PLC1 and *At*PLC3, was named *Sl*PLC6, 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.

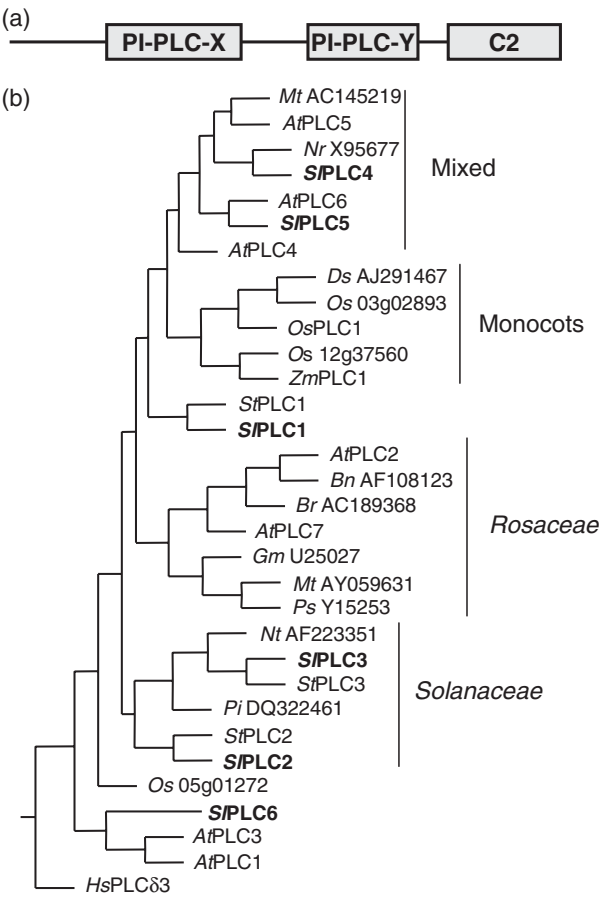

Figure 1. Characterisation of the tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) protein family.
(a) Schematic representation of the PI-PLC protein structure. PI-PLC-X and PI-PLC-Y domains are the conserved X and Y boxes of the catalytic domain, respectively. C2, also known as CaLB (calcium-dependent lipid-binding domain), is a conserved regulatory domain.
(b) Maximal parsimony consensus tree derived from an alignment (shown in Figure S1) of PI-PLC protein sequences from various species. *Hs*PLCδ3 was used as an outgroup. In cases where sequence names were not available, accession numbers are indicated. Abbreviations of species names: *At, Arabidopsis thaliana*; *Bn, Brassica napus*; *Br, Brassica rapa*; *Ds, Digitaria sanguinalis*; *Gm, Glycine max*; *Hs, Homo sapiens*; *Mt, Medicago truncatula*; *Nr, Nicotiana rustica*; *Nt, Nicotiana tabacum*; *Os, Oryza sativa*; *Pi, Petunia inflata*; *Ps, Pisum sativum*; *Sl, Solanum lycopersicum*; *St, Solanum tuberosum*; *Zm, Zea mays*.

SlPLC gene expression patterns

In order to identify *SlPLC* genes that are potentially involved in the resistance response of tomato to *C. fulvum* in the leaves, we first investigated basal *SlPLC* 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 *SlPLC* genes. *SlPLC3* is the most abundantly expressed *PLC* gene. Its average expression level corresponds to 20% of the tomato actin (*SlACT*) Ct value, whereas *SlPLC5* 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 *SlGAPDH*. 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 *SlPLC1*, *SlPLC2*, *SlPLC4* and *SlPLC5* transcripts significantly increased. This increase was transient for *SlPLC1* and *SlPLC4*, as their expression levels decreased again at day 10 to reach the same levels as in the mock-treated plants. Interestingly, *SlPLC2* and *SlPLC5* reached their maximum expression levels at day 7. The concise regulation of *SlPLC* 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 *SlPLC* genes in the resistance response. However, the induction of the *SlPLC* transcripts does not seem to be a direct response of the *Cf-4* plants to the Avr4 effector, as in the compatible interaction *SlPLC1*, *SlPLC4* and *SlPLC5* transcript accumulation follows similar kinetics as in the incompatible interaction. *SlPLC2*, *SlPLC3* and *SlPLC6* transcript accumulation shows slightly different kinetics in the compatible as compared with the incompatible interaction.

SlPLC4 and *SlPLC6* encode catalytically active enzymes that convert phosphatidylinositol into diacylglycerol

SlPLC4 and *SlPLC6* show distinct expression patterns in resistant *Cf-4* plants upon inoculation with *C. fulvum*. *SlPLC4* is a representative of the group whose expression peaks at day 7, whereas *SlPLC6* expression is not affected.

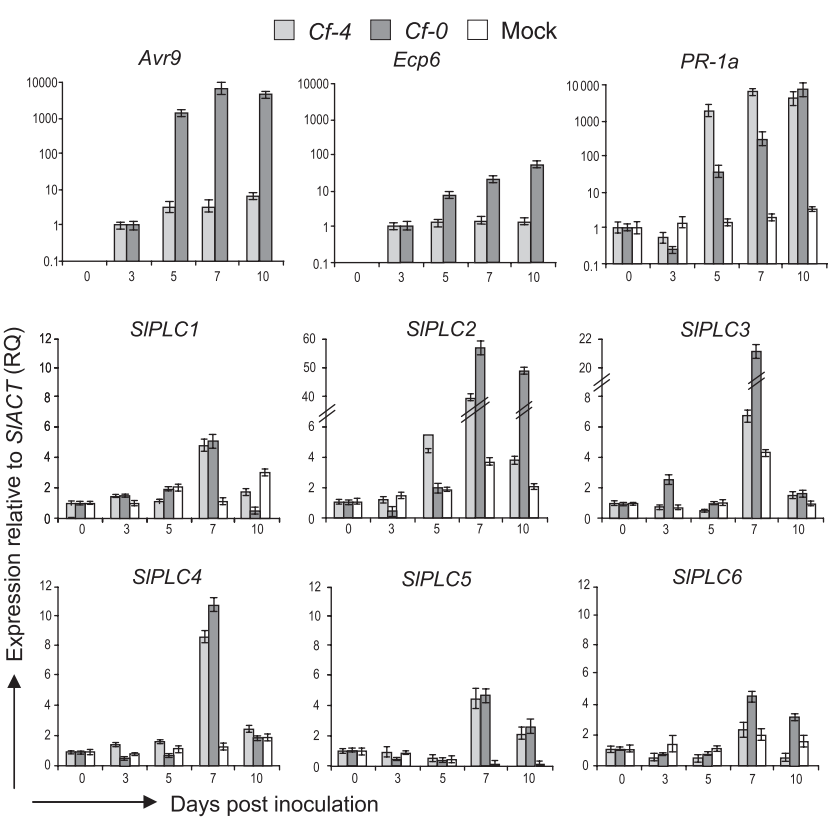

Figure 2. Expression patterns of *Avr9*, *Ecp6*, *PR-1a* and the *SlPLC* genes during the interaction between tomato and *Cladosporium fulvum*. The *Cf-4* and *Cf-0* tomato plants were inoculated with a strain of *C. fulvum* expressing Avr4 or mock-treated with water. Leaflets were taken at the indicated 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.

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 *Sl*PLC4 and *Sl*PLC6, 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-*Sl*PLC4 and 92 kDa for GST-*Sl*PLC6. For the GST-only control the expected GST band of 27 kDa was observed (results not shown). Both GST-*Sl*PLC4 and GST-*Sl*PLC6 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-*Sl*PLC4 in Figure 3a. Interestingly, the enzymatic activity of both enzymes increased when decreasing the pH of the reaction buffer (Figure 3b). For GST-*Sl*PLC4 and GST-*Sl*PLC6 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-*Sl*PLC4 nor GST-*Sl*PLC6 hydrolysed PIP_2 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 PIP_2 (Kopka *et al.*, 1998). Furthermore, we tested the ability of GST-*Sl*PLC4 and GST-*Sl*PLC6 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.

*Sl*PLC4 is required for Avr4/Cf-4-induced HR

After having shown that both SlPLC4 and SlPLC6 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 *SlPLC4* and *SlPLC6* 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 *SlPLC4* (grey arrows) and *SlPLC6* (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 *SlPLC4* and *SlPLC6* 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 *SlPLC2* 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

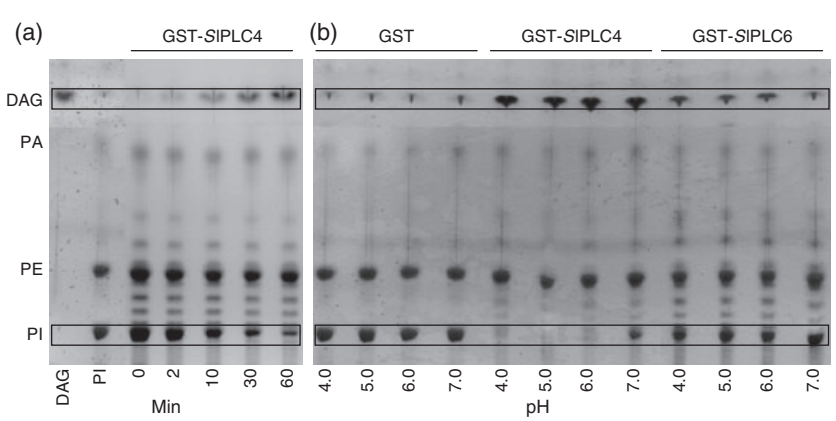

Figure 3. GST-*Sl*PLC4 and GST-*Sl*PLC6 are catalytically active phosphatidylinositol-specific phospholipase-Cs (PI-PLCs) that hydrolyse phosphatidylinositol (PI), thereby generating diacylglycerol (DAG).
(a) GST-*Sl*PLC4 hydrolyzes PI and generates DAG in a time-dependent manner.
(b) Both GST-*Sl*PLC4 and GST-*Sl*PLC6, but not GST-only purified from the empty vector-transformed *Escherichia coli* culture, display an increase in catalytic activity when decreasing the pH of the reaction buffer.

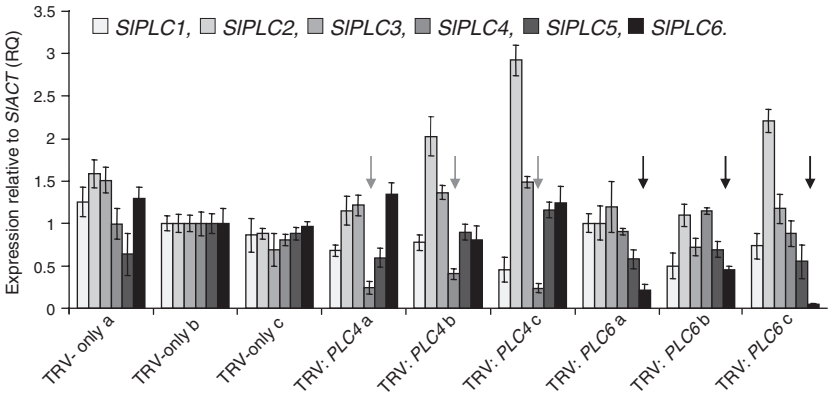

Figure 4. Specificity of virus-induced gene silencing (VIGS) of *SlPLC4* or *SlPLC6* in tomato. Quantitative PCR analysis on cDNA from three different leaflets (indicated with a, b and c), harvested from tomato plants 3 weeks after inoculation with the indicated tobacco rattle virus (TRV) silencing constructs. Expression levels were calculated relative to *SlACT* (RQ) and sample TRV-only b was used as the calibrator. The grey arrows point to the *SlPLC4* expression levels in the TRV:*PLC4*-inoculated plants and the black arrows point to the *SlPLC6* expression levels in the TRV:*PLC6*-inoculated plants. Error bars represent standard deviations of two quantitative PCR samples from the same cDNA archive.

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 *Sl*PLC4 protein was confirmed by western blot analysis of extracts of leaves infiltrated with a 4× cMyc-tagged version of *Sl*PLC4 in the same vector backbone. The molecular weight of the tagged *Sl*PLC4 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 *Sl*PLC4 and *Sl*PLC6 are involved in Cf-4-mediated resistance to *C. fulvum*

Having established that *Sl*PLC4 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

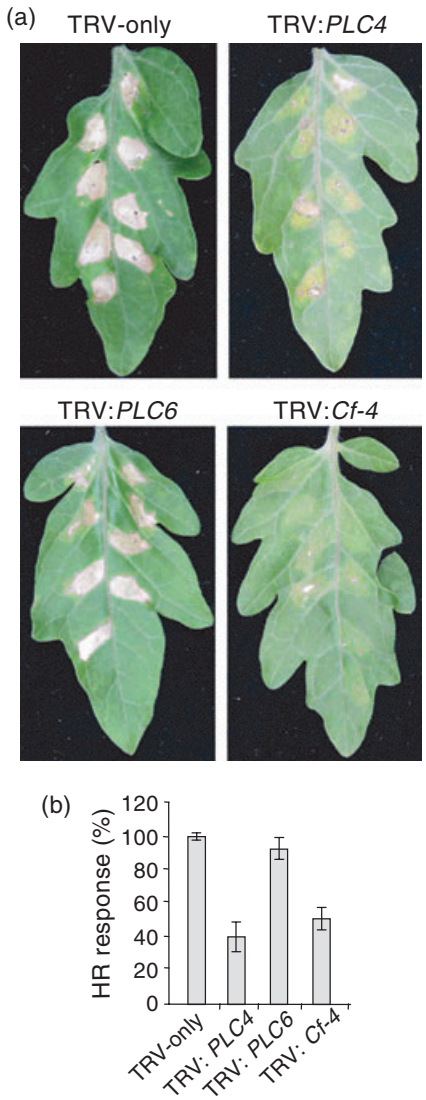

Figure 5. *SlPLC4,* but not *SlPLC6,* is required for the Avr4/Cf-4-induced hypersensitive response (HR).
(a) Leaflets of *Cf-4* tomato plants, inoculated with the indicated tobacco rattle virus (TRV) strains, were injected with Avr4 at eight sites. Pictures were taken from representative leaflets 4 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.

indicated colonisation of the intercellular spaces of the leaflets by fungal mycelial structures in the TRV:*Cf-4*-inoculated plants, and also in the TRV:*PLC4*- and TRV:*PLC6*-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 *Sl*PLC4 and *Sl*PLC6 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 *Sl*PLC4 and *Sl*PLC6 are required for full Cf-4-mediated resistance.

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

So far, we have studied the requirement of the *Sl*PLCs 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

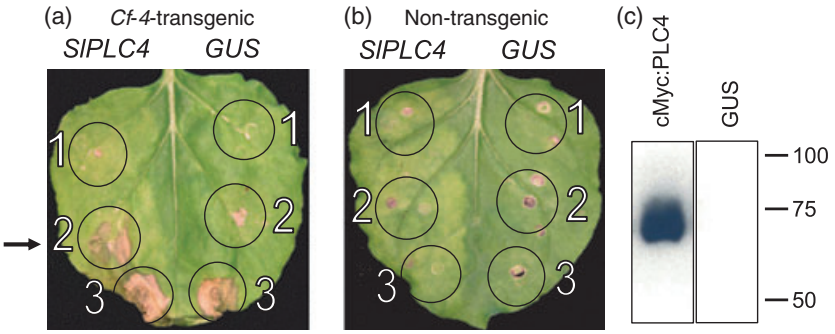

Figure 6. Ectopic expression of *Sl*PLC4 in *Nicotiana benthamiana* causes enhanced Cf-4-mediated sensitivity to Avr4.
A *35S:SlPLC4* construct was agroinfiltrated into the left leaf halves and a *35S:*GUS construct was agroinfiltrated into the right leaf halves of (a) *Cf-4*-transgenic or (b) non-transgenic *N. benthamiana* plants. Three days later, 5 and 50 mg ml^{-1} Avr4 protein was injected at positions 2 and 3, respectively. At position 1, only infiltration medium was injected. Pictures were taken 4 days after injection.
(c) Leaves were agroinfiltrated with a *35S:4xcMyc:SlPLC4* construct. Three days after agroinfiltration proteins were extracted and equal amounts of protein were subjected to SDS-PAGE. Subsequently, cMyc antigenic proteins were detected on a western blot. Sizes of the molecular weight markers are shown at the right (kDa). The molecular weight of the tagged *Sl*PLC4 protein is predicted to be 70.5 kDa, being 4.5 kDa for 4× cMyc-tag and 66 kDa for the *Sl*PLC4 protein itself.

inoculated onto *Pto*- and *Prf*-expressing tomato plants and 3 weeks later the plants were inoculated with *Pst* 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 *Pst* after 4 days (Figure 8d). This is in agreement with the intensity of the speck symptoms observed (Figure 8c). We conclude that *Sl*PLC6 is required for full function of both transmembrane and intracellular R proteins. Since no role for *Sl*PLC4 was found in Ve1- and Pto/Prf- mediated resistance and because the role of *Sl*PLC4 appeared to be most pronounced in the Avr4/Cf-4-induced HR (Figure 5a,b) we speculated that *Sl*PLC4 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 *Sl*PLC4, in contrast to *Sl*PLC6, 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 *Sl*PLC2 and *Sl*PLC3 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 Ca^{2+} 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).

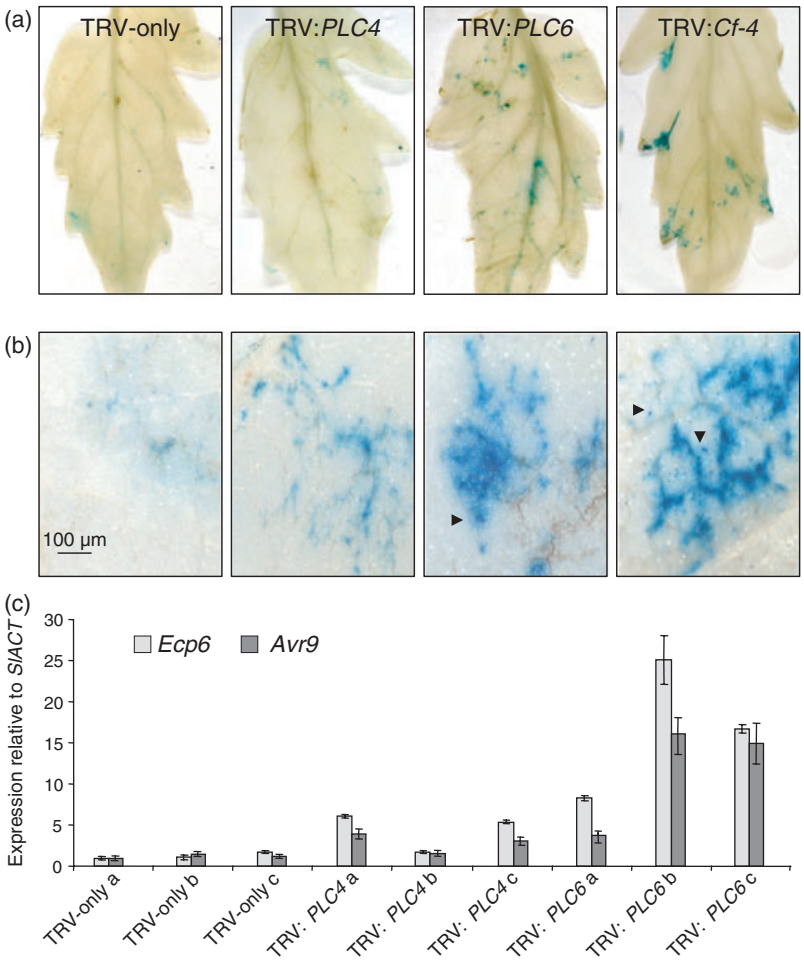

Figure 7. Silencing of *SlPLC4* or *SlPLC6* compromises Cf-4-mediated resistance.
(a) *Cf-4* tomato plants were inoculated with the indicated tobacco rattle virus (TRV) strains. After 3 weeks the plants were inoculated with *Cladosporium fulvum* expressing *Avr4* and the GUS marker gene. Two weeks after *C. fulvum* inoculation the leaflets were stained for GUS activity revealing fungal growth in the plant.
(b) Microscopic pictures of the leaves shown in (a). Arrowheads indicate positions where fungal stroma accumulates underneath the stomata.
(c) Plants were inoculated as described under (a) and 2 weeks after inoculation with *C. fulvum* leaflets were collected for quantitative PCR analysis to reveal the expression of *C. fulvum*-derived transcripts. Expression levels in independent leaflets (-a, -b and -c) were calculated relative to *SlACT* (RQ). Sample TRV-only-a was used as the calibrator. Error bars represent standard deviations of two quantitative PCR samples from the same cDNA archive.

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 *SlPLC1*, *SlPLC2*, *SlPLC3*, *SlPLC4* and *SlPLC5* 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 *SlPLC4* and *SlPLC6* open reading frames encode enzymatically active PI-PLCs, as the heterologously expressed recombinant GST-*Sl*PLC4 and GST-*Sl*PLC6 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 *SlPLC4* and *SlPLC6* was shown to be effective as the expression of the target genes was knocked down to 5–50% of the levels in the control

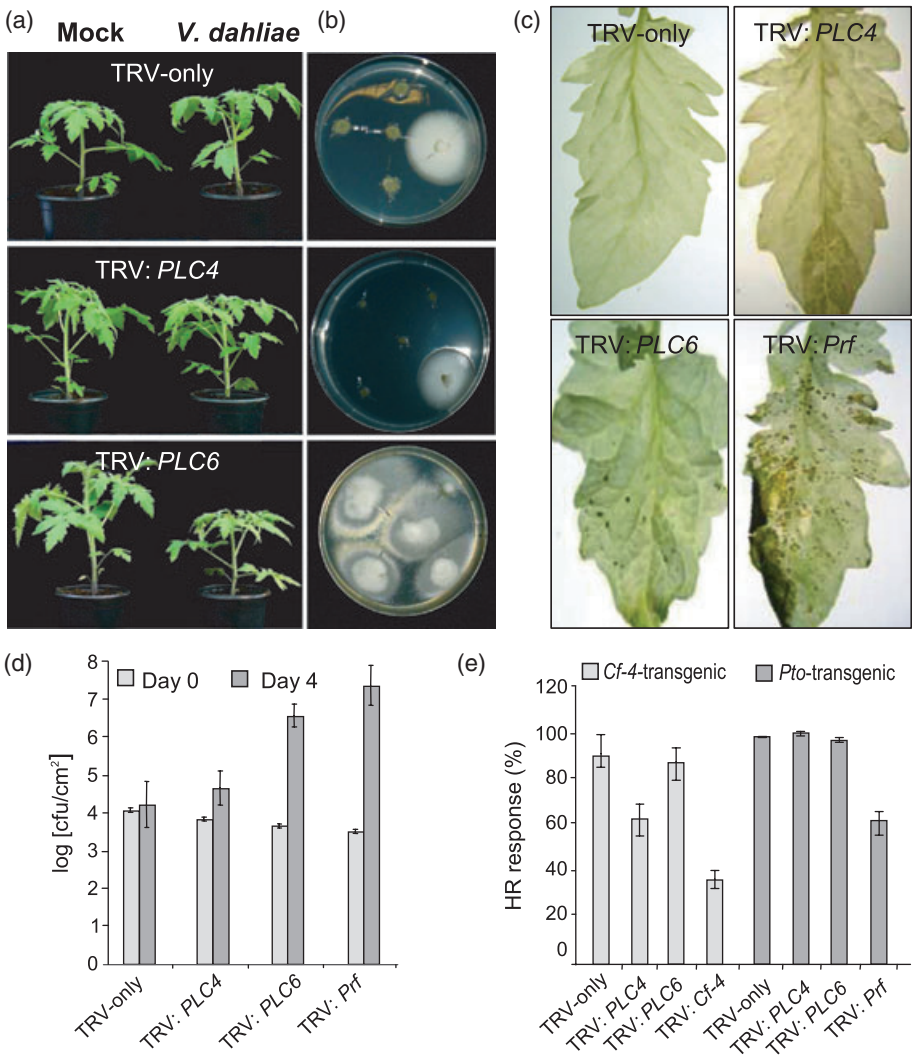

Figure 8. Silencing of *SlPLC6,* but not *SlPLC4*, compromises Ve1- and Pto/Prf-mediated resistance. Inoculation with the indicated virus-induced gene silencing (VIGS) constructs was followed by inoculation with *Verticillium dahliae* (a, b) or *Pseudomonas syringae* pv *tomato* DC3000 (c, d).
(a) *Verticillium dahliae*-induced stunting was visible at 14 days post-inoculation in tobacco rattle virus (TRV):*PLC6*-inoculated plants.
(b) Fungal colonization of the plants shown in (a) was assessed by plating stem sections onto potato dextrose agar (PDA) plates. Pictures were taken 2 weeks later.
(c) Bacterial speck symptoms had clearly developed at day 5, and pictures were taken at day 7.
(d) At day 0 and at day 4 samples were taken from the plants of which leaflets are shown in (c) to determine the number of colony forming units (cfu).
(e) Quantification of the Avr4/Cf-4- and AvrPto/Pto-induced hypersensitive response (HR) in *Nicotiana benthamiana*. The various TRV constructs were inoculated onto *Cf-4*- and *Pto*-transgenic *N. benthamiana* plants and after 3 weeks the plants were agroinfiltrated with *Avr4* and *AvrPto* constructs, respectively. Infiltrated sites that developed a HR were counted and the average response was expressed as a percentage of the maximum average response. Error bars represent the standard deviation of the average of five independent experiments.

plants (Figure 4). 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 *Sl*PLC4 is involved in the Avr4/Cf-4-induced HR. Our finding that *Sl*PLC4 is not involved in the *Pto/Prf*-mediated HR (Figure 8e) shows that *Sl*PLC4 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 *SlPLC4* and *SlPLC6* accounts for these different functions. An increased expression of *SlPLC4*, 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 *SlPLC4* (Figure 6).

We find that both *Sl*PLC4 and *Sl*PLC6 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 (conidiophore 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 *SlPLC4* and *SlPLC6* genes and/or functional redundancy with other *PLC* genes. *SlPLC4* and *SlPLC6* are possibly involved in different aspects of the resistance response. This is supported by our finding that *SlPLC4* is more important for mounting the HR, while *SlPLC6* 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 *At*PLC2 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 *At*PLC2 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 *Sl*PLC4 is the serine residue that is phosphorylated in *At*PLC2 conserved, while in *Sl*PLC6, for example, this domain is absent. This also indicates that *Sl*PLC4 and *Sl*PLC6 can be subject to different types of regulation.

*Sl*PLC6 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 *Sl*PLC4. However, knock down of *SlPLC6* 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 *Sl*PLC6 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 (Boyes *et al.*, 1998) where PIP_2, 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 PIP_2 hydrolysis and the formation of the second messengers IP_3 and DAG, which eventually evoke downstream signalling responses. In plants, however, the phosphorylated forms of IP_3 and DAG, which are IP_6 and additional derivatives and PA, respectively, seem to be important signalling molecules (Zonia and Munnik, 2006). Certain plant PI-PLCs can hydrolyse PI4P and $PI(4,5)P_2$ equally well *in vitro*, but the *in vivo* substrate is unknown. Also, since plant PLCs mostly resemble the PLCζ type of isoenzymes (Tasma *et al.*, 2008), and it is completely unknown how these are regulated (Cockcroft, 2006), it remains elusive which phosphoinositide is the *in vivo* substrate. Interestingly, as PI4P and $PI(4,5)P_2$ are also emerging as signalling molecules themselves, PLC might also function as an attenuator of their signalling capacity.

The phosphorylated products of IP_3 may be involved in the release of Ca^{2+} from internal stores or from the apoplast, thereby inducing transient spikes in cytoplasmic Ca^{2+} 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 *At*PDK1. Binding to PA activates *At*PDK1, which subsequently results in activation of the AGC kinase *At*AGC2-1 (Anthony *et al.*, 2004). *At*AGC2-1 is also known as OXI1 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.* (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 SlPLC cDNA sequences

Expressed sequence tags (ESTs) were selected from the SOL and TIGR EST databases using a tBLASTn search with the Arabidopsis PLC1 protein (*At*PLC1). Primers were designed based on the selected sequences preceding the potential start codon (Table S2) and, using a poly A-tail primer (5′-TTGGATCCTCGAGTTTTTTTTT-TTTTTTTTV-3′), 3′-rapid amplification of cDNA ends (RACE) was performed on tomato Cf0 cDNA. Because a potential start codon for *SlPLC6* could not be found, we first cloned the *SlPLC6* genomic DNA using the genome-walker technique (primers used: 5′-CCA-CACCTTCAAGAAAAAGTAGCTCAA-3′, 5′-TTGATCAAATAGTTAC-CCTCCGTGACG-3′ and 5′- AGACTGATGAGCAAAGTTATGTTCACC-3′). Three consecutive 'walks' produced a region of 980 bp of genomic DNA (accession no. EU099601). It contained a predicted exon with the potential start codon for *SlPLC6*. Using a primer (5′-ATGTCTAATGGTAAGCAACA-3′) just upstream of the predicted start codon and a primer on the 3′ end of the *SlPLC6* cDNA (5′-TGAGCTACTTTTTCTTGAAGGTGTGG-3′), a PCR was performed on cDNA derived from Cf0, producing a 650-bp product. This PCR product represented the 5′-end of the *SlPLC6* cDNA since it overlapped with the 3′-RACE product of *SlPLC6*. The PCR products were eventually cloned into pGEMT (Promega, http://www.promega.com/) and at least two independent clones were sequenced for each *PLC* cDNA by MWG Biotech AG (http://www.mwg-biotech.com/).

For the phylogenetic analysis of the *Sl*PLC protein sequences, sequences of full-length PI-PLCs from other plant species were searched using BLASTp and tBLASTn (Altschul *et al.*, 1997) at NCBI, The Arabidopsis Information Resource, TIGR or the Rice Genome Research Program. The collection of sequences was focused at completed genome sequences (Arabidopsis and rice), the agronomically important Solanaceae and Papilionoideae and monocots. All sequences were checked for the presence of PI-PLC hallmarks using PROSITE (Hulo *et al.*, 2006). Sequences were manually truncated just after the potential transit peptides and prior to the predicted α-helices, thereby corresponding to the sequence of mature *At*PLC1. Protein sequences were subjected to a first alignment by T-Coffee (Notredame *et al.*, 2000). Phylogeny was performed using PHYLIP v.3.6.1-2 (Felsenstein, 1989). A single most parsimonious tree was constructed using the HsPLCδ3 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 *pGPD:*GUS. 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 further 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-7300SDS v.1.3.1 relative quantification software was used to calculate relative quantities (RQ) of cDNA. *SlACT* was used as endogenous control.

Heterologous expression of recombinant SlPLC4 and SlPLC6 and phospholipase activity assays

First, the full-length *SlPLC6* cDNA was amplified from cDNA derived from *Cf-4*- 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 *SlPLC6* expression (data not shown). The complete *SlPLC6* cDNA was obtained in two steps. First, by PCR using primer (5′-TCCCACATATAAATTGAAC-ATTAAACA-3′) on the 5′-untranslated region (UTR) and primer (5′-TGGGATTGAGGAAGATTAATTAAGTAGTG-3′) spanning the stop codon and the 3′-UTR. Second, by a nested PCR using the primers (5′-TTCTAGATATGTCTAATGGTAAGCAACATTTCCA-3′) on the predicted start codon and primer (5′-ACTCGAGTTAAGTAG-TGAAGTCGAAACGCAT-3′) on the stop codon. These two primers also introduced *Xba*I and *Xho*I sites to the 5′- and 3′- ends of *SlPLC6*, respectively, and these sites were used for subsequent in-frame cloning of *SlPLC6* into the pGEX-KG plasmid resulting in a GST-*Sl*PLC6 fusion (Guan and Dixon, 1991). For the GST-*Sl*PLC4

fusion, *SlPLC4* was amplified from a plasmid containing full-length *SlPLC4* using the primers (5′-TTCTAGATATGGGGAATTATAGGGTATGTGT-3′) and (5′-ACTCGAGTCAGATAAACTCAAAGCGCATGAG-3′), cloned into pGEMT and then isolated by digestion with *Xba*I and *Xho*I. The pGEX:*SlPLC4* and pGEX:*Sl*PLC6 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-1-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 Na_2HPO_4, 1.8 mM KH_2PO_4). After centrifugation, pellets were resuspended in 1/16 of the initial culture volume using cold extraction buffer [50 mM 2-amino-2-(hydroxymethyl)1,3-propanediol (TRIS)-HCl, pH 7.5, 150 mM NaCl, 1 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 dithiothreitol (DTT). Cells were lysed using a French press (SLM Instruments, http://www.pegasusscientific.com) and after centrifugation (23 000 **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-*Sl*PLC4, GST-*Sl*PLC6 or GST-only protein in 50 mM TRIS/maleate (pH 6.25), 10 μM Mg^{2+} and 10 mM Ca^{2+}, when phosphatidylinositol (PI), phosphatidylcholine (PC) or phosphatidylethanolamine (PE) were used as the substrate. With PIP_2 as the substrate, 10 μM Ca^{2+} was used (Kopka *et al.*, 1998). Substrates were added as a micellar-lipid solution, made of one of the following substrates: 30 μg PI-mixture (L-α-phosphatidylinositol; also including PE and PA) (Sigma), 10 μg PIP_2 (1,2-dipalmitoylphosphatidylinositol-4,5-diphosphate) (Sigma) or 20 μg PC (Lα-phosphatidylcholine) (Sigma). As a standard, 12 μg diacylglycerol (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.

Reaction 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:H_2O (12:2:3:10, v/v/v/v)], then plates were allowed to dry before a full run in the second solvent [hexane: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:*Cf-4* and TRV:*Prf* have been described before (Ekengren *et al.*, 2003; Gabriëls *et al.*, 2006). The insert for TRV:*PLC4* was amplified using primers 5′-GTGGATCCGGTGTACCCCAAAGGTACTAG-3′ and primer 5′-GTGGTACCCTTCATAACCTCATCAGCAGGT-3′. For TRV:*PLC6* primers 5′-CAGGATCCCAAATGTGCTCTTCACCATCTG-3′ and 5′-ACGGTACCTTGAAAGCCATAAAGGAGGATG-3′ were used on MM-Cf0 cDNA as a template. The PCR products were ligated into the *Asp*718 and *Bam*HI restriction sites in pYL159. The integrity of the inserts of the resulting clones was confirmed by DNA sequencing. The cotyledons of seedlings were agroinfiltrated (OD_{600} = 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*, *Pst* DC3000, injected with Avr4 protein or agroinfiltrated with *Avr4* or *AvrPto*.

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 10^6 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 are plated onto potato dextrose agar (five slices per plate) and incubated for 2 weeks at 22°C. Inoculation and determination of colonisation with *Pst* DC3000 was performed as described by Ekengren *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 *AvrPto* 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′-CACTCGAGCATGGGGAATTATAGGGTAT-3′) and a reverse primer overlapping the stop codon (5′-TGCGCTTTGAGTTTATCTGAAGCTTTGACCCTAGACTTGT-3′). The PIN1 transcriptional terminator sequence was fused downstream by overlap extension using forward primer 5′-CACTCGAGCATGGGGAATTATAGGGTAT-3′ and reverse primer 5′-GTTCTGTCAGTTCCAAACGT-3. The product was ligated into the *Xho*I and *Eco*RI restriction sites downstream of the 35S promoter of a pMOG800-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 PLCδ3.

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 *SlPLC4*.

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.

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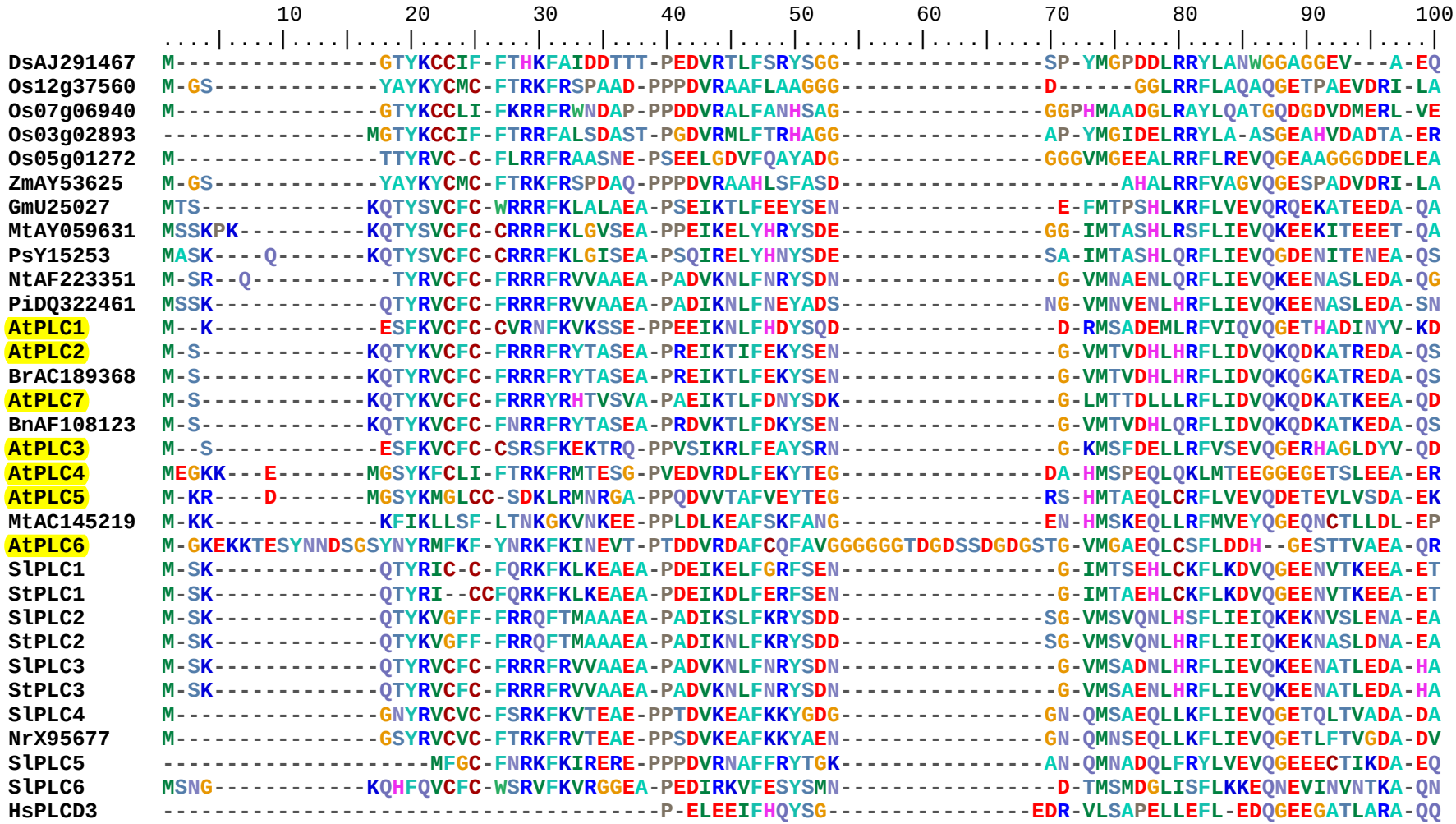
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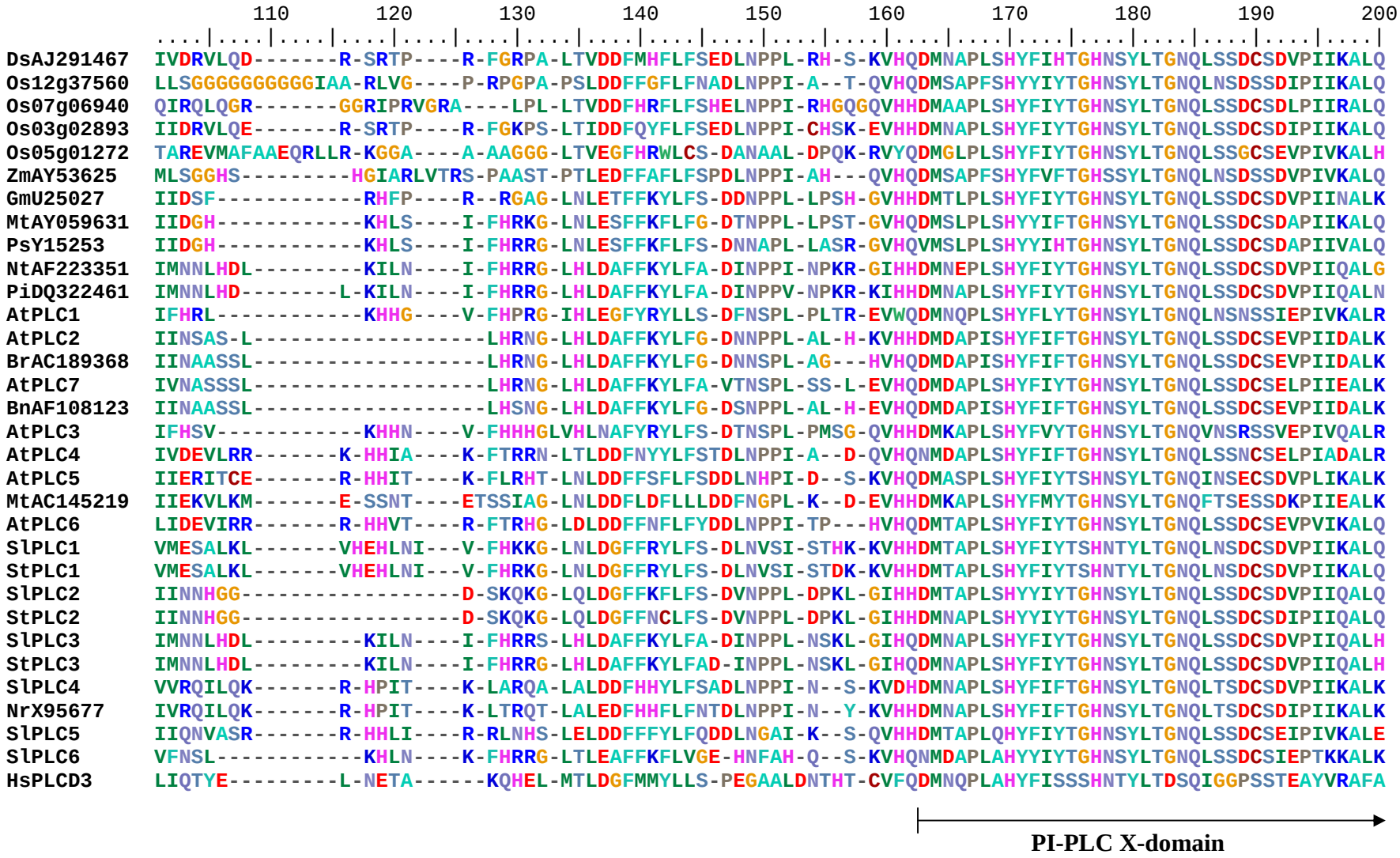
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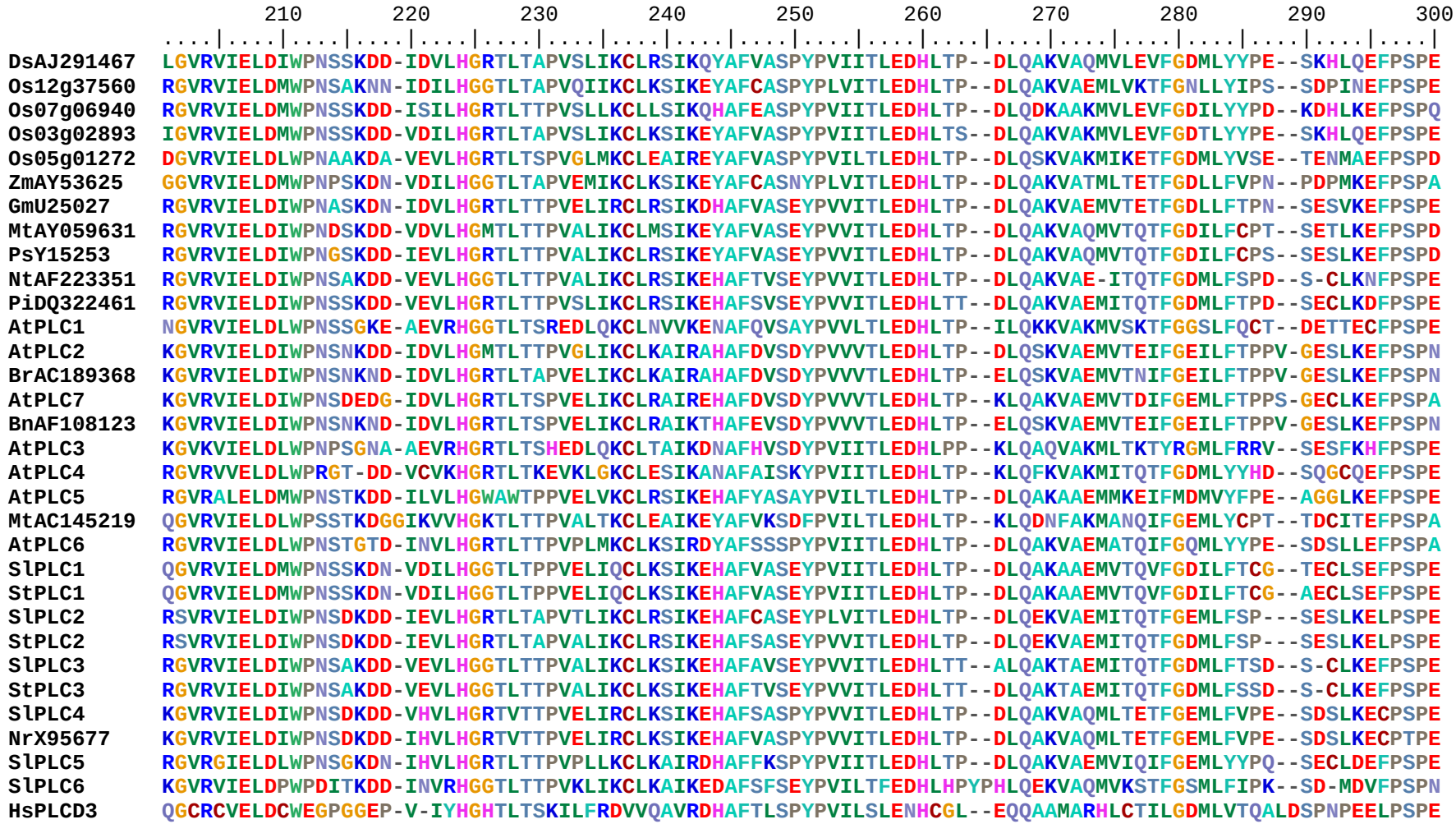

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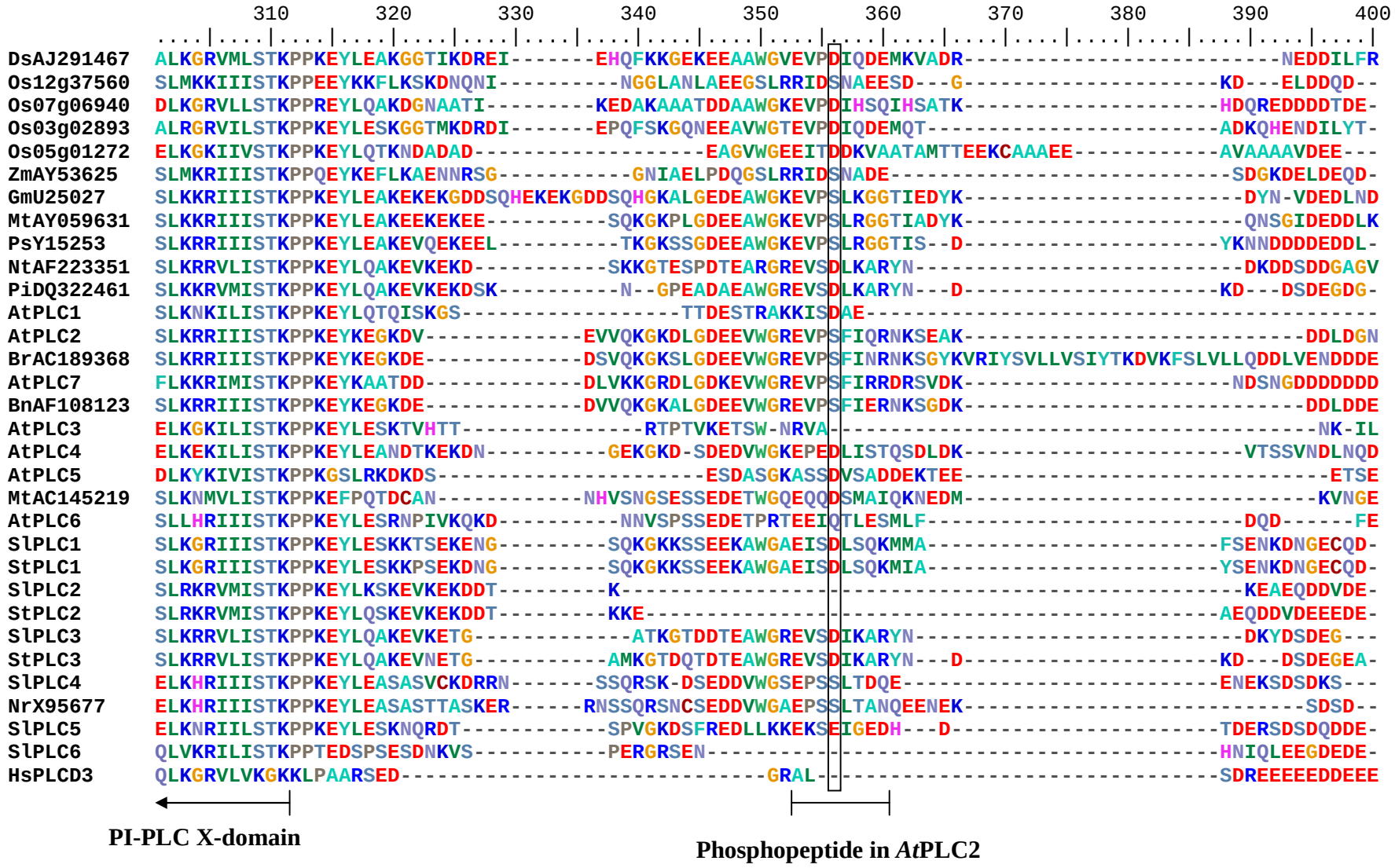
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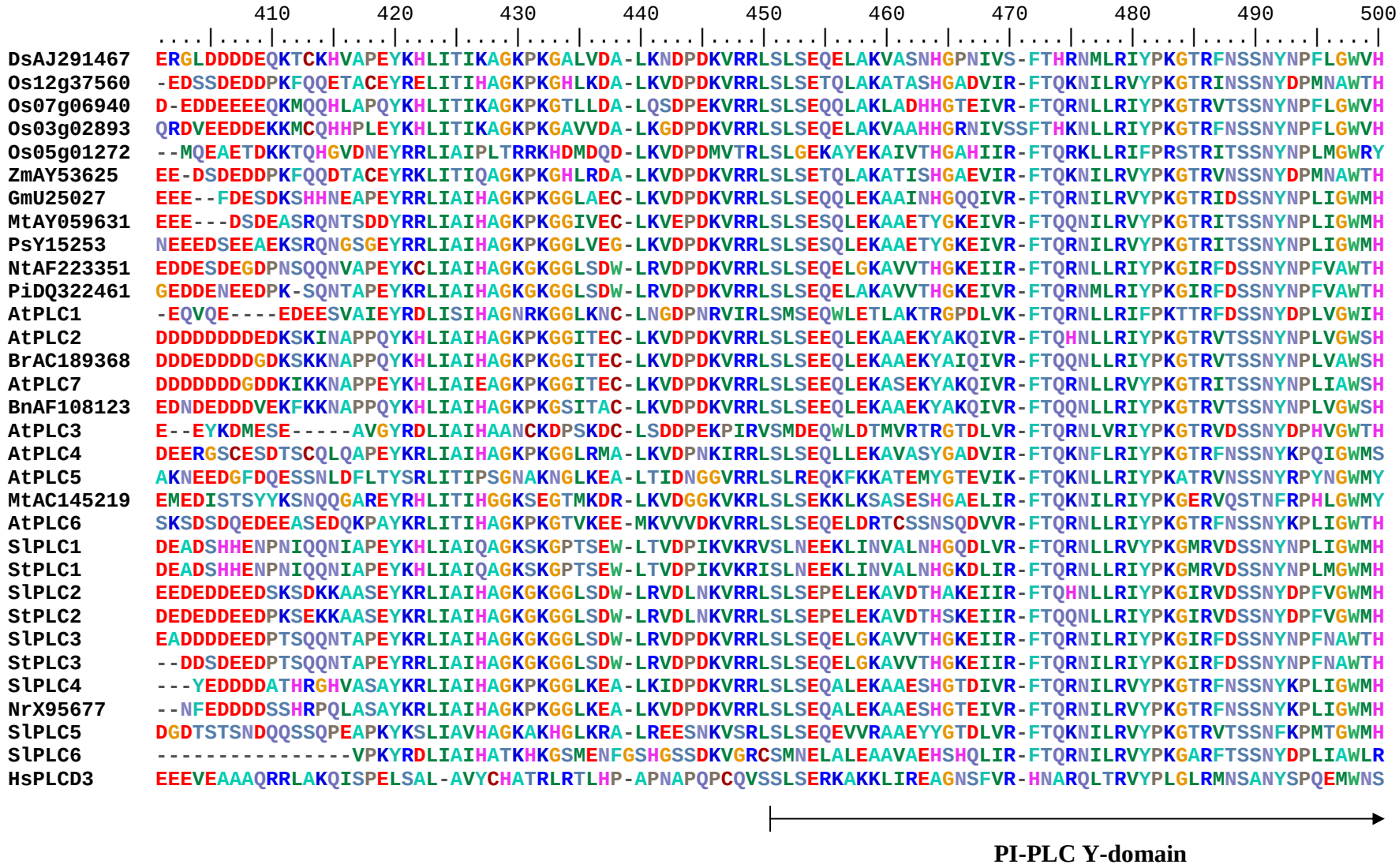
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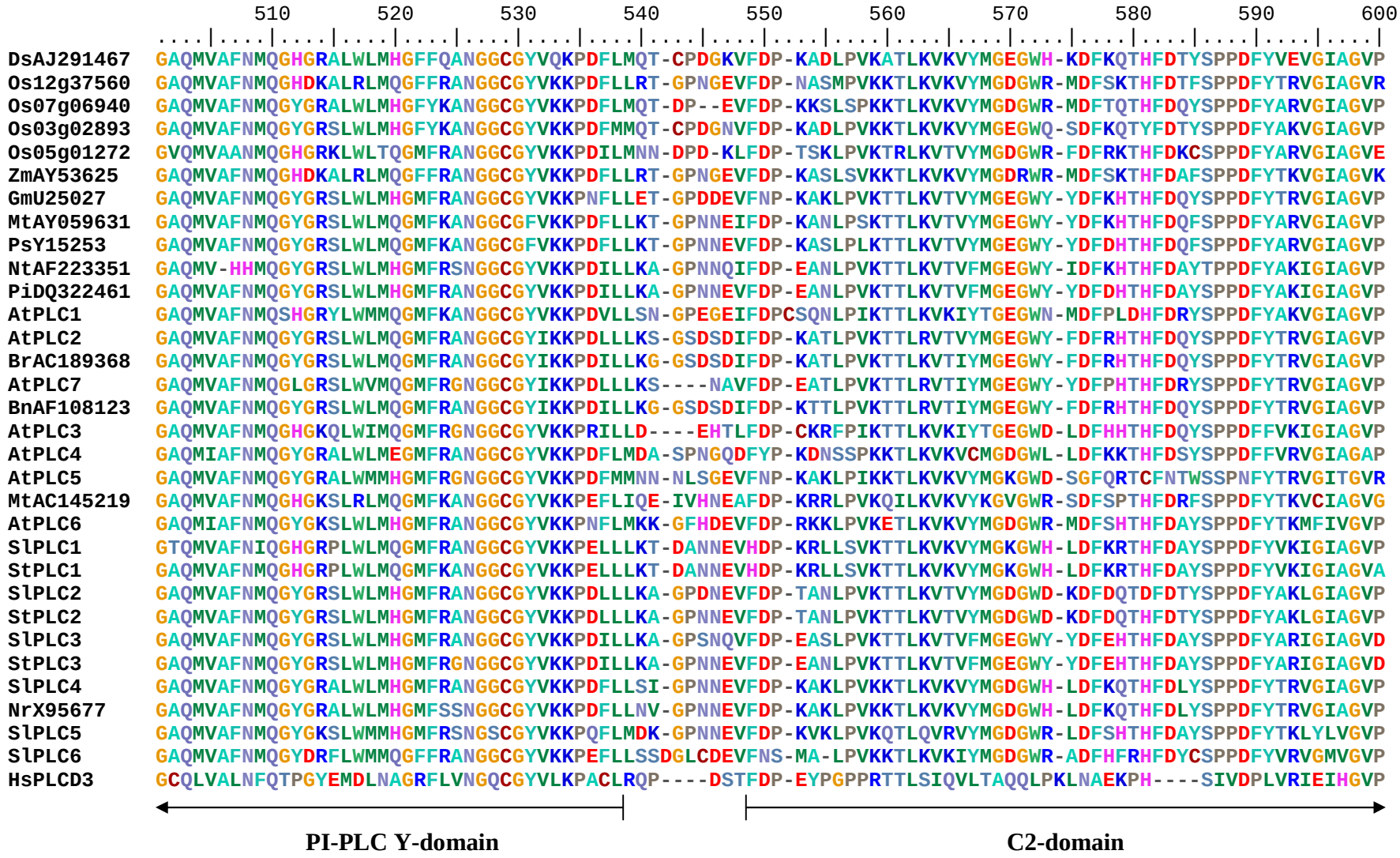
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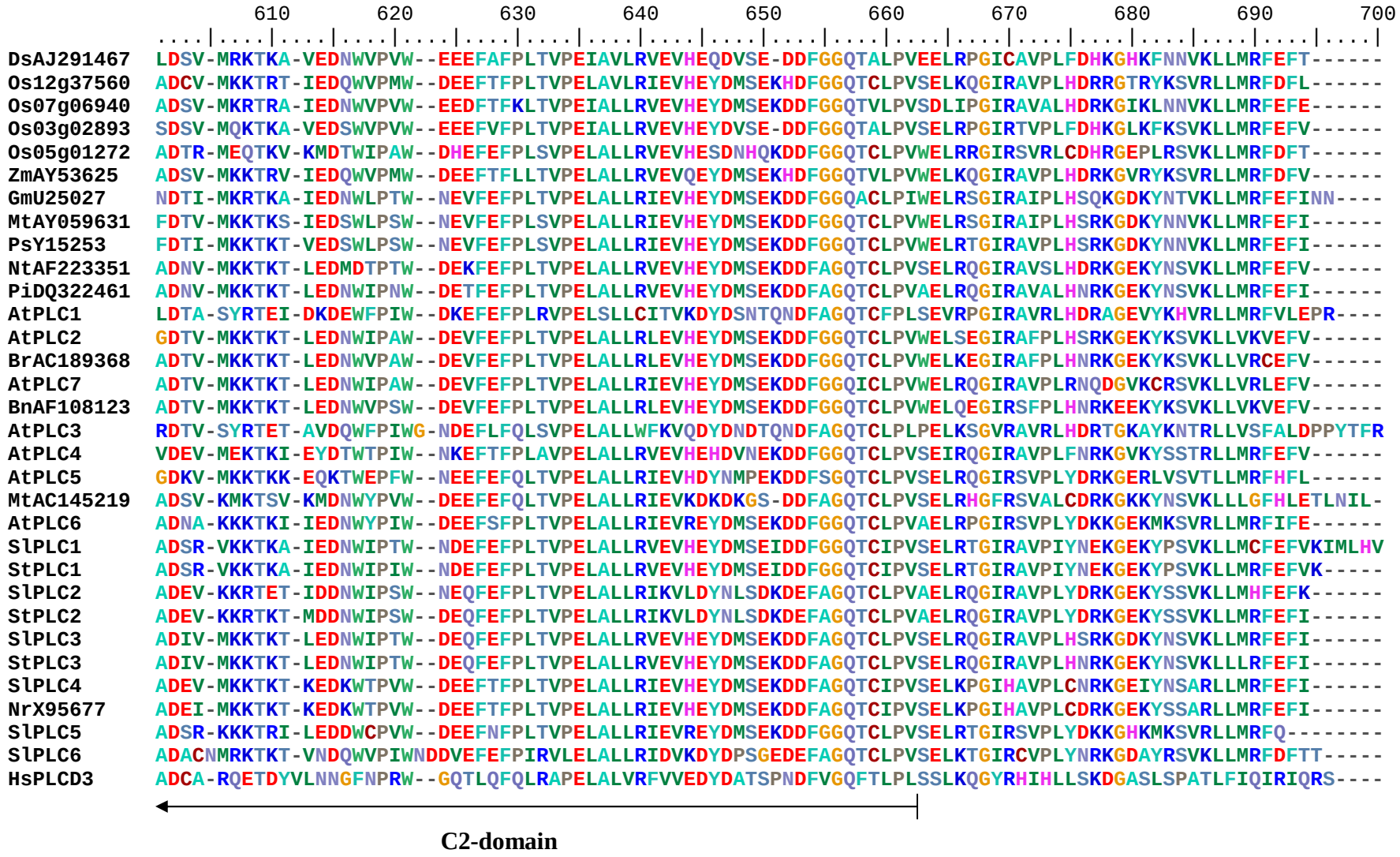
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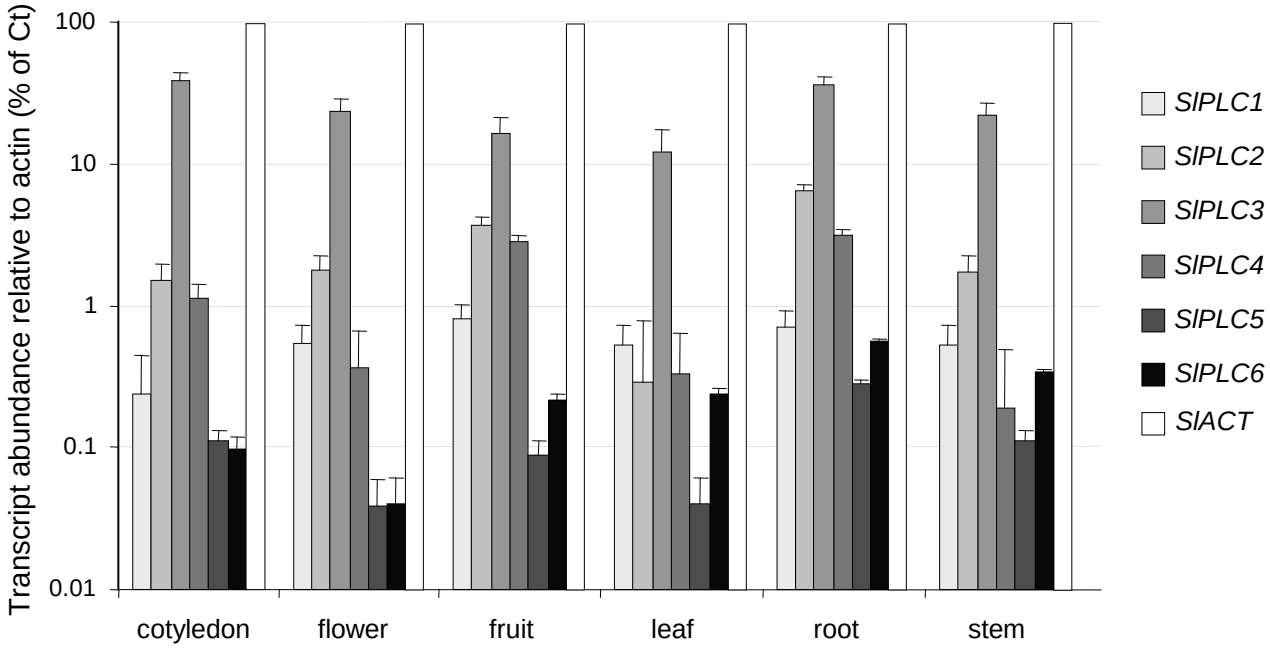
Transcript abundance relative to actin (% of Ct)
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10
1
0.1
0.01
cotyledon
flower
fruit
leaf
root
stem
SlPLC1
SlPLC2
SlPLC3
SlPLC4
SlPLC5
SlPLC6
SlACT

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of PI-PLC protein sequences from various plant species and human PLCδ3.

The various conserved domains (X, Y and C2) are indicated, as well as the position of a peptide derived from a phosphorylated form of *At*PLC2 (as described in Nühse *et al*., 2007). For species abbreviations see legend of Figure 1. Similar amino acids are shown in the same colour according to the Dayhoff PAM similarity matrix.

Figure S2. Relative transcript abundance of *PI-PLC* genes in different organs of tomato plants.

Threshold values (Ct) from Q-PCR analysis were determined for *SlACT* and the *PI-PLC* genes in cDNA archives from indicated tomato organs. The relative transcript abundance is expressed as a percentage of the *SlACT* Ct values, which were set to 100% in each sample. Each *PLC* gene is expressed in every tested organ, however, clear differences are observed in the transcript abundance of the individual *SlPLC* genes. *SlPLC3* is the most abundantly expressed *PLC* gene. Its average expression level corresponds to 20% of the *SlACT* Ct value, whereas *SlPLC5* transcripts show the lowest abundance in each organ (about 0.1% of *SlACT*).

Table S1. Quantification of Avr4-induced HR in *N. benthamiana* plants transiently expressing *SlPLC4*.

Experiments were performed as described in Figure 6. The constructs indicated in the second row were agro-infiltrated into *Cf-4*-transgenic plants or into non-transgenic plants. Recombinant Avr4 protein was injected three days post agro-infiltration.

Table S2. EST sequence data and primer sequences used for the cloning of tomato *PLC* cDNAs. The names as used in this report and the accession numbers, under which the cDNA sequences have been deposited, are indicated in the first and fourth column, respectively.

Table S3. Primers and probes used for Q-PCR.

Table S1. Quantification of Avr4-induced HR in *N. benthamiana* plants transiently expressing *SlPLC4*.

Experiments were performed as described in Figure 6. The constructs indicated in the second row were agro-infiltrated into *Cf-4*-transgenic plants or into non-transgenic plants. Recombinant Avr4 protein was injected three days post agro-infiltration.

Concentration of Avr4 (µg/ml)	*Cf-4*-transgenic	*Cf-4*-transgenic	non-transgenic	non-transgenic
	35S:SlPLC4	*35S:GUS*	*35S:SlPLC4*	*35S:GUS*
0*	0/15**	0/15	0/5	0/5
5	14/15	2/15	0/5	0/5
50	14/15	12/15	0/5	0/5

* Here infiltration medium was injected.

** Infiltration zones that developed HR were counted and are indicated in front of the slash. The total number of infiltrations is indicated behind the slash.

Table S2. EST sequence data and primer sequences used for the cloning of tomato *PLC* cDNAs. The names as used in this report and the accession numbers, under which the cDNA sequences have been deposited, are indicated in the first and fourth column, respectively.

Name	EST numbers	Primer sequences for 3'-RACE (5'- to -3')	Accession number
SlPLC1	TC167030, AW98481, SGN-U238053, SGN-U224659	GACTCGAGCATGTCTAAACAAACATACAGAATCTG	EU099600
SlPLC2	BI931651, SGN-U242093	CACTCGAGCATGTCGAAACAAACGTACAAAGTC	EU099599
SlPLC3	TC164753, TC159091, SGN-U222589, SGN-U221131	CACTCGAGCATGTCCAAACAGACGTACAGA	EU099598
SlPLC4	TC166538, TC159661, AW53869, SGN-U230684, SGN-U234333, SGN-U220392	CACTCGAGCATGGGGAATTATAGGGTAT	EU099597
SlPLC5	TC166008, SGN-U224897	CACTCGAGCATGTTTGGGTGTTTCAACCGT	EU099596
SlPLC6	BG132098, SGN-U238098	GTCACGGAGGGACACTAACA	EU099595

Table S3. Primers and probes used for Q-PCR.

Target cDNA	Forward primer (5'- to 3'-)	Reverse primer (5'- to 3'-)	Gene-specific, dual labelled probe
SlACT	GCTCCACCAGAGAGGAAATACAGT	CATACTCTGCCTTTGCAATCCA	
SlPLC1	ATTTTGGTGGACAAACTTGCATT	GTTTACGATTAAAGATGCAGTTTGCTA	CTCAGAACAGGAATTCG AGCTGTGCCTATATACA
SlPLC2	TTTGCTGGCCAAACATGTCTAC	GCAAAGCTCCCTTTTCAAGATCTA	
SlPLC3	AACTAAGACAAGGTATTCGAGCAGTAC	AGCTTGCAAATCATGGCAAA	
SlPLC4	CGGAGCTGAAACCTGGTATACAT	GAAAGCTCAAGAAGCACACAACA	
SlPLC5	GGATGATTTTGGTGGACAAACA	AAATTGAAGCATGTGTATATAGGAAAG TAACT	CTCAGAGTTGAGAACAG GGATCCGATCA
SlPLC6	CGTTGCTTCGGATTGATGTTAAA	TGGGATTGAGGAAGATTAATTAAGTAG TG	TGGTGAAGATGAATTTG CAGGACAAACATG
CfAvr9	GAGCTTGCTCTCCTAATTGCTACTACT	AACTTCGTCGAGCGGTTACACA	
SlPR-1a	TGGTGGTTCATTTCTTGCAACTAC	ATCAATCCGATCCACTTATCATTTTA	
CfEcp6	GCTCAAGGTTGGTCAGCAGAT	TTCACACCTGACAGATCACTTATGC	