The Role of Phosphatidylinositol-Specific Phospholipase-C in Plant Defense Signaling

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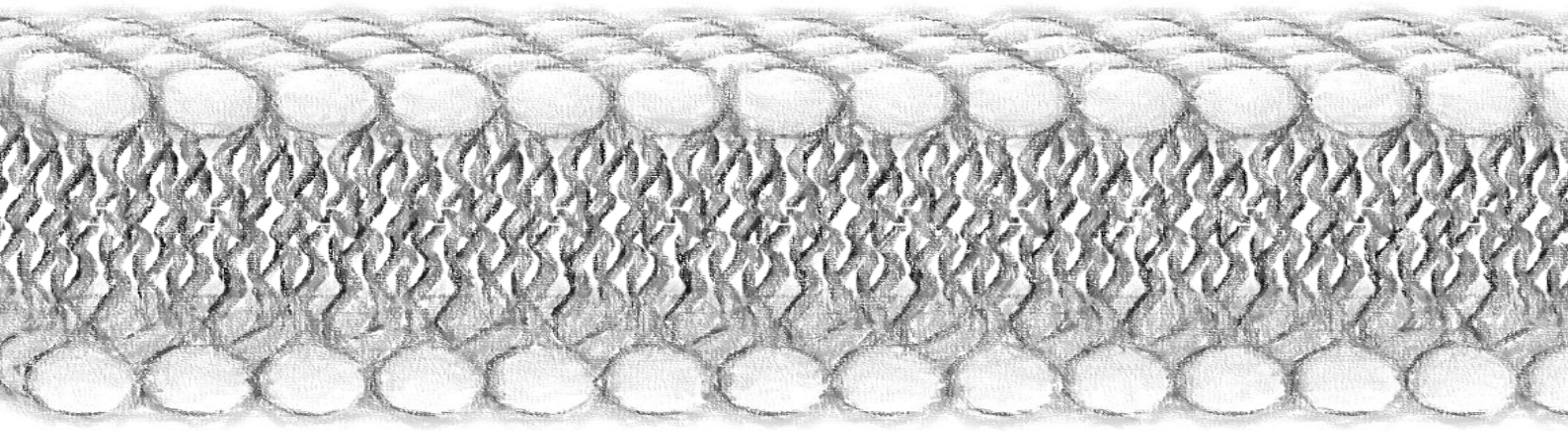
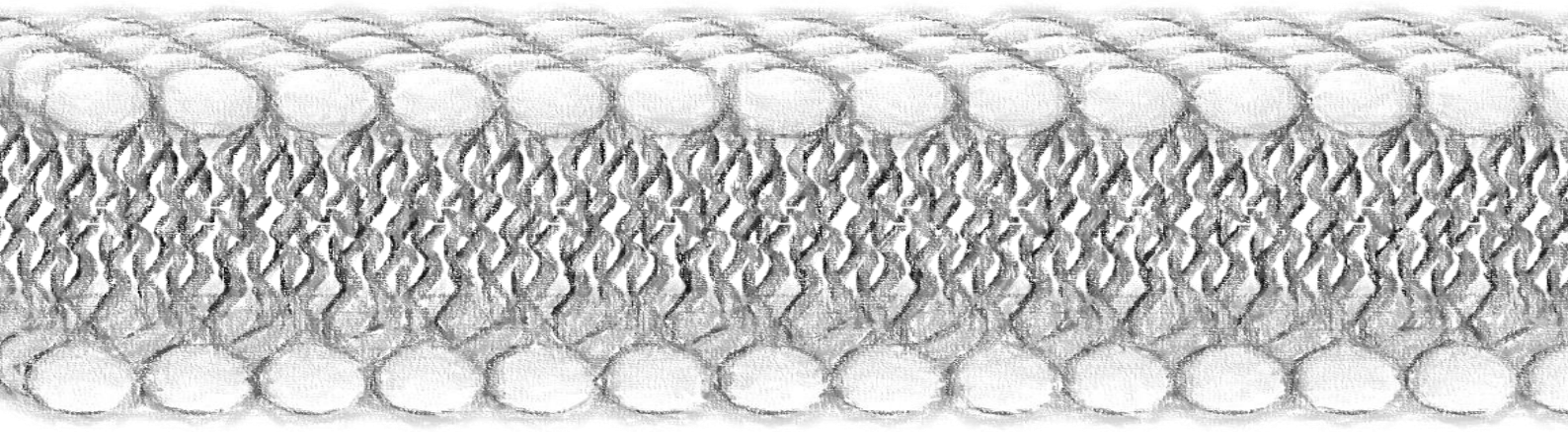


CHAPTER 1

General Introduction & Thesis Outline



Introduction

Plants utilize an efficient innate immune system to compensate for their lack of adaptive immunity (1,2). The absence of circulating immune cells is compensated by the ability of almost every individual cell of a plant to execute an array of antimicrobial defense-strategies. Plants have developed various fast-responding defense mechanisms, which enable them to reduce their vulnerability and resist attackers. Most of these mechanisms are based on the ability of plants to discriminate between self and non-self molecules.

The plant innate immune system is activated once an invading microbe succeeds in bypassing the first layer of defense. This layer includes physical and chemical barriers like the plant waxy cuticle, (lignified) cell walls, antimicrobial metabolites and proteins, enzyme inhibitors and detoxifying enzymes. The second layer of defense involves the induction of defense responses at the cellular level, such as cell wall reinforcement, synthesis and accumulation of antimicrobial metabolites like Reactive Oxygen Species (ROS), phytoalexins and pathogenesis-related proteins like chitinases, beta-glucanases, proteases and peroxidases. Moreover, plant cells are able to execute a rapid cell death response, which usually requires the activation of resistance proteins (also referred to as immune receptors). The process of rapid cell death is termed the Hypersensitive Response (HR) and is considered to be a type of Programmed Cell Death (PCD), which is specific for plants. The HR is thought to limit growth of biotrophic pathogens and prevents them from advancing into healthy tissue (3-6).

Types of resistance

The presence of microbes can be sensed by Pattern Recognition Receptors (PRRs), which are present at the surface of plant cells. PRRs are localized at the Plasma Membrane (PM) and recognize conserved structural microbial molecules termed Microbe-Associated Molecular Patterns (MAMPs) that are exposed or released during interaction of microbes with plants. Examples of MAMPs are lipopolysaccharide (LPS) and flagellin from bacteria, and chitin and glycans of fungi. PRRs mediate what is commonly known as MAMP-Triggered Immunity (MTI, also known as Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI)) (1,7).

Resistance that is caused by the recognition of MAMPs is commonly referred to as non-host resistance and the affected microbes are considered to be non-adapted pathogens (8). In general, MTI occurs during the interaction of plants with all types of microbes, also when they can cause disease (in the case of a pathogen) or engage in a mutualistic relationship (in the case of a symbiont). However, adapted pathogens are generally able to exert different degrees of suppression of MTI by secreting effector proteins, which either actively suppress plant immunity or perturb immunity by other strategies like diminishing their footprints, making pathogens semi-invisible to PRRs (9). Even with adapted pathogens, which are able to successfully suppress plant immunity and cause disease, plants still have some level of “basal resistance”, meaning that there is still some control of pathogen proliferation by the defense machinery of the plant.

On the other hand, plant immunity commonly known as "host resistance" represents a stronger type of resistance that involves the recognition of either highly specific- or less-conserved effector molecules that are secreted by the pathogen, by different types of resistance proteins. It has been suggested that host resistance has developed in plants in response to the successful break-down of non-host resistance by effectors of the pathogen (3,10). In contrast to multilayered non-host resistance, host resistance depends on proteins encoded by polymorphic resistance genes (*R* genes). R proteins function as immune receptors, which recognize matching effectors (8). These effectors function in virulence, but are also recognized by R proteins inducing plant immunity. The recognition of effectors by R proteins and R protein-mediated defense is referred to as Effector-Triggered Immunity (ETI). All the information provided above clearly shows that the innate immune system of plants is able to respond to different types of microbe-derived molecules (3,11). It is therefore difficult to come to an absolute classification of immune responses, as some microbe-derived molecules cannot clearly be classified as being either a MAMP or an effector. Furthermore, MTI and ETI of plants triggered by MAMPs and effectors, respectively, are less distinct than initially proposed and they even partly overlap (11).

Plant immune receptors

In general, there are two types of plant immune receptors (1,3,8). The first type encompasses receptors that are located at the surface of the cell. These include the Receptor-Like Proteins (RLPs), such as the tomato (*Solanum lycopersicum*) Cf-resistance proteins that confer resistance to strains of the extracellular fungal pathogen *Cladosporium fulvum* expressing the matching effectors (12,13). In addition to RLPs, Receptor-Like Kinases (RLKs) are present at the cell surface, like Flagellin Sensing 2 (FLS2) that mediates recognition of the bacterial MAMP flagellin. FLS2 recruits additional cell surface RLKs to initiate defense responses upon perception of flagellin (14-16). The second type of immune receptors comprises cytoplasmic proteins known as Nucleotide-Binding Leucine-Rich Repeat (NB-LRR) proteins. NB-LRR proteins have a tripartite domain organization and are classified into two subfamilies based on the presence of either a Toll and Interleukin-1 Receptor (TIR)-like domain or a Coiled-Coil (CC) domain at their N-terminus (17). Plants possess hundreds of different cell-surface and cytoplasmic immune receptors and it is conceivable that many of these receptors recruit similar downstream defense signaling networks.

Phospholipids as defense signals

Activation of immune receptors leads to the initiation of multiple downstream signaling events. These essentially include the opening and closing of specific ion channels (18-20), a transient increase of the Ca^{2+} levels in the cytoplasm (calcium spiking), the activation of Mitogen-Activated Protein Kinases (MAPKs) (19-23) and Calcium-Dependent Protein Kinases (CDPKs) (24,25) and the production of ROS (26,27). Moreover, the results of an increasing number of studies on innate immunity in plants suggest that plants are similar to animals in having the ability to exploit modifications of PM phospholipids as a means to

rapidly relay defense signals. The generation of phospholipid signals occurs shortly after receptor activation (28-32). Phosphoinositide-specific phospholipase C (PI-PLC) enzymes are essential for generating these phospholipid signals in both plants and animals and PI-PLCs play a central role in coordinating cell signaling during plant defense (33-40). PI-PLC enzymes are considered to be signal transducers and signal amplifiers, primarily due to the signaling roles ascribed to their substrates and reaction products. The activation of an immune receptor initiates a defense signaling cascade which eventually culminates in resistance against a particular pathogen. In general, plant defense consists of early and late signaling components. Early signaling events occur very rapidly after elicitation (within seconds to minutes) and determine the success of the defense reaction. PI-PLC enzymes take part in the early signaling events where they control a transient increase in cytosolic free Ca^{2+}, thereby mediating the simultaneous generation and depletion of important lipid signaling molecules (Figure 1). The canonical PI-PLC signaling pathway reported in animals involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$ or PIP2) by PI-PLCs at the PM, thereby generating diacylglycerol (referred to as DAG or DG) and $Inositol(1,4,5)P_3$ ($InsP_3$) (41). DAG remains anchored to the PM, of which a considerable amount is rapidly phosphorylated to phosphatidic acid (PA) by the action of DAG-kinases (DGKs), which is eventually further phosphorylated to diacylglycerol pyrophosphate (DGPP) by PA-kinases. These reactions are very dynamic and the amounts of the various lipid molecules are also controlled by specific lipid-phosphatases which catalyze reactions in the opposite direction (Figure 1). Essentially, all these lipid species are believed to function as signaling molecules in plant cells by directly activating signaling components or recruiting them to signaling complexes at the PM (36). In contrast to DAG, $InsP_3$ diffuses from the PM into the cytosol where, in animal cells, it transiently induces the release of Ca^{2+} from intracellular stores (42,43). Also in plants, both $InsP_3$ and its phosphorylation product $InsP_6$ are reported to trigger Ca^{2+} release from intracellular stores (32,44,45). The turnover of the signaling lipid molecules by PI-PLCs and the subsequent release of Ca^{2+}, in addition to changes in the cytosolic pH (18), are thought to be essential factors that together determine the type and magnitude of the resistance response of plants. Interestingly, the discovery of a nuclear phosphoinositide cycle and the presence of PI-PLC enzymes in the nucleus of animal and plant cells (Abd-El-Haliem et al, unpublished data), suggest that the mechanism by which PI-PLC signaling regulates cellular responses extends beyond the PM interface.

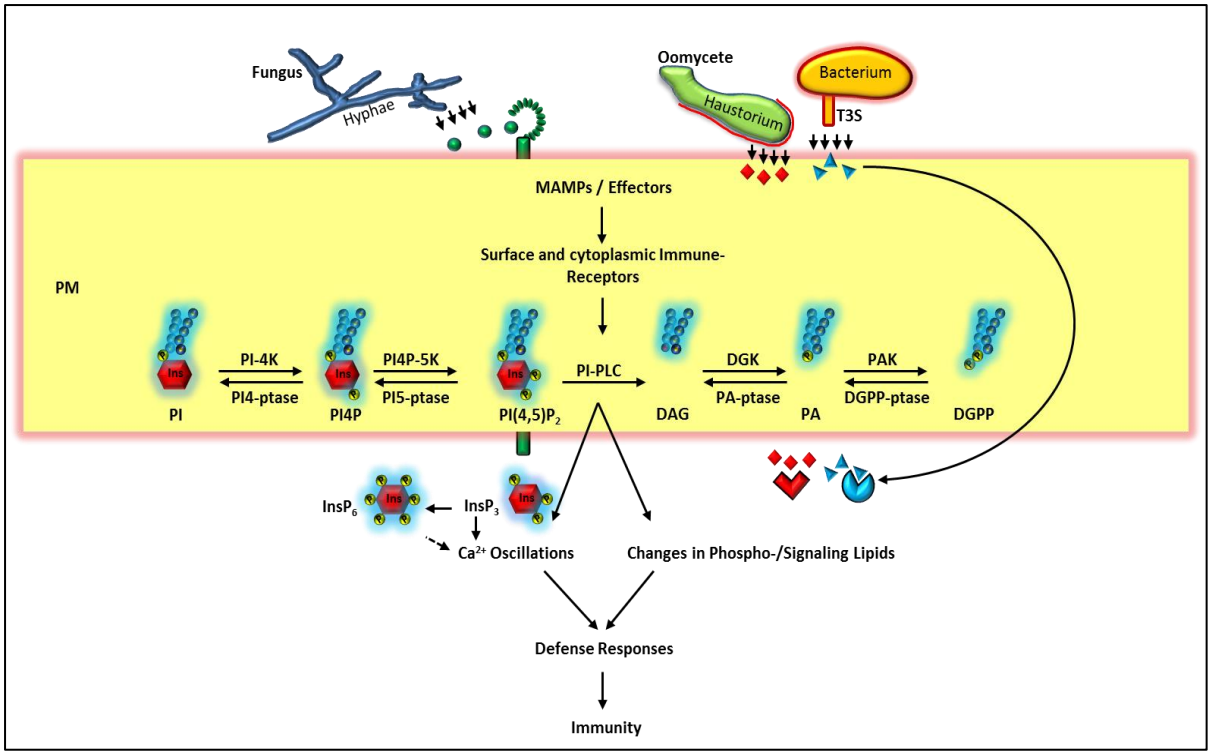


FIGURE 1. **The possible role of the phosphoinositide-specific phospholipase C (PI-PLC) signaling pathway in plant immunity.**

Upon activation of plant immune receptors by MAMPs or effectors, PI-PLCs are translocated to the PM and activated, leading to the hydrolysis of $PI(4,5)P_2$, thereby generating DAG and $InsP_3$. DAG is converted to PA by various DGKs and it can be phosphorylated to DGPP by PA-kinases. The fast dynamic changes in the levels of the various signaling lipid molecules and the release of Ca^{2+} are assumed to determine the type and magnitude of the immune response of plants to pathogens. (See text for further details). T3S, Type III secretion system; MAMPs, Microbe-Associated Molecular Patterns; PM, Plasma Membrane; PI, Phosphatidylinositol; PI4P, Phosphatidylinositol-4-phosphate; $PI(4,5)P_2$, Phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; PA, Phosphatidic Acid; DGPP, Diacylglycerol pyrophosphate; $InsP_3$, Inositol(1,4,5)P_3; PI4K, Phosphatidylinositol-4-phosphate-kinase; PI4P-5K, Phosphatidylinositol-4-phosphate 5-kinase; PI-PLC, Phosphoinositide-specific phospholipase C; DGK, Diacylglycerol kinase; PAK, Phosphatidic Acid-kinase; DGPP-ptase, Diacylglycerol pyrophosphate phosphatase; PA-ptase, Phosphatidic Acid-phosphatase; PI5-ptase, Phosphatidylinositol-5-phosphate phosphatase; PI4-ptase, Phosphatidylinositol-4-phosphate phosphatase.

Differences between plant and animal PI-PLC-mediated defense signaling

Although the various activation mechanisms and downstream targets of PI-PLCs have been extensively studied in animals, this knowledge cannot directly be extrapolated to plants, despite the clear similarities that exist between PI-PLC-mediated signaling in animals and plants. This is partly due to differences in the modular composition between animal and plant

PI-PLCs. All plant PI-PLCs contain the core domains that are specific for this type of enzymes, whereas most of the animal counterparts are equipped with additional domains required either for interaction with the PM or with other key signaling proteins. Furthermore, animal PI-PLCs consist of six different classes while all plant PI-PLC enzymes identified to date resemble the animal PI-PLCζ class. Plants also lack the orthologues of important downstream signaling targets that are known in animals. Accordingly, the main differences between PI-PLC signaling in plants and animals can be summarized as follows: (i) plants do not seem to widely employ G-proteins to activate PI-PLCs upon triggering of immune receptors; (ii) plants lack orthologues for the conventional animal $InsP_3$-receptors ($InsP_3$-Rs), which are $InsP_3$-gated channels responsible for Ca^{2+} release from intracellular stores and; (iii) plants lack protein kinase C (PKC) enzymes, which in animals are activated by DAG downstream of surface receptors and are involved in triggering an array of receptor-mediated responses. Other phospholipid signals also exist in animals and plants and their structure depends on the type of lipid from which they are generated and the enzyme(s) responsible for their production. In addition to PI-PLC, these enzymes include inositol-lipid synthases, inositol-lipid kinases and phosphatases, Phospholipase A1 and -A2, Phospholipase B (PLB) and Phospholipase D (PLD).

The *C. fulvum* – tomato model system utilized in this study

The interaction between *C. fulvum* and tomato is a model system that has extensively been studied in order to obtain a better insight into the molecular basis of resistance and susceptibility in plant-pathogen interactions. In this interaction, tomato Cf proteins confer resistance to strains of *C. fulvum* secreting the matching effectors, here referred to as avirulence proteins (Avrs) (12,46). For example tomato immune receptor Cf-4 provides resistance to strains of *C. fulvum* that secrete Avr4 and earlier observations have shown the involvement of PI-PLC signaling in defense triggered by Avr4 and mediated by the Cf-4 protein. The PI-PLC pathway was found to be activated rapidly after the recognition of Avr4 by Cf-4 and inhibition of PI-PLC activity blocked the Avr4-induced oxidative burst, indicative for the suppression of defense responses (33). Cf-4 is a suitable plant immune receptor for studying the role of PI-PLCs in resistance, as it is a PM-associated RLP and PI-PLCs are cytoplasmic enzymes of which the substrates are mainly located at the PM. Therefore, it is likely that PI-PLC signaling is essential for the function of PM-localized immune-receptors, rather than for cytoplasmic immune-receptors. However, PI-PLC activity was recently shown to be also involved in defense responses that are activated after triggering of the cytoplasmic NB-LRR immune receptors, RPM1 and RPS2 (38).

Aim of the thesis

As discussed above, plant innate immunity depends on the function of cellular immune receptors that sense the presence of microbes. Activation of different types of immune receptors has been extensively studied and an important response pattern that follows their activation is the generation of a set of downstream signals that is crucial for the plant cell in order to adequately execute the various protective defense reactions. A rapid and transient increase in the levels of free Ca^{2+} ions in the cytoplasm serves as a universal master signal that is generated after the activation of immune receptors. This increase is either due to an influx of Ca^{2+} ions from the apoplast or the result of a release of trapped Ca^{2+} ions from intracellular compartments (47,48). The latter is known to occur rapidly after receptor activation in both plants and animals and controls downstream responses. PI-PLC activity was demonstrated to be important for several types of plant defense reactions. However, it is not known how PI-PLC participates in defense to achieve resistance against pathogens. Likewise, it is not clear why plants possess several PI-PLC isoforms and whether these isoforms have distinct or overlapping roles in signaling. In fact, information on how triggering of immune receptors subsequently activates plant PI-PLCs, is lacking. As mentioned earlier, the activation of PI-PLCs leads to the generation of two types of signals. These signals are the transient increase in cytosolic Ca^{2+} and the turnover of phospholipid signaling molecules that exist in minute amounts in the PM. Both are early signals which are rapidly generated after activation of immune receptors and have proven to be vital for downstream signaling in both animals and plants. The previous observations imply that PI-PLCs play a central role in coordinating major downstream signaling during immune reactions in plants.

This study aimed at providing genetic evidence for the involvement of PI-PLC signaling in plant defense against pathogens. Tomato was used as a model to determine the structure of a plant PI-PLC family, to clone the different isoforms of the PI-PLCs and to study the function of each individual isoform during the defense response. The second goal was to confirm that the predicted tomato PI-PLCs show enzymatic activity and possess different substrate specificities. The latter would allow linking the specific enzyme activities of different PI-PLC isoforms to phenotypes obtained based on functional analysis of the encoding genes. Specific properties of the enzymes were obtained by studying the various requirements for optimum enzyme activity of a number of PI-PLC isoforms and by investigating the role of PI-PLC activity in immune receptor-activated responses. The generated information was combined with data from the literature to draw a model that specifically explains the role of PI-PLC-mediated signaling in plant defense and resistance. The model illustrates how plant immune receptors utilize PI-PLC signaling to relay defense signals in order to establish resistance against pathogens.

Thesis outline

In **Chapter 2**, the identification and partial characterization of the tomato *PI-PLC* gene family is reported. Based on expression profiling of this gene family during the onset of the resistance response, *PI-PLC4* and *PI-PLC6* were selected for their possible role in resistance to multiple pathogens. The effects of down-regulation (by Virus-Induced Gene Silencing) and transient over-expression (by agroinfiltration) of both genes on the HR and resistance against multiple pathogens were studied. Tomato PI-PLC4 was found to be required for the Cf-4/Avr4-induced HR, whereas PI-PLC6 appeared to play a role in resistance of tomato, not only to *C. fulvum*, but also to the vascular fungal pathogen *Verticillium dahliae* and the bacterial pathogen *Pseudomonas syringae*. Heterologous expression, combined with activity assays of the encoded enzymes, was used to prove that the studied *PI-PLC4* and *PI-PLC6* genes encode active enzymes, suggesting that the observed phenotypes depend on their activity.

Chapter 3 describes the transcriptional regulation of the various tomato *PI-PLC* genes upon initiation of the HR. *PI-PLC* gene regulation was studied in transgenic tomato seedlings expressing both the Cf-4 and Avr4 proteins that resulted from crossing plants carrying *Cf-4* with plants carrying the *Avr4* gene. In these *Cf-4*/*Avr4* F1 plants, the HR was activated in a synchronized way by lowering both the ambient temperature and humidity, as the Cf-4-mediated HR is both temperature- and humidity-sensitive, being suppressed at elevated temperatures and 100% relative humidity. *PI-PLC3* and, to a lesser extent, *PI-PLC6* were transcriptionally up-regulated in response to elevated temperatures in all seedlings, including the control parental lines only expressing Cf-4 or Avr4. This up-regulation of *PI-PLC* gene expression was accompanied by a rapid increase in the levels of phosphatidic acid and a decrease in the levels of PI and PI-phosphate, suggesting the involvement of the enzymes encoded by *PI-PLC3* and *PI-PLC6* in the response of the seedlings to elevated temperatures. The levels of structural phospholipids, such as phosphatidylcholine and phosphatidylglycerol, decreased during recovery of the seedlings from the elevated temperature, indicating the involvement of phospholipase D activity and a change in the composition of phospholipids in the PM of the plant cells.

In **Chapter 4**, the roles of tomato *PI-PLC* genes are re-evaluated based on the recently published tomato genome sequence. Interestingly, yet another tomato *PI-PLC* gene (*PI-PLC7*) was identified. The tomato *PI-PLC* gene sequences were used as a query to identify all related *PI-PLC* sequences in other plant genomic sequences available to date in the public sequence database at NCBI. Subsequently, a phylogenetic analysis was performed in order to relate the tomato *PI-PLC* genes to their orthologues in other plant species. Based on recent observations described in the literature, it is proposed that the X/Y-linker region of the plant PI-PLC enzymes plays a role in their activation. In this chapter, the enzyme activity of recombinant proteins encoded by all tomato *PI-PLC* genes, except for *PI*-PLC7, was studied. The optimum PI-PLC activity requirements, with regard to the pH and Ca^{2+} concentration of the incubation medium and substrate-preference, were determined for three

PI-PLC isoforms. This was achieved by performing *in vitro* PI-PLC enzyme assays using non-radioactive techniques to visualize the substrates and reaction products. It is demonstrated that the PI-PLC inhibitor U73122 blocks activity of these three PI-PLC enzymes *in vitro*. The same inhibitor was subsequently used to study the requirement of PI-PLC activity in ETI and PTI mediated by different cell surface immune receptors, using medium alkalization as a read-out. The effect of the inhibition of PI-PLC activity on the internalization of the receptor-like kinase Flagellin Sensing 2 (FLS2), in response to treatment with the MAMP flg22 was also monitored.

Chapter 5 provides an overview and a discussion of the current status of what is known about PI-PLC defense signaling in plants. It starts by describing the discovery of the PI-PLC pathway in animals and plants and discusses the main differences and similarities of the PI-PLC enzymes in these two kingdoms. Subsequently, the differences between PI-PLCs and other types of PI-PLC enzymes in eukaryotes and prokaryotes are discussed. The notion emerges that the essential role of PI-PLC enzymes in phospholipid signaling involves both depletion of specific substrates and generation of the respective reaction products, which are all involved in signaling. The findings described in this thesis, as well as those reported by others in the literature, are placed in a broader perspective and provide clues for the involvement of phospholipid molecules in different defense response pathways. Based on the available data, the different activation mechanisms of plant PI-PLC enzymes, following the triggering of immune receptors, are described. Finally, a model is put forward that summarizes all the information that has been discussed in this chapter. It proposes a central role for PI-PLC signaling downstream of plant immune receptors.

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CHAPTER 2

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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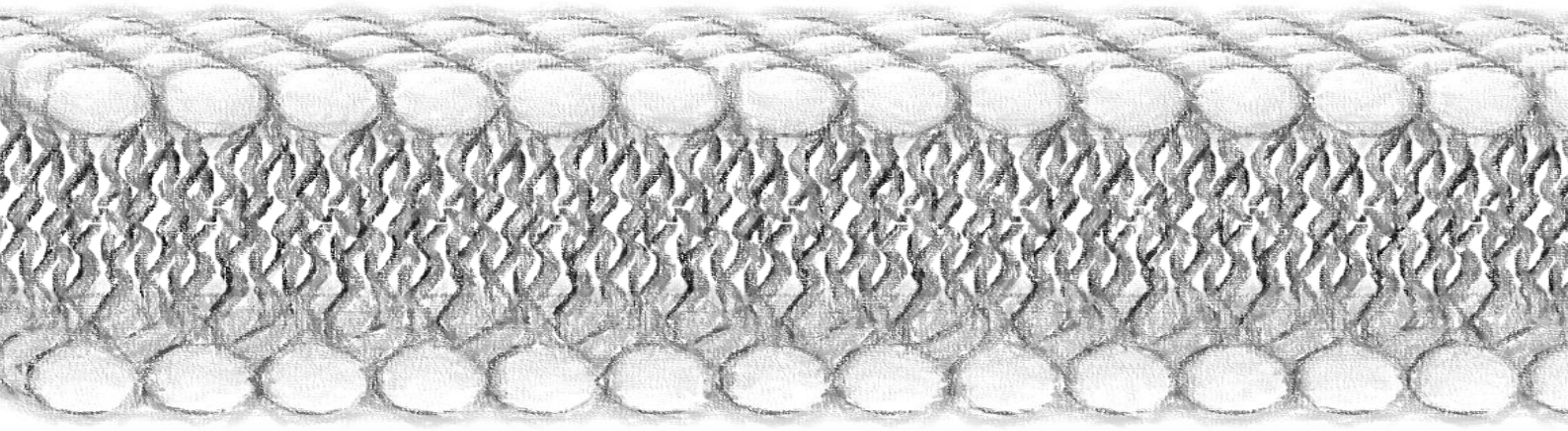


Abstract

The perception of pathogen-derived elicitors by plants has been suggested to involve phosphatidylinositol-specific phospholipase-C (PI-PLC) signaling. Here we show that PLC isoforms are required for the hypersensitive response (HR) and disease resistance. We characterized 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 colonization 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 colonization 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 signaling.

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)] (1,2). Using such a wide repertoire of receptors, plants are able to recognize a broad spectrum of extracellular and intracellular elicitors. Recognition results in the activation of a complex set of defense responses and can result in microscopically or macroscopically visible cell death, the so-called hypersensitive response (HR), that contributes to resistance to pathogens (3). 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 (4)). Upon stimulation, these receptors either directly or indirectly activate phospholipid-hydrolyzing 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 signaling function in animal cells. Phosphatidylinositol (4,5)-bisphosphate provides a docking site for various proteins and is a key regulator of actin organization 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 organization and membrane traffic appears to be quite similar to that in animal cells (5-7). 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 (8-13). Many plant genomes encode PI-PLCs (14-18) 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 (13,15,19)).

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 (20,21) 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 (22). 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 (23). A role for PLC has been implicated not only in elicitor recognition processes but also in downstream disease resistance signaling. 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 (24,25).

In several processes, such as ABA perception (26), pollen tube growth (6,27), cytokinin signaling (28) and drought tolerance (29), 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 characterization 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. *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 defense responses. In addition, the two PLCs are shown to have non-overlapping roles in disease resistance.

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-TTTTTTTTTV-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′-CCACACCTTCAAGAAAAAGTAGCTCAA-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 (30) 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 (31). 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 (32). Phylogeny was performed using PHYLIP v.3.6.1-2 (33). A single most parsimonious tree was constructed using the HsPLCδ3 as an out-group 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 (34). 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* (35). 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 (35). 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′-TCCCACATATAAATTGAACATTAAACA-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 (36). For the GST-*Sl*PLC4 fusion, *SlPLC4* was amplified from a plasmid containing full-length *SlPLC4* using the primers (5′-TTCTAGATATGGGGAATTATAGGGT-ATGTGT-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 in (37,38)and (14). 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 (14). 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 (37), 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 (39). The pTRV-RNA2-derived constructs TRV:*Cf-4* and TRV:*Prf* have been described before (35,40). 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 (41). Colonization 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-sterilized. Five slices are plated onto potato dextrose agar (five slices per plate) and incubated for 2 weeks at 22°C. Inoculation and determination of colonization with *Pst* DC3000 was performed as described by (40).

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 (35).

SlPLC4 expression in N. benthamiana – The *SlPLC4* expression construct was made using a forward primer overlapping the start codon (5′-CACTCGAGCATGGGGAATTA-TAGGGTAT-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′-CACTCGAGCATGGGG-AATTATAGGGTAT-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 (42). 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 (43).

Results

Characterization 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 organization (44), 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 Supplemental Information). The PI-PLC-X and PI-PLC-Y domains together form a barrel-like structure containing the active site residues (45). The C2 domain is expected to have a regulatory function in response to Ca^{2+} and phospholipids (46). Using PSORT, a potential N-terminal mitochondrial import signal was found in the *Sl*PLC2 and *Sl*PLC3 proteins. No obvious subcellular localization could be predicted for the other PLC proteins.

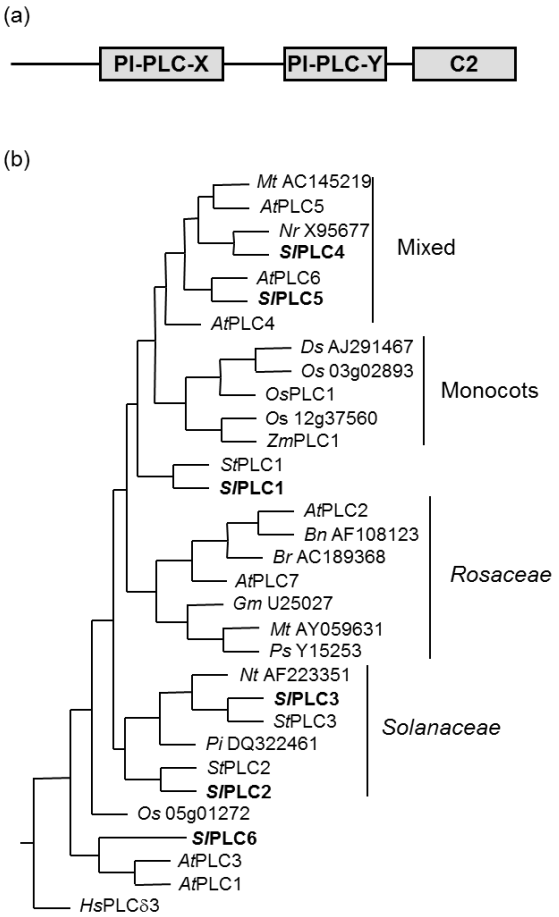


FIGURE 1. **Characterization of the tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) protein family.** (a) Schematic representation of the PI-PLC protein structure. PI-PLC-X and PI-PLC-Y domains are the conserved X and Y boxes of the catalytic domain, respectively. C2, also known as CaLB (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.*

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.

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 (22). 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* (47) and *Ecp6* (48) showed that colonization 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 defense marker *PR-1a*, whereas in *Cf-0* these transcripts accumulated more slowly. These kinetics are typical for an incompatible and a compatible interaction, respectively (49). 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.

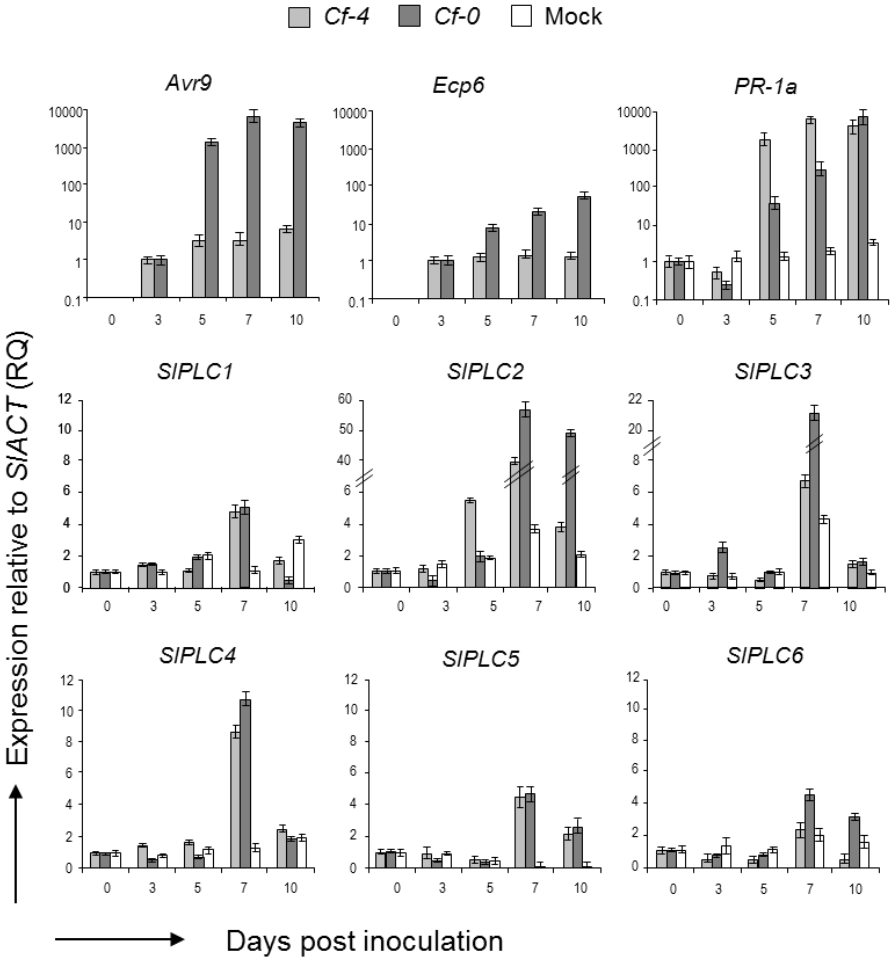


FIGURE 2. **Expression patterns of *Avr9, Ecp6, PR-1a* and the *SlPLC* genes during the interaction between tomato and *Cladosporium fulvum*.** The *Cf-4* and *Cf-0* tomato plants were inoculated with a strain of *C. fulvum* expressing Avr4 or mock-treated with water. Leaflets were taken at the indicated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

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. Therefore in our further studies we decided to focus on the role of these two genes in defense. 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 (36). To exclude interference of possible co-purifying endogenous PI-hydrolyzing 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 hydrolyze 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-hydrolyzing activity of *E. coli* itself, as there is no enzymatic activity present in the GST-only control.

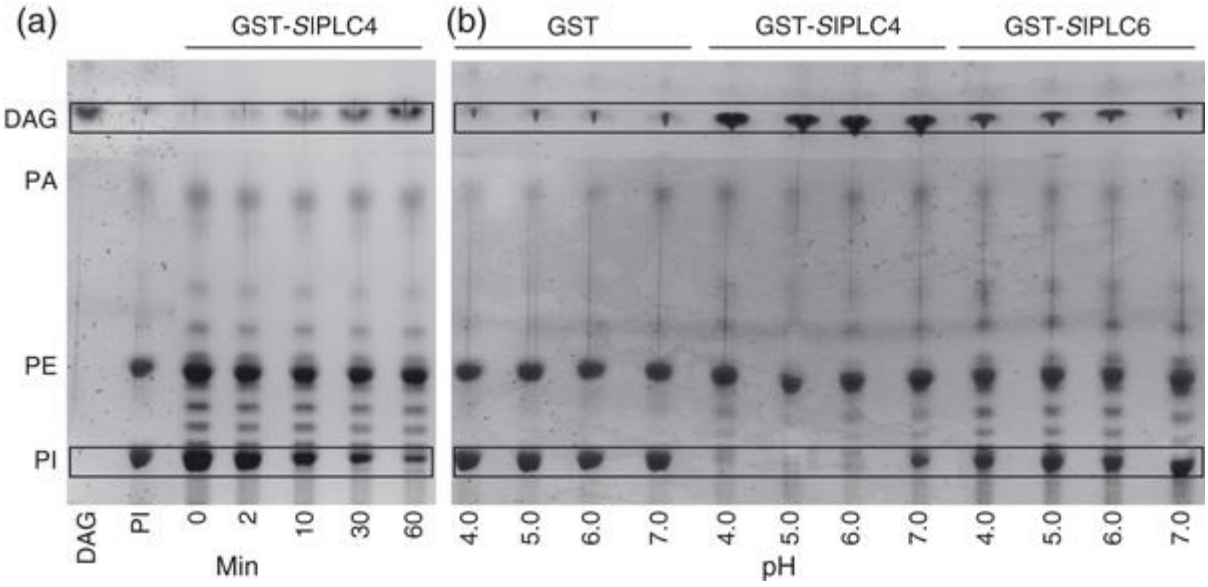


FIGURE 3. **GST-*Sl*PLC4 and GST-*Sl*PLC6 are catalytically active phosphatidylinositol-specific phospholipase-Cs (PI-PLCs) that hydrolyze phosphatidylinositol (PI), thereby generating diacylglycerol (DAG).** (a) GST-*Sl*PLC4 hydrolyzes PI and generates DAG in a time-dependent manner. (b) Both GST-*Sl*PLC4 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.

Unexpectedly, neither GST-*Sl*PLC4 nor GST-*Sl*PLC6 hydrolyzed 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 hydrolyze both PI and PIP_2 (14). 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.

SlPLC4 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.

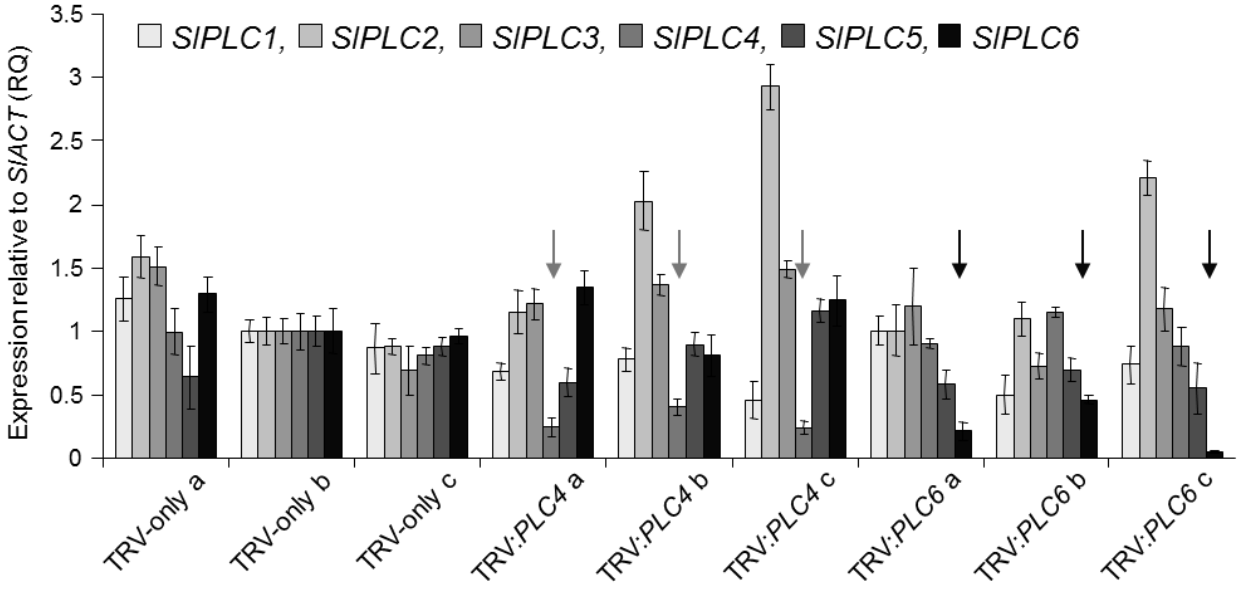


FIGURE 4. **Specificity of virus-induced gene silencing (VIGS) of *SlPLC4* or *SlPLC6* in tomato.** Quantitative PCR analysis on cDNA from three different leaflets (indicated with a, b

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.

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 (50) 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.

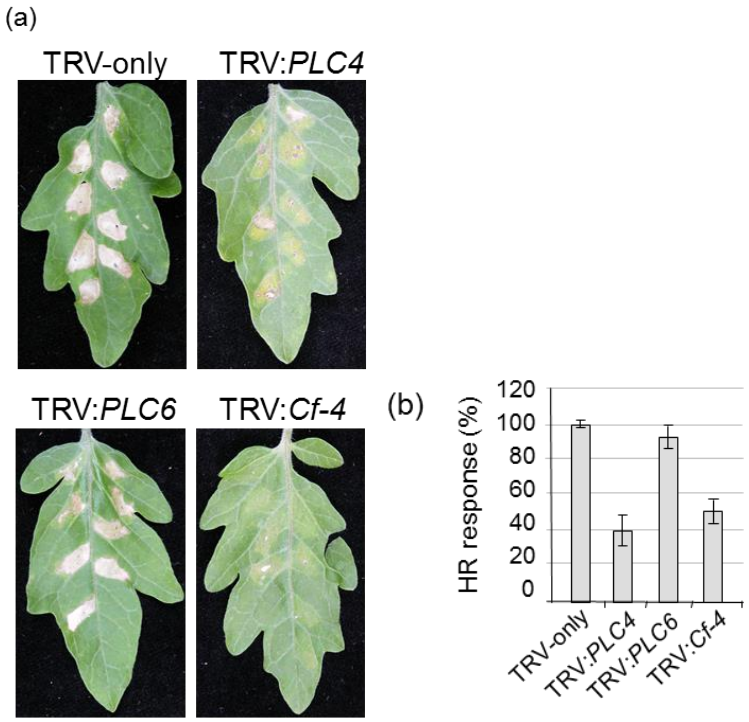


FIGURE 5. ***SlPLC4*, but not *SlPLC6*, is required for the Avr4/Cf-4-induced hypersensitive response (HR).** (a) Leaflets of *Cf-4* tomato plants, inoculated with the indicated tobacco rattle virus (TRV) strains, were injected with Avr4 at eight sites. Pictures were taken from representative leaflets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

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 (51,52). 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 (35). 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.

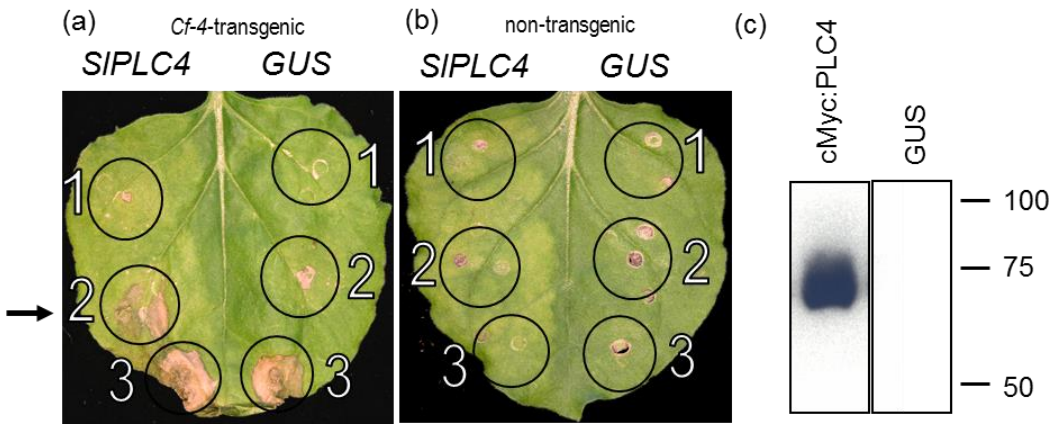


FIGURE 6. **Ectopic expression of *Sl*PLC4 in *Nicotiana benthamiana* causes enhanced Cf-4-mediated sensitivity to Avr4.** A *35S:SlPLC4* construct was agroinfiltrated into the left leaf halves and a *35S:*GUS construct was agroinfiltrated into the right leaf halves of (a) *Cf-4*-transgenic or (b) non-transgenic *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.

Both SlPLC4 and SlPLC6 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 colonized the tomato leaflets, the transgenic GUS marker was used. Blue staining clearly indicated colonization 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.

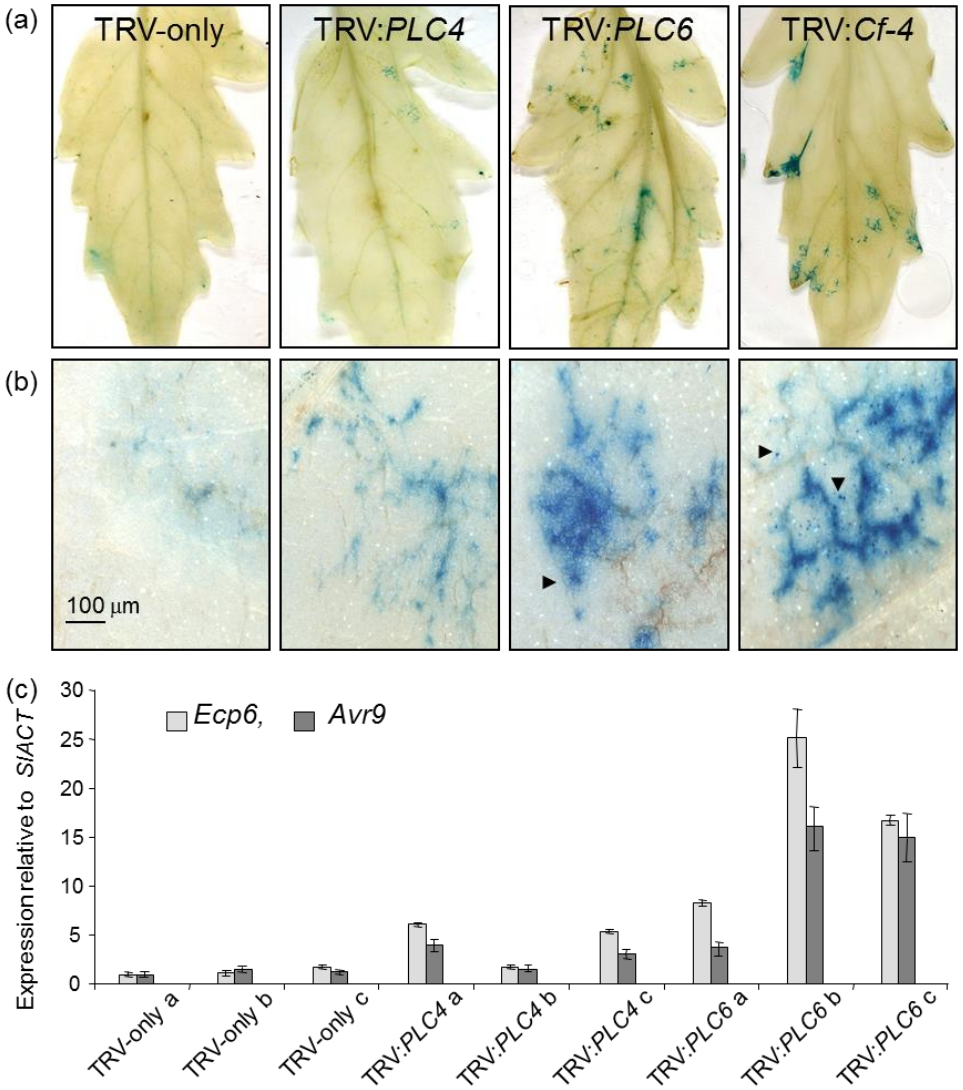
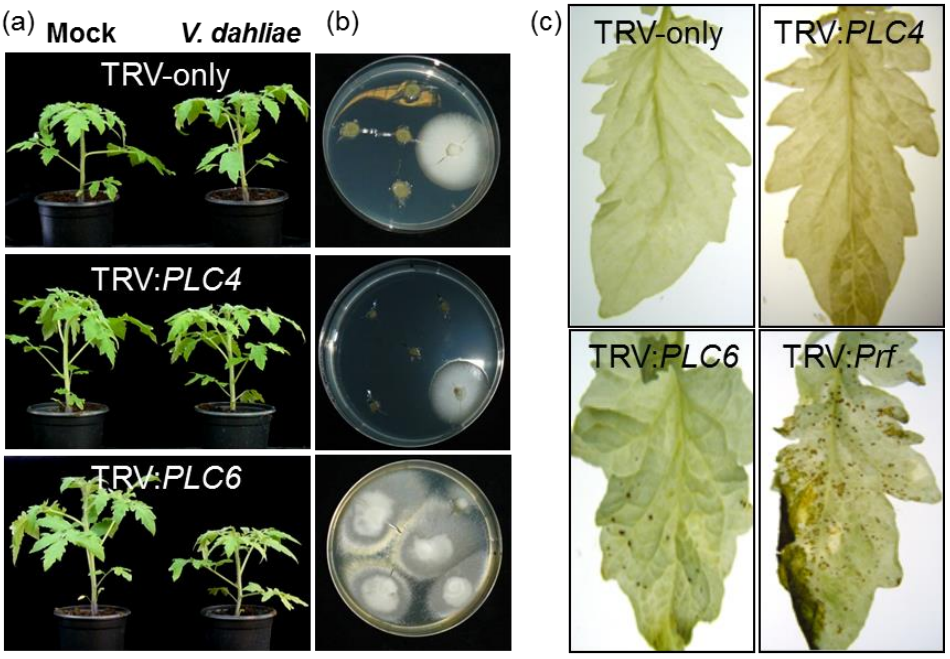


FIGURE 7. **Silencing of *SlPLC4* or *SlPLC6* compromises Cf-4-mediated resistance.** (a) *Cf-4* tomato plants were inoculated with the indicated tobacco rattle virus (TRV) strains. After 3 weeks the plants were inoculated with *Cladosporium fulvum* expressing *Avr4* and the GUS marker gene. Two weeks 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.

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.

SlPLCs 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 (53,54). 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 colonization as compared with the TRV-only- and TRV:*PLC4*-inoculated plants (Figure 8b).



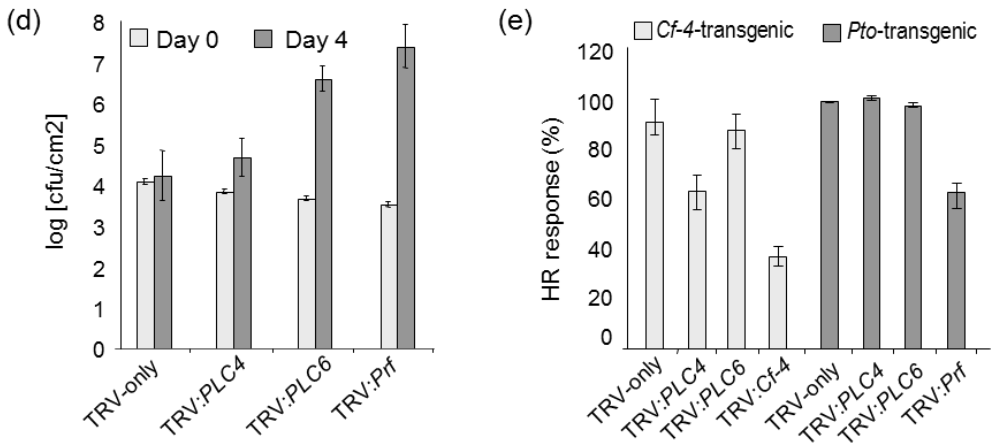


FIGURE 8. **Silencing of *SlPLC6,* but not *SlPLC4,* compromises Ve1- and Pto/Prf-mediated resistance.** Inoculation with the indicated virus-induced gene silencing (VIGS) constructs was followed by inoculation with *Verticillium dahliae* (a, b) or *Pseudomonas syringae* pv *tomato* DC3000 (c, d). (a) *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.

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 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 colonization 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 colonization 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 *SI*PLC4, in contrast to *SI*PLC6, is specifically required for Cf-4-mediated resistance responses.

Discussion

The PLC gene family – We have identified and characterized six cDNAs from tomato encoding different PLC proteins (Figure 1). The encoded proteins show a domain organization that is typical for plant PI-PLCs (15). 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 localization 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 (55). 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 (55).

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

(56-60). Interestingly, a recent report shows that the transcript levels of *OsPLC1* in rice cell suspensions respond to BTH and *X. oryzae* (25). 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 hydrolyze 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 defense response (61). 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 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 recognized and (partial) defense 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 colonization 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 localization 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 (62). 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.

SlPLC6 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 signaling 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 localize to the inner leaflet of the plasma membrane (63) where PIP_2, a potential PLC substrate, is present (5,12). 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 signaling response.

The PLC signaling 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 signaling 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 signaling molecules (11). Certain plant PI-PLCs can hydrolyze 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 (60), and it is completely unknown how these are regulated (64), it remains elusive which phosphoinositide is the *in vivo* substrate.

Interestingly, as PI4P and PI(4,5)P_2 are also emerging as signaling molecules themselves, PLC might also function as an attenuator of their signaling 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 (18). Dependent on the subcellular location, lag time, amplitude and frequency, a specific calcium signature is generated that further specifies downstream signaling (65-67). 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 signaling. One report describes the identification of several PA-binding proteins, among which is Hsp90 (68). Hsp90 plays an important role in pathogen perception since it is required for the activity of both intracellular and transmembrane R proteins(35,69-73). 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 (74). *At*AGC2-1 is also known as OXI1 kinase, which was identified as an important mediator of oxidative burst signaling (75). The kinase acts upstream of a MAP kinase cascade involved in basal resistance against *Hyaloperonospora arabidopsidis*. Recently, an AGC kinase from tomato, Adi3, was identified which inhibits a MAP kinase cascade involved in disease resistance-associated cell death (76). Despite these opposite functions, it is apparent that PDKs and AGC kinases form a link between phospholipid signaling and downstream MAP kinase cascades involved in disease resistance (77). 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 (40,41,78-81). In line with the observations described above, (82) 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 defense signaling events.

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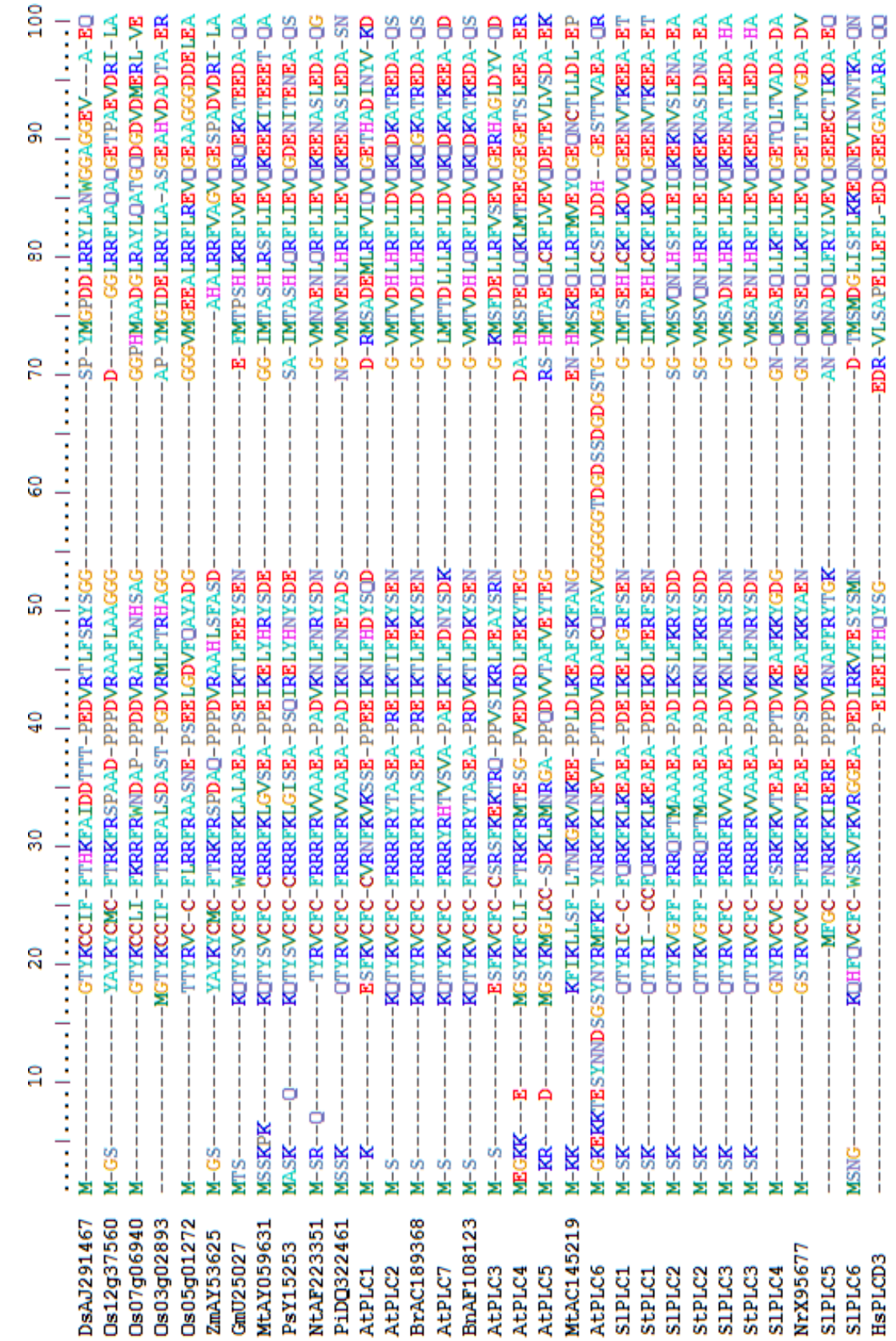
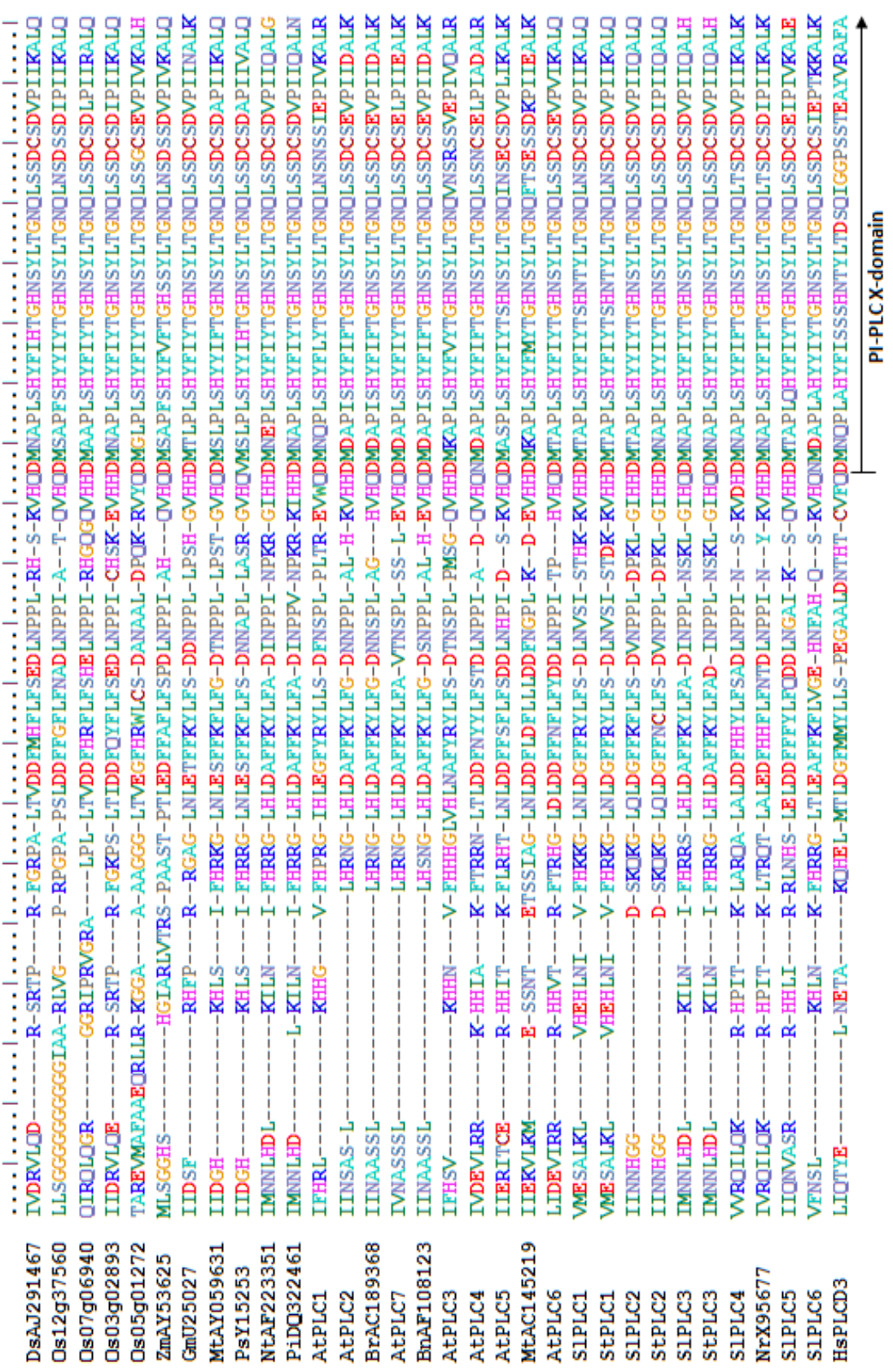
Supplemental information

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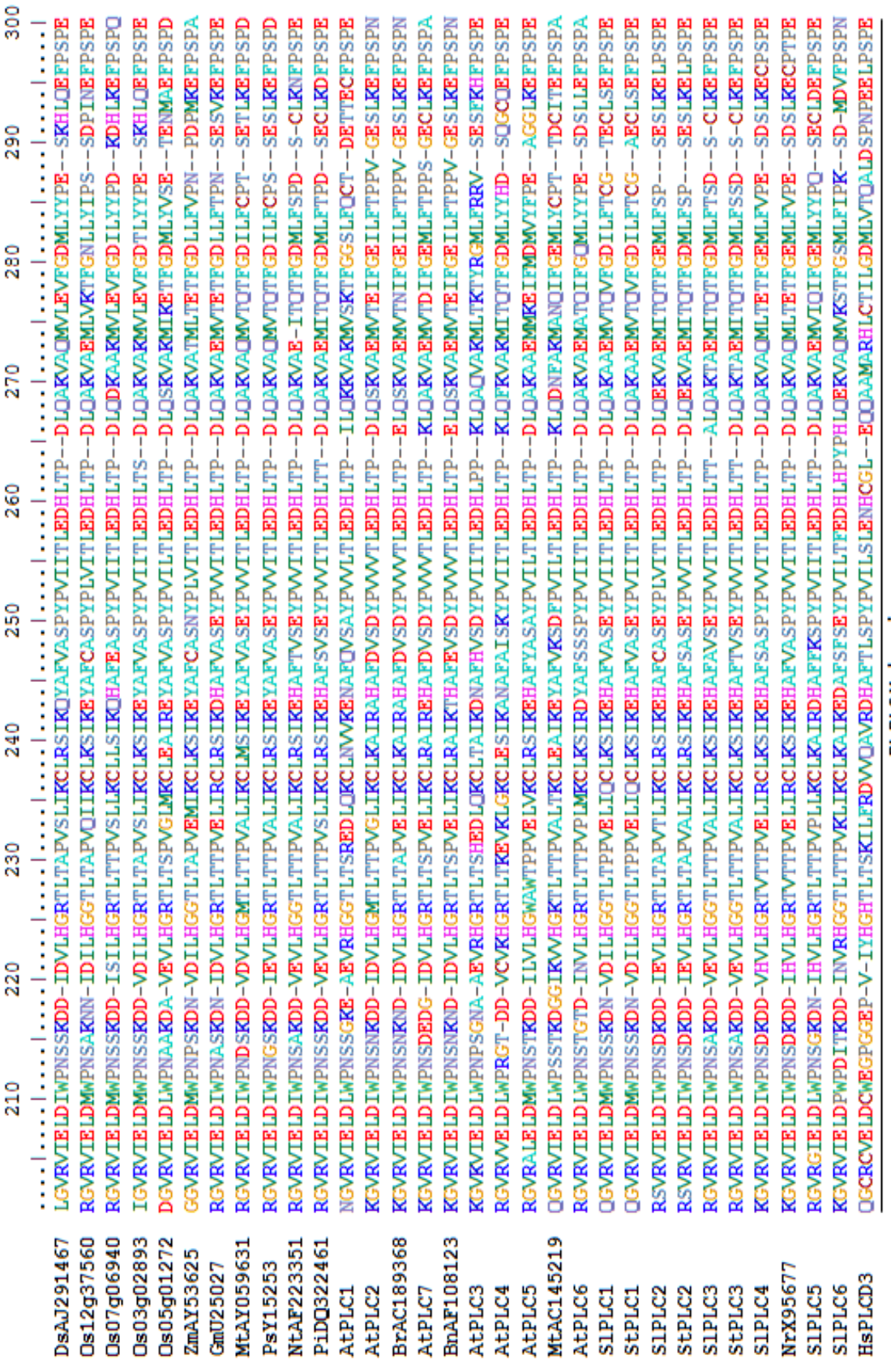
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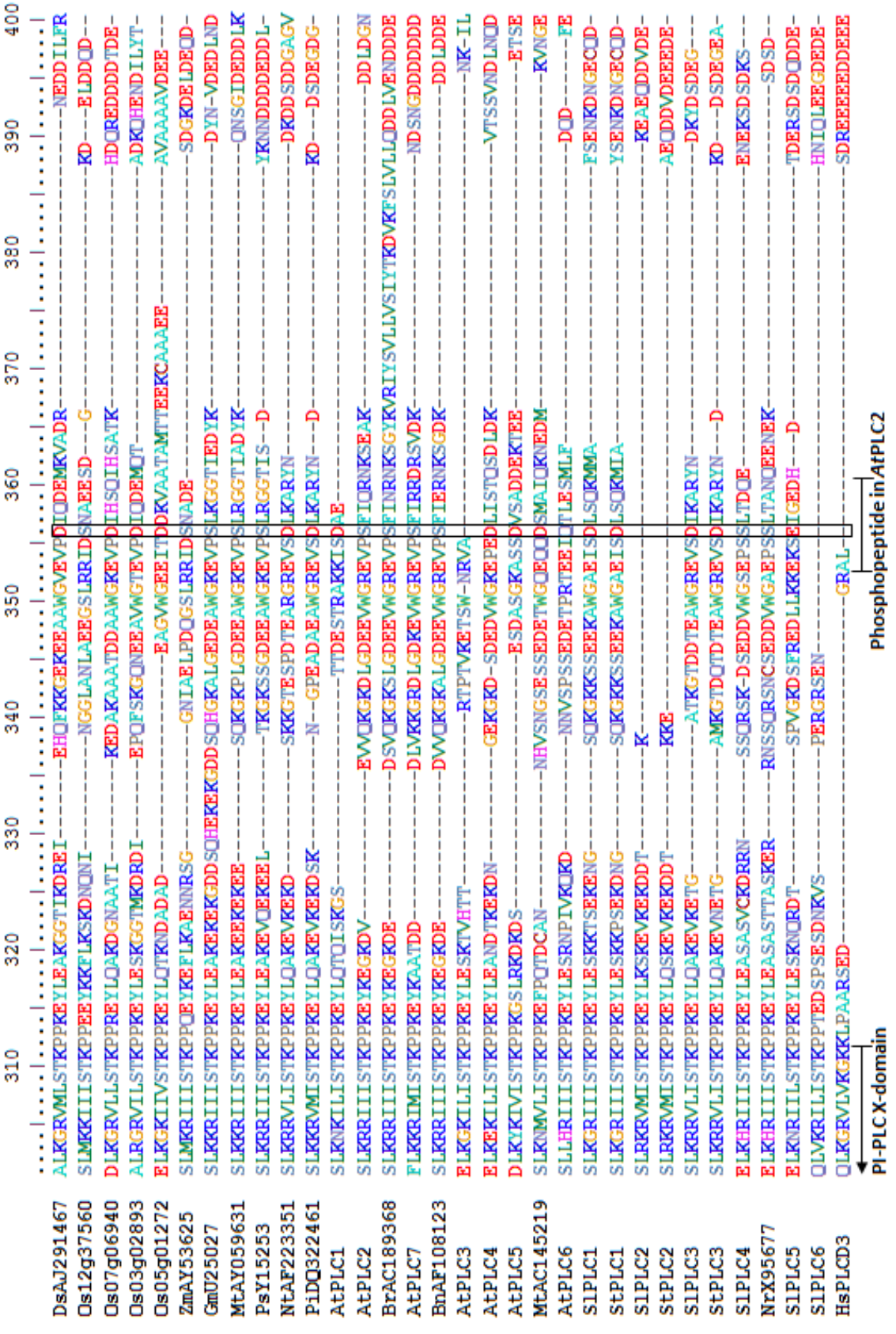
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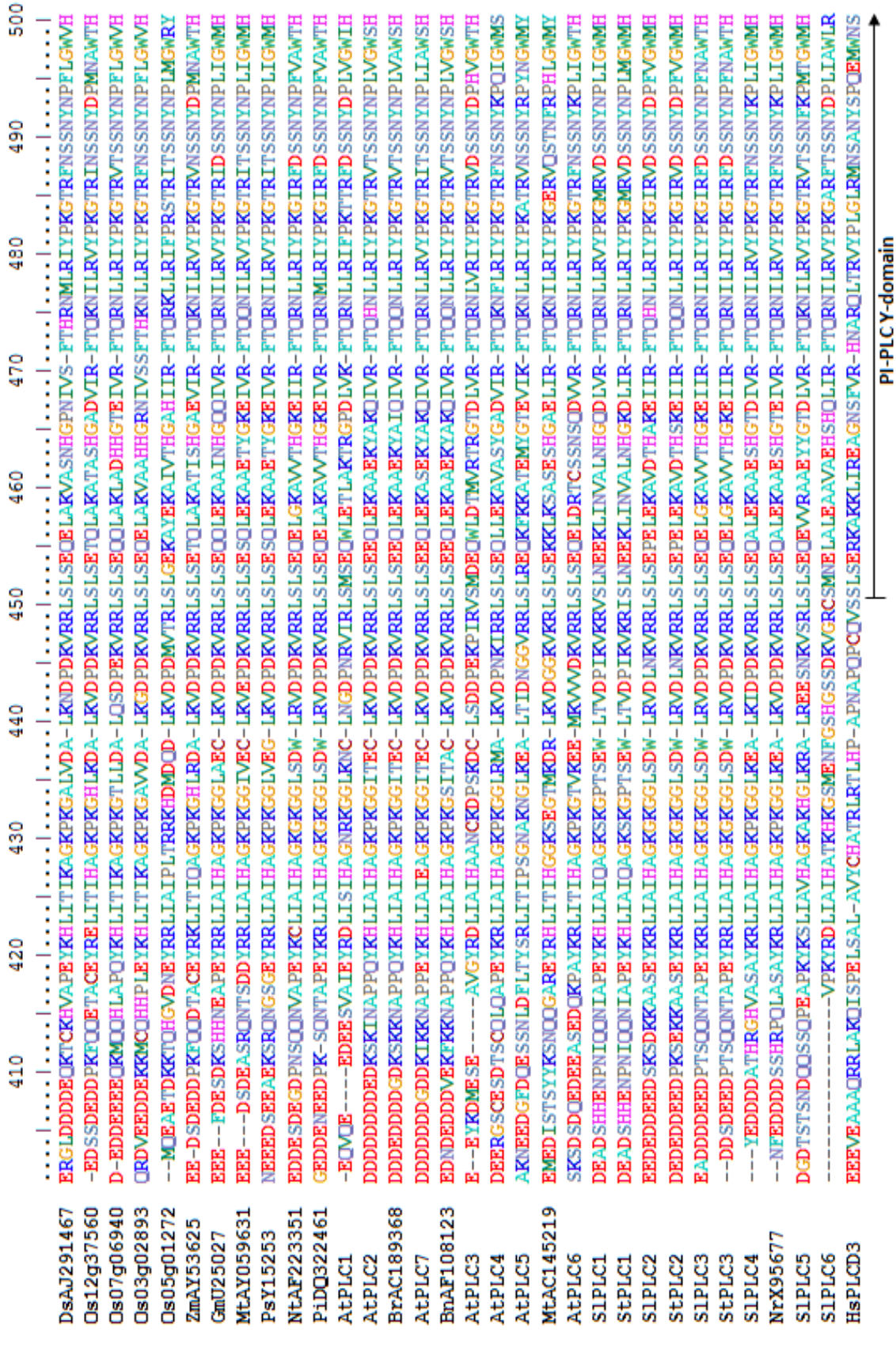
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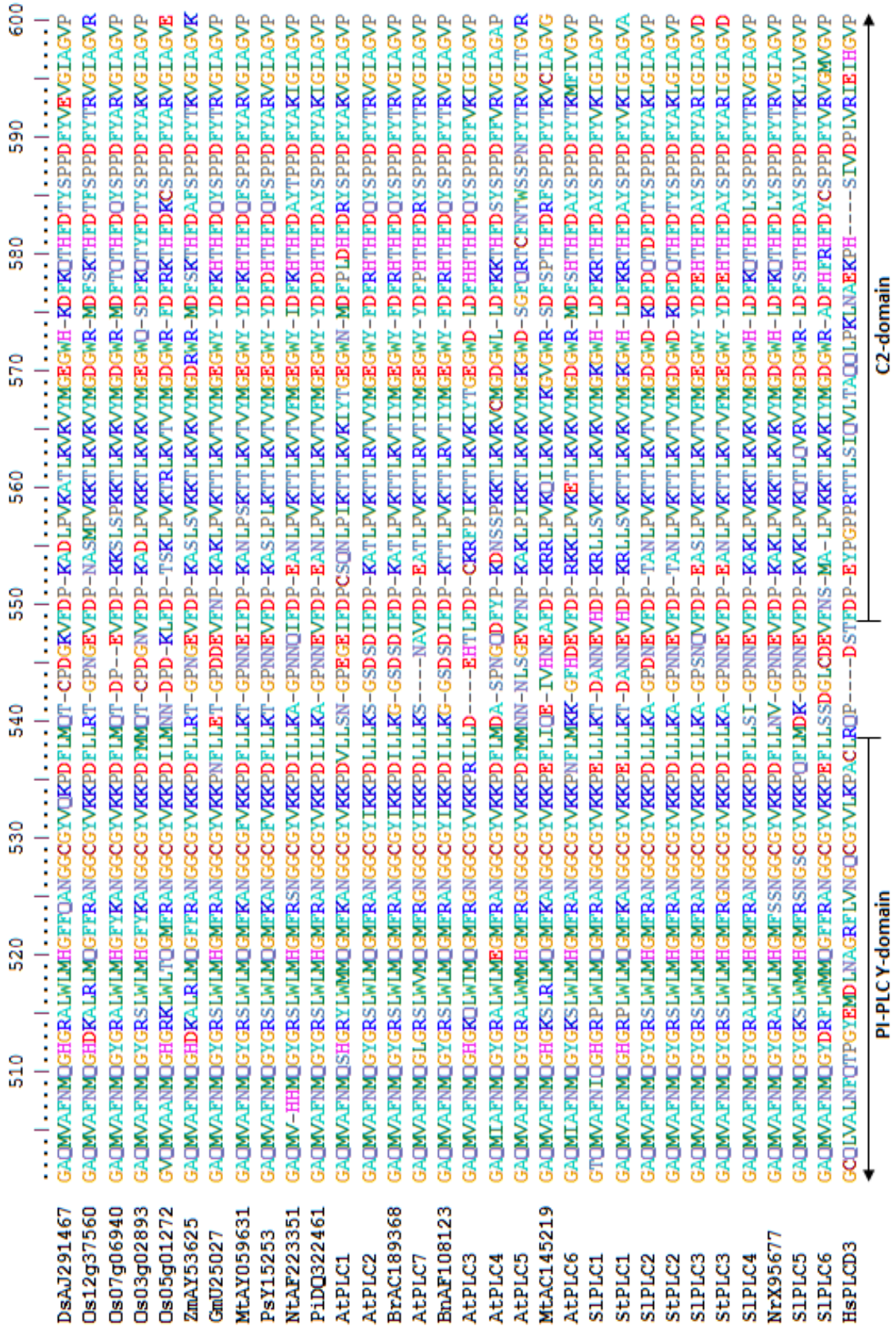
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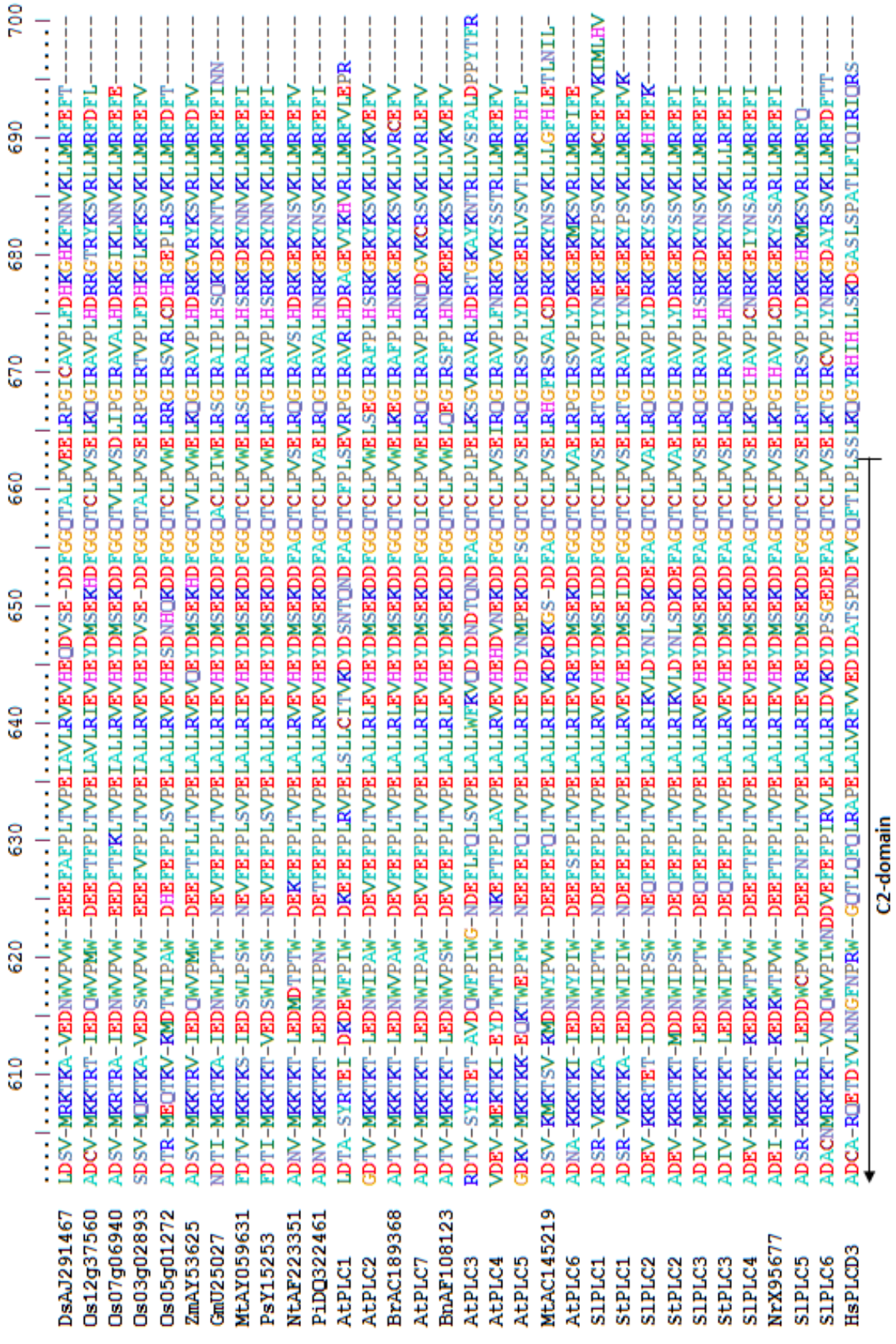

FIGURE **S1. Alignment of PI-PLC protein sequences from various plant species and human PLCδ3.** The various conserved domains (X, Y and C2) are indicated, as well as the position of a peptide derived from a phosphorylated form of *At*PLC2 (as described in Nühse

et al., 2007). For species abbreviations see legend of Figure 1. Similar amino acids are shown in the same color according to the Dayhoff PAM similarity matrix.

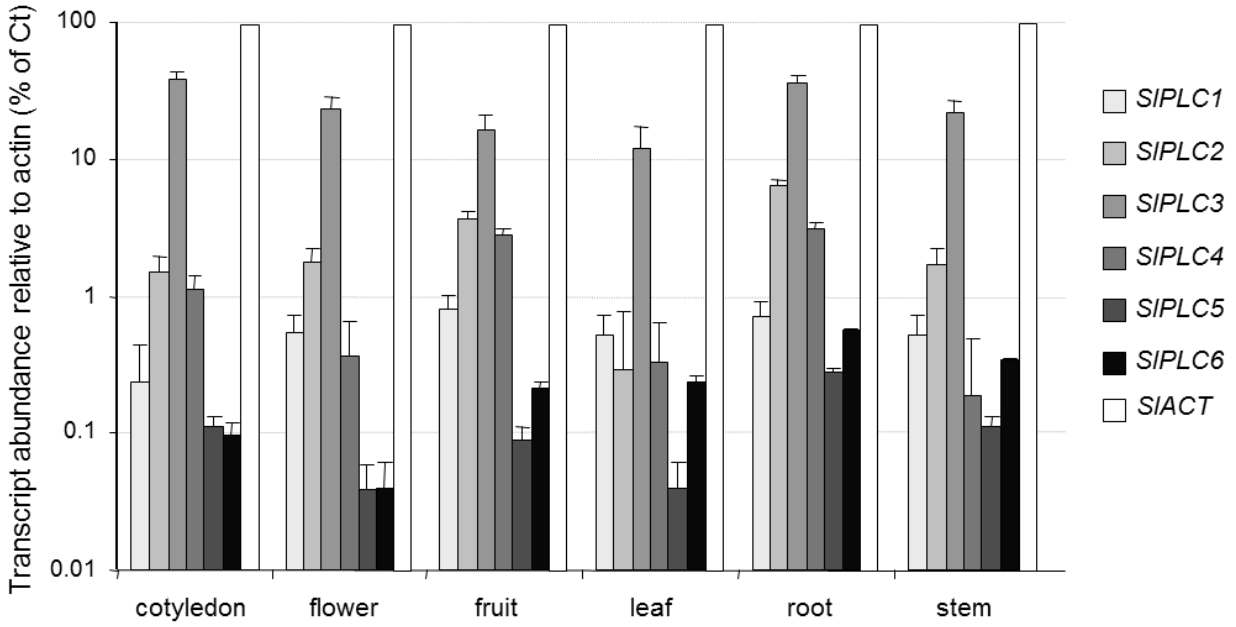

FIGURE S2. **Relative transcript abundance of *PI-PLC* genes in different organs of tomato plants.** Threshold values (Ct) from Q-PCR analysis were determined for *SlACT* and the *PI-PLC* genes in cDNA archives from indicated tomato organs. The relative transcript abundance is expressed as a percentage 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.

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	GTTTACGATTAAAGATGCAGTTTG CTA	CTCAGAACAGGAATTCGAGCTGTGC CTATATACA
SlPLC2	TTTGCTGGCCAAACATGTCTAC	GCAAAGCTCCCTTTTCAAGATCTA	
SlPLC3	AACTAAGACAAGGTATTCGAGCAGTAC	AGCTTGCAAATCATGGCAAA	
SlPLC4	CGGAGCTGAAACCTGGTATACAT	GAAAGCTCAAGAAGCACACAACA	
SlPLC5	GGATGATTTTGGTGGACAAACA	AAATTGAAGCATGTGTATATAGG AAAGTAACT	CTCAGAGTTGAGAACAGGGATCCGA TCA
SlPLC6	CGTTGCTTCGGATTGATGTTAAA	TGGGATTGAGGAAGATTAATTAA GTAGTG	TGGTGAAGATGAATTTGCAGGACAA ACATG
CfAvr9	GAGCTTGCTCTCCTAATTGCTACTACT	AACTTCGTCGAGCGGTTACACA	
SlPR-1a		TGGTGGTTCATTTCTTGCAACTAC	ATCAATCCGATCCACTTATCATTTTA
CfEcp6		GCTCAAGGTTGGTCAGCAGAT	TTCACACCTGACAGATCACTTATGC

CHAPTER 3

Defense Activation Triggers Differential Expression of *Phospholipase-C* (*PLC*) Genes and Elevated Temperature Induces Phosphatidic acid (PA) Accumulation in Tomato

Ahmed Abd-El-Haliem, [1,†] Harold J.G. Meijer, [1] Wladimir I.L. Tameling, [1] Jack H. Vossen, [2] and Matthieu H.A.J. Joosten [1]. **Defense activation triggers differential expression of phospholipase-C (PLC) genes and elevated temperature induces phosphatidic acid (PA) accumulation in tomato.** *Plant Signal Behav* (2012) 7, 1073-1078

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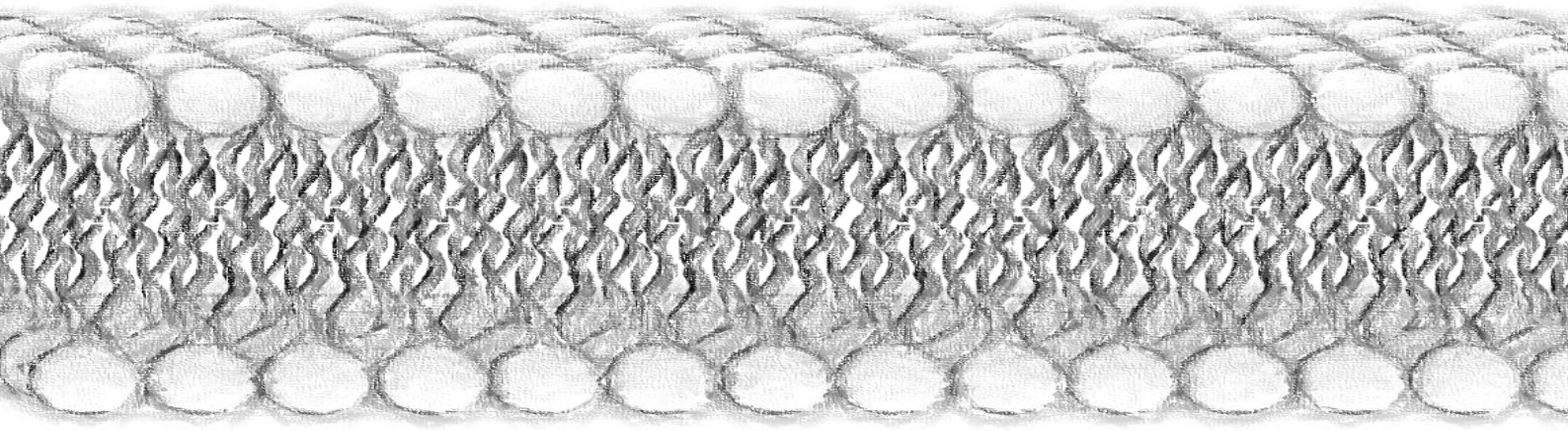
Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PLC, phospholipase C; PLD, phospholipase D; DGK, diacylglycerol kinase; PAMPs, pathogen-associated molecular patterns; R gene, resistance gene; Avr gene, avirulence gene; HR, hypersensitive response; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIP2, phosphatidylinositol-4,5-bisphosphate; DG, diacylglycerol; IP3, inositol-1,4,5-triphosphate; PKC, protein kinase C; DGPP, diacylglycerol pyrophosphate; TLC, thin layer chromatography; RH, relative humidity.



Abstract

Recently, we provided the first genetic evidence for the requirement of tomato *PLC4* and *PLC6* genes in defense activation and disease resistance. The encoded enzymes were catalytically active as they were able to degrade phosphatidylinositol (PI), thereby producing diacylglycerol (DG). Here we report differential *PLC* gene expression following the initiation of defense signaling by the interaction between *Cladosporium fulvum* resistance (R) protein Cf-4 and its matching effector Avr4 in tomato hybrid seedlings that express both Cf-4 and Avr4. Furthermore, we observed that *PLC3* and *PLC6* gene expression is upregulated by elevated temperature in the control seedlings. This upregulation coincides with an increase in the levels of phosphatidic acid (PA) and a decrease in the levels of PI and phosphatidylinositol phosphate (PIP). The decrease in PI and PIP levels matches with the activation of PLC. In addition, the levels of the structural phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) declined transiently during recovery after the exposure to elevated temperature., Further studies will be required to explain the mechanism causing the sustained accumulation of PA during recovery, combined with a reduction in the levels of structural phospholipids.

Introduction

In an incompatible interaction between a host plant and a pathogenic microbe, in which the plant is resistant and the pathogen is avirulent, the matching products of a resistance (*R*)-avirulence (*Avr*) gene combination trigger immune signaling in the plant. The interaction between R and Avr proteins occurs immediately upon pathogen ingress and allows a swift detection of the invader and immediate execution of effective defense responses by the host plant, including the induction of a hypersensitive response (HR) during which programmed cell death takes place (1,2). The activation of the innate immune system of the plant eventually results in the restriction of pathogen outgrowth. Recent studies on plant innate immunity revealed that, similar to mammals, plants have the ability to exploit membrane phospholipid modifications as a means to relay defense signals rapidly after pathogen recognition. Phospholipid-modifying enzymes are therefore considered to be key players in successful defense of plants against intruders (3-5).

Several studies showed that cellular defense signaling requires phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme activity. PI-PLC enzymes are considered to be signal transducers, mainly due to the signaling roles attributed to their substrates and reaction products. PLC activity essentially leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) into diacylglycerol (DG) and inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) (6,7). Subsequent metabolism of these products affects the final outcome of the responses. Ins(1,4,5)P_3 is essential for calcium entry from internal and external stores in mammals. In plants, Ins(1,4,5)P_3 and its further phosphorylated forms IP_4, IP_5 and IP_6, of which the latter is referred to as phytate, might have similar cellular functions, in addition to a role in auxin signaling (8-10). DG can be phosphorylated by diacylglycerol kinase (DGK) to generate phosphatidic acid (PA). DG is known to activate protein kinase C (PKC) in mammals while PA also plays an important role in cellular signaling in mammals and appears to have similar role in plants (11-16). Phospholipase D (PLD) enzymes also hydrolyze phospholipids, thereby generating PA. For PLDs this occurs in a single step, by hydrolysis of structural phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (6,17). PLDs are involved in plant growth and development, in addition to a multitude of stress responses including biotic and abiotic stress (6,17-19). Phosphorylation of PA by PA kinase (20) occurs in plants and leads to the generation of diacylglycerol pyrophosphate (DGPP) (21). DGPP is a common plant phospholipid which is present at trace amounts under resting conditions and accumulates under general stress conditions, in the presence of elicitors and during pathogen infection processes (3,4,22,23).

Several years of extensive research in mammalian systems has expanded our understanding of the important role of PLCs in different aspects of cellular signaling, including immune responses (24-26). Likewise, we recently discovered that also in plants effective innate-immunity requires catalytically active PLC enzymes. Two *PLC* genes from tomato (*Solanum lycopersicum*) were found to be essential for the efficient arrest of different types of plant pathogens and for the initiation of immunity-driven cell death (5). Furthermore, differential regulation of gene expression was observed for six *PLC* genes upon infection of tomato plants by the fungus *Cladosporium fulvum*, the causal organism of tomato leaf mold

disease. This was deduced from infections of susceptible tomato plants, lacking a resistance gene to *C. fulvum* (Cf-0), resulting in a compatible interaction, and resistant plants carrying the *Cf-4* resistance gene, with a *C. fulvum* strain secreting the Avr4 effector, resulting in an incompatible interaction. Although we observed differential expression patterns for all *PLC* genes in both susceptible and resistant plants, these patterns were distinct between the incompatible and compatible interactions (5).

The interaction between plants and their pathogens is very complex and moreover at first contact, defense signaling will only occur locally at the infection sites, which furthermore appear non-synchronously. As a consequence, only a few cells in the infected tissue will induce a local defense response in a rather non-synchronized way. Therefore, we here studied the expression of the tomato *PLC* genes in a more synchronized and homogeneous system, where the pathogen is absent and defense signaling is activated solely by the R/Avr interaction, and more importantly, in all cells of the plant. For this purpose we used tomato seedlings expressing both the *Cf-4* resistance gene and the matching fungal avirulence gene *Avr4* (27,28). The immune response in these *Cf-4*/*Avr4*-expressing hybrid seedlings is suppressed by growing them at 33°C and 100% relative humidity (RH). Subsequently, defense signaling is synchronously activated by transferring the plants to 20°C and 70% RH. This shift in growth conditions releases the immune response blockade and triggers defense signaling throughout the whole seedling, eventually culminating into the death of the seedling as a result of systemic HR (29). We germinated seeds of a 1:1 mixture of the parental lines (control) and the hybrid *Cf-4*/*Avr4* line in closed transparent containers that were placed in an incubator set to 20°C with a 16 h/day light regime. After one week, one set of seedlings (15–20 seedlings) from the control was left at 20°C to be used as a reference for the effect of the elevated temperature. Other sets, with equal numbers (15–20 seedlings) of the control and *Cf-4*/*Avr4* seedlings, were transferred to a rescue condition, being 33°C/100% RH (16 h/day light regime) and incubated for a period of 2 weeks. Subsequently, the seedlings were shifted from 33°C/100% RH to 20°C/70% RH (16 h/day light regime) which then triggers the Cf4/Avr4-mediated immune responses. Control and *Cf-4*/*Avr4* seedlings were harvested between 0 to 6 h after the temperature and humidity shift by immersing them directly into liquid N_2. Total RNA was extracted and cDNA was generated, which was subsequently used for expression analysis of the individual tomato *PLC* genes as described before (5).

Differential expression patterns were observed for most tomato *PLC* genes upon initiating Cf-4/Avr4-mediated defense activation by the shift in growth conditions (Fig. 1). For *PLC2*, *PLC4*, *PLC5* and *PLC6* matching expression patterns, although within a different time-frame, were previously observed for Cf-4 plants inoculated with an avirulent Avr4-expressing strain of *C. fulvum* (5). In the case of the *Cf-4*/*Avr4* seedlings, transcriptional upregulation of the indicated *PLCs* occurs at 1.5 h after the temperature and humidity shift. In contrast, such expression patterns were obtained at 5 d after inoculation in Cf-4 tomato inoculated with an avirulent strain of *C. fulvum* mentioned above. The similarity between these *PLC* expression profiles is expected to be a consequence of plant defense activation specifically due to the Cf-4/Avr4 interaction and subsequent triggering of downstream signaling. In the *Cf-4*/*Avr4* seedlings, *PLC* expression might be enhanced due to the activation of defense throughout the plant. Remarkably, changes in *PLC* expression in the *C.*

fulvum-inoculated plants appeared to be transient, whereas in the *Cf-4*/*Avr4* seedlings, probably due to continuous triggering of defense signaling, these changes become even more pronounced at later time points (Fig. 1). In the *Cf-4*/*Avr4* seedlings, effector recognition occurs simultaneously in every cell as compared with its occurrence in only a limited number of cells present at the infection sites in the *C. fulvum*-inoculated Cf-4 plants. Furthermore, in *C. fulvum*-inoculated Cf-4 plants these *PLC* expression patterns correlate to the fungal growth arrest at 5–7 d upon inoculation (5) (Fig. 1).

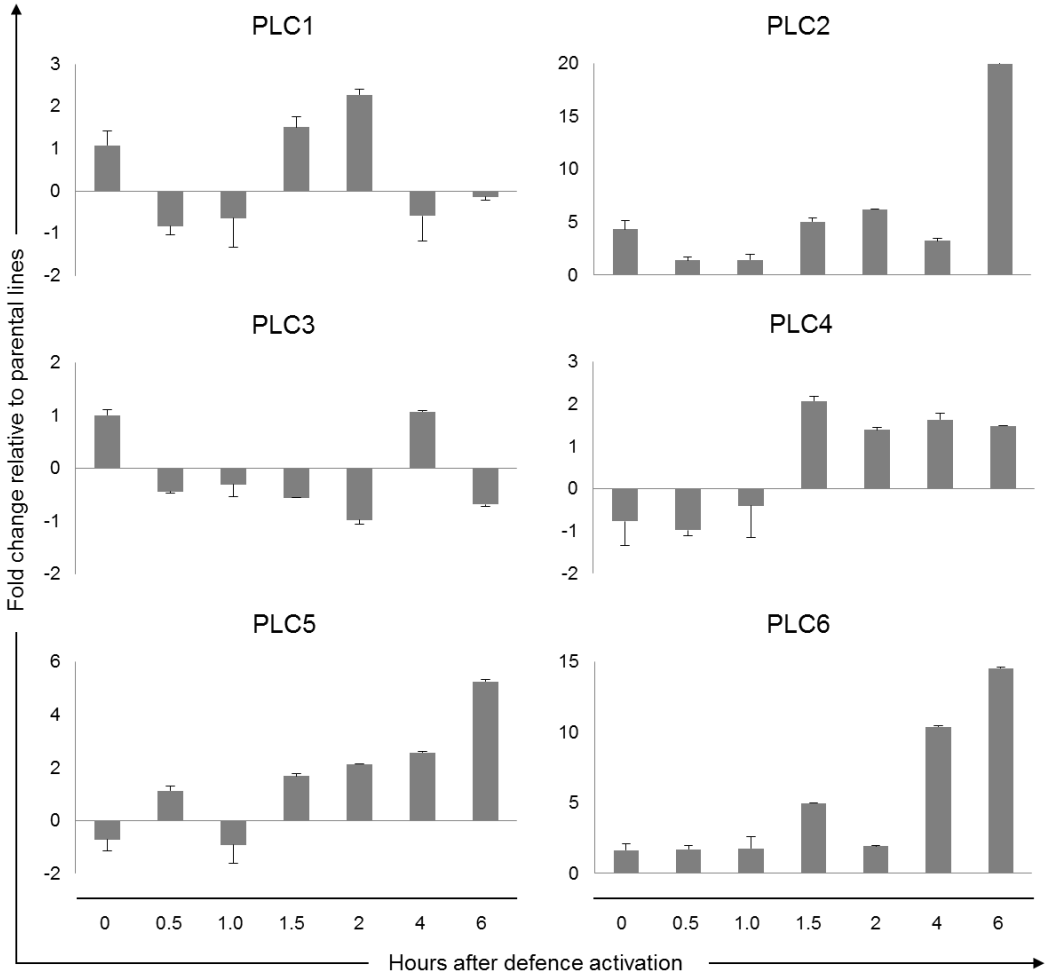


FIGURE 1. ***PLC* expression during the activation of defense in the *Cf-4*/*Avr4* tomato seedlings.** In *Cf-4*/*Avr4* seedlings, defense signaling is activated by the interaction between the resistance protein Cf-4 and the matching *C. fulvum* effector Avr4. Defense is initiated by a shift in the growth conditions of the plants (see text for details). The *Cf-4*/*Avr4* seedlings and a 1:1 mixture of the seedlings from the parental *Cf-4*- and *Avr4*-carrying lines (control) were harvested at the indicated time points after defense initiation and expression of the different tomato *PLCs* was analyzed by quantitative Real-Time PCR. The relative *PLC* expression levels in the *Cf-4*/*Avr4* plants were calculated using the expression of the tomato *Actin* gene as an internal reference to normalize for the amount of template present in the different samples. *PLC* expression in the parental lines was used as a control to calculate the fold change in the *Cf-4*/*Avr4* plants for a given time point. Relative expression is presented for the six tomato *PLC* genes (*PLC1 to -6*). Error bars represent the standard error of two

quantitative PCR samples from the same cDNA archive. The experiment was performed twice, with similar results.

The effect of the shift in growth conditions on the expression of the tomato *PLCs* in the absence of recognition of Avr4 by Cf-4 was also examined. Now only the 1:1 mixture of the parental seedlings was used, which was either incubated at 33°C/100% RH or at 20°C/70% RH and (16 h/day light regime) after germination at 20°C over a period of one week. After two weeks of subsequent incubation at 33°C, *PLC3* expression levels were about 6-fold higher and *PLC6* expression levels were about 2-fold higher than those found in seedlings grown at 20°C (n = 2). Other *PLC* genes were apparently not majorly affected in their expression (Fig. 2). This indicates that the expression of *PLC3* and *PLC6* is constitutively upregulated due to the shift to the elevated temperature and humidity (Fig. 2).

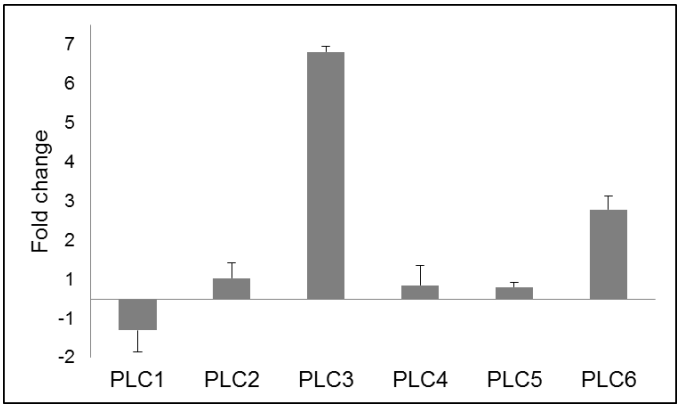


FIGURE 2. **Effect of elevated temperature on *PLC* expression in tomato seedlings.** Relative expression of the six tomato *PLC* genes (*PLC1 to -6*) is presented for a 1:1 mixture of seedlings of the parental lines which were maintained at 33°C. Relative expression was calculated using the expression of the tomato *Actin* gene as an internal reference and the *PLC* expression in the seedlings which were maintained at 20°C as control. This allowed determining the changes in *PLC* expression as a result of elevated temperature. Error bars represent the standard error of two quantitative PCR samples from the same cDNA archive. The experiment was performed twice, with similar results.

These findings prompted us to study whether we could observe changes in phospholipid profiles under the different growth conditions for the *Cf-4*/*Avr4*- and control seedlings. Cold and heat treatments are known to affect phospholipid profiles in Arabidopsis and tobacco cell suspensions (18,30). Initially, we were interested to determine whether PLCs are activated after Cf-4/Avr4-mediated changes in *PLC* gene expression (Fig. 1). For this, we metabolically labeled the phospholipids in seedlings by feeding them with the radioactive isotope orthophosphate-32 (^{32}P), prior to the temperature shift. Radioactive labeling was conducted by cutting 10-d-old seedlings, again germinated at 20°C, directly above the soil and placing them into a 24 wells micro-titer plate, containing 1 ml of tap water and 100 µCi ^{32}P per well. The plate was incubated overnight at either 20°C or 33°C with a 16 h light/day regime. The phospholipids in the seedlings are ^{32}P-labeled overnight to such an extent that no

major changes were anticipated to occur during the period of the sampling (11,31). Seedlings incubated at 33°C were shifted to 20°C and equal numbers of seedlings (3-4 seedlings) were collected after distinct time points. Control samples were collected at the same time points from ^{32}P-fed seedlings which were continuously kept at 20°C. Collected seedlings were flash frozen in a mortar filled with liquid nitrogen and each sample was transferred promptly to a precooled micro tube and placed in liquid nitrogen. Subsequently, phospholipid isolation was performed as described before with minor modifications (see Supplemental Method) (3). The phospholipids were dissolved in chloroform and separated by one dimensional thin layer chromatography (TLC) using alkaline conditions. After completion of the run, TLC plates were allowed to dry at room temperature and radioactivity was detected by autoradiography (KODAK T-MAX 100) (22).

Initial experiments indicated that no differences existed between the phospholipid patterns of the *Cf-4*/*Avr4* and control seedlings when subjected to the temperature shift from 33°C to 20°C, as in both types of seedlings the PA levels increased (data not shown). This increase could result from either the activation of the PLC/DGK pathway or could be caused by increased phospholipase D (PLD) activity. Most likely, both pathways contribute simultaneously to the observed increase in PA levels. From these results we concluded that the effect of the temperature shift dominates the defense-related effects (4) by several orders of magnitude, which makes it difficult to distinguish the latter. To study the phospholipid profile changes upon the temperature shift in more detail, 10-d-old control seedlings germinated at 20°C and grown for three days at 33°C/100% RH and 16 h/day light regime were pre-labeled overnight with ^{32}P at 33°C and then transferred to 20°C/70% RH and 16 h/day light regime. Seedlings were sampled at 0, 1, 2, 4 and 6h after the temperature shift, phospholipids were extracted and analyzed by TLC as described above. Phospholipid profiles were compared with those obtained from seedlings that were kept at 20°C at all steps and that were harvested at the 6h time point. Figure 3 shows that seedlings maintained at 33°C (t = 0) have low levels of phosphatidylinositol (PI) and phosphatidylinositol phosphate (PIP), whereas the levels of the phospholipids PA, DGPP, PC, PE and PG were elevated in comparison to the control seedlings that were maintained at 20°C (Fig. 3; compare the left and right lanes). These results strongly indicate that the phospholipid profile of plants is highly temperature-dependent even at these moderate conditions. After shifting the seedlings from 33°C to 20°C, the levels of PI gradually increased toward the basal levels of plants kept at 20°C. PA levels, already relatively high at 33°C, increased significantly after the temperature downshift. In contrast, PA was only present at very low levels in the seedlings which were maintained at 20°C and harvested at the 6h time point. Most remarkably, we observed a rapid decrease in the levels of the structural phospholipids. Within 2 h, the levels of PC, PE and PG decreased to background levels, whereas under control conditions these typically represent the major radiolabelled species. Their decrease coincided with an increase in PA and DGPP. PLD activation during temperature shifts is a common phenomenon in plants, as demonstrated in various plant systems by PA accumulation and reduced PLD substrate levels (18,30). For example, a recent study showed that PLD activity increased when the temperature was raised. However, in this case this was accompanied by a significant increase in PtdIns(4,5)P_2, a phospholipid species that lacks in our observations,

probably due to labeling restrictions in whole plants (18). The effects observed also resemble the triggering of PLD activity during water deficiency in whole plants (32), although we anticipated an opposite effect since recovery from an elevated temperature by shifting the seedlings to 20°C is not expected to simulate drought stress. However, it should be noted that the 33°C to 20°C temperature shift is also accompanied by a 100% RH to 70%RH shift. As the seedlings have their stomata fully opened at 33°C/100%RH (results not shown), cutting off the seedlings and lowering the temperature and humidity results in an instantaneous water loss and wilting (results not shown). The seedlings soon recover, however, this short period of drought stress might be the cause of PLD activation. In conclusion, the rapid decrease of PC, PE and PG levels suggests that PLD is involved in the adaptation or recovery process (Fig. 3).

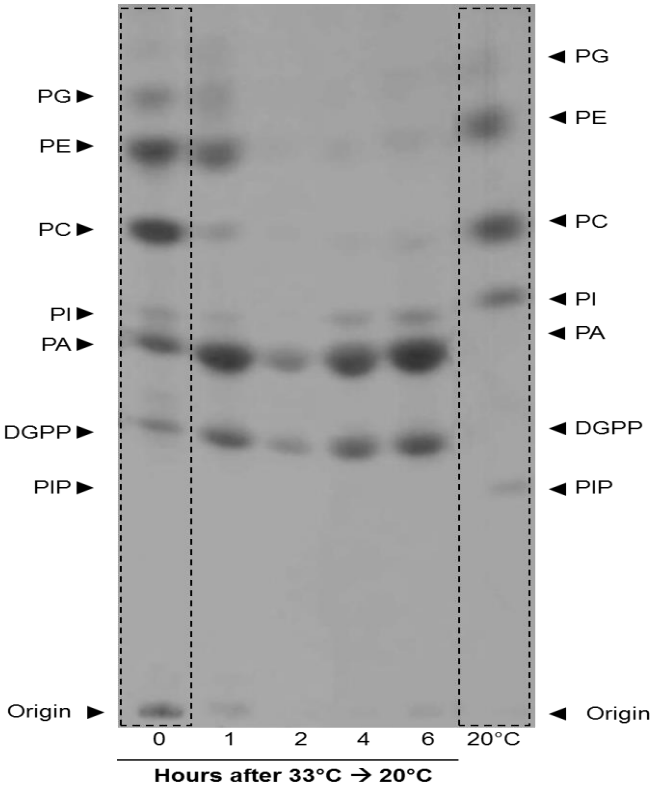


FIGURE 3. **Changes in the phospholipid profile are triggered by lowering the temperature.** Total phospholipids were labeled in vivo using ^{32}P, isolated and analyzed by alkaline TLC and detected using autoradiography. Alkaline TLC profiles of total phospholipids are shown from a 1:1 mixture of seedlings of tomato parental lines (control) grown at either 20°C (right lane) and 33°C (left lane) and those shifted from 33°C to 20°C, at 1, 2, 4 and 6 h after the shift. The control seedlings were sampled together with the 6 h shifted seedlings. Note the differences in the levels of PA, DGPP, PI and PIP between the seedlings which were grown at 20°C or at 33°C. Also note that after the temperature shift form 33°C to 20°C there is a massive increase in the amount of PA and DGPP and a decrease in PC, PE and PG.

In conclusion, the upregulation of the gene expression of *PLC3* and *PLC6* at 33°C (Fig. 2) shows that PLCs might be involved in the cellular responses of tomato to elevated temperatures. Together with our previous finding that PLC6 is able to degrade PI and

produce DG,[5] which can be phosphorylated by DGK to generate PA, we conclude that an elevated temperature leads to the activation of the PLC/DGK pathway in tomato and that this is accompanied by transcriptional upregulation of *PLC3* and *PLC6*. Supporting this conclusion is the reduced PI level and depletion concealment of PIP, which is accompanied by increased levels of PA and DGPP in the seedlings growing at 33°C, as compared with those growing at 20°C. We emphasize that PLD activity also might contribute to the PA increase, since the levels of the structural phospholipids PC, PE and PG, which are all PLD substrates, decrease significantly upon the temperature shift, an effect also shown for cold acclimation of Arabidopsis cells (30). This supports a role for PLD in the temperature adaptation process, most likely to replace a pool of structural phospholipids which was formed during high temperature conditions and allowing the synthesis of structural phospholipids with different properties and thus more suitable for membrane stability and fluidity at lower temperatures. Further studies are required to confirm this increased PLD activity, for example by using a transphosphatidylation assay (33). In such an assay, the phosphatidate moiety is preferentially transferred from a structural phospholipid to a primary alcohol leading to the formation of the corresponding phosphatidyl alcohol which is metabolically stable and readily detectable by TLC techniques. We anticipate that molecular differences exist in the structural phospholipid pool generated at various temperatures due to changes in the composition of the acyl chains, as has been reported during cold exposure in Arabidopsis (30). This will modulate membrane properties and the interaction with membrane-localized proteins. It will be intriguing to determine whether such structural membrane changes are required to maintain plant immune responses modulated by temperature. The observed overall increase in PA content as a result of elevated temperature might be a mechanism by which plants recruit different types of PA-interacting protein complexes to be able to cope with acute changes in the ambient temperature. This suggestion is supported by the finding that heat shock protein 90 (Hsp90) was identified as a PA-binding target in plants (34).

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CHAPTER 4

Biochemical Characterization of the Tomato Phosphatidylinositol-Specific Phospholipase C (PI-PLC) Family and Its Role in Plant Immunity

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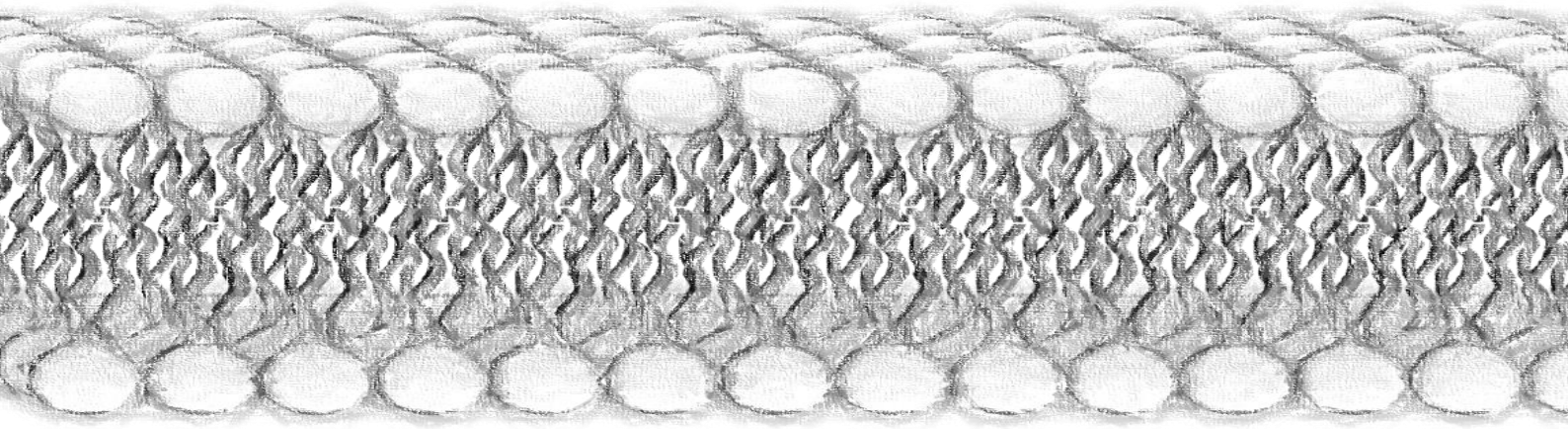

Abstract

Plants possess effective mechanisms which allow them to respond quickly to microbial attacks. The rapid activation of Phosphatidylinositol-Specific Phospholipase C (PI-PLC or PLC) enzymes is an early event following the stimulation of plant immune-receptors. PLC activation results in the transduction of defense signals by controlling the turnover of soluble and membrane-bound second messenger molecules. This leads to the release of cytosolic free-Ca^{2+} and the modulation of the plasma membrane interface, respectively. As a follow-up of our previous study on tomato PLCs, we scanned the recently completed tomato genome sequence and identified an additional PLC member which we named *SlPLC7* (GenBank accession, KM210340). Phylogenetic analysis shows that multiple PLC homologs in different plant species exist and that PLC isoforms from monocots and dicots group separately. We analyzed the tomato PLC enzymes and predicted the involvement of the X/Y-linker region and protein phosphorylation in the regulation of their activity. All recombinant tomato PLC enzymes, except SlPLC7, were successfully produced in *Escherichia coli* and *in vitro* analysis of PLC activity showed that they are all catalytically active. Biochemical studies on *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 enzymes showed distinct requirements for Ca^{2+} ions and the pH for both their optimum activity and substrate preference. Finally, we show that PLC activity is required for both Effector-Triggered Immunity (ETI) and PAMP-Triggered Immunity (PTI), in addition to the flagellin (flg22)-mediated internalization of the corresponding receptor, Flagellin Sensing 2 (FLS2). Altogether, our data suggest an important role for PLC in plant defense signaling downstream of immune receptors and indicate that the PLC isoforms may have both overlapping and distinct roles in signal-transduction leading to the resistance of plants against pathogens.

Introduction

Just like animals, plants also possess an immune system to recognize compounds that are non-self and subsequently trigger immune responses. However, plants lack adaptive immunity and possess only an innate immune system which has similarities but also differences with its counterpart in animals. Animal innate immunity is considered as a generic or non-specific immune system which forms the first line of defense (1,2). The innate immune system of plants can however respond to both a general and a more specific stimulus, compensating for the lack of adaptive immunity (3,4). In fact, an absolute separation between the immune response triggered by general on the one and more specific stimuli on the other, does not exist (4). General structural compounds of microbes that are recognized as non-self are referred to as Pathogen- or Microbe-Associated Molecular Patterns, PAMPs or MAMPs respectively, and the type of immunity that is activated is commonly known as PAMP- or MAMP-triggered immunity (PTI or MTI, respectively). The response to specific pathogen-derived compounds is referred to as effector-triggered immunity (ETI), as it is initiated upon recognition of microbe- or race-specific, so-called effector proteins which usually have a function in virulence of the pathogen (3,4). In both PTI/MTI and ETI a cellular signal transduction cascade is being activated upon pathogen perception, which eventually leads to mounting an effective defense barrier preventing colonization of host tissues by the pathogen.

Phosphatidylinositol-phospholipase C (PI-PLC or PLC) enzymes play an important role in intracellular signaling in animal cells by hydrolyzing inositol lipids in the membranes. For example, this type of PLC enzymes hydrolyze phosphatidylinositol (PtdIns)4,5-bisphosphate (PIP_2), to produce inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ diffuses from the membrane into the cytosol and triggers the release of Ca^{2+} from intracellular stores upon binding to specific $InsP_3$ receptors (5), while DAG resides in the membrane and is responsible for the activation of protein kinase C (PKC) (6). The depletion of PIP_2 and the generation of the reaction products are interpreted by the cell as signals that serve in specific cellular processes, usually in response to external stimulation. PLC activity was shown to be involved in innate immunity (7-10) and adaptive immunity (11) in animals. Likewise, evidence on the involvement of PLC activity in plant innate immunity is accumulating (12-19). For example, a cell culture expressing the *Cf-4* gene, providing resistance to the fungal tomato pathogen *Cladosporium fulvum* (20), shows PLC activation and rapid accumulation of phosphatidic acid (PA) upon treatment with Avr4, which is the effector matching the Cf-4 protein (13). Most of the generated PA results from the phosphorylation of PLC-produced DAG by DAG kinases (DGKs). PA is considered to be an important signaling lipid in the response of plants to biotic and abiotic stresses (16,21). Moreover, we demonstrated previously that tomato PLC4 and PLC6 play a role in resistance to microbial pathogens (12). Animal PLCs exist in six different classes, each characterized by the presence of class-specific domains, in addition to the standard X and Y catalytic domains and the C2 lipid-binding domain (21-23). In contrast, all plant PLC enzymes identified until know belong to a single class which is PLCζ that has recently been discovered in sperm (24) and acts as an important signaling component in egg activation by controlling calcium

oscillations (25). Moreover, plants lack the downstream targets PKC and the Ins_3P-receptors that are present in animals, and it is therefore still unclear how signals are relayed after activation of plant PLCs.

As a follow-up of our previous study (12), we scanned the recently completed tomato genome sequence (26) and identified an additional PLC family member, *SlPLC7* (GenBank accession, KM210340), and discovered an extension for the coding sequence of the previously cloned *SlPLC5* gene at the N-terminus (GenBank accession, KM587010). Phylogenetic analysis revealed that many plant species possess multiple PLC isoforms and there is a distinction between PLC isoforms from monocots and dicots. Prediction analysis suggests the regulation of the tomato PLC enzymes by an auto-inhibition mechanism and phosphorylation, similar to what has been demonstrated in recent studies (27,28). Heterologous expression of the tomato *PLC* genes in *Escherichia coli* revealed that six out of seven PLCs encode catalytically active enzymes. We made use of a non-radioactive phospholipase-activity assay (12) and studied the conditions under which three representative tomato PLC enzymes (*Sl*PLC2, *Sl*PLC4 and *Sl*PLC5) show optimum activity and we studied their substrate preference. We found that both the concentration of Ca^{2+} and the pH value differentially affected the enzyme activity and substrate preference. Furthermore, inhibition of PLC activity inhibited defense in both ETI and PTI. Interestingly, the inhibition of PTI in case of the interaction between the immune receptor flagellin sensing 2 (FLS2) and flagellin (flg22) coincided with the suppression of flg22-mediated internalization of FLS2. Our data suggest an important role for PLC activity in plant defense via immune receptors and indicate that the PLC isoforms may have both overlapping and distinct roles in defense signal-transduction.

Experimental procedures

Inventory of Plant PLC Sequences and Construction of a Phylogenetic Tree – Amino acid sequences of tomato PLC1 to PLC7 were successively used for tBLASTn (29) on the NCBI public database. All matching nucleotide sequences were retrieved by their GI number and subsequently, sequences were translated and then filtered to exclude duplicated or truncated entries. Domain prediction by SMART (30,31) was used to identify full-length proteins possessing the distinctive domains of a PLC enzyme. Multiple amino acid sequence alignments were carried out using ClustalX, in which the filtered unique PLC sequences, in addition to the PLC sequences of all tomato isoforms, were included. The generated alignment was used to draw a phylogenetic tree using the program Treeview (32) and utilizing the Bootstrap N-J Tree procedure where the random generator seed was set to 111 and the number of bootstrap trials was set to 1,000. Human PLCδ3 was used as an out-group and *Hs*PLCζ1 was included in the alignment as it resembles the mammalian counterpart for plant PLCs.

Cloning and Heterologous Expression of Recombinant Tomato PLC Enzymes – The open reading frames (ORFs) of the previously isolated tomato *PLC* genes (12) were amplified from plasmids in which the encoding cDNA was inserted, and N-terminally tagged with GST

by cloning them in the pGEX-KG vector (33). Accordingly, the primers (forward, F) 5`-TTCTAGATATGTCTAAACAAACATACAGAATCTG-3` and (reverse, R) 5`-TCTCGAGCTATACATGTAACATTATTTTTACAAAT-3' were used to add *XbaI* / *XhoI* restriction sites (respectively underlined in the primer sequences) by PCR amplification, and subsequently insert the ORF of *SlPLC1* in pGEX-KG. Primers (F) 5`-TTCTAGATATGTCGAAACAAACGTACAAAGTC-3` and (R) 5`-TCTCGAGTTATTTAAACTCGAAATGCATGAGAAG-3` were used for amplification and cloning of the ORF of *SlPLC2*, whereas the ORF of *SlPLC3* was cloned in pGEX-KG using *SalI* / *XhoI* restriction sites after amplification with the primers (F) 5`-TGTCGACTAGATATGTCCAAACAGACGTACAGAGTC-3` and (R) 5`-TCTCGAGTTAGATAAATTCGAAACGCATAAGTAG-3`, with the respective restriction sites underlined. The ORF of *SlPLC5* was cloned using *NcoI* / *XhoI* restriction sites after amplification using the primers (F) 5`-TCCATGGTTATGTTTGGGTGTTTCAACCGTAAAT-3` and (R) 5`-TCTCGAGTCAAAGAAATTGAAATCGCATGAGAAG-3`, with the respective restriction sites underlined. The *SlPLC7* ORF was amplified from tomato cDNA using the primers (F) 5`-TTCTAGAAATGGGTAGTTACAATTATTACAAAGTG-3' and (R) 5`-TCTCGAGTCAGATGAATCGAAATTGCATAAGCAGCCTAACA-3`, which add the *XbaI* / *XhoI* restriction sites (respectively underlined in the primer sequences), for cloning into pGEX-KG. Induction of the expression of the recombinant PLC proteins in *E. coli* and subsequent affinity purification of the GST-tagged PLCs were carried as described previously (12).

Phospholipase Activity Assays – *In vitro* PI-PLC activity assays were essentially carried out as described previously (12), except that synthetic PIP_2 was replaced by PIP_2 from a natural source (L-α- PtdIns 4,5-bisphosphate, Brain, Porcine, ammonium salt, Cat# 840046P, Avanti Polar Lipids). Furthermore, PI4P (L-α- PtdIns 4-phosphate, Brain, Porcine, ammonium salt, Cat# 840045P, Avanti Polar Lipids), was included as a substrate. Synthetic PIP_2 with different lengths of acyl chains, PtdIns 4,5-P_2 (1,2-dihexanoyl), Cat# 10007762; PtdIns 4,5-P_2 (1,2-dioctanoyl), Cat# 64910 and PtdIns 4,5-P2 (1,2-dipalmitoyl), Cat# 10008115, were obtained from Sigma or Cayman.

To determine the optimum Ca^{2+} concentration required for the activity of a specific PLC, phospholipase reaction mixtures were prepared in series in which the Ca^{2+} concentration in the buffer of each reaction was adjusted using EGTA (34,35). The concentration of all other components in the mixture was fixed and incubation proceeded for 60 min when PI was the substrate and for 30 min when PIP_2 or PI4P were the substrates, both at 25°C. Similarly, the requirement of Mg^{2+} was studied by adjusting the Mg^{2+} concentration in the buffer of each reaction, but without adding EGTA. In this case, reactions were incubated for 60 min at 25°C. The optimum pH required for PI-PLC activity was determined at the previously determined optimum Ca^{2+} concentration for each tested PLC, by pre-adjusting the pH of the buffer used for each reaction. Reactions were initiated by the addition of the substrate as a micellar-lipid solution and incubation at 25°C for 60 min in case PI was the substrate and for 5 minutes in case PIP_2 was the substrate. A shorter incubation time for PIP2 hydrolysis was used, compared with 30 minutes for the determination of optimum Ca2+

concentrations, which allowed the visual differentiation between the generated levels of DAG in each reaction. The inhibition of *in vitro* PLC activity was studied by incubating 5µg of each tested recombinant *Sl*PLC enzyme with either 10, 50 or 100 µM of the chemical compound U73122 (Cat# U6756, Sigma-Aldrich) or its structural analog U73343 (Cat# U6881, Sigma-Aldrich), for 5 min. Subsequently, reactions were initiated by adding the substrate (PI) as a micellar-lipid solution and incubation at 25°C for 30 min. Termination of the reactions and analysis of the reaction products were carried out as described previously (12).

Computational Analysis of Charge and Putative Phosphorylation Sites in the X/Y-linker – X/Y-linker regions were determined as the regions located between the PLC X and PLC Y catalytic domains in each PLC using SMART domain prediction. The number of negatively charged aspartic acid (D), and glutamic acid (E) residues present in either the full-length PLC protein or its X/Y-linker region, was determined using Vector NTI (Invitrogen). Putative phosphorylation sites in the PLC protein or its X/Y-linker were predicted using the NetPhos 2.0 server (36), where the threshold for the prediction score was set to > 0.5. A score between 0.5 and 1.0 was regarded as significant. Note that 0.5 is the threshold and a score between 0.5 and 1.0 reflects confidence of the prediction and the higher the similarity to one or more of the phosphorylation sites used in training the method.

Inhibition of the Medium Alkalization Response Related to ETI and PTI by the PLC Inhibitor U73122 in Tobacco Cell Suspensions – 2.5 ml aliquots of a cell suspension generated from transgenic tobacco expressing the *Cf-4* gene (13,37) were transferred to the wells of 12-well cell culture plates (Greiner Cellstar, Cat# 665102) and allowed to recover for 2 hours at room temperature on a flat shaker (LaboTech, Muttenz, Switzerland) set to 240 rpm. Subsequently, 10 µl of a 5 mM stock of U73122 or U73343, dissolved in dimethylsulfoxide (DMSO), was added to obtain a final concentration of 20 µM. As a control treatment, 10 µl of DMSO was added to a well. Cells were then incubated for 10 min, after which the elicitors were added. For testing the effect on ETI-related responses, 20 µg of Avr4 was added to elicit Cf-4/Avr4-triggered medium alkalization (pH shift) (13). Similarly, flg22 peptide (EZBiolab, Carmel, USA) was added to a final concentration of 1, 10 or 20 nM, whereas chitin was added in the form of chitohexaose (Seikagaku Corporation, Tokyo, Japan), to a final concentration of 1 µM. The pH shift due to alkalization of the cell suspension medium after perception of Avr4, flg22 or chitin was monitored using a micro pH meter (InLabMicro, Mettler-Toledo), with a digital recording device (Seven Multi, Mettler Toledo).

Plasmid Construction and Overexpression of Tomato PLC Genes In Nicotiana benthamiana – Full length mRNA transcripts were amplified to add restriction sites used for inserting them in destination plasmids under transcriptional control of the 35S promoter. *SlPLC1* and *SlPLC3* were amplified from plasmids (12) using primers (F) 5`-ACTCGAGATGTCTAAACAAACATACAGAATCTG-3` and (R) 5`-TGGTACCCTATACATGTAACATTATTTTTACAAATTCG-3` for *SlPLC1* and (F) 5`-ACTCGAGATGTCCAAACAGACGTACAGAGTCTGTTTCTG-3` and (R) 5`-

TGGTACCCTATTAGATAAATTCGAAACGCATAAGTAGC-3` for *SlPLC3* and cloned in a pMOG800-based expression vector (12) using *XhoI* and *KpnI* restriction sites (underlined in the respective primers) (38). *SlPLC2* and *SlPLC5* (12) were amplified using (F) 5`-ACTCGAGATGTCGAAACAAACGTACAAAGTCGGATTTT-3` and (R) 5`-AGAATTCTTATTTAAACTCGAAATGCATGAGAAGCTTC-3` for *SlPLC2* and (F) 5`-ACTCGAGATGTTTGGGTGTTTCAACCGTAAATTTAAG-3` and (R) 5`-AGAATTCTCAAAGAAATTGAAATCGCATGAGAAGTCTAAC-3` for *SlPLC5* and cloned using *XhoI* and *EcoRI* restriction sites (underlined in the respective primers) in the same vector, following a similar strategy as was previously used for *SlPLC4* (12). *SlPLC6* was amplified using (F) 5`-TTCTAGAATGTCTAATGGTAAGCAACATTTCCAGGTTTG-3` and (R) 5`-AGGATCCGCCGTTACATACAATTTTTCCTATTATCTATG-3` and cloned between the *XbaI* and *BamHI* restriction sites (underlined in the respective primers) in the expression vector pBIN61 (39). *SlPLC7* was cloned into pSol2092 (40) by plasmid overlap extension using the primers (F) 5`-GACCTGCAGGCGGCCGCACTAGTTCTAGAAATGGGTAGTTACAATTATTACAAA GTG-3` and (R) 5`-GCCGCGGGATATCACCACTTTGTACACTCGAGTCAGATGAATCGAAATTGCATAA GCAGCCTAACA-3`, followed by digestion with *DpnI* and transformation to *E. coli*. All generated constructs were transformed to *Agrobacterium tumefaciens* strain GV3101. HR enhancement assays were carried out by infiltration of *A. tumefaciens* in *Nicotiana benthamiana* leaves to transiently express Avr4 at final OD_{600} = 0.03 (41), together with 35S:GUS (12) as a control on one leaf half or with one of the seven tomato *PLC* genes, all at final OD_{600} = 1.0, on the other half of the leaf. The development of HR was scored at 3 to 6 days post infiltration.

Suppression of flg22-mediated internalization of the FLS2 receptor by the PLC inhibitor U73122 – Quantification of FLS2 endosomes was essentially done as previously described (Beck et al. Plant Cell 2012). Briefly, detached cotyledons of two weeks old seedlings were vacuum infiltrated for 1h in 20 µM U73122 and U73342 solutions. After 60 min incubation, 10 µM flg22 was added to the inhibitor solution followed by 40 min incubation, and imaging was done using the high-throughput confocal automated Opera microscope (PerkinElmer Cellular Technologies, Germany). Excitation of the samples was performed at 488 nm for GFP; the emission spectrum for GFP was captured using the 540/570 band pass filter. Images of a consecutive series of 21 planes with a distance of 1 µm were taken and analyzed with the EndomembraneQuantifier script (Beck et al. Plant Cell 2012).

Results

Current View on the Tomato Phospholipase C (PLC) Gene Family – We have previously identified six tomato *PI-PLC* genes (*SlPLC1* to *SlPLC6*) using EST database searches and subsequently cloned the full-length cDNA sequences of the corresponding genes (12). The

recent completion of the tomato genome sequence (26) now allowed us to verify whether we have documented the entire PLC family of tomato. Interestingly, a new search resulted in the identification of one additional PLC isoform, which we designated *SlPLC7* (GenBank accession number KM210340). Furthermore, we had to correct the *Sl*PLC5 protein sequence which has 9 additional amino acids at its N-terminal end (Solyc06g051630.2). We extended our search to include all available plant PLC protein sequences in the public database on the NCBI website and we retrieved 98 full-length plant PLC sequences, including the tomato PLC family, together representing 29 different plant species. Subsequently, we generated a multiple alignment of the retrieved PLC amino acid sequences (Fig. S1*A*) and used this to construct a phylogenetic tree which was rooted using human *Hs*PLCδ3 as an out-group (Fig. S1*B*). *Hs*PLCζ1 was included in the alignment as it closely resembles the domain architecture of common plant PLCs (42).

The analysis shows that almost each tomato PLC protein is represented by one or more ortholog(s) in other plant species. Extending the sequence dataset generated additional information compared to our previous observations. Accordingly, PLC proteins from dicots and monocots clustered separately and most plant species in the current analysis appeared to possess multiple PLC isoforms. *Arabidopsis thaliana At*PLC1 and *At*PLC3, and their orthologs from close relatives, clustered together and emerged from a higher node in the phylogenetic tree, separating them from all other plant PLC proteins and suggesting that these diverged early. An ortholog for *Sl*PLC1 was identified in *Torenia fournieri* (*Tf*, wishbone flower), in addition to the previously reported ortholog from potato (*Solanum tuberosum*). *Sl*PLC2 and *Sl*PLC3 grouped together and they were exclusively represented by orthologs in species belonging to the *Solanaceae*. The phylogenetic analysis also shows that *Sl*PLC4 now clusters separately from *Sl*PLC5, which clusters together with the newly identified *Sl*PLC7. Furthermore, *Sl*PLC6 was located on a separate branch in the phylogenetic tree compared to all other *Sl*PLC isoforms and two orthologs of *Sl*PLC6 were identified in *Ricinus communis* (*Rc*, castor oil plant) and *Populus trichocarpa* (*Pt*, black cottonwood). Interestingly, we previously concluded that this PLC isoform might be unique for tomato. However, *Sl*PLC6 is still the least represented isoform in the phylogenetic tree. Furthermore, we analyzed the domain architecture to reevaluate all conserved domains in the *Sl*PLC protein family using recent updates in domain prediction databases and the completed *Sl*PLC family. Domain prediction using the SMART server (http://smart.embl-heidelberg.de/), combined with HMM (http://hmmer.janelia.org/) and Pfam searches (http://pfam.sanger.ac.uk/) confirmed the general PI-PLC protein domain organization as indicated by Vossen and Abd-El-Haliem and co-workers for all *Sl*PLCs (12), and now including *Sl*PLC7 (Fig. S1*C*). All *Sl*PLCs contain the typical catalytic X and Y domains that are known to form together a distorted Triose Phosphate Isomerase TIM barrel structure, containing the active-site residues (27). Furthermore, all *Sl*PLC isoforms, including *Sl*PLC7, contain a C2 domain at their C-terminal end (12). Such a C2 domain has been shown to be crucial for the enzymatic activity of PLCδ1 from rat (43,44) and is required for the binding of Ca^{2+} ions (45). For example, the C2 domain of mammalian synaptotagmin I, a phospholipid-binding protein of synaptic vesicles, has been shown to bind different anionic phospholipids and this requires the presence of Ca^{2+} (22,46). In plants, it was recently demonstrated that the C2 domain of a PLC from rice (see

phylogram, GI 32974942) is responsible for targeting the enzyme to the plasma-membrane in response to Ca^{2+} (23). Running domain prediction at the Pfam database identifies a structurally conserved EF hand-like domain at the N-terminus of all *Sl*PLCs (Fig. S1*C*). EF hand-like domains have coordination properties for Ca^{2+} ions similar to classical EF hand domains, but differ in that they either contain deviations in the secondary structure of the flanking sequences and/or variation in the length of the Ca^{2+}-coordinating loop (http://pfam.sanger.ac.uk/family/PF00036). Similar domains were found in mammalian PLCδ isoforms and were shown to be important for enzyme activity and Ca^{2+} binding (45,47). Interestingly, a relatively short coiled coil (CC) domain is now predicted in the linker region between the X and Y domains (X/Y-linker) of *Sl*PLC2, which distinguishes *Sl*PLC2 from the other tomato PLC isoforms.

Analysis and Prediction of the Activation Mechanism of Tomato PLC Enzymes – Little is known about the molecular mechanisms involved in the activation of plant PLC enzymes. The activity of specific mammalian PLC enzymes was found to be regulated by G-proteins (48), Ras-like GTPases (49) or tyrosine kinases (44,50,51). Interestingly, recent analysis of the crystal structure of human PLC-β2 showed that the X/Y-linker region acts as an auto-inhibitory module which blocks the catalytic core, thereby inhibiting undesired contact with membrane phospholipid substrates in the resting state (27). Furthermore, the authors provided experimental evidence supporting this auto-inhibition hypothesis for two other mammalian PLC enzymes. It was suggested that the auto-inhibition mechanism is released by electrostatic repulsion of the X/Y-linker, which is enriched with dense clusters of negatively charged amino acid residues, when the PLC enzyme molecule approaches the negatively charged membrane. Therefore, we examined the X/Y-linker region of the various *Sl*PLC proteins to determine whether such a mechanism may also be regulating their activity. Alignment of the X/Y-linker regions of tomato *Sl*PLC1 to *Sl*PLC7 (Fig. 1*A*) indeed shows enrichment with clusters of the negatively charged amino acid residues aspartic acid (D) and glutamic acid (E), compared to their overall distribution along the full-length protein sequence (Fig. 1*B*). This enrichment was relatively the highest in *Sl*PLC2 and the lowest in *Sl*PLC1. This suggests that the hydrolytic activity of *Sl*PLC proteins may be controlled by auto-inhibition mechanisms similar to those observed in human PLCs. The implication of PLC activation by releasing auto-inhibition is that cellular signaling and other cellular processes are required to bring the PLC molecules to the vicinity of the membrane.

Tyrosine and serine phosphorylation of mammalian PLCs has been previously reported (50,52-54). In these cases, phosphorylation occurred within the X/Y-linker. Furthermore, *Arabidopsis At*PLC2 was found to be phosphorylated at a serine residue, within seven minutes after treatment of a cell suspension with flagellin (28). Also here, the phosphorylated serine was located within the X/Y-linker region. Therefore, we predicted the number of phosphorylation sites in *Sl*PLC1 to *Sl*PLC7 using the NetPhos 2.0 server and examined their distribution along the full-length protein. We found a significantly higher density of predicted phosphorylation sites in the X/Y-linker region, when compared to the full-length protein (Fig. 1*A* and 1*C*). This density was the highest in *Sl*PLC1 and *Sl*PLC4 and was the lowest in *Sl*PLC6. The relatively high density of predicted phosphorylation sites in the X/Y-linker regions indicates that phosphorylation of *Sl*PLC proteins may indeed play a

role in their activation, either directly or by allowing interaction with other proteins when their phosphorylation status is altered. Alternatively, phosphorylation is also expected to increase the total negative charge and thereby the repulsion efficiency of the X/Y-linker. This will liberate the catalytic core or will enforce an active confirmation, allowing interaction with the phospholipid substrate in cellular membranes.

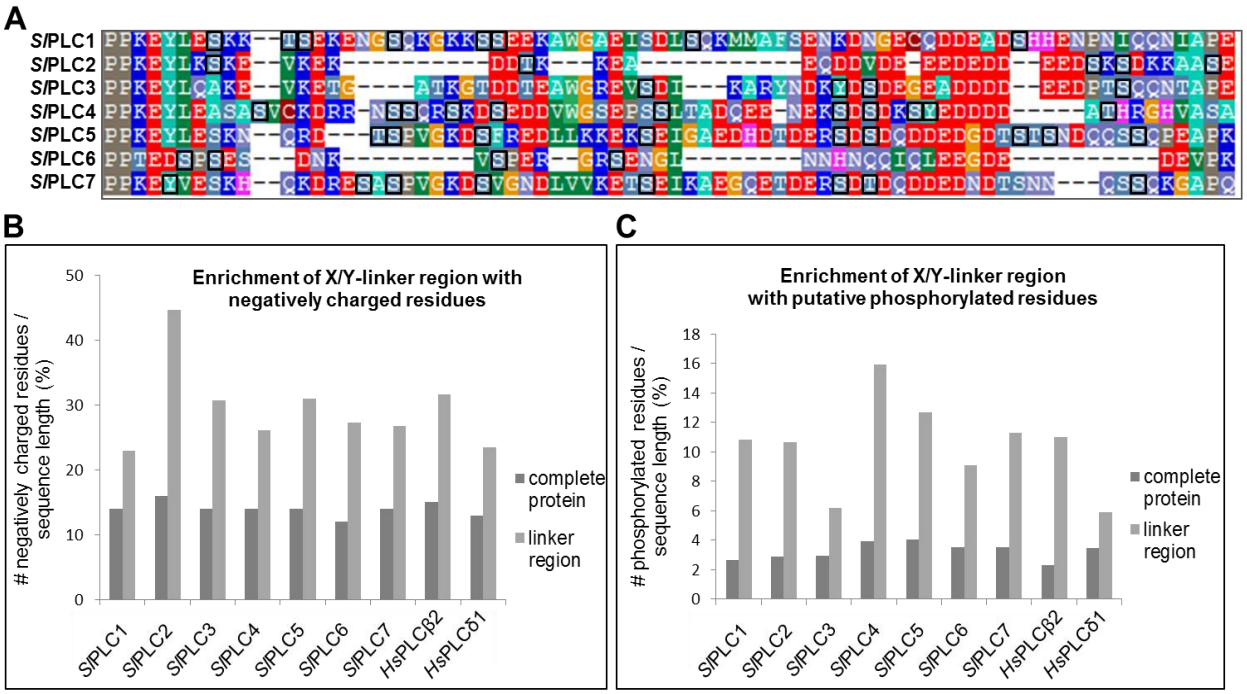

FIGURE 1. **Enrichment of the X/Y-linker region of tomato PLC with negative charges and phosphorylation sites.** *A,* section of a multiple sequence alignment of the *Sl*PLC family of tomato, showing only the linker region between the catalytic X and Y domains of the *Sl*PLCs (see Fig. 1). Negatively charged aspartic acid (D) and glutamic acid (E) residues are highlighted in red. *B,* enrichment of the X/Y-linker region with negatively charged residues. The relative occurrence of the amino acid residues D and E, as determined by their total amount divided by the sequence length, in the full-length sequence of the *Sl*PLC proteins (dark grey columns) or only in its X/Y-linker sequence (light grey columns) is shown. *C,* enrichment of X/Y-linker region with putative phosphorylated residues. Dark grey columns represent the ratio between the length of the sequence of the X/Y-linker and the length of the corresponding full-length *Sl*PLC, while light grey columns represent the ratio between the number of predicted phosphorylation sites in the X/Y-linker region and the full sequence length of the corresponding *Sl*PLC protein. The predicted phosphorylated residues are surrounded with boxes in Fig. 2A. The NetPhos 2.0 server was used for prediction of the phosphorylation sites, and a score between 0.5 and 1.0 was regarded as significant. Note that 0.5 is the threshold and a score between 0.5 and 1.0 reflects confidence of the prediction and the higher the similarity to one or more of the phosphorylation sites used in training the method.

Production and Initial Characterization of Recombinant Tomato PLC Enzymes – Previously, enzymatically active versions of *Sl*PLC4 and *Sl*PLC6 were produced by tagging them with GST through insertion of their coding sequences into the pGEX-KG plasmid and expressing them in *Escherichia coli* (12). Here, we set out to also produce all other *Sl*PLC isoforms and investigate whether they also encode catalytically active PLC enzymes. The induction of protein expression, followed by affinity purification was successful for all *Sl*PLCs, with the exception that *Sl*PLC7 did not accumulate, possibly due to its instability in *E. coli* (Fig. 2*A*). The migration of the observed bands representing the different GST-tagged *Sl*PLC proteins matched their calculated molecular weights (Fig. 2*A*).

Studies on the substrate specificity and activity requirements of plant PLCs allow us to understand the specific conditions under which the enzymes are active. This information is crucial to link the changes occurring in the plant cells, after stimulation by perception of a MAMP or an effector of a pathogen, with the specific role of PLC enzymes in the defense response against invading microbes. Previously, we used a non-radioactive phospholipase C assay to show that tomato *Sl*PLC4 and *Sl*PLC6 are catalytically active PLC enzymes, as they are able to hydrolyze PI, resulting in the accumulation of DAG (12). However, we could not show hydrolysis of PIP_2 which is considered to be the primary substrate for PI-PLC enzymes (12). This failure to hydrolyze PIP_2 was irrespective of the acyl chain length of the substrate, as we tested different PIP_2 preparations with different lengths, but no hydrolysis occurred (results not shown). The PI substrate that was successfully hydrolyzed originated from a natural source, while the PIP_2 preparation that we tested was synthetic. As the reaction conditions that we applied were very similar to those reported in other studies showing the hydrolysis of ^{32}P-labeled natural PIP_2, and the generation of $InsP_3$ by plant PLC enzymes (55,56), we concluded that *Sl*PLC4 and *Sl*PLC6 might not be able to hydrolyze PIP_2. Here, we introduced non-synthetic PIP_2, from porcine brain, as a PI-PLC substrate and tested this on a preparation of the *Sl*PLC4 enzyme. We now observed a time-dependent hydrolysis of the substrate, as visualized by a decrease in the amount of PIP_2, in combination with generation of the reaction product DAG (Fig. 2*B*). The hydrolysis of PIP_2 was also more efficient than previously observed for PI (12), as PIP_2 was already nearly depleted after 60 min of incubation with the *Sl*PLC4 enzyme which indicates that PIP_2 is the preferred substrate, compared with PI. Furthermore, PIP_2 hydrolysis occurred here at a 1,000 times lower concentration of Ca^{2+} than was used in the experiments involving the hydrolysis of PI (10 µM versus 10 mM, respectively). In addition to *Sl*PLC4, also recombinant *Sl*PLC2 and *Sl*PLC5 proteins displayed catalytic activity (using natural PIP_2 as a substrate), which was retained for several days after their purification (results not shown). In contrast, *Sl*PLC1, *Sl*PLC3 and *Sl*PLC6 displayed variable degrees of phospholipase activity and the activity was often lost shortly after their purification. Therefore, we set out to test the activity requirements and substrate specificity of *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 and in some cases we included the other *Sl*PLCs. In addition to showing the highest enzyme stability, *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 each represent a separate clade in the phylogenetic tree of the plant PLCs (Fig. S1*B*), making these enzymes interesting candidates for further characterization.

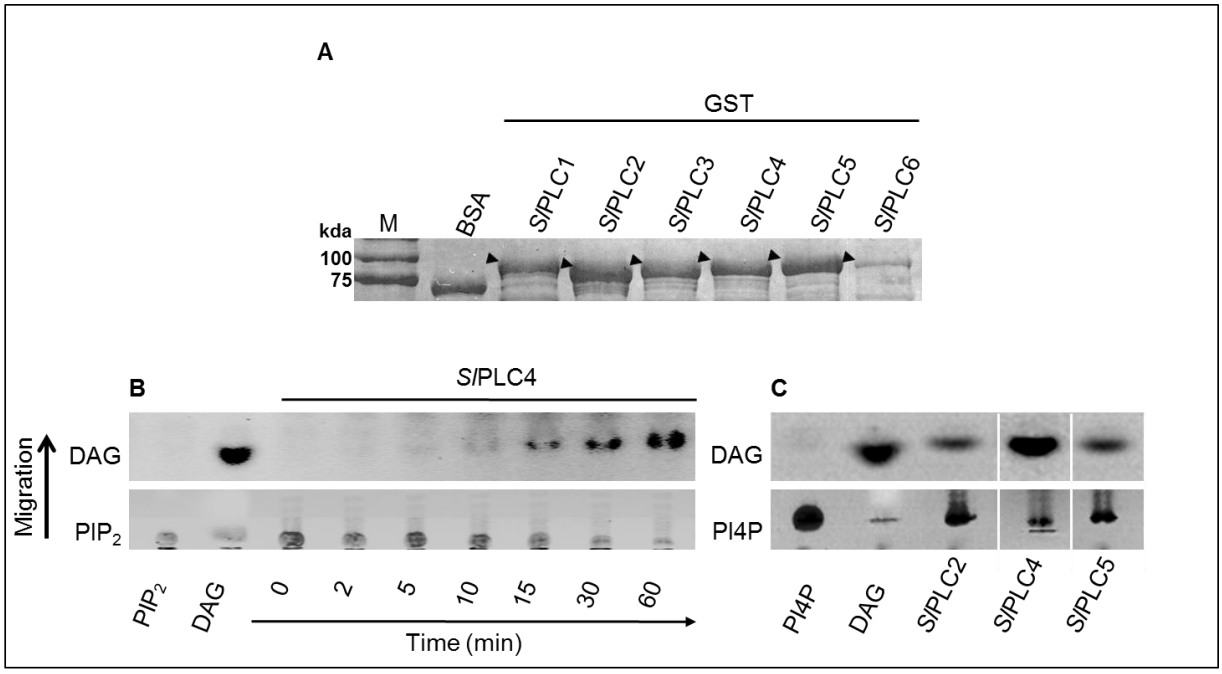

FIGURE 2. **Heterologous expression and affinity purification of tomato PLC proteins and hydrolysis of PIP_2 and PI4P.** *A,* affinity purification of six tomato PI-PLCs (*Sl*PLC1 to *Sl*PLC6), each fused to glutathione S-transferase (GST). Tagged versions of the proteins were expressed in *Escherichia coli* and recombinant proteins were affinity-purified as described by Vossen et al. (2010) (12). 5µg of each of the *Sl*PLC proteins was analyzed by SDS-PAGE, next to 2.5µg of BSA as a reference and stained with Coomassie Brilliant Blue (CBB). Arrowheads indicate the position of the purified GST-*Sl*PLC fusion proteins. The calculated molecular weights of GST-*Sl*PLC1 to GST-*Sl*PLC6 are 95.5, 91.0, 94.3, 93.6, 95.0 and 92.0 kDa, respectively. *B,* recombinant tomato *Sl*PLC4 hydrolyses phosphatidylinositol (4,5)-bisphosphate (PIP_2) in a time-dependent manner**.** The *in vitro* phospholipase-C assay was carried out using affinity-purified recombinant *Sl*PLC4 (GST-*Sl*PLC4). For each time point, 10 µg of PIP_2 (as micellar-lipid solution) were incubated with the enzyme. Reactions were carried out using 10 µM Ca^{2+}, at pH 5.0. Reaction products were extracted and analyzed by thin layer chromatography (TLC) as described under "Experimental Procedures". Note the simultaneous decrease in the amount of the substrate, PIP_2, and increase in the amount of the reaction product, 1,2-diacylglycerol (DAG) over time. The second product of the phospholipase reaction, inositol 1,4,5-triphosphate ($InsP_3$), is lost in the water phase during the lipid extraction process. The first lane (from the left) was loaded with 10 µg of PIP_2 and the second lane with 30 µg of DAG, to serve as migration references. *C,* hydrolysis of phosphatidylinositol-4-phosphate (PI4P) by recombinant tomato *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 at the same reaction conditions used for PIP_2 hydrolysis, as described under "Experimental Procedures". For each reaction, 10 µg of PI4P, provided as micellar-lipid solution, were incubated for 15 minutes with each recombinant enzyme, after which reaction products were extracted and analyzed as mentioned above. Note the decrease in the amount of the substrate, PI4P, which correlates with the increase in the amount of the reaction product, 1,2-diacylglycerol (DAG). The second product of the phospholipase reaction, inositol 1,4-diphosphate (IP_2), is lost during extraction. The first lane (from the left) was

loaded with 10 µg PI4P and the second lane with 30 µg of DAG, to serve as migration references.

Biochemical Characterization of Recombinant Tomato PLC Enzymes – We found that the three enzymes, *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5, were all able to hydrolyze natural PI4P (Fig. 2*C*), when tested under the same reaction conditions as used for PIP_2 hydrolysis by *Sl*PLC4. *Sl*PLC3 was also able to hydrolyze both PI4P and PIP_2 (Fig. S2*A* and S2*B*, respectively), while *Sl*PLC1 was active when tested using PIP_2 as a substrate (Fig. S3). *Sl*PLC1 and *Sl*PLC3 are also able to hydrolyze PI (Fig. S4 and S5). Several studies have reported the importance of the divalent cations Mg^{2+} and Ca^{2+} for the activity of both mammalian and plant PLC enzymes (35,55,57-60). Accordingly, Mg^{2+} was required for PLC enzyme activity in plant microsomal fractions (35,57,58,61). However, Mg^{2+} was shown to be dispensable for the *in vitro* activity of heterologously-expressed *St*PLC1, *St*PLC2 and *St*PLC3 of potato (55), which are very close orthologs of *Sl*PLC1, *Sl*PLC2 and *Sl*PLC3, respectively (Fig. S1*b*). We here tested the *in vitro* requirement for Mg^{2+} for the activity of two recombinant *Sl*PLC enzymes, *Sl*PLC3 and *Sl*PLC4 using a concentration range of Mg^{2+} of up to 1000 µM but we observed no effect on their activity, as in all cases a similar activity was observed (Fig. S6). We proceeded with testing the requirement for Ca^{2+} and determining the optimum Ca^{2+} concentration needed for the activity of *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5, using either PI or PIP_2 as substrates. Ca^{2+} ions were provided in the reaction mixture either as $CaCl_2$ alone, in case PI was used as a substrate, or combined with EGTA to buffer for lower Ca^{2+} concentrations when PIP_2 was the substrate (see "Experimental Procedures"). All three enzymes were able to hydrolyze PI with an optimum activity at a Ca^{2+} concentration of around 1000 µM (Fig. 3*A*). At a Ca^{2+} concentration of 10 mM, *Sl*PLC2 and *Sl*PLC4 retained efficient hydrolysis of PI, while the activity of *Sl*PLC5 was strongly suppressed. In fact, *Sl*PLC5 activity was only detected at a narrow range of Ca^{2+} concentrations, which was between 100 µM and 1000 µM (Fig. 3*A*). On the other hand, efficient PIP_2 hydrolysis was observed at a much wider Ca^{2+} concentration range for all three enzymes and occurred between 50 and 1000 µM (Fig. 3*B*). In some cases, substrate hydrolysis was even observed without addition of Ca^{2+}, as the accumulation of DAG was for example clearly detected upon incubation of PI with *Sl*PLC4, and PIP_2 with *Sl*PLC2. This indicates that these two enzyme preparations probably retained enough Ca^{2+} ions bound within the enzyme to catalyze substrate hydrolysis. It also suggests a specific affinity of *Sl*PLC2 and *Sl*PLC4 for different types of phospholipid substrates, in the absence of added Ca^{2+}.

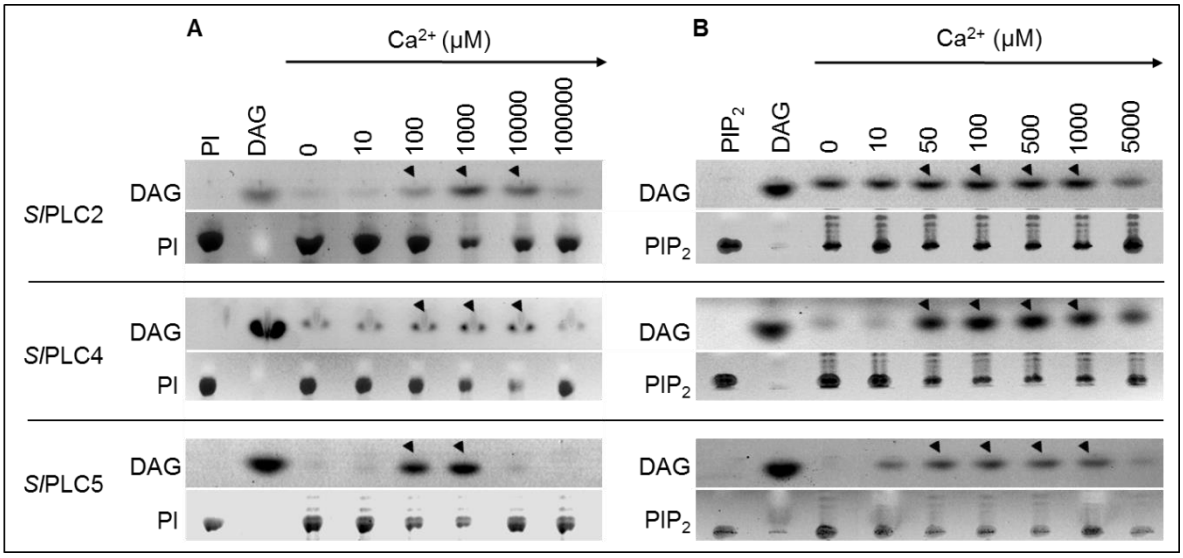

FIGURE 3. **Ca^{2+}-dependent hydrolysis of phosphatidylinositol (PI) and phosphatidylinositol (4,5)-bisphosphate (PIP_2) by tomato PLC2, PLC4 and PLC5 enzymes.** *A, in vitro* phospholipase activity of affinity-purified recombinant *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 was tested at the indicated Ca^{2+} concentrations. For each reaction, 30 µg of PI preparation (as micellar-lipid solution) were used as a substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol-phosphate (IP) when hydrolysis takes place. Depletion of the substrate, PI, (lower panels) and accumulation of one of the reaction products, DAG, (upper panels) were monitored by extraction of the reaction products followed by thin layer chromatography (TLC) analysis, after 60 minutes of incubation. The first two lanes (from the left) were loaded with 30 µg of PI and 30 µg of DAG and serve as migration references. The second product of the reaction, inositol phosphate (IP), was lost during the lipid extraction process. Arrowheads indicate the accumulation of DAG. *B*, PLC activity assay using PIP_2 as a substrate at the indicated Ca^{2+} concentrations. For each reaction, 10 µg of PIP_2 (as micellar-lipid solution) were used which is converted into DAG and inositol 1,4,5-triphosphate ($InsP_3$) upon its hydrolysis. Reaction products were extracted and analyzed by TLC after 30 minutes of incubation, as described under "Experimental Procedures". The first two lanes (from the left) were loaded with 10 µg of PIP_2 and 30 µg of DAG and serve as migration references. The second product of the reaction, $InsP_3$, was lost during the lipid extraction process. Arrowheads indicate the accumulation of DAG.

We also tested the effect of the pH on the hydrolytic activity of *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 enzymes on PI and PIP_2 as substrates (Fig. 4*A* and Fig. 4*B* respectively). We found that *Sl*PLC2 hydrolyzed PI at pH values of 5 to 9, having an optimum activity at pH 6, whereas optimal hydrolysis of PIP_2 by *Sl*PLC2 occurred at pH 6 to 8 and *Sl*PLC2 activity decreased only slightly at pH 9. *Sl*PLC4 hydrolyzed PI at all tested pH values, pH 4 to 9, but with an optimum activity at pH 5. Interestingly, the pH optimum for hydrolysis of PIP_2 by *Sl*PLC4 appeared more or less inversed compared to the pH optimum for PI, as PIP_2 was hydrolyzed efficiently at pH 5 to 9, with a peak activity at pH 8 and slowest hydrolysis at pH 5. This suggests that the substrate specificity of *Sl*PLC4 might be controlled by the pH of the reaction environment. *Sl*PLC5 hydrolyzed PI at a pH of 5 to 9, with optimal hydrolysis occurring at

pH 6, while it hydrolyzed PIP_2 at a relatively narrow pH range of 6 to 8, with maximum activity at pH 7.

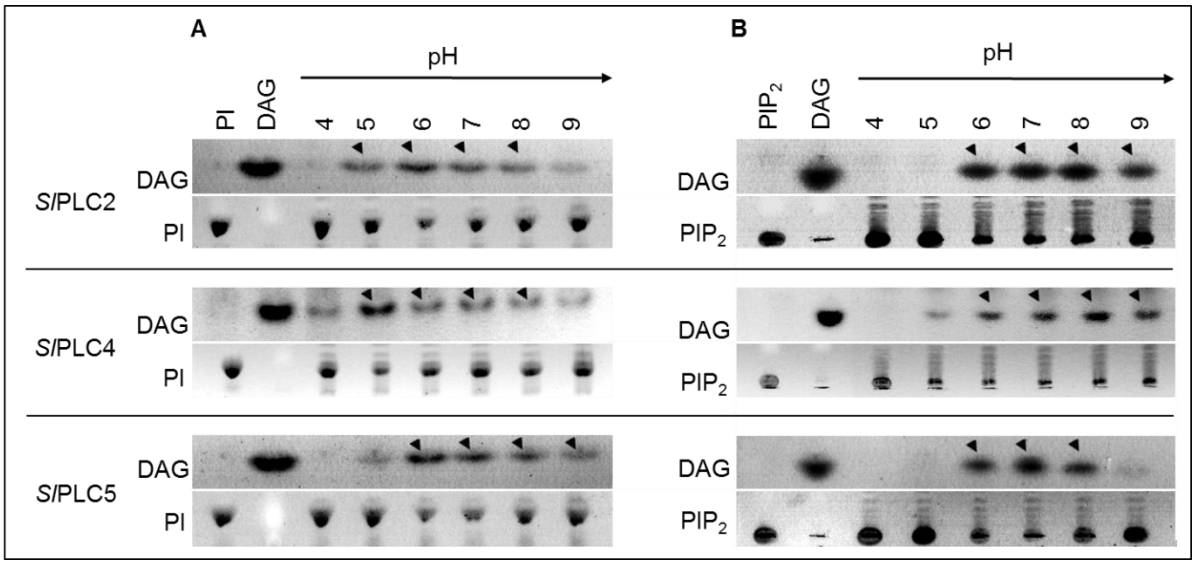

FIGURE 4. **pH-dependent hydrolysis of phosphatidylinositol (PI) and phosphatidylinositol (4,5)-bisphosphate (PIP_2) by tomato PLC2, PLC4 and PLC5.** *A, in vitro* phospholipase activity of affinity-purified recombinant *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 was tested at the indicated pH values. For each reaction, 30 µg of PI preparation (as micellar-lipid solution) were used as a substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol-phosphate (IP) when hydrolyzed. Depletion of the substrate, PI, (lower panels) and accumulation of one of the reaction products, DAG, (upper panels) were monitored by extraction of the reaction products, followed by thin layer chromatography (TLC) analysis, after 60 minutes of incubation. The first two lanes (from the left) were loaded with 30 µg of PI and 30 µg of DAG and serve as migration references. The second product of the reaction was inositol phosphate (IP) and was lost during the lipid extraction process. Arrowheads indicate the accumulation of DAG. *B,* PLC activity assay using PIP_2 as a substrate at the indicated pH values. For each reaction, 10 µg of PIP_2 (as micellar-lipid solution) were used, which is converted into DAG and inositol 1,4,5-triphosphate ($InsP_3$) when hydrolyzed. Reaction products were extracted and analyzed by TLC after 5 minutes of incubation as described under "Experimental Procedures". The first two lanes (from the left) were loaded with 10 µg of PIP_2 and 30 µg of DAG and serve as migration references. The second product of the reactions was $InsP_3$ and was lost during the lipid extraction process. Arrowheads indicate the accumulation of DAG.

Inhibition of In Vitro Activity of Recombinant Tomato PLC by the PLC Inhibitor U73122 – Pharmacological approaches are often used to manipulate PLC activity and thereby study the role of these enzymes in several cellular processes and physiological responses. Although recently conflicting data appeared (62), the chemical compound U73122 is well established as an effective inhibitor of PLC activity and its specific inhibitory effect is often compared to that of the structural analog U73343, which is considered not to inhibit PLC activity (56,63,64). U73122 was previously shown to significantly inhibit cellular processes mediated

by PLC-activity in mammals (63,65,66) and in plants (56,67,68), in addition to the inhibition of the *in vitro* activity of recombinant human PLC (64,66) and a *Nicotiana rustica* PLC (67) in cell-free systems. We set out to verify whether U73122 is able to suppress the *in vitro* activity of *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5. For this, equal amounts of the recombinant PLC enzymes were pre-incubated for 5 min with 10, 50 or 100 µM of either the inhibitor U73122 or its structural analog U73343, as a control. Subsequently, we analyzed *Sl*PLC activity using PI as a substrate. We observed significant inhibition of *Sl*PLC activity due to the incubation with U73122, which was evident by PI remaining present at the original levels and the absence of the reaction product, DAG (Fig. 5). Interestingly, the three *Sl*PLC enzymes that were tested showed differential sensitivity towards the inhibitor, despite the fact that they all catalyze the same reaction. Accordingly, the activities of *Sl*PLC2 and *Sl*PLC4 were hardly affected at a concentration of 10 µM of U73122, while *Sl*PLC5 activity was already significantly suppressed at that concentration (Fig. 5). At a concentration of 50 µM of the inhibitor, the hydrolytic activities of all three *Sl*PLC enzymes were suppressed to levels approaching 100%. Indeed, the inhibitory effect of U73122 proved to be specific, as incubation with the inactive analog, U73343, only caused some minor suppression of PLC activity at 50 µM and 100µM upon incubation with *Sl*PLC2 and *Sl*PLC4. *Sl*PLC5 appeared to be completely insensitive to incubation with U73343 (Fig. 5).

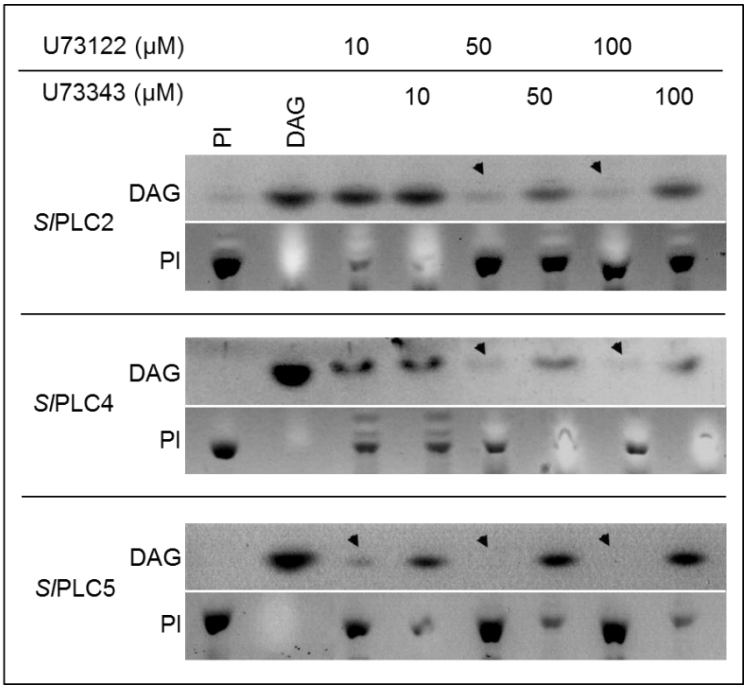

FIGURE 5. **Inhibition of the phospholipase activity of tomato PLC2, PLC4 and PLC5 by the PLC inhibitor U73122, and not by its close structural analog U73343.** *In vitro* phospholipase activity of affinity-purified *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 was assessed after pre-incubation of the enzymes with the indicated concentrations of either the PLC inhibitor U73122, or its inactive structural analog, U73343. For each reaction, 30 µg of phosphatidylinositol (PI) preparation (as micellar-lipid solution) were used as a substrate, which is hydrolyzed into the reaction products 1,2-diacylglycerol (DAG) and inositol-

phosphate (IP). The depletion of the substrate (PI, lower panels) and production of one of the reaction products (DAG, upper panels) was monitored by TLC analysis after 60 minutes of incubation, as described under "Experimental Procedures". The second product of the phospholipase reaction, inositol phosphate (IP), is lost during the lipid extraction process. Arrowheads indicate strong reduction, or even complete prevention of DAG formation, indicative of an inhibition of PLC activity upon incubation with U73122. Note that U73343 does not, or only partly, inhibit PLC activity. The first two lanes (from the left) were used as migration references for PI and DAG.

Inhibition of ETI and PTI by the PLC Inhibitor U73122 – After having established the specific inhibitory effect of U73122 on the activity of the three different *Sl*PLC enzymes, *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5, we examined the effect of the inhibitor on defense activation *in vivo*. As mentioned earlier, PLC enzymes have been shown to be required for mounting defense reactions in plants (12-15,17,18) and we first tested the effect of the inhibitor on ETI, which is immunity triggered by specific effectors of pathogens that are perceived by matching resistance proteins. For this, we used a transgenic tobacco cell-suspension expressing the tomato Cf-4 protein, providing resistance to Avr4-expressing strains of the fungal pathogen *C. fulvum* (13). In this system, ETI is induced upon addition of pure Avr4 protein to the cell suspension. Addition of Avr4 induces a swift alkalization of the cell-suspension medium, which is considered to be an early read-out of defense activation by plant cells (69,70). Before the addition of Avr4, cell suspensions were pre-incubated with either U73122 or U73343, or a similar volume of their solvent (DMSO) as a control, for 10 min. After the addition of Avr4, the pH gradually increased after a lag-phase of about 6 min in both U73343- and DMSO-treated cell suspensions, whereas in the ones pre-incubated with U73122 this rise in pH did not occur (Fig. 6*A*). This indicates that U73122 treatment completely blocks Avr4-triggered defense activation in the Cf-4 tobacco suspension cells. These results prompted us to study whether PTI, which is activated upon perception of conserved structural microbial compounds, is also suppressed by the PLC inhibitor. Accordingly, we used a similar setup and instead of Avr4 we added the PAMPs flg22, a peptide derived from bacterial flagellin (71), or the oligosaccharide elicitor chitohexaose, which is derived from chitin that forms a structural component of fungal cell walls (72). Both molecules were shown before to induce a medium alkalization response in plant cell suspensions (71-73). Similarly, we observed that tobacco cells recognize these molecules and activate PTI, leading to an increase in the pH of the medium. Again, the pH increase after addition of flg22 or chitin was fully suppressed when the cells had been pre-treated with U73122, indicating that in addition to ETI also the PTI response was suppressed (Fig. 6*B* and 6*C*). The structural analog U73343 also weakly suppressed the pH increase after the addition of Avr4 or chitin (Fig. 6*A* and 6*C*). However, the suppression effect after treatment with the PLC inhibitor U73122 was much more pronounced. Earlier, we showed that *Sl*PLC proteins are required for the activation of defense responses (12). The results obtained here are in accordance with these earlier findings and match the observed differential effects of the inhibitory compound and its non-functional analogue on *in vitro Sl*PLC activity. Therefore, we conclude that the PLC inhibitor U73122 suppresses defense activation related to both ETI

and PTI and that this suppression is caused by inhibition of the hydrolytic activity of the different PLCs.

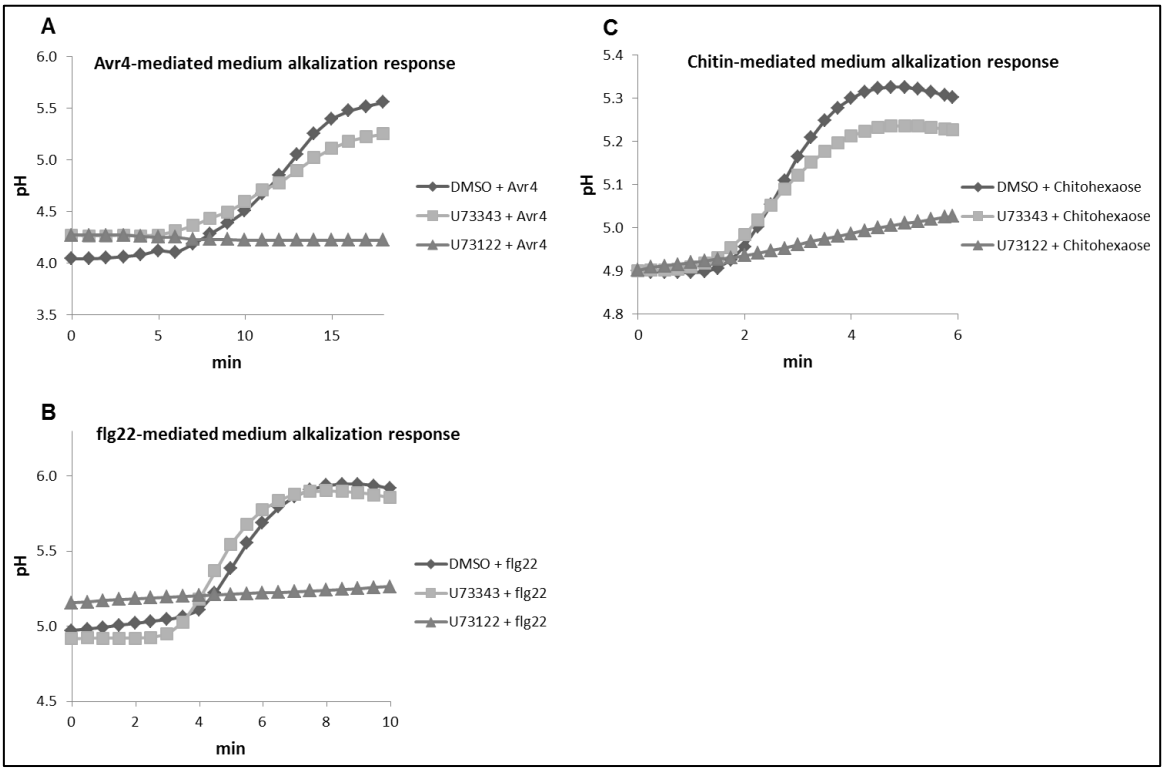

FIGURE 6. **Suppression of early defense activation, in relation to both effector- and PAMP-triggered immunity (ETI and PTI), by the PLC inhibitor U73122.** Early defense activation, due to recognition of the microbial effector Avr4, or the PAMPs flg22 and chitin, induces medium alkalization of a cell suspension. *A*, tobacco cells expressing Cf-4 were pre-incubated with the PLC inhibitor U73122, its inactive analog U73343 or their solvent, DMSO. After 10 min, Avr4 was added (to a final concentration of 10 µg/ml) and the pH of the medium was monitored over a period of 20 min. *B*, tobacco cells were pre-incubated with the PLC inhibitor U73122, its inactive analog U73343 or their solvent, DMSO. After 10 min, the PAMP flg22 was added (to a final concentration of 1 nM) and the pH of the medium was monitored over a period of 10 min. *C*, tobacco cells were pre-incubated with the PLC inhibitor U73122, its inactive analog U73343 or their solvent, DMSO. After 10 min, chitohexaose was added (to a final concentration of 1 µM) and the pH of the medium was monitored over a period of 10 min. Note that in all cases the increase of the pH was specifically suppressed upon pre-treatment with the PLC inhibitor U73122, both in case of the ETI and the PTI response. Data represent one representative experiment out of two repetitions.

Transient Overexpression of Tomato PLCs in N. benthamiana Enhances the Avr4-triggered Hypersensitive Response – Based on the current results and previous findings (12), we hypothesized that particular *Sl*PLC isoforms with variable roles in the activation of defense against pathogens may exist. Accordingly, we used agro-infiltration to transiently co-express each individual *SlPLC* gene and Avr4 in transgenic *N. benthamiana* plants expressing

the Cf-4 immune receptor (12). Expression of Avr4 in these plants induces a hypersensitive response (HR) in the form of local cell death at the infiltrated area. However, the induction of the HR is highly dependent on the optical density (OD_{600}) of the *A. tumefaciens* suspension delivering *Avr4*. In our co-expression experiments we used an OD_{600} near the threshold that is required to induce an HR. This allowed us to determine whether the HR is enhanced due to the co-expression of Avr4 with an individual *SlPLC* gene, as compared to co-expression with the *GUS* gene as a control. Out of the seven tested *SlPLC* genes, *SlPLC1* to *SlPLC7*, we found that co-expression with *SlPLC3* and, to a lesser extent with *SlPLC1*, clearly enhanced the Cf-4/Avr4-triggered HR (Fig. 7). In this assay co-expression of the other tomato *PLCs*, including *SlPLC4* (12), did not enhance the HR. Altogether, our results strongly support the requirement of PLC activity for the early activation of plant defense and the induction of the HR. This is in line with earlier studies showing rapid accumulation of phosphatidic acid (PA) via the PLC/DGK pathway in response to perception of Avr4 by Cf-4 (13) and confirm the role of PLCs in disease resistance (12).

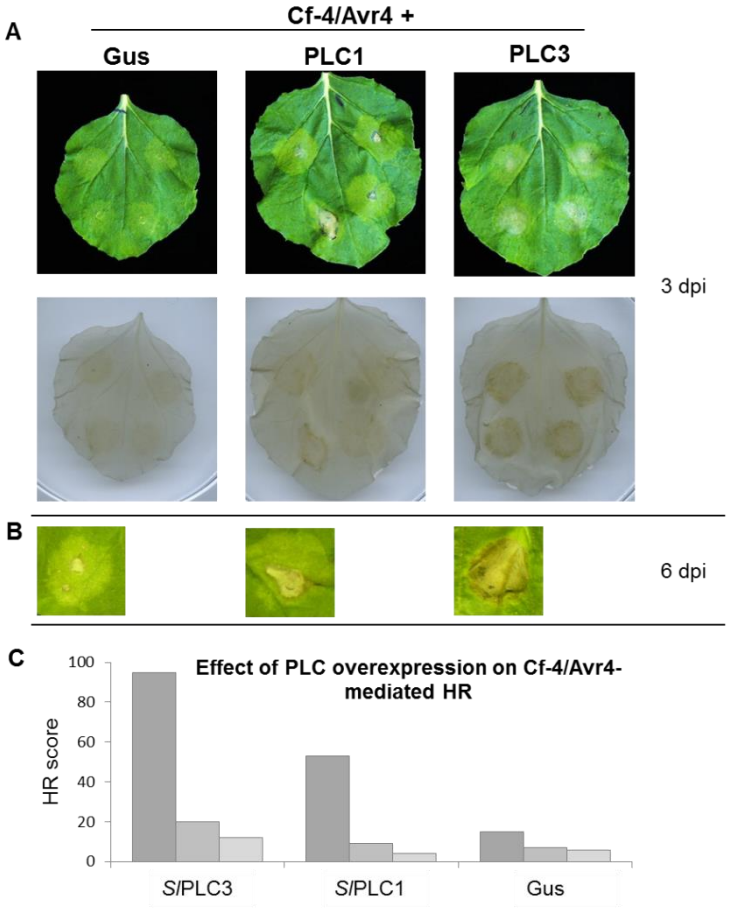

FIGURE 7. **Effect of *Sl*PLC over-expression on Cf-4/Avr4-triggered HR.** Transgenic *Nicotiana benthamiana* plants expressing the *Cf-4* resistance gene were agro-infiltrated to transiently express Avr4 at a final $O.D._{600}$ of 0.03, together with either GUS or *Sl*PLC1 or *Sl*PLC3, at a final $O.D._{600}$ of 1.0. *A*, (upper panels) a hypersensitive response (HR) was observed at 3 days post infiltration when either *Sl*PLC1 or *Sl*PLC3 was co-expressed with Avr4. The HR was not apparent when GUS was co-expressed with Avr4. After de-staining in ethanol, brownish dead cells are visible in the leaves in which *Sl*PLC3, and to a lesser extent,

*Sl*PLC1 was co-expressed, whereas cell death was not visible in the leaves in which GUS was co-expressed (lower panels). *B,* The HR becomes more pronounced at 6 days post infiltration and the differences become more apparent. *C,* quantitation of the effect of over-expressing *Sl*PLC1 and *Sl*PLC3 on the induction of the Cf-4/Avr4-triggered HR. The HR was scored in three independent experiments, each represented by one of the three columns in each treatment. HR was visually scored according to intensity on a scale of 0 to 5.

Suppression of flg22-triggered Internalization of the FLS2 Receptor by the PLC Inhibitor U73122 – The suppression of flg22-triggered medium alkalization by the PLC inhibitor U73122 prompted us to investigate whether perception of flg22 is altered. The suppression was very effective, even at a higher concentration of flg22 (Fig. S7). Flg22 is perceived by the cell surface receptor Flagellin Sensing 2 (FLS2) (74,75) and this perception involves binding of flg22 to the receptor (75) and subsequent internalization of FLS2 via endocytosis (76). Phosphorylation and ubiquitination of FLS2 (76,77), in addition to the recruitment of the regulatory co-receptor BRI1-Associated Kinase (BAK1)/Somatic Embryogenesis Related Kinase 3 (SERK3) (78), were shown to be crucial for the internalization of FLS2 and activation of downstream defense-responses. Therefore, we here used the internalization of FLS2 as a read-out to study the effect of the PLC inhibitor U73122 on FLS-mediated defense activation. Accordingly, cotyledons of transgenic *Arabidopsis* Col-0 expressing FLS2-GFP (79) were pre-incubated in water containing either U73122, U73343 (both at a concentration of 20 uM) or an equal volume of their solvent (DMSO) as a control, for 60 min, and then treated with 10 uM of flg22 after which confocal micrographs were acquired by high throughput imaging and computational detection of FLS2-GFP endosomes. The number of FLS2-containing endosomes was similar in both control and U73343 treatments, while their number significantly decreased when plant material was treated with the PLC inhibitor U73122 (Fig. 8). These results show that the previously observed suppression of flg22-mediated medium alkalization coincides with an impairment of FLS2-internalization.

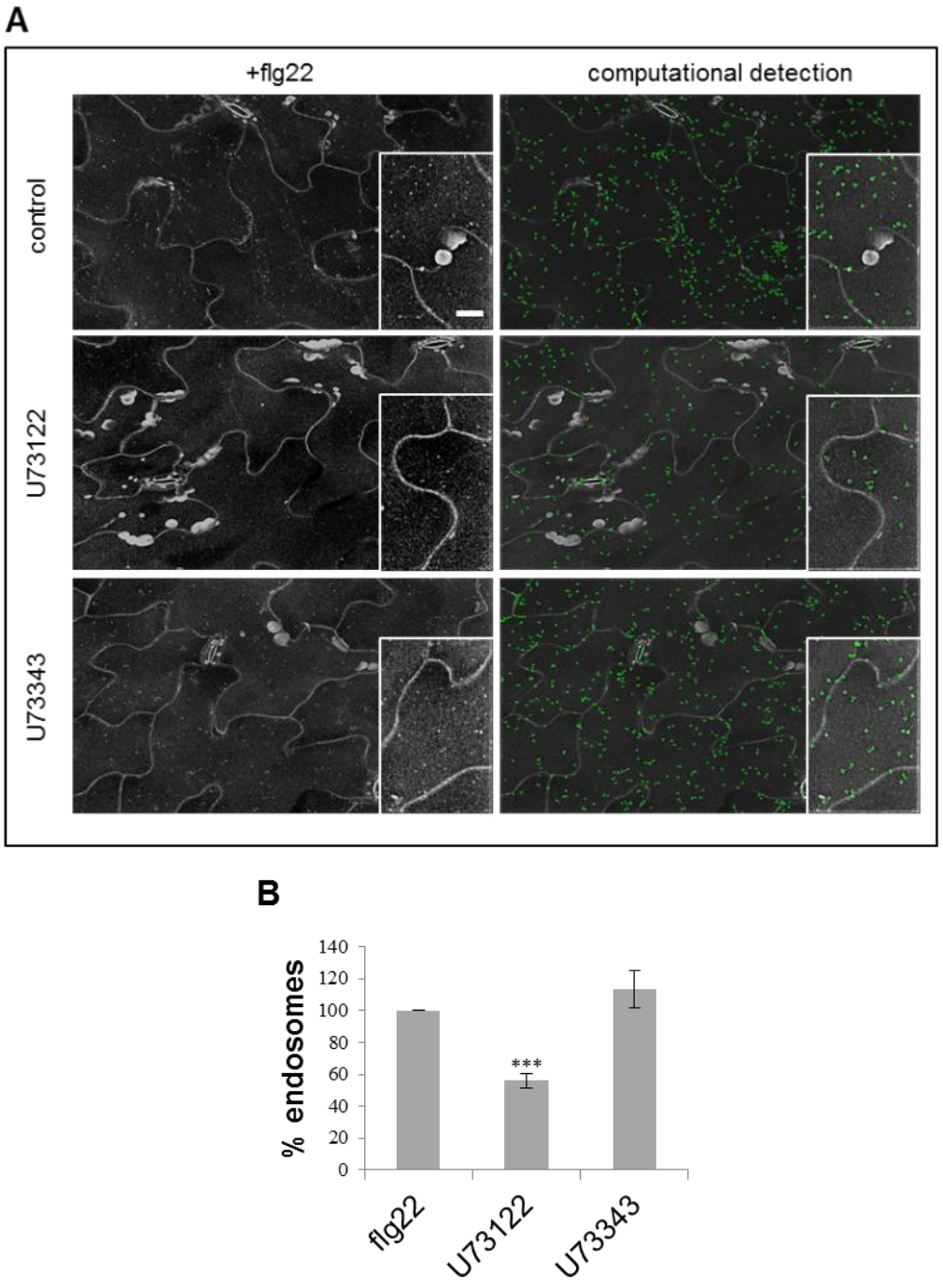

FIGURE 8. **Impaired flg22-induced endocytosis of FLS2 by the PLC-inhibitor U73122**. *A,* left panel, confocal micrographs of transgenic *Arabidopsis* lines expressing FLS2-GFP show merged z-stacks of cross-sections of cotyledon epidermis treated with flg22 after incubation with the PLC inhibitor U73122, its structural analog U73343 or their solvent (DMSO). Right panel, computational spot detection by EndomembraneQuantifier. Inset pictures (white boxes) show detailed details of FLS2-GFP endosome detection; bar = 15 µm. *B,* quantification of FLS2-GFP endosomal numbers per image area in presence of flg22; values are mean ± SD. Means are produced from multiple images of DMSO-treated + flg22

(*n* = 129), U73122-treated + flg22 (*n* = 90) and U73343-treated + flg22 (*n* = 122) cotyledons. Asterisks indicate statistical significance of p-value ≤ 0.001 based on student t-test analysis.

Discussion

Although different roles were established for mammalian PLC enzymes in receptor-mediated cellular signaling, understanding how PLC contributes to stress signaling in plants is still in its infancy. The main domain architecture required for PLC activity is conserved in both plants and mammals. However, additional specialized domains are common only in mammalian PLCs (21). This observation and the absence of specific PLC-related signaling components in plants, like PKC, $InsP_3$ receptors and multiple G protein-encoding genes, hamper knowledge transfer of PLC signaling from mammalian to plant systems. Recently we provided evidence that *Sl*PLC4 and *Sl*PLC6 are catalytically active PI-PLCs and that both are required for mounting defense responses in tomato (12). Our current study identifies *Sl*PLC7 which is highly similar (72% identical) to *Sl*PLC5. The observed distinction between PLC enzymes from monocots and dicots (Fig. S1B) suggests a link between PLC evolution and the early genetic divergence between monocots and dicots (80). Alternatively, PI-PLC enzymes may have been evolved to be regulated or function differently during signaling in monocots and dicots. The existence of multiple PLC isoforms suggests a distinct function for each isoform in a specific signaling event or providing functional redundancy between closely related isoforms.

The observed high density of negatively charged amino acid residues (Fig. 1A and 1B) in the X/Y-linker region of tomato PLC enzymes suggests the existence of an auto-inhibition mechanism similar to what was reported recently for animal PLCs (27). This should allow suppressing PLC activity in the resting state and enhancing it following interaction with the negatively charged plasma membrane. Studies on mouse PLCζ, which has a domain structure similar to plant PLCs, showed that binding to membrane phospholipids depends on a cluster of basic residues located at the X/Y-linker (81). Such a cluster was not identified in any of the tomato PLC proteins which excludes that tomato PLCs relies on similar feature for binding to the cell membrane. Instead, sequence comparison indicates that it is more likely that the C2 domain in tomato PLC binds membranes in response to Ca^{2+} signals as was demonstrated for rice PLC (23). Enrichment in putative phosphorylation sites within the X/Y liker region in tomato PLC suggests that protein phosphorylation may regulate enzyme activity as has been described previously in both mammals (50,52-54) and plants (28). Phosphorylation could facilitate PLC activation by either allowing the interaction with other activating proteins or increasing the negative charge of the X/Y-linker to facilitate the release of eventual auto-inhibition.

Expression of the *Sl*PLC7 enzyme in *E. coli* was unsuccessful, possibly due to its instability. All other heterologously-expressed tomato PLCs encoded active enzymes and they were able to hydrolyze natural but not synthetic phosphoinositides irrespective of the length of their acyl chain. This selectivity could be due to the difference in the saturation of the acyl chains between natural and synthetic substrates. The two acyl chains are saturated in

commercial synthetic phosphoinositides, while in natural phosphoinositides isolated from plants or animals, usually one of the two acyl chains is saturated and the other is not. Although we found no reports indicating this type of substrate preference for PLCs, there are studies showing that other phosphoinositide-metabolizing enzymes select their substrate based on the saturation state of the acyl chain (82,83). Further confirmation requires chemical modification of these synthetic preparations to make them resemble the natural ones and testing whether they will be hydrolyzed by tomato PLCs. Moreover, the encountered difference in enzyme stability between the tomato PLC isoforms suggests that the less-stable enzymes *Sl*PLC1, *Sl*PLC3 and *Sl*PLC6 might require additional factors provided in the plant cell to maintain their activity. Several reports indicated that Mg^{2+} was only required for PLC activity analyzed in membrane fractions or partially purified from plant material (35,57-60,84) and not for the *in vitro* activity of recombinant plant PLCs (55). All tomato PLCs that we tested so far did not require Mg^{2+} to stimulate their *in vitro* activity (Fig. S7), suggesting a similar indirect role for Mg^{2+} in the stimulation of tomato PLC *in vivo* but not *in vitro.* In contrast, varying Ca^{2+} concentration and the pH value differentially regulated the *in vitro* activity and substrate preference of the tested tomato PLCs. This suggests different roles for each PLC enzyme in signaling and phosphoinositide homeostasis. Possibly, specific PLC isoforms have redundant functions under unperturbed conditions in the cell, but will significantly differ in their activity and substrate preference when these conditions change, for example upon microbial stimulation.

Application of the PLC inhibitor U73122 shows that PLC activity is essential for inducing medium alkalization in response to microbial molecules during ETI and PTI. This indicates that PLC is upstream of the regulation of plasma-membrane proton pumps and downstream of stimulated surface immune receptors. This is in line with our previous finding that *SlPLC4* and *SlPLC6* are required for receptor-mediated defense and resistance (12). Previously, we have shown that ectopic expression of *Sl*PLC4 enhances the HR in transgenic *N. benthamiana* expressing Cf-4, upon infiltrating purified Avr4 protein (12). We investigated if other *SlPLC* genes could play a similar role by transiently expressing Avr4 and each tomato *PLC* by agro-infiltration in Cf-4 background. Surprisingly, *Sl*PLC3, and to a lesser extent, *Sl*PLC1 enhanced the Cf-4/Avr4-triggered HR whereas *Sl*PLC4 did not. Assuming that all genes were expressed to similar levels, it could be that *SlPLC3* and *SlPLC1* enhance PTI induced by *A. tumefaciens* (3,85), in addition to their enhancement of the Cf-4/Avr4-triggered HR. Accordingly, this generates an earlier and more pronounced HR. This idea is supported by the observed weak chlorosis that occurs at the site of agro-infiltration when higher ODs are used to express *SlPLC3* alone (results not shown). Importantly, the HR enhancement-effect of ETI and PTI by PLC-overexpression matches our inhibitor studies showing that PLC activity is required for both ETI and PTI. To understand how the PLC inhibitor U73122 actually suppresses the FLS2/flg22-mediated defense activation, we investigated whether FLS2 internalization is affected by the inhibitor. FLS2 internalization massively occurs upon flg22 treatment, which is tightly linked to subsequent activation of PTI (76). We found that treatment with the PLC inhibitor significantly suppresses the flg22-induced internalization of FLS2, indicating that PLC activity plays a role in the internalization process upon ligand binding. Earlier studies, using either FLS2 mutants or an inhibitor of its kinase activity, demonstrated that the phosphorylation of the kinase domain of

FLS2 is a prerequisite for its internalization and the subsequent activation of flg22-mediated defense (76). It is therefore intriguing to decipher whether PLC activity is involved directly in the internalization of FLS2, or interferes with its phosphorylation upon perception of flg22. It will also be interesting to determine whether PLC activity affects Cf-4 localization in a similar way as FLS2 signaling is affected, as it was recently observed that Cf-4 undergoes endocytic internalization similar to FLS2, upon its activation by the matching ligand Avr4 (Postma and Robatzek, unpublished data).

The mechanism by which PLCs are activated upon stimulation of surface immune receptors by microbial molecules is still unclear. However, the sensitivity of PLC activity to increasing Ca^{2+} concentrations and to altering pH, in addition to the differences among the studied PLC isoforms in their responsiveness to these two components, suggest an indirect receptor-mediated regulation of PLC activity. Accordingly, the perception of pathogens would trigger a local Ca^{2+} influx that persists according to the amount of perceived signal and the number of stimulated receptor molecules. When this is combined with changes in the cytosolic pH, it might result in a controlled activation of specific PLC isoforms and the hydrolysis of a specific combination of phosphoinositide substrates. The outcome of this altered PLC activity would be a very specific change in the phosphoinositide composition of the targeted membrane. As a result, DAG or its phosphorylated form, PA, will occupy the sites of the hydrolyzed substrates. This should allow the recruitment of different sets of proteins to these modified membranes, appropriate for the defense event that will follow (86,87). It is therefore important to study the subcellular localization of the various PLC isoforms and their preferred substrates under resting conditions and upon stimulation by microbial compounds. Another consequence of PLC activation is the generation of $InsP_3$, which can be further phosphorylated to become $InsP_6$. Both $InsP_3$ and $InsP_6$ stimulate the intercellular release of Ca^{2+} from endo-membrane compartments in plants (88-92). The released calcium functions as a master signal which coordinates different aspects of defense signaling. Altogether, PLC plays a central role in plant immunity and specifically early after the activation of immune receptor. This is due to the control of generation and turnover of several distinct signaling molecules. We are just beginning to appreciate this role and future studies should elucidate more about the mechanisms by which PLC influence immune reactions and contribute to resistance. This will eventually allow us to generate plants with an enhanced degree of resistance to detrimental pathogens.

Acknowledgments

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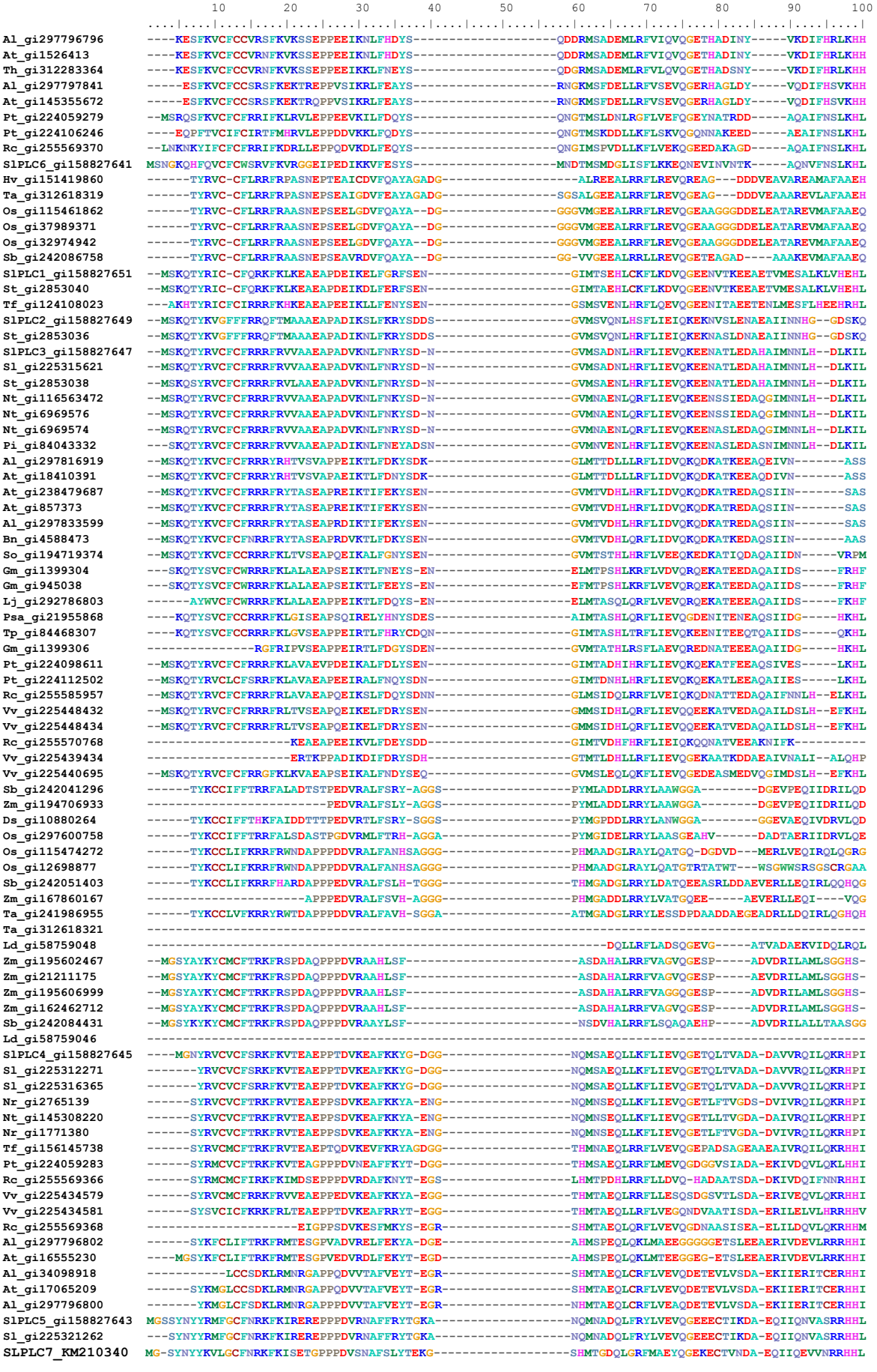

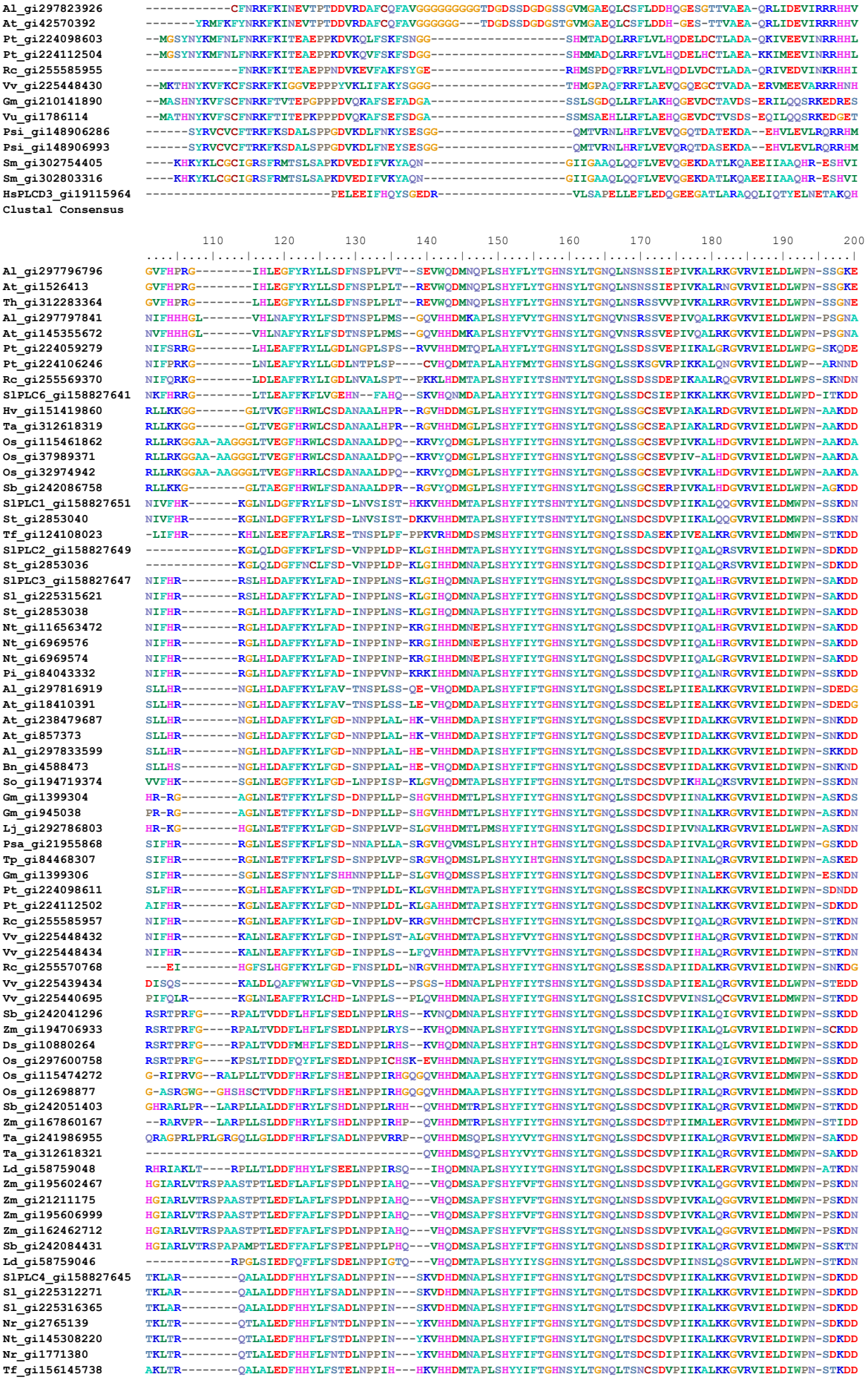
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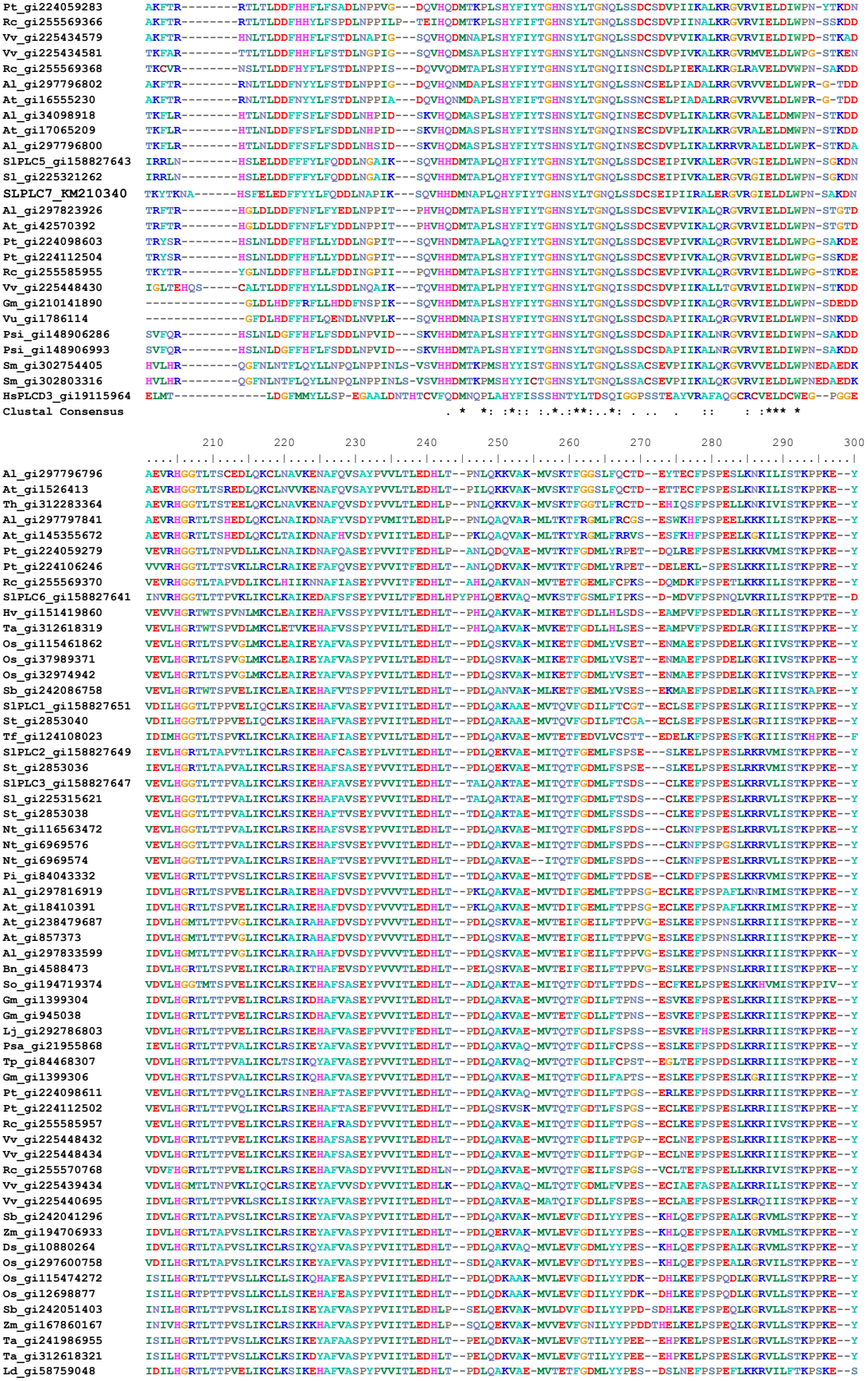
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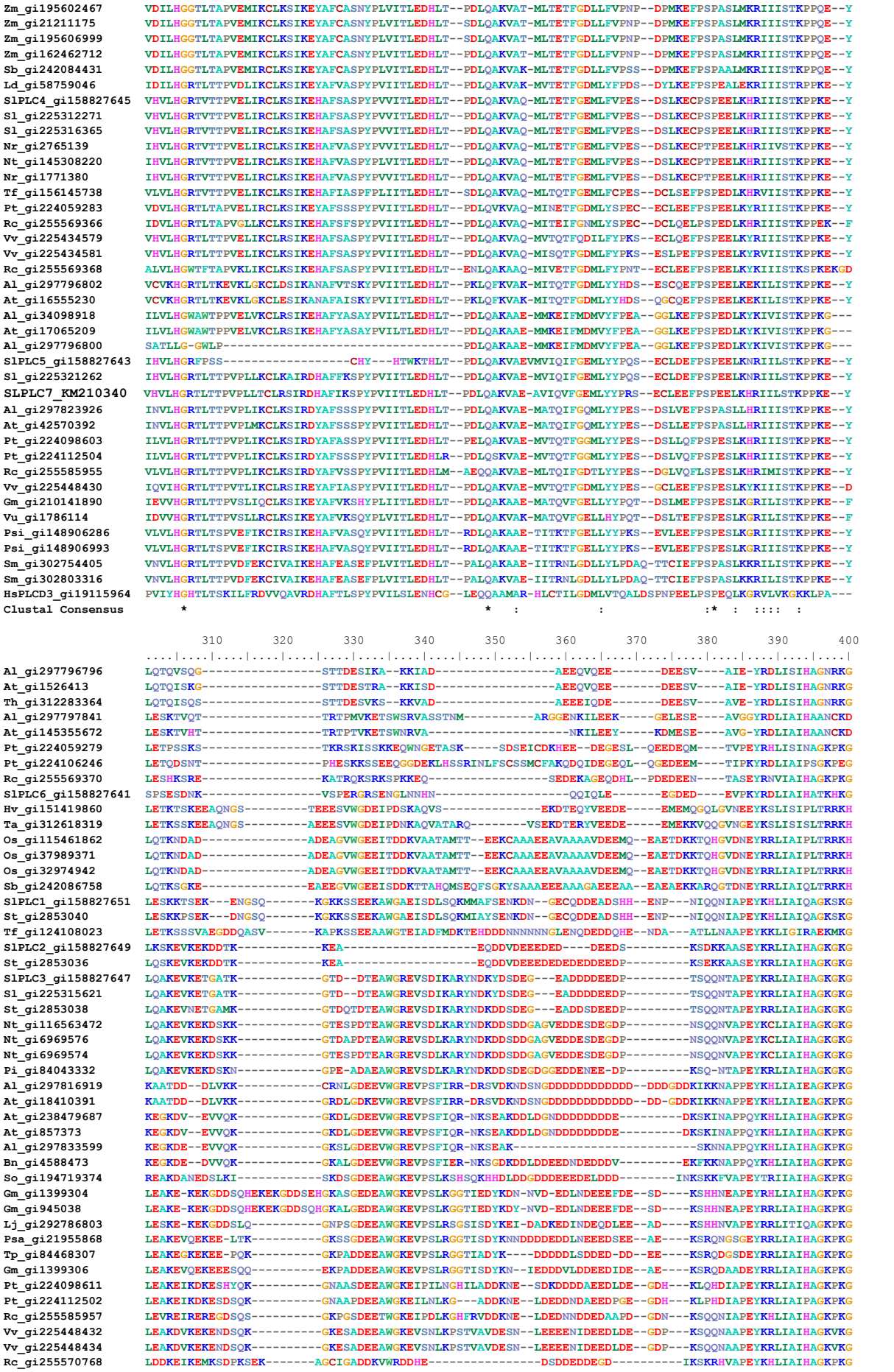
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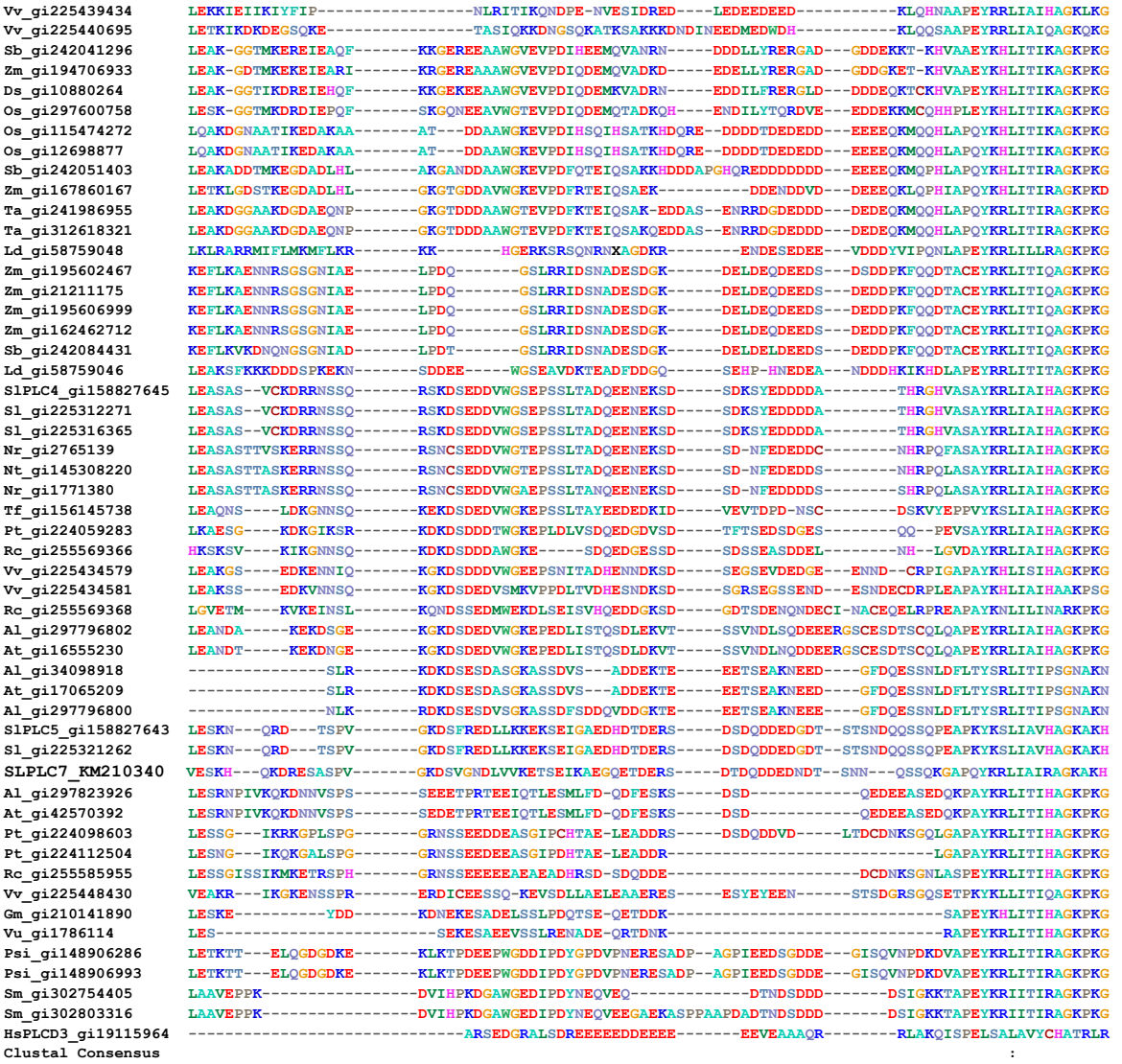

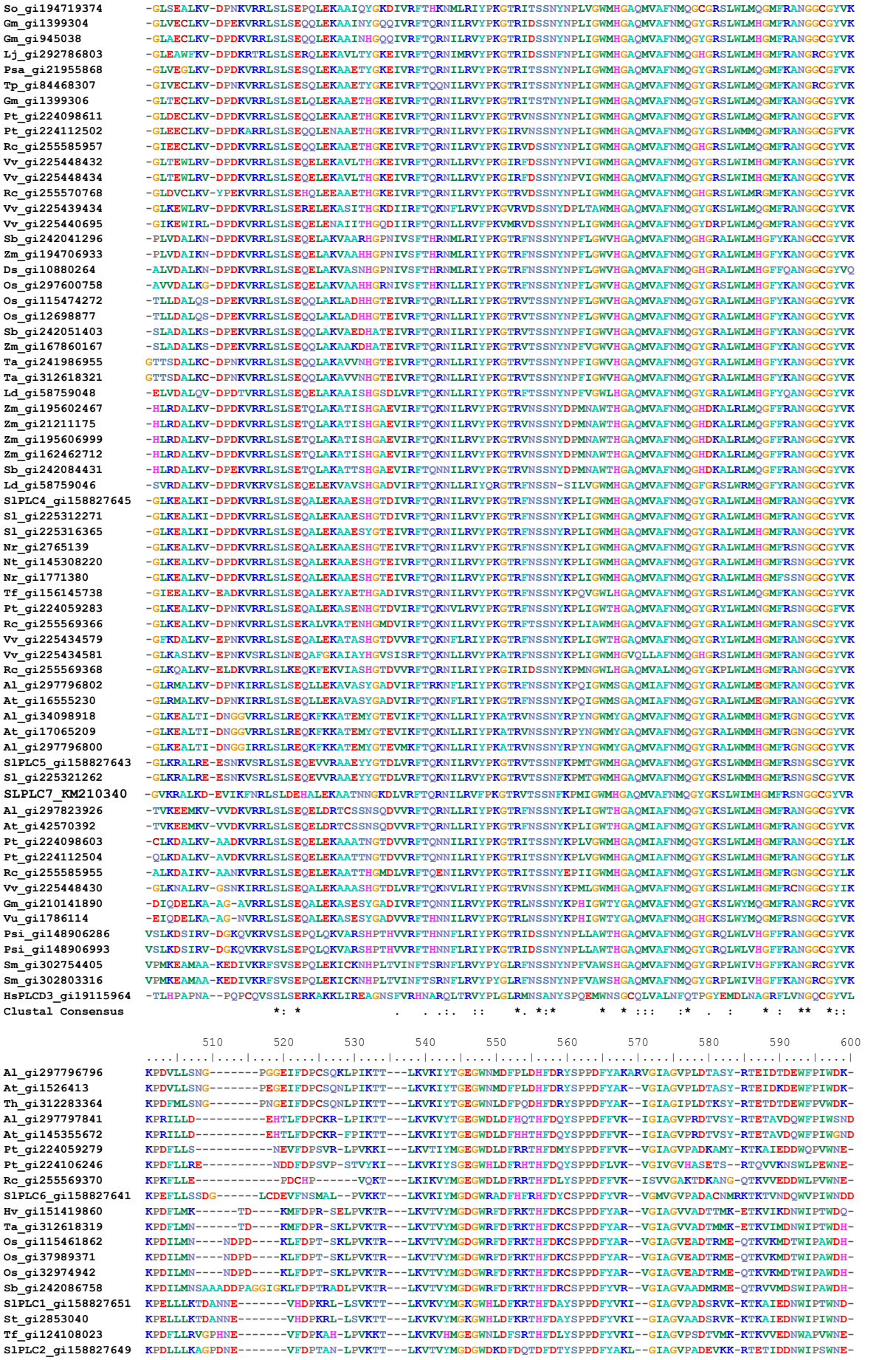
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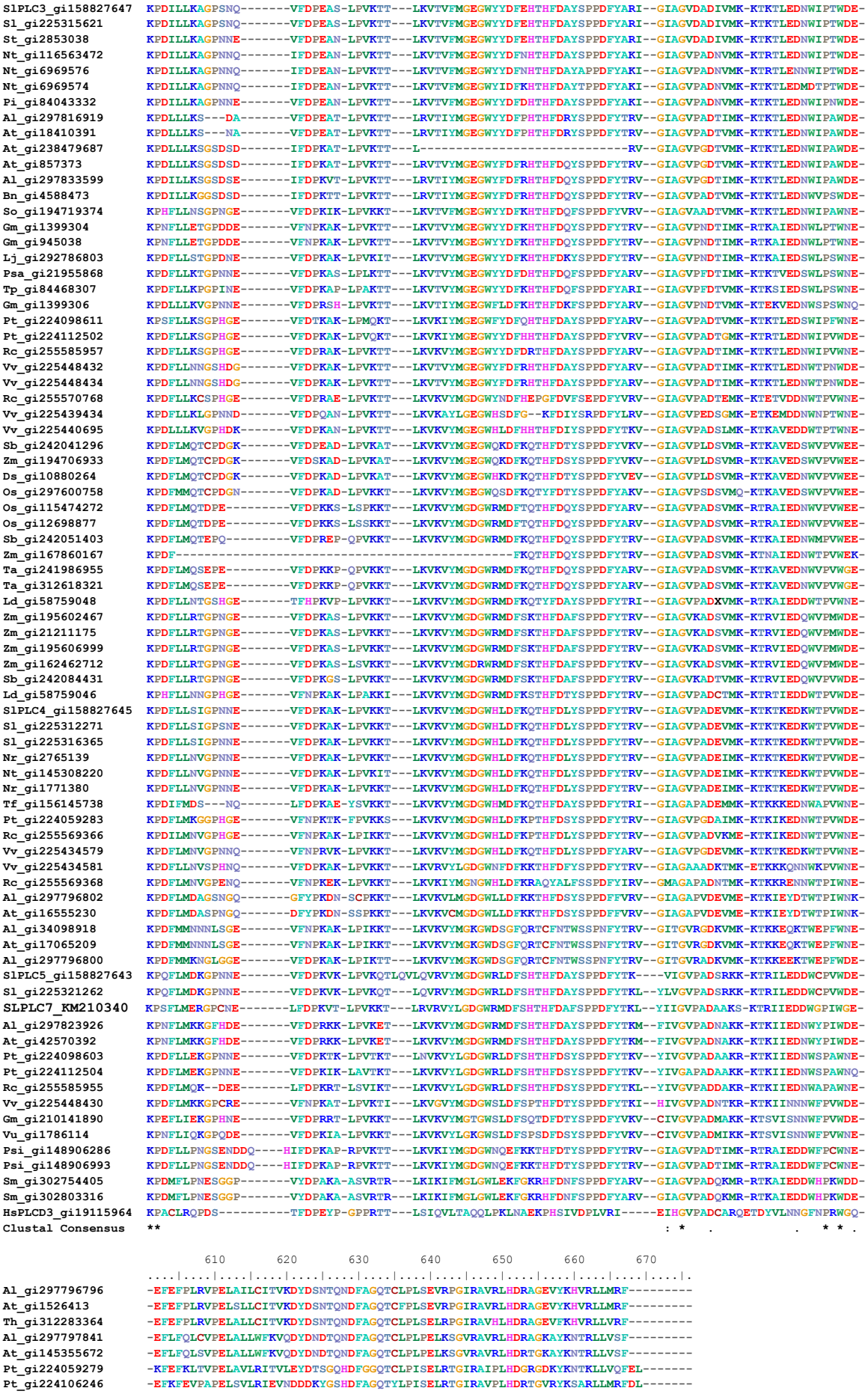
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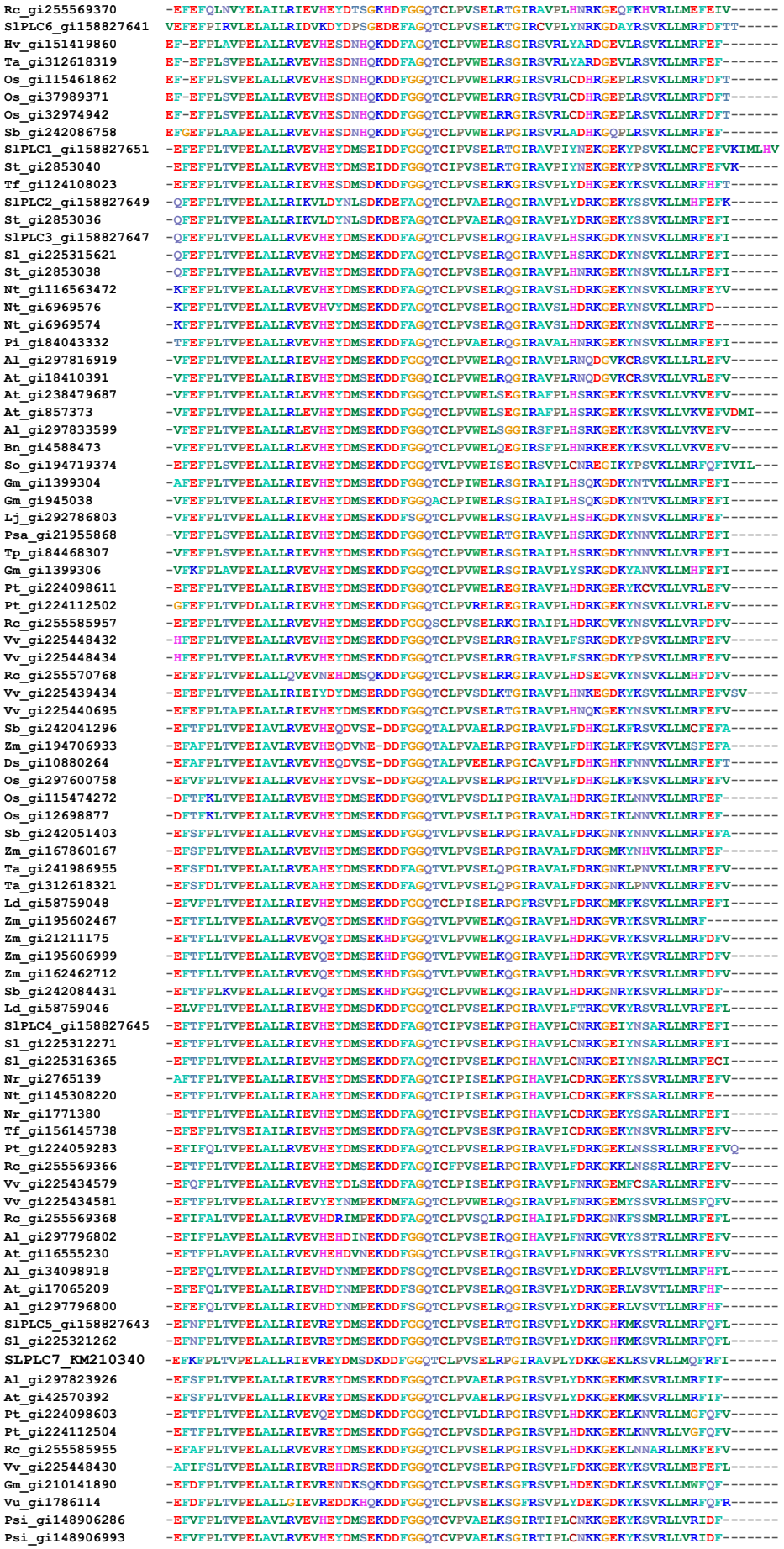
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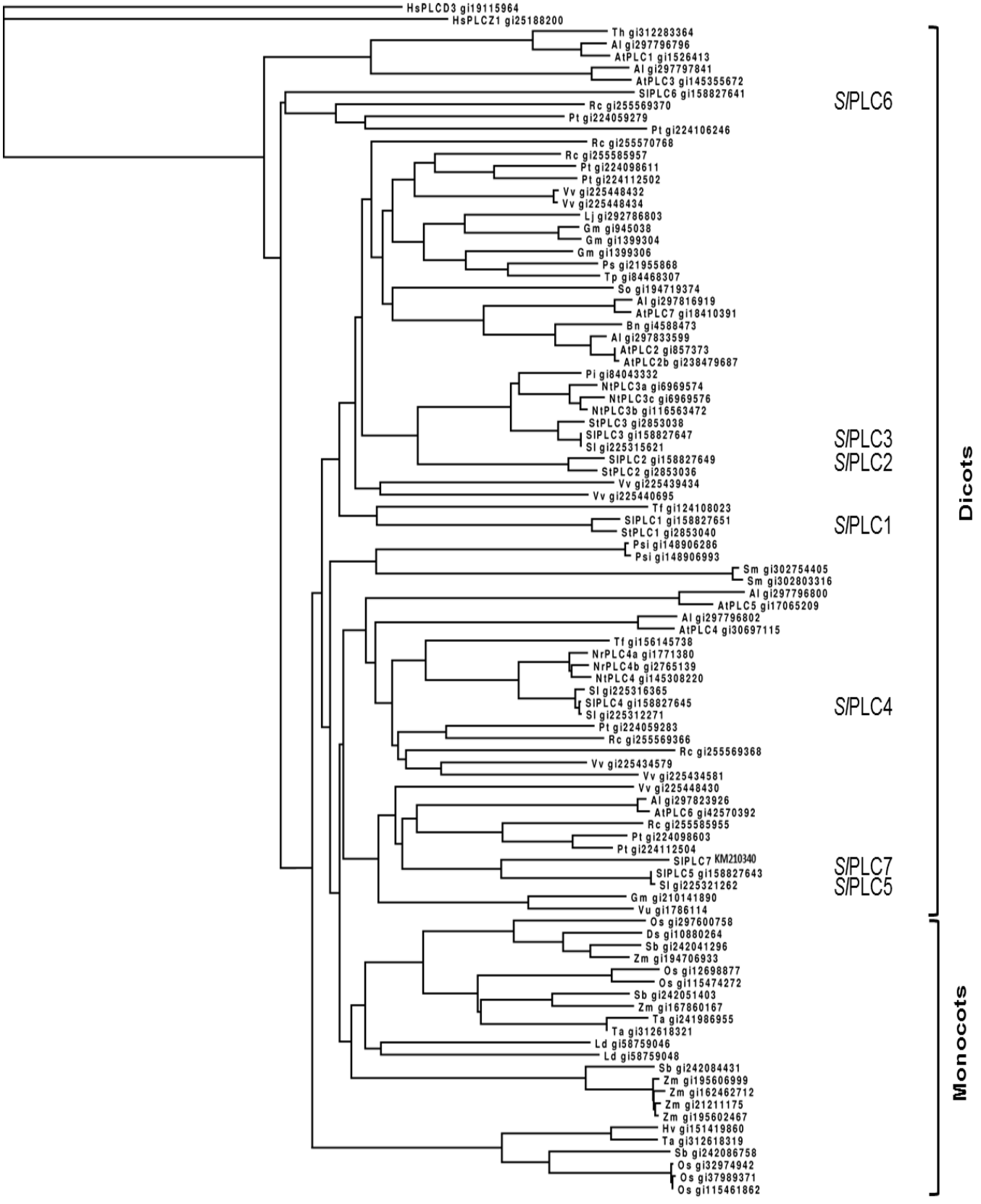
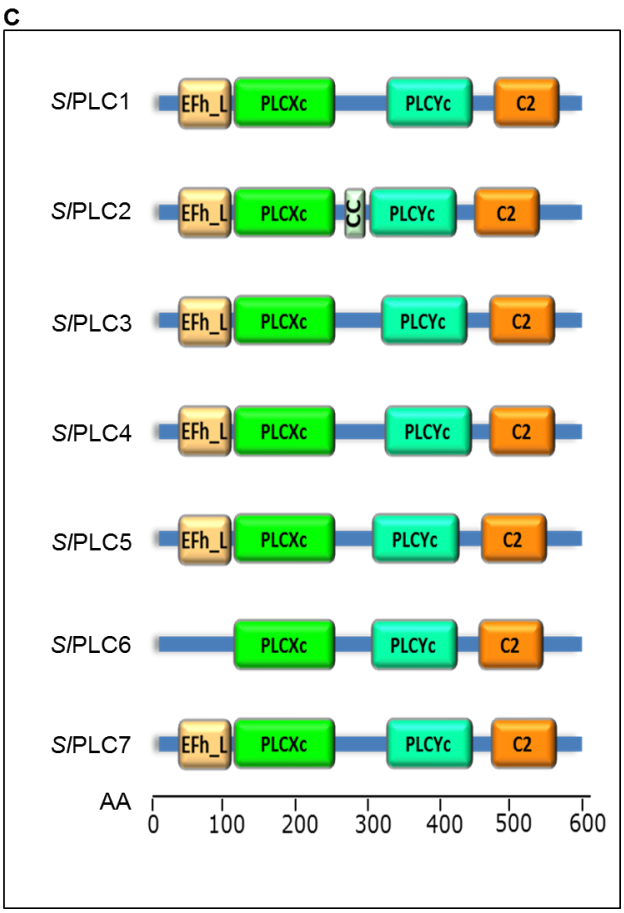
S1A



S1B



S1C

FIGURE S1. **Phylogenetic analysis of plant PI-PLCs.** *A*, Clustal X alignment of the amino-acid sequence of 98 PLC proteins from 29 different plant species, including the tomato PLC family. PLC sequences were retrieved from the public database at NCBI (http://www.ncbi.nlm.nih.gov/). *B*, a phylogram generated from the alignment in *A*. The tree was generated using the Bootstrap N-J Tree procedure, in which *Hs*PLCδ3 was used as an out-group. Abbreviations of species names: *Al, Arabidopsis lyrata; At, Arabidopsis thaliana; Bn, Brassica napus; Br, Brassica rapa; Ds, Digitaria sanguinalis; Gm, Glycine max; Hs, Homo sapiens; Hv, Hordeum vulgare; Ld, Lilium davidii; Lj, Lotus japonicus; Nr, Nicotiana rustica; Nt, Nicotiana tabacum; Os, Oryza sativa; Pi, Petunia inflata; Ps, Pisum sativum; Psi, Picea sitchensis; Pt, Populus trichocarpa; Rc, Ricinus communis; Sb, Sorghum bicolor; Sl, Solanum lycopersicum; Sm, Selaginella moellendorffii; So, Spinacia oleracea; St, Solanum tuberosum; Ta, Triticum aestivum; Tf, Torenia fournieri; Th, Thellungiella halophila; Tp, Trifolium pratense; Vu, Vigna unguiculata; Vv, Vitis vinifera; Zm, Zea mays. C,* schematic representation of the domain architecture for the different members of the tomato PLC family. Colored blocks indicate the evolutionary conserved domains predicted in the *Sl*PLC amino acid sequences using SMART prediction (http://smart.embl-heidelberg.de/) combined with HMM and Pfam searches. EFh_L, EF hand-like domain; PLCXc, PLC catalytic domain part X containing the active-site residues; CC, coiled-coil; PLCYc, PLC catalytic domain part Y; C2, protein kinase C conserved region 2.

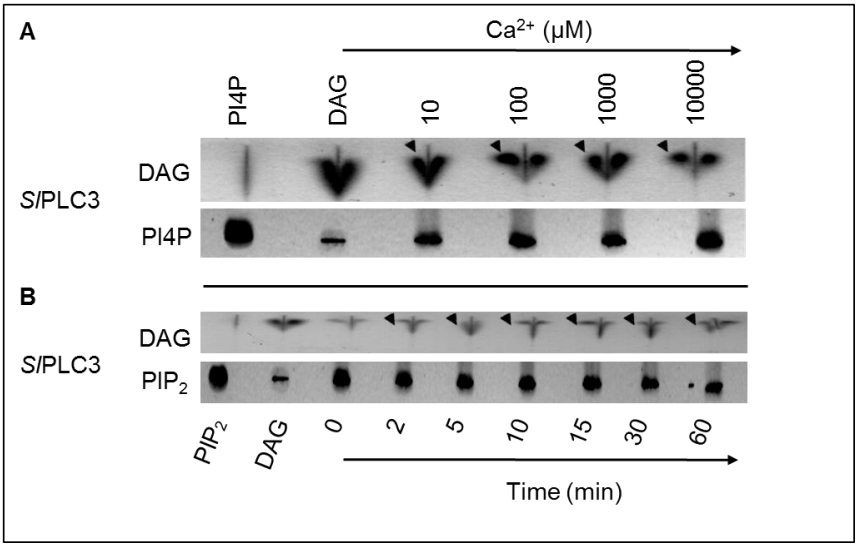


FIGURE S2. **Hydrolysis of phosphatidylinositol mono- (PI4P) and bis-phosphate (PI(4,5)P2 by *Sl*PLC3.** *A, in vitro* phospholipase activity of affinity-purified recombinant *Sl*PLC3 tested at the indicated Ca^{2+} concentrations. For each reaction, 10 µg of PI4P preparation (as micellar-lipid solution) were used as substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol-1,4-diphosphate (IP2) when hydrolyzed. Hydrolysis of the substrate, PI4P, (lower panel) and accumulation of one of the reaction products, DAG, (upper panel) was monitored by extraction of the reaction products followed by thin layer chromatography (TLC) analysis, after 60 minutes of incubation. The first two lanes (from the left) were loaded with 10 µg of PI4P and 30 µg of DAG and serve as migration references. *B*, time-dependent PLC activity assay using PI(4,5)P2 (PIP_2) as a substrate. For each reaction, 10 µg of PIP_2 (as micellar-lipid solution) were used which was converted into DAG and inositol 1,4,5-triphosphate ($InsP_3$) upon its hydrolysis. Reaction products were extracted and analyzed by TLC after incubation for the indicated time points, as described under "Experimental Procedures". The first two lanes (from the left) were loaded with 10 µg of PIP_2 and 30 µg of DAG and serve as migration references. The second product of the phospholipase reaction was inositol phosphate ($InsP_2$) in *A*, and $InsP_3$ in *B*. These compounds were lost during the lipid extraction process. Arrowheads indicate the accumulation of DAG.

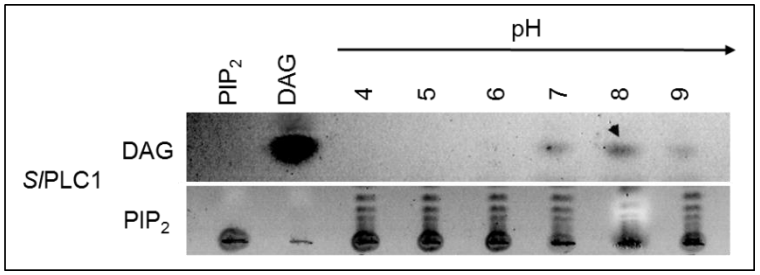


FIGURE S3. **pH-dependent hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) by tomato PLC1.** *In vitro* phospholipase activity of affinity-purified tomato *Sl*PLC1 was tested at the indicated pH values. For each reaction, 10 µg of PIP_2 were used as a substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$)

when hydrolyzed. The depletion of the substrate (PIP_2) (lower panel), and accumulation of one of the reaction products (DAG) (upper panel) was monitored by TLC analysis after 60 minutes of incubation as described under "Experimental Procedures". The second reaction product, $InsP_3$, is lost during the lipid extraction process following the phospholipase reaction. The arrowhead indicates the accumulation of DAG. The first two lanes (from the left) were loaded with 10 µg of PIP_2 and 30 µg of DAG and serve as migration references.

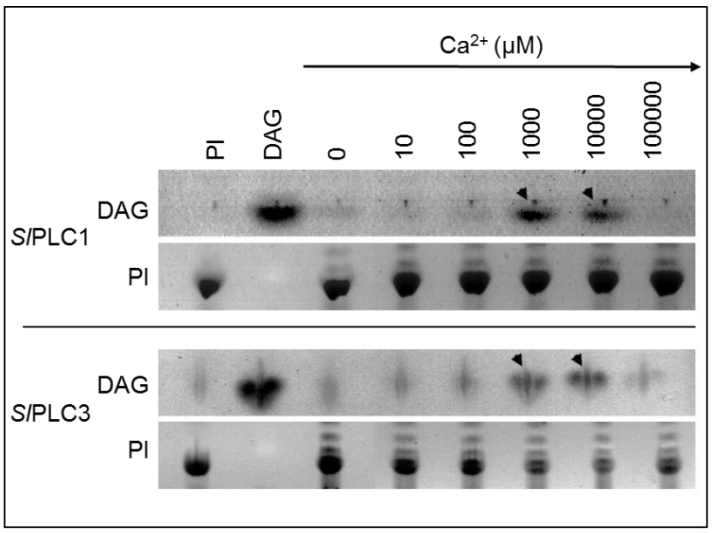


FIGURE S4. **Ca^{2+}-dependent hydrolysis of phosphatidylinositol (PI) by tomato PLC1 and PLC3.** *In vitro* phospholipase activity of affinity-purified *Sl*PLC1 and *Sl*PLC3 was tested at the indicated Ca^{2+} concentrations. For each reaction, 30 µg of PI preparation were used as a substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol-phosphate (IP) when hydrolyzed. The depletion of the substrate (PI) (lower panels) and accumulation of one of the reaction products (DAG) (upper panels) were monitored by TLC analysis after 60-70 minutes of incubation as described under "Experimental Procedures". The second reaction product, IP, is lost during the lipid extraction process following the phospholipase reaction. Arrowheads indicate the accumulation of DAG. The first two lanes (from the left) were loaded with 30 µg of PI preparation and 30µg of DAG and serve as migration references. Note that both *Sl*PLC1 and *Sl*PLC3 have a similar Ca^{2+} concentration optimum of about 1 to 10 mM.

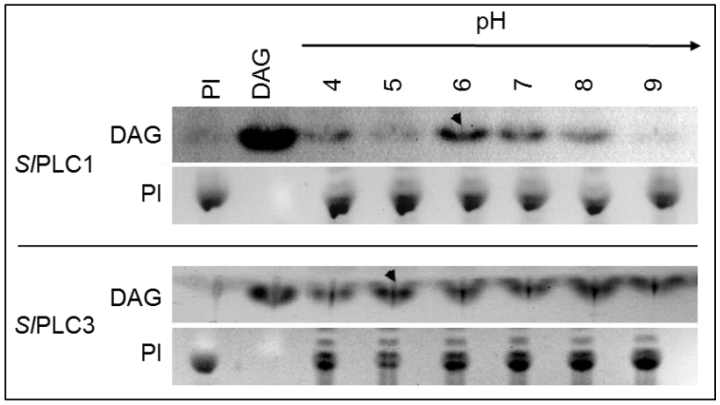


FIGURE S5. **pH-dependent hydrolysis of phosphatidylinositol (PI) by tomato PLC1 and PLC3.** *In vitro* phospholipase activity of affinity-purified *Sl*PLC1 and *Sl*PLC3 was tested at the indicated pH values. For each reaction, 30 µg of PI preparation were used as substrate, which is converted into 1,2-diacylglycerol (DAG) and inositol-phosphate (IP). The depletion of the substrate (PI) (lower panels) and accumulation of one of the reaction products (DAG) (upper panels) were monitored by TLC analysis after 60-70 minutes of incubation as described under "Experimental Procedures". The second reaction product, IP, is lost during the lipid extraction process following the phospholipase reaction. Arrowheads indicate the accumulation of DAG. The first two lanes (from the left) were loaded with 30 µg of PI preparation and 30 µg of DAG and serve as migration references. Note that *Sl*PLC1 has a pH optimum of around 6-7, whereas the pH optimum for *Sl*PLC3 is around pH 5. In the *Sl*PLC3 assay a contamination is present that migrates at the same height as DAG.

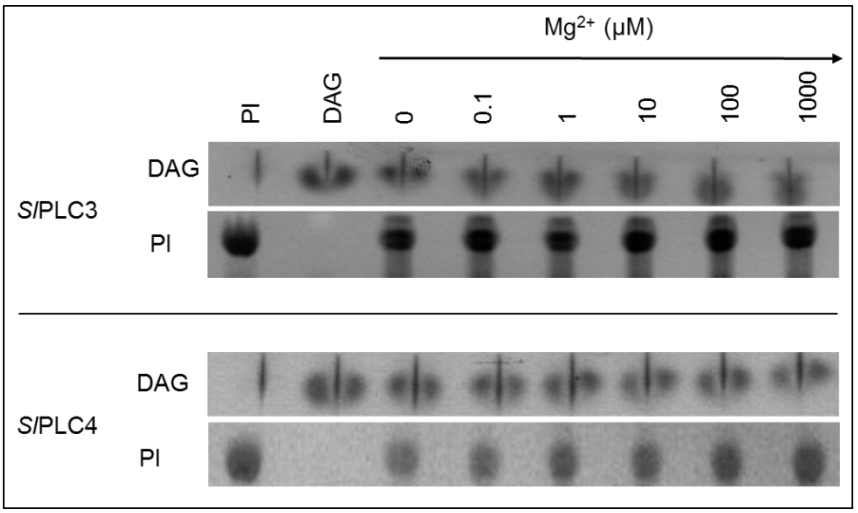


FIGURE S6. **Mg^{2+} is not required for the hydrolysis of phosphatidylinositol (PI) by tomato PLC3 or PLC4.** *In vitro* phospholipase activity of affinity-purified *Sl*PLC3 and *Sl*PLC4 was tested at the indicated Mg^{2+} concentrations. For each reaction, 30 µg of PI preparation (as micellar-lipid solution) were used as substrate, from which DAG is generated. The first two lanes (from the left) were loaded with 30 µg of PI and 30 µg of DAG and serve as migration references.

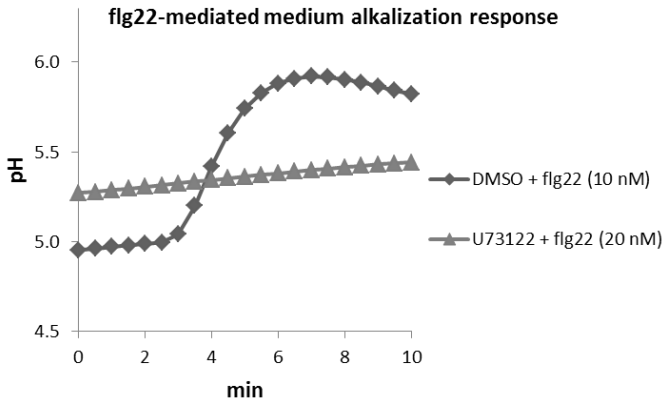


FIGURE S7. **Suppression of defense activation by PLC inhibitor U73122, after elicitation with a high concentration of flg22.** Tobacco cells were pre-incubated with the PLC inhibitor U73122 and after 10 min the PAMP flg22 was added to a final concentration of 20 nM. Flg22, added to a concentration of 10 nM, served as a control. The pH of the medium was then monitored over a period of 10 min. Note that suppression of flg22-mediated medium alkalization by U73122 still occurs after the addition of 20 µM of flg22.

Table S1. **Number of negatively charged residues and putative phosphorylation sites in the full-length PLC proteins and their X/Y-linker regions.** *A*, the ratio between the total number of negatively charged amino acid residues and the total amount of amino acids of both the full-length *Sl*PLC proteins and their X/Y-linker regions. *B*, the ratio between the total number of predicted phosphorylation sites and sequence length for both the full-length *Sl*PLC proteins and the corresponding X/Y-linker regions. The NetPhos 2.0 server was used for predicting phosphorylation sites. A score between 0.5 and 1.0 was regarded as significant. Note that 0.5 is the threshold and a score between 0.5 and 1.0 reflects confidence of the prediction and the higher the similarity to one or more of the phosphorylation sites used in training the method.

A

Enzyme	Complete protein				X/Y-linker			
	# D and E	length (AA)	charge at pH 7.0	Ratio	# D and E	length (AA)	charge at pH 7.0	Ratio
***Sl*PLC1**	82	602	-7.2	14	17	74	-7	23
***Sl*PLC2**	90	562	-16.9	16	21	47	-12	45
***Sl*PLC3**	82	584	-10.5	14	20	65	-13	31
***Sl*PLC4**	84	587	-5.4	14	18	69	-10	26
***Sl*PLC5**	81	574	-1.5	14	22	71	-12	31
***Sl*PLC6**	70	566	-2.5	12	12	44	-8	27
***Sl*PLC7**	81	595	-0.5	14	19	71	-10	27
***Hs*PLCβ2**	178	1185	-13.3	15	26	82	-23	32
***Hs*PLCδ1**	98	756	-8.1	13	12	51	-6	24

Table *1A:* Enrichment of the X/Y-linker region with negatively charged residues.

B

Protein	Complete protein			Linker		
	# predicted phosphorylation sites	Length (AA)	Ratio	# predicted phosphorylation sites	Length (AA)	Ratio
***Sl*PLC1**	**16**	**602**	**3**	8	74	11
***Sl*PLC2**	**16**	**562**	**3**	5	47	11
***Sl*PLC3**	17	584	3	4	65	6
***Sl*PLC4**	23	587	4	11	69	16
***Sl*PLC5**	23	574	4	9	71	13
***Sl*PLC6**	20	566	4	4	44	9
***Sl*PLC7**	21	595	4	8	71	11
***Hs*PLCβ2**	27	1185	2	9	82	11
***Hs*PLCδ1**	26	756	3	3	51	6

Table *1B:* Enrichment of the X/Y-linker region with putative phosphorylated residues.

CHAPTER 5

(General Discussion) Plant Phosphatidylinositol-Specific Phospholipase C (PI-PLC): Activation, Regulation and Function in Receptor-Mediated Defense Signaling Against Microbes

Plant Phosphatidylinositol-Specific Phospholipase C (PI-PLC): Activation, Regulation and Function in Receptor-Mediated Defense Signaling Against Microbes

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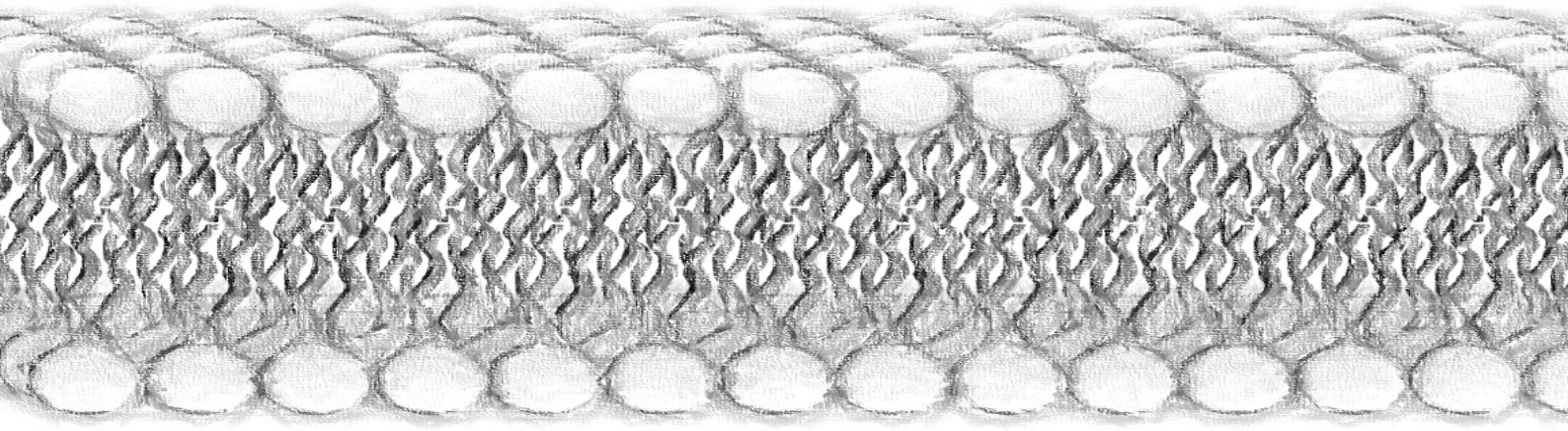

Abstract

A better understanding of plant defense and resistance to pathogenic microbes requires detailed studies of the molecular mechanism by which the plant innate immune-reaction is executed. A growing body of evidence places the phosphoinositide-specific phospholipase C (PI-PLC) enzymes immediate downstream of activated immune receptors and the early defense responses controlled by them. Examples for these responses involve the regulation of cytoplasmic levels of free Ca^{2+}, the cytoplasmic pH, and the oxidative burst. The central role for PI-PLC seems to be shaped by its biochemical activity, which allows it to control the hydrolysis of phospholipid signaling molecules and the subsequent generation of amplifying second messengers, required for fast and robust cellular defense signaling. Here, an inventory of plant PI-PLCs will be provided. Furthermore, their regulation, activation, and role in immune signaling through the degradation of specific substrates and the subsequent generation of signaling-competent reaction products, will be discussed.

1. Introduction

Plants are sessile organisms that need to respond quickly to environmental stresses, including microbial invasion. The innate immune-system of plants allows them to sense the presence of pathogens and induce defense responses that are sufficient to preclude or overcome infection. Plants recognize microbe-derived molecules or secreted effectors of invading pathogenic microbes by surface- or intracellular immune receptors (1-3). Once an immune receptor is triggered by the matching ligand, a defense signaling cascade is initiated that culminates in resistance to the invader. The signaling cascade can be roughly divided into early- and late signaling events. Early signaling events determine the outcome of the defense reaction and occur within minutes after immune receptor activation. Here I focus on discussing the central role of phosphoinositide-specific phospholipase C enzymes (PI-PLCs) in early defense signaling of plants. I will briefly discuss why these enzymes have received so much attention in animal and plant studies including domain structure and phylogeny of plant PI-PLCs and will illustrate the main differences with their animal counterparts. The substrates of plant PI-PLCs and their distribution in the membrane system of host cells, in addition to their reaction products are described in relation to their mechanism of modulating plant defense signaling. Furthermore, I will describe all known mechanisms of plant PI-PLCs activation upon triggering immune receptors, followed by an inventory of data showing the involvement of the PI-PLC pathway in plant defense. Finally, a model depicting the current view for PI-PLC signaling in plant immunity is presented.

2. The PI-PLC signaling pathway

2.1. The discovery of the PI-PLC signaling pathway

In the early 1950s, researchers discovered a rapid turnover in the pool of inositol phospholipids (phosphoinositides, PIs) after stimulation by the neurotransmitter acetylcholine and during the secretion of digestive enzymes from pancreatic slices (4,5). Phosphoinositides are composed of phosphoglycerides, in which a D-myo-inositol (Ins) ring, or its phosphorylated form, is linked at its 1-carbon position via a phosphodiester bond to the 3-carbon position of the glycerol backbone of the diacylglycerol moiety. (Figure 1).

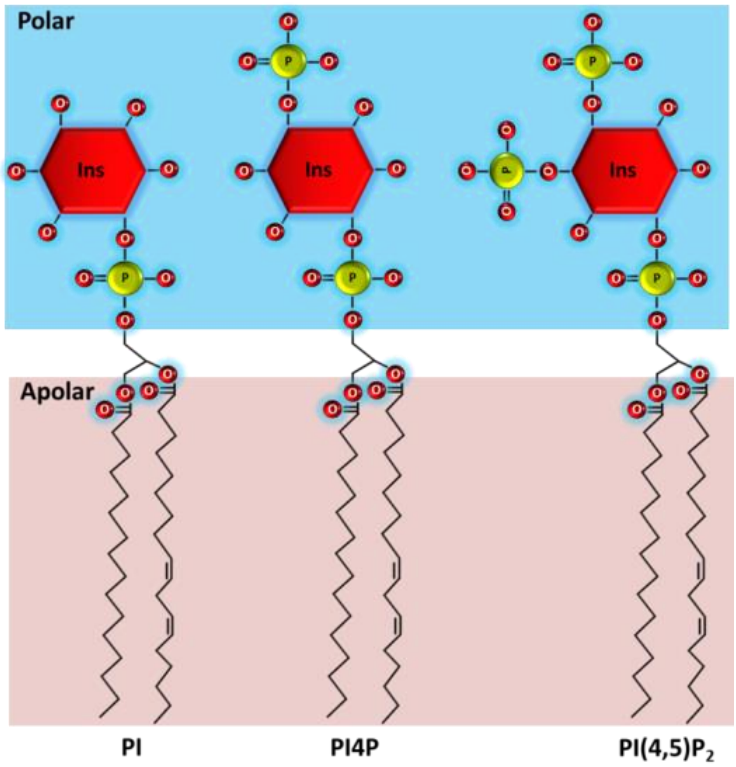

FIGURE 1. **The chemical structure and amphipathic property of three phosphoinositide species: phosphatidylinositol, PI,PI-4-phosphate, PI4P, and PI4,5-bisphosphate, PI(4,5)P_2.**

An important reason for the interest of scientists in PI-turnover is its association with receptor-mediated release of cytoplasmic Ca^{2+} (6). The generated Ca^{2+} is released from intracellular stores and functions as a second messenger in cellular signaling and is considered to be a hallmark that precedes downstream responses following receptor activation. This conditional release of Ca^{2+} is vital, as it coordinates cellular responses towards an array of external cues in both animals (7,8) and plants (9-11). Later, it was found that PIs are hydrolyzed to produce inositol (12) which stimulates the release of free Ca^{2+}. Indeed, phosphodiesterase (later named phospholipase C, PLC) activity was discovered in both animals (13) and plants (14,15) responsible for hydrolysis of phosphoinositides *in vitro* and *in vivo*, especially after receptor stimulation.

2.2. Other phospholipases

In addition to PI-PLCs that cleave the phosphodiester bond at the stereospecific position number 3 (sn-3) between diacylglycerol and phosphate of PIs, additional phospholipase enzymes, cleaving PIs at other positions, exist in plants and animals (Figure 2). The activity of these phospholipases affects both the composition and properties of cellular membranes, in addition to regulating cellular processes by producing signaling molecules (16-18). Phospholipase A1 (PLA1) and -A2 (PLA2) release the acyl chains from the sn-1 and sn-2 positions, respectively, of the diacylglycerol moiety (Figure 2). Phospholipase B (PLB) has multiple activities, as it can hydrolyze phospholipids at the sn-1 and sn-2 positions

simultaneously to produce two free fatty acids and glycerol attached to the head group. PLB can also hydrolyze lyso-glycerophospholipids, produced by PLA1 or PLA2, thereby releasing the remaining acyl chain (Figure 2). PLB was also shown to possess both acyl-transferase and acyl-hydrolase activities (19,20). PLD is another phospholipase, which cleaves the terminal phosphodiester bond in glycerophospholipids. However, PLD catalyzes the hydrolysis of structural phospholipids (e.g. phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE), leading to the formation of membrane-localized phosphatidic acid (PA) and the release of the soluble head group (Figure 2).

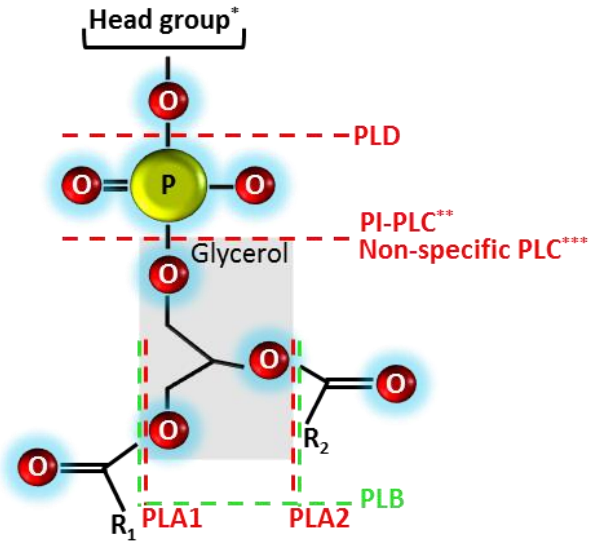

FIGURE 2. **Depiction of a general phospholipid molecule with a variable head group and the sites of cleavage for different types of phospholipases.**

*Head group can be: Ins(4,5)P_2, Ins4P, Ins, choline, ethanolamine, serine or glycerol

**Hydrolyzes PI, PI4P and PI(4,5)P2

***Hydrolyzes phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) and has low affinity for the substrates phosphatidyl serine (PS) and phosphatidyl glycerol (PG).

2.3. The importance of the PI-PLC signaling pathway in animals and plants

Currently, PI-PLC is considered to be essential in cellular signaling. It is known to function in the canonical PI-pathway, where it hydrolyzes PI(4,5)P_2 to produce *sn*-1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate ($InsP_3$ or Ins(1,4,5)P_3). Both reaction products are important second messengers in animals (21,22). DAG resides in the plasma membrane where it recruits several protein kinase C (PKC) isoforms in animals and, together with Ca^{2+}, activates them. Activated PKC phosphorylates several downstream targets involved in cellular signaling, allowing the cell to execute responses appropriate to the perceived stimulation. The released $InsP_3$ diffuses into the cytosol and gates Ca^{2+}channels in the endoplasmic reticulum (ER) of animal cells to release Ca^{2+} into the cytoplasm (23). Several mechanisms exist for the generation of Ca^{2+} signals, but the one requiring PI-PLC activity is crucial in cell signaling. Accordingly, receptor-mediated activation of the canonical PI-PLC pathway affects three different second messengers simultaneously. These are the PI-PLC

substrate $PI(4,5)P_2$ and its products, DAG and $InsP_3$ (24). PI-PLC activation is therefore considered crucial to link receptor activation with downstream responses.

2.4. The core domain structure of eukaryotic PI-PLCs

Eukaryotic PI-PLCs are multi-domain proteins in contrast to prokaryotic PI-PLCs which are single domain proteins. The core structure of a PI-PLC consists of an EF-hand domain (referred to as EF-hand-like domain in plants) at the N-terminus, central X and Y domains and a C-terminal C2 domain (Figure 3). The EF-hand-like domain is similar to the canonical EF-hand domain in its coordination properties towards Ca^{2+} ions. Yet, the former has a predicted secondary structure which resembles that of only the second loop of the EF-hand domain found in animal PI-PLCδ1 (25). EF-hand-like domains are essentially found in proteins encoded by bacterial and viral genomes, where they bind Ca^{2+} especially in proteins involved in Ca^{2+} signaling and homeostasis (26). The EF-hand-like domain is essential for plant PI-PLC activity (25). The X and Y domains together form the catalytic core, in which several residues are highly conserved among eukaryotic PI-PLCs (27). In both eukaryotic and prokaryotic PI-PLCs, the X and Y domains are separated by a peptide linker (X/Y-linker). As discussed later, this linker is highly divergent in length and sequence and has recently been shown to play a role in regulating PLC activity (27,28). The C2 domain is crucial for the enzymatic activity, as was shown for rat (*Rattus norvegicus*) PLCδ1 (29,30). It can specifically bind Ca^{2+} ions and negatively-charged phospholipids (31). Recently, it was demonstrated that the C2 domain of a PI-PLC enzyme from rice (*Oryza sativa*) is responsible for targeting the enzyme to the plasma-membrane (PM) in response to Ca^{2+} (32).

2.5. Domain structure of plant PI-PLCs compared to that of animal PI-PLCs

Most plant PI-PLC enzymes identified to date possess only the aforementioned core domain structure. In contrast, most animal PI-PLC enzymes possess additional domains like the pleckstrin homology (PH) domain in PI-PLCδ, in addition to other regulatory domains (see Figure 3). Animal PI-PLCζ has a structure similar to PI-PLCδ but lacks the PH domain. Accordingly, all plant PI-PLCs resemble PI-PLCζ.

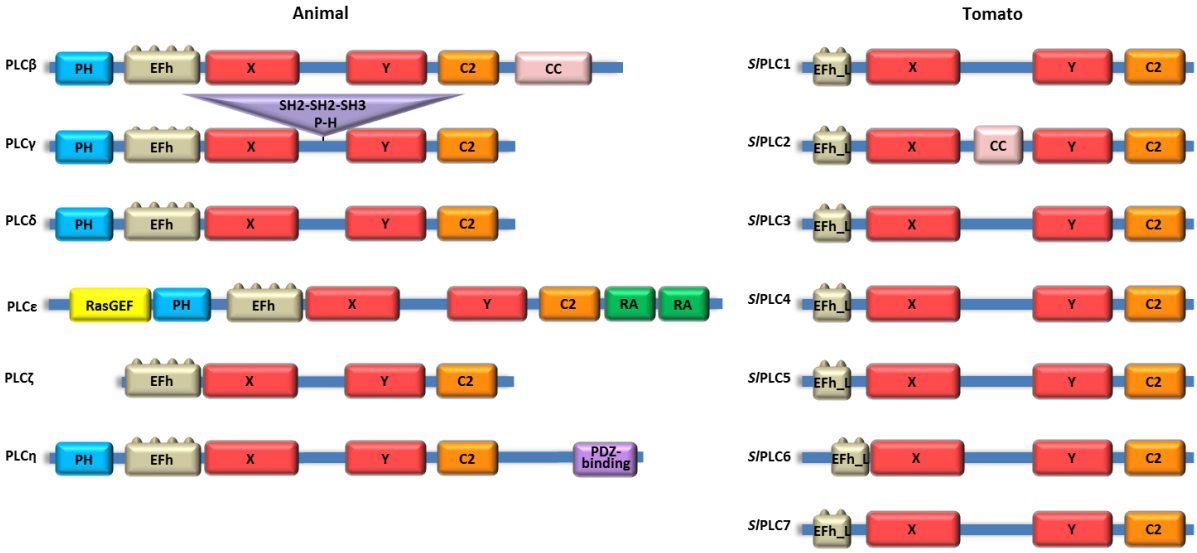

FIGURE 3. **Depiction of the six classes of animal PI-PLCs (left) and the complete PI-PLC family of tomato (right), showing domain structure and organization.** Abbreviations: PH, pleckstrin homology; EFh, EF-hand; EFh_L, EF-hand-like domain, X and Y, catalytic domains; C2, PKC conserved region 2; CC, coiled coil structure; SH2 and SH3, Src homology 2 and -3 respectively; RasGEF, guanine nucleotide exchange factor for Ras-GTPase; RA, Ras-binding domain; PDZ, ubiquitous protein-protein interaction domain of PSD-95, Discs-large and ZO-1 proteins (PDZ)-binding motif.

2.6. Phylogeny of PI-PLCs in higher plants

There is limited information about the evolution and diversity of PI-PLCs from higher plants. Phylogenetic studies on the *Arabidopsis thaliana* (Arabidopsis) *PI-PLC* gene family shows a complex evolution of this family via a number of duplication and/or relocation events on five different chromosomes (33). In addition, homology searches in public sequence databases, using the amino acid sequences of different tomato PI-PLC isoforms (shown to be enzymatically active) as queries, identified several unique orthologs distributed among various plant species (34). This is in accordance with previous studies showing that multiple PI-PLC isoforms exist in potato (*Solanum tuberosum*) (35), Arabidopsis (33) and tomato (36). Moreover, PI-PLC proteins from dicots and monocots cluster separately (34), suggesting a link between their evolution and the early genetic divergence between monocots and dicots (37). Alternatively, PI-PLCs may have evolved to be regulated differently or play different (signaling) roles in monocots and dicots.

2.7. Other types of plant PLCs

2.7.1. Non-specific PLCs

Non-specific PLC is different type of PLC which seems to exist only in plants (38,39). It shares sequence homology with bacterial phosphatidylcholine-PLC (PC-PLC) (16) and it mainly hydrolyze structural phospholipids like PC, phosphatidylethanolamine (PE) and has lower affinity towards phosphatidylserine (PS) and phosphatidylglycerol (PG) (39,40). In addition to the difference in substrate specificity between plant PI-PLC and non-specific PLC, the latter do not share any conserved domains with PI-PLC (41).

2.7.2. PI-PLC-like proteins

PI-PLC-like proteins are found in both animals and plants (28,42). This type of PLC proteins contains only one part of the catalytic core of PI-PLC, usually the X domain, and therefore is unable to hydrolyze PIs. Interestingly, a similar protein encoded by a gene named *DNF2* was recently shown to play a positive role during symbiosis in *Medicago truncatula*, presumably, by indirectly suppressing defense reactions induced by the nitrogen-fixing Rhizobium bacterium. It is speculated that it might be involved in attenuating defense signaling by binding PIs and preventing its hydrolysis by PI-PLC (42).

3. Components of the PI-PLC signaling pathway

The activity of PI-PLCs is required for receptor-mediated immune responses in animals and plants (34,36,43). Several suggestions were made regarding the characteristics of PI-PLC substrates and corresponding reaction products and their possible signaling role in the response of plants to biotic and abiotic stresses (44-48). Accordingly, the signaling functions of the PI-PLC substrates and their reaction products in plant immunity will be discussed.

3.1. Eukaryotic PI-PLC and its substrates

PI-PLC from plants and animals uses only three PI species as substrates. These are, in the order of preference, PI(4,5)P2, PI4P and PI (35,49-51). In plants, PI is the most abundant substrate, followed by PI4P and then by PI(4,5)P_2 (52,53). PI species which have a phosphate group at the D3 position of the inositol ring (i.e. PI3P and PI(3,5)P_2) do not serve as substrates for PI-PLC. The active site of eukaryotic PI-PLCs contains several basic amino acid residues which are required for the interaction with the phosphate groups at the D4 or D4 and D5 position(s) of PI4P and PI(4,5)P_2, respectively. This is an important difference compared with prokaryotic PI-PLC, which can only hydrolyze PI that lack these basic residues (23,49,54). Another consequence of this substrate preference and -specificity of eukaryotic PI-PLCs is that the hydrolysis of PI *in vitro* requires a much higher Ca^{2+}

concentration as compared to the hydrolysis of PI(4,5)P_2 or PI4P (35,36). The additional Ca^{2+} ions act as co-factors during the hydrolysis of PI (29).

3.2. PIs as membrane phospholipids

In order to understand the dynamics of PI-PLC signaling during defense activation, it is important to determine PI-PLC substrate preference *in vivo*. Biological membranes consist of glycerol-containing phospholipids (i.e. glycerophospholipids), in addition to other lipid types such as sphingolipids and sterols (55). Glycerophospholipids comprise PIs, structural phospholipids such as PC, PS, PE and PG and other types of phospholipids, e.g. glycolipids. The amphipathic nature of phospholipids (Figure 1) allows them to form the membrane bilayer. PIs constitute only a minor fraction, less than 10% of the total cellular lipids (56) and less than 15% of the total phospholipids of eukaryotic cells (57). PI4P and PI(4,5)P_2 form together approximately 10% of the total PIs and therefore it is unlikely that they have a structural role in the membrane (56,58). PI4P and PI(4,5)P_2 mainly reside at the inner leaflet of the PM in animal cells (58). A network of phospholipid phosphatases exists in animals and plants and these enzymes catalyze the reverse reaction which converts the PI-PLC substrates PI(4,5)P_2 to PI4P and PI4P to PI. Together with phospholipid kinases, they are responsible for a tight regulation of the levels of PI-PLC substrates in the membrane during the resting state and after receptor stimulation (59-61).

3.3. PI-PLC substrates present in membranes

The ratio between PI4P and PI(4,5)P_2 significantly differs between animals and plants. In plants, the percentage of PI4P compared to the total amount of phospholipids was found to be fairly similar to that in animals, which is 0.3% and 0.5%, respectively. The levels of PI(4,5)P_2 are, however, 10 to 20 fold lower in plants as compared to animals or lower plants, such as *Chlamydomonas reinhardtii* (62-64) and are 0.02% and 0.2%, respectively (65,66). The low amounts of PI(4,5)P_2 are possibly caused by a lower activity of the plant variants of PI phosphate kinase (PIPK) enzymes, which catalyze the generation of PI(4,5)P_2 from PI4P (64,67). This means that about 10 times more PI(4,5)P_2 needs to be rapidly synthesized in plants to serve as a PI-PLC substrate in order to produce sufficient amounts of the Ca^{2+}-releasing signal $InsP_3$ as compared to animal cells. It is therefore suggested that PI4P could be the prime substrate for PI-PLC in plants (47) or more likely serves as a signaling substrate for PI4P5 kinase (PI4P5K) to rapidly form PI(4,5)P_2 after receptor stimulation (50,68). Until this can be shown *in vivo*, the former suggestion faces a number of challenges to prove. First, despite of the low levels of PI(4,5)P_2 in plants, the molecule was shown to be functional as a substrate for PI-PLC *in vivo* during pollen tube growth in petunia (69) and tobacco (*Nicotiana tabacum*) (70). Also, despite the low level of PI(4,5)P_2 in tomato suspension-cultured cells, its amount rapidly decreased after treatment with the elicitor xylanase, and subsequent activation of defense responses, due to PI-PLC activation (71). Second, it is generally accepted that PIs, including PI(4,5)P_2,are not uniformly distributed throughout the PM but are concentrated in micro-domains known as lipid rafts (68,72). The local concentration of the PI(4,5)P_2 pool in these micro-domains is relatively high and its localized hydrolysis by PI-PLC is likely

sufficient to drive signaling at these specific locations in the membrane. Third, PI4P and $PI(4,5)P_2$ from plants and animals were shown to be hydrolyzed *in vitro* with an equal efficiency and under similar reaction conditions (34,73). However, the major cellular $PI(4,5)P_2$ pool is located at the PM both in animals (58) and plant cells (66), whereas the major PI4P pool is located at the Golgi (58) and PM (74,75) in animals and plants. This all suggests that the subcellular location of PI-PLC activity, which often changes after stimulation, in combination with the transient localized changes in substrate levels, will determine which PI-PLC substrate is used during defense signaling. Likewise, PI cannot be excluded from acting as a PI-PLC substrate during signaling under specific conditions as it exists in relatively high amounts in plants, when compared to PI4P and $PI(4,5)P_2$ (53,76). The hydrolysis of PI by PI-PLC *in vitro* requires higher Ca^{2+} levels (34,35), suggesting that PI might be secondarily used *in vivo* as a PI-PLC substrate when intracellular Ca^{2+} levels have already increased at a later stage after immune receptor activation.

4. PI-PLC substrates as signaling molecules

Several studies have shown that PIs, including the PI-PLC substrates PI, PI4P and $PI(4,5)P_2$, are involved in cellular signaling (77,78). This signaling function is mainly due to the physical and chemical properties of these molecules, as determined by the chemical composition and charge of their inositol head groups and the nature of their fatty acid tails. The mechanism by which the PI-PLC substrates exert their function in the cell is suggested to involve modulation of the membrane properties, like membrane packing, curvature and fluidity, thereby modulating global cellular processes such as vesicle trafficking and actin cytoskeleton organization (79). This change in membrane properties also affects the distribution of signaling proteins and ion-channels along the membrane. For example, $PI(4,5)P_2$ activates a PLA2 by increasing its catalytic activity through increased membrane penetration (80). Furthermore, the specific binding of signaling proteins to different PI species can lead to their translocation in response to stimulation (81).

4.1. $PI(4,5)P_2$ and PI4P as substrates during PI-PLC signaling

$PI(4,5)P_2$ functions as a substrate for PI-PLC and its cleavage results in the formation of the signaling molecules DAG and $InsP_3$. The synthesis of $PI(4,5)P_2$ from PI4P by PI4P5K is proposed to be limiting for the flux of $InsP_3$ resulting from PI-PLC-mediated $PI(4,5)P_2$ cleavage (68). Remarkably, a type of mono-phosphatidylinositol and not $PI(4,5)P_2$ was reported to be hydrolyzed *in vitro* by PI-PLC upon recognition of two type III-secreted bacterial effectors, AvrRpm1 and AvrRpt2 from *Pseudomonas syringae*, by the nucleotide-binding leucine-rich repeat (NB-LRR)-type of resistance proteins RPM1 and RPS2, respectively (82). As PI4P is more abundant and is the only mono-phosphatidylinositol that can be hydrolyzed by PI-PLC (34,35), we anticipate that the observed decrease in mono-phosphatidylinositol levels was caused by the degradation of PI4P. As the hydrolysis of PI4P would produce $InsP_2$, which is not known to function in internal Ca^{2+} release,

phosphorylation of $InsP_2$ by InsP-kinases to $InsP_3$ or to further phosphorylated isoforms, is expected to be responsible for the observed increase in free cytoplasmic Ca^{2+}. Moreover, elicitor treatment of suspension-cultured soybean (*Glycine max*) cells led to the accumulation of $InsP_3$ and the simultaneous reduction in the levels of both PI4P and $PI(4,5)P_2$, suggesting an activation of PI-PLC and the hydrolysis of the former two substrates *in vivo* (83). It was suggested that PI4Kα1 and PI4Kβ1 enzymes from Arabidopsis are responsible for distinct PI pools (68). The $PI(4,5)P_2$ pool essentially containing saturated and mono-unsaturated fatty acids was found to be associated with clathrin coated vesicles (CCVs), indicating a positive role for this pool in endocytosis and membrane recycling during hyperosmotic stress (84). In contrast to this finding, inhibition of PI-PLC activity by the PI-PLC inhibitor U73122 in Arabidopsis suspension-cultured cells treated with the flagellin-derived peptide flg22, suppressed effector-mediated endocytosis of the matching receptor-like kinase flagellin sensing 2 (FLS2), thereby blocking the immune response (Abd-El-Haliem et al., 2014). Furthermore, pretreatment with U73122 also suppressed the defense reaction in response to flg22 which was observed as a suppression of proton efflux in tobacco suspension-cultured cells. This suggests that lower levels of $PI(4,5)P_2$ are required for defense signaling and flg22-mediated endocytosis of FLS2. A similar observation was found in animals where the activity of PLCγ was required for the internalization of Toll-like Receptor 4 (TLR4) in response to lipopolysaccharide (LPS) (43,85,86). Moreover, suppression of membrane endocytosis and rhizobia internalization into the host cells was also observed after treatment of the roots of *Medicago truncatula* with the PI-PLC inhibitor U73122. This treatment also suppressed the rhizobia-induced oxidative burst and reduced the curling of root hairs and the formation of infection threads (87). This all indicates a positive role for the hydrolysis of $PI(4,5)P_2$ by PI-PLC on receptor-mediated endocytosis.

In yeast (*Saccharomyces cerevisiae*), it is suggested that distinct pools of PI4P and $PI(4,5)P_2$ are generated by two PI4K enzymes that act on different subcellular membranes (88). PM-localized $PI(4,5)P_2$ was also found to control gene transcription by binding certain transcription factors and thereby sequestering them to the PM, away from the nucleus. This was demonstrated for the animal transcription factor Tubby, which binds $PI(4,5)P_2$ in the PM via a specialized C-terminal "tubby" domain. After receptor-mediated activation of PI-PLCβ at the PM by G protein α_q, $PI(4,5)P_2$ is hydrolyzed and tubby is released and targeted by its nuclear localization domain to the nucleus where it initiates gene transcription (89). A similar mechanism of regulation was also identified for tubby-like protein 3 (TULP3) in plants (90,91) and tubby-like proteins in animals (89). It is therefore conceivable that PI-PLC activity regulates gene transcription during different plant responses, including those triggered by receptor stimulation, and the mechanism of transcriptional regulation could be similar.

Furthermore, ion-channel control by $PI(4,5)P_2$ is very important in signaling as it links receptor activation with several very early down-stream responses. It also postulates that several PI-metabolizing enzymes, including PI-PLCs, may control the activity of different ion channels by modulating the levels of $PI(4,5)P_2$ in the PM. In plants, the levels of $PI(4,5)P_2$ were shown to control the efflux of K^+ from plant cells by regulating the activity of the Shaker-like outward-rectifying K^+ channel *Nt*ORK in *Nicotiana tabacum* (92). This is similar to the findings from previous studies which confirm the inverse correlation between $PI(4,5)P_2$

levels in the membrane and the activity of Arabidopsis SKOR-like (Shaker-like outward-rectifying K^+) channels in guard cells (93-96). In line with these findings, in guard cells of *S. tuberosum* both $InsP_3$, which is released by PI-PLC activity, and its derivative $InsP_6$ inhibited the K^+-influx via an inward-rectifying K^+ channel (97). The inhibition, which mimics the effects of ABA and results in stomatal closure, was dependent on Ca^{2+}-mobilization from endomembrane stores (98). Increasing the accumulation of $PI(4,5)P_2$ by using the PI-PLC inhibitor U73122 led to a decrease in NtORK activity (92). This suggests that PI-PLC is involved in regulating cytoplasmic K^+ levels by controlling the hydrolysis of $PI(4,5)P_2$ and the synthesis of $InsP_3$.

A close look at the previous findings in the context of immune signaling may allow us to envisage the early steps of immune receptor signaling. For example, ABA-mediated hydrolysis of $PI(4,5)P_2$ upon the activation of PI-PLC in guard cells will cause a depletion of K^+ ions as a result of opening the *Nt*ORK channel, a subsequent reduction of water uptake and loss of turgor pressure of the guard cells, with closing of the stomata as a result (99). On the other hand, stimulation of plant immune receptors in plant cell types other than guard cells is also suggested to activate PI-PLC to hydrolyze of $PI(4,5)P_2$. This will then lead to the activation of outward-rectifying K^+ channels and the depletion of K^+ ions form the cytoplasm (92). The drop in K^+ levels by these cells is likely to be interpreted differently than in guard cells. It was shown that plant cells have to maintain a threshold level of K^+ ions in order to re-enter the cell cycle (100). Failure to reach this threshold results in cell cycle arrest (100,101), which is known to be an early event that precedes the execution of programmed cell death (PCD) in animal cells (102). The depletion of K^+ ions from tobacco BY-2 protoplasts led to a decrease of the cytoplasmic pH and both events were important for the cell cycle to continue and for the cells to divide (103). This is in line with the observation that increasing the pH of the protoplast medium leads to the hydrolysis of $PI(4,5)P_2$ and increases the activity of NtORK (92).

Interestingly, activation of plant immune receptors by corresponding elicitors creates a similar situation, in which a simultaneous medium alkalization and acidification of the cytoplasm occurs. These conditions are for example observed when the resistance protein Cf-4 is activated by the matching effector Avr4 (34,104). Cf-4 is a PM-localized receptor-like protein (RLP) that confers resistance to the fungal pathogen *Cladosporium fulvum* in tomato plants by recognizing the secreted fungal effector Avr4 (105). Cf-4/Avr4 interaction culminates in local cell death, known as the hypersensitive response (HR). Indeed, we found that pretreatment of tobacco suspension-cultured cells expressing Cf-4 by the PI-PLC inhibitor U73122, blocks the medium alkalization response and prevents cell death (34). At the same time, transient over-expression of tomato *SlPLC3* and *SlPLC4* was found to significantly enhance cell death in *N. benthamiana* leaves (34,36). Furthermore, silencing of *SlPLC4* in tomato plants expressing Cf-4 using virus-induced gene-silencing (VIGS) was found to suppress cell death upon transient expression of Avr4. All these findings strengthen the link between PI-PLC activation and the hydrolysis of $PI(4,5)P_2$ to generate Ca^{2+}-releasing signals, trigger the pH-shift and lowering the cellular level of K^+ ions during plant immune responses.

Although PI-PLC activation is utilized in both ABA- and defense-related signaling, there is another distinguishing feature between ABA- and defense-related responses, which is

the cytoplasmic pH. The cytoplasm is known to become alkaline in response to ABA (106,107) and this is crucial to initiate downstream ABA responses, including the regulation of the transcription of ABA-related genes. In contrast, cytoplasmic acidification occurs after the activation of immune receptors, a process that is phosphorylation-dependent and essential for the continuation of the defense signal downstream of the receptor leading to the execution of appropriate defense actions (108-111). Therefore, it reasonable to conclude that the activation of PI-PLC is an essential early defense response regulating the cytosolic levels of free Ca^{2+} and together with cytosolic pH determine the final cellular response.

Interestingly, RxLR effectors of oomycete pathogens shown to bind PI3P and weakly to PI4P, thereby facilitating the delivery of the effectors into the cell (112). More recently, it was demonstrated that the RxLR effector AVR3a of *Phytophthora infestans* binds to PI3P, PI4P and PI5P, which increases the stability of the effector protein *in planta* and stimulates its accumulation in the host cells. Binding is not required for recognition by the NB-LRR resistance protein R3a but enhances the virulence function of the effector, which is inhibiting the target E3 ubiquitin ligase CMPG1and suppressing INF1-triggered cell death (113). This indicates that the recognition of effectors by NB-LRR receptors may occur with higher sensitivity, requiring lower amounts of the effector protein than the amount of effector molecules required to effectively suppress defense. Therefore, PI-PLC activity may play a positive role in suppressing effector function in virulence by reducing the levels of PI3P, PI4P and PI5P, thereby negatively affecting effector stability.

4.2. PI as a substrate during PI-PLC signaling

PI acts as a substrate for numerous enzymes involved in cell signaling. Its importance in signaling is mainly due to the fact that it can be phosphorylated by distinct kinases at the D3, D4 and D5 positions of the myo-inositol ring to produce PI3P, PI4P and PI5P, respectively. The generated PI4P can be further phosphorylated to produce $PI(4,5)P_2$. As mentioned earlier, PI, PI4P and $PI(4,5)P_2$ were shown to function as PI-PLC substrates *in vitro* (34-36). However, it has yet to be demonstrated that PI itself can be directly utilized as a PI-PLC substrate *in vivo*. PI plays an essential role in determining the nature of the substrate pools of PI4P and $PI(4,5)P_2$, which are used during PI-PLC signaling. This is because the enzymes responsible for PI synthesis, PI synthase 1 (PIS1) and PIS2 from Arabidopsis, use different cytidinediphospho-diacylglycerol (CDP-DAG) species with different fatty acid compositions as a substrate and thus generate different PI pools. Moreover, PI seems to play an effective but indirect role in PCD by acting as a precursor for the synthesis of inositolphosphorylceramide (IPC) by IPC synthase (IPCS). IPC is a sphingolipid species that is found in plants and other organisms (114,115). IPCS transfers the inositol phosphate (IP) head group from PI to ceramide in order to form IPC (116,117). Failure of the synthesis of IPC, due to either the malfunction of IPCS (115) or the unavailability of the precursor PI (118), leads to an increase in the levels of ceramide, which induces cell death in plants (118,119). It will be intriguing to determine whether the levels of PI in plant cells mounting a defense response is controlled by PI-PLC activity and whether this affects the levels of ceramide to finally control PCD.

5. Products of the PI-PLC pathway as signaling molecules

The canonical pathway of PI-PLC signaling in animal cells involves the hydrolysis of $PI(4,5)P_2$, occurring mainly at the PM, thereby generating the second messenger molecules $InsP_3$ and DAG. As mentioned earlier, $InsP_3$ is considered as an intracellular mobile signal which is water-soluble so it diffuses into the cytoplasm to remotely cause an increase in the levels of intracellular free-Ca^{2+} via binding to an $InsP_3$-gated channel in the ER. In contrast, DAG forms an immobile signal as it remains attached to the membrane via its acyl chains. The same holds for other DAG-related metabolites like PA and diacylglycerol pyrophosphate (DGPP). This means that the generated immobile signals are exclusively responsible for the part of the PI-PLC signaling which occurs at the PM.

5.1. $InsP_3$ and $InsP_6$ as signaling molecules

Early studies showed that Ca^{2+} release occurs through specific Ca^{2+} channels in the vacuolar membrane after stimulation of red beet cells with micro-molar concentrations of $InsP_3$ ($Ins(1,4,5)P_3$) (120,121). On the other hand, using up to 20-fold higher concentrations of $Ins(1,4)P_2$, $Ins(1,3,4)P_3$, and $Ins(1,3,4,5)P_4$ had no effect (121). Because $InsP_3$ is diffusible, its concentration in the cytoplasm is likely to be critical for the activation of the corresponding $InsP_3$-receptor ($InsP_3$-R) and the subsequent release of free Ca^{2+} ions in the cytoplasm. $InsP_6$ was found to be a ~100-fold more potent modulator of this channel than $InsP_3$ (97). As the levels of $PI(4,5)P_2$ are 10- to 20 fold lower in plants than in animals, the transient formation of sufficient levels of $InsP_3$ during PI-PLC signaling may require a significant increase in the level of $PI(4,5)P_2$. Indeed, $PI(4,5)P_2$ was shown to increase in plants during the response to abiotic stress. However, this increase was not observed upon exposure to biotic stress, which often involves the activation of immune receptors (52), except for one report where the levels of $PI(4,5)P_2$ increased after treating pea with an elicitor from the fungus *Mycosphaerella pinodes* (122). As both types of stresses are known to involve enhanced PI-PLC activity, it is questionable how intracellular Ca^{2+} release and the activation of downstream responses are controlled by the generally anticipated low levels of $InsP_3$, generated from hydrolysis of $PI(4,5)P_2$ by PI-PLC or by its subsequent phosphorylation product, $InsP_6$. An answer to this question could be that maximum levels of $InsP_3$ should be produced close to its receptor, $InsP_3$-R, which resides in the ER of animal cells. This can be achieved by localizing the $InsP_3$-R to the PM-ER anchoring points (123-125), and specifically to the lipid microdomains, which are inriched in PI4P and $PI(4,5)P_2$ (72,126,127). Previous findings are in line with this suggestion, as $InsP_3$-R in animal cells associates with the detergent-resistant membrane fraction via its C-terminal membrane anchoring domain (128,129). Alternatively, PI-PLC might use the more abundant PI and PI4P as substrates to produce InsP and $InsP_2$, respectively, that can be phosphorylated by InsP-kinases to produce the $InsP_3$-R activators $InsP_3$ and $InsP_6$. $InsP_3$ production was shown to be supplied mainly by the unsaturated pool of PIPs. This was observed in transgenic plants which were disrupted in phosphoinositide metabolism, confirming the existence of two signaling pools of either saturated or unsaturated PIPs (130).

It is still unclear how PI-PLC signaling via $InsP_3$ or $InsP_6$ functions in plants, as the receptors of these molecules have not been identified in the Plant Kingdom. The availability of the full genome sequences of many plant species encouraged us to carry out a basic homology search by tBLASTn on the NCBI Blast search site (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). For this, the amino acid sequences of $InsP_3$-R from fruit fly (*Drosophila melanogaster*, accession P29993) and mouse (*Mus musculus*, accession P11881) were used as a query. None of the BLAST search results revealed the presence of orthologs for $InsP_3$-R in higher plants. Interestingly, a putative $InsP_3$-R ortholog was found in the single-cell green alga *C. reinhardtii*. The existence of $InsP_3$-R in *C. reinhardtii* and not in higher plants correlates with higher $PI(4,5)P_2$ levels in *C. reinhardtii*, similar to animal cells (52,66,76,131), suggesting significant differences in PI-PLC signaling by soluble signals between higher and lower plants. On the other hand, numerous studies have demonstrated the intracellular release of Ca^{2+} by $InsP_3$ in several plant species (132). This occurred in response to an array of different biotic and abiotic stimuli, including hyperosmotic stress, cold, heat, gravi-stimulation and treatment with several plant pathogen-derived elicitors. For reviews see: (132-135). This accumulating evidence has led to the conclusion that $InsP_3$ acts as a positive regulator of many signaling pathways in plants (132).

In addition to modulating signaling via swift changes in the levels of $InsP_3$, the sustained production of $InsP_3$ seems to play an important role in signaling as well (136-139). $InsP_3$ appears to function in plants as a ligand that is perceived by ligand-gated Ca^{2+}-ion-channels (140). Alternatively, the release of intracellular Ca^{2+} could occur indirectly via another mechanism, which does not involve direct binding of $InsP_3$ to a receptor (Coté and Crain, 1993). It is also unknown whether in plants $InsP_3$ and $InsP_6$ stimulate the same receptor or each stimulates a separate one. $InsP_6$ has long been known for its role as a phosphate-storing molecule, especially during seed development (141,142). Furthermore, $InsP_6$ was found recently to molecularly associate with the F-box proteins TIR1 and COI1, suggesting a function in regulating auxin and jasmonic acid (JA) responses, respectively (143,144). Moreover, a role for $InsP_6$ in mRNA export from the nucleus in yeast and animals was discovered, although a similar role in plants has yet to be demonstrated (145).

A reduction of the level of $InsP_6$ in Arabidopsis carrying a mutation in either *L-myo-inositol 1-phosphate synthase 1* or *-2* (*atips1* or *atips2*), was found to have different effects on basal resistance (146). Recently, *atips1* mutant plants were shown to express enhanced basal resistance to *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*), which was dependent on the accumulation of salicylic acid (SA) (147). This observation led to the hypothesis that distinct subcellular pools of $InsP_6$ are required for pathogen resistance (146). It is therefore likely that PI-PLC activity positively affects resistance to multiple pathogens by controlling the levels of $InsP_3$ and InsP6. However, more studies are required to investigate this in detail.

5.2. DAG and PA as signaling molecules

In addition to the release of soluble $InsP_3$, PI-PLC activity simultaneously results in the accumulation of DAG in the PM. This DAG is generated by the hydrolysis of any of the previously discussed substrates PI, PI4P or $PI(4,5)P_2$. DAG can also be produced by the dephosphorylation of PA by lipid phosphate phosphatase (LPP), by PA-phosphatases (PAP)

or by the activity of sphingomyelin synthase (SMS), which catalyzes the transfer of phosphocholine from PC to ceramide, thereby producing sphingomyelin and DAG. Moreover, DAG can be generated by the hydrolysis of structural phospholipids like PC, PE and PS by non-specific PLC enzymes (39,40).

5.2.1. Signaling via PI-PLC-generated DAG

DAG plays a role as an intermediate for lipid metabolism, especially galactolipids, but certain DAG species function as important second messenger molecules in animal cells (38,148). Here the role of sn-1,2-diacylglycerol, referred to as DAG, which has low abundance in animal (149) and plant cells (148) will be discussed. This type of DAG is extensively studied in animals due to its demonstrated function in cellular signaling. These DAG molecules mainly contain polyunsaturated fatty acids (38,150). Thus, the type of their fatty acids reflects the composition of their precursor phospholipids and indicates whether the generated DAG pool is involved in signaling. Based on this, not all sn-1,2-diacylglycerols are equally important signaling molecules. For example, PLD produces PA by hydrolyzing the membrane structural phospholipids such as PC and PE. The activity of PAP on the generated PA produces DAG, which predominantly contains saturated fatty acids as in the structural phospholipids that are hydrolyzed. It was previously suggested that the pool of PA that is generated by diacylglycerol kinase (DGK) may differ from that produced by PLD with respect to the fatty acyl composition and the two pools may be functionally unrelated (150,151). Similarly, the transferase activity of SMS leads to the production of DAG that contains saturated fatty acids. This type of DAG is likely to be channeled towards the metabolism of cellular and membrane lipids rather than playing a direct role in early signaling (38). Most of the PI-PLC-generated DAG is rapidly phosphorylated by DGK to produce PA. Remarkably, most studies focused on changes in the levels of PA rather than changes in DAG levels and studying the effects of these alterations.

5.2.2. Why is the role of DAG in signaling underestimated?

The lack of studies on DAG accumulation in plants in response to external stimulation and its role as a second messenger molecule in plants is often explained by the low abundance of DAG and its rapid conversion to PA or to other lipids (131,148,152-154). In general, technical difficulties in measuring the DAG concentrations and distinguishing between the different molecular species of DAG in the cell may have also hindered studying DAG as a signaling molecule in plants. Another reason for the lack of DAG studies as a signaling molecule is likely the absence of structural homologs of PKC from the genomes of sequenced plants. This is while several PKC family members function as important initiators of signaling downstream of several cellular receptors in animals (155). PKC is recruited to the PM via its cysteine-rich C1 domain, which binds a signaling pool of DAG leading to a conformational change and allosteric activation of the enzyme (156,157). PKC activation tends to prefer polyunsaturated- rather than saturated DAG species (150). The lack of PKC in plants relates to the fact that plants lack structural homologs of $InsP_3$-R. This led to the notion that PI-PLC signaling in plants might be mediated by different signaling molecules than in

animals, in which $InsP_6$ and PA are the signaling molecules instead of $InsP_3$ and DAG that play a central role in plants (47).

5.2.3. Signaling functions of DAG and PA in the PI-PLC/DGK pathway

Intriguingly, although PKC and $InsP_3$-R are lacking in plants, both the effects of $InsP_3$ on the release of cytoplasmic Ca^{2+} (132) and the existence of PKC-like activities have been observed (47). Phorbol esters are tetracyclic diterpenoids, which are stable analogs of DAG that cannot be converted to PA. They mimic the action of DAG, in activating PKC-like signaling in different plant species (158,159). Phorbol esters were also found to stimulate elicitor-mediated synthesis of defense metabolites such as phytoalexins, whereas inhibition of PI-PLC- and PKC-like activity blocks this response (160). This suggests that PI-PLC-generated DAG plays a positive role in plant defense. Application of either DAG or phorbol ester to suspension-cultured *Rubus fruticosus* cells was found to induce defense, which is displayed as an increase in the activity of the defense enzyme laminarinase in the absence of oligo- and polysaccharide elicitors. Application of a PKC inhibitor also blocked this response, suggesting the involvement of PKC-like signaling in plant defense (161). Before the genomic era and the discovery of hundreds of plant kinases, it was believed that it would be a matter of time before the first ortholog of PKC would be discovered in plants. This was especially the case after the study of R. Subramaniam et al. (159), who used PKC-inhibitors and antibodies, in addition to the detection of phosphorylation of a PKC-specific substrate, to demonstrate the existence of PKC activity in *S. tuberosum*. Soon it became clear that the described plant kinases are fundamentally different from animal PKC, as none of them require phospholipids like PS to be activated. Moreover, none of these plant kinases was activated by DAG in the presence of low concentrations of Ca^{2+}, as was known for conventional PKC from animals (65,162,163). The described PKC-like activities of plants are currently thought to be due to different families of plant kinases that do not exist in animals.

Another role of DAG in plant defense was demonstrated in rice protoplasts, in which simultaneous silencing of multiple *DGK* isoforms by introduced double-stranded RNA leads to suppressing xylanase-induced expression of the pathogenesis-related gene *OsNPR1*and the defense-related transcription factor *OsWRKY71* (164). Silencing of DGK is expected to increase the accumulation of DAG, produced as a result of the activation of PI-PLC and to simultaneously suppress the accumulation of PA. It is however not clear which of these two signaling molecules is responsible for the observed transcriptional changes. Interestingly, inhibition of DGK activity in pea (*Pisum sativum*) epicotyl tissues by the compound R59022 prevented the conversion of PI-PLC-generated DAG to PA and thereby enhanced elicitor-mediated accumulation of the phytoalexin pisatin. It also enhanced the transcription and activity of the defense-related enzyme phenylalanine ammonia-lyase (PAL) (165). These findings suggest a negative role for DGK activity in defense, similar to such a role in animals (166). It also indicates that production of PA via the PI-PLC/DGK pathway can suppress plant defense, and that production of DAG via PI-PLC activity enhances defense, at least in the studied systems. Moreover, suppression of PA accumulation via PLD by silencing the rice *OsPLDβ1* gene induced the accumulation of reactive oxygen species (ROS), transcription of defense-related genes, accumulation of the phytoalexin momilactone-A and

caused a marked increase in resistance of rice to the blast fungus, *Pyricularia grisea*, and the bacterial blight pathogen, *Xanthomonas oryzae* (167). This all suggests that PA, including the PA pool generated by the activity of PLD, plays a negative, rather than positive role, in plant defense to microbes. In contrast, overexpression of the rice DGK gene *OsBIDK1* in tobacco enhanced resistance to tobacco mosaic virus (TMV) and *Phytophthora parasitica* var. *nicotianae* (168). This discrepancy may be explained by the existence of different signaling pools of DAG and PA with contrasting effects on resistance, or by the activation of distinct PI-PLC and DGK isoforms in response to infection by various types of pathogens.

In animals, application of phorbol esters and DAG can also mediate cellular responses by the activation of non-PKC proteins that possess a DAG-binding C1 domain and which displays a high affinity for binding the phorbol ester (156). Similarly, many C1-containing proteins are predicted in plants (152). Since these proteins are not kinases, the complexity of DAG signaling seems to exceed the activation of PKC or PKC-like kinases. The PA pool that is produced by the PI-PLC/DGK pathway accumulates very early upon the triggering of immune responses in plants (52,71,82). Nevertheless, only a small portion of the accumulated PA was attributed to the activity of PLD during effector triggered immunity (ETI) (52,82) and after elicitation of defense, referred to as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), with the fungal elicitor xylanase (71). Interestingly, elicitation of tomato suspension-cultured cells with a chitin analog or with flg22 activated only the PI-PLC/DGK pathway (71). On the other hand, nitric oxide (NO) was found to control PLD-mediated PA accumulation by chitin but not by xylanase (169).This indicates that activation of defense by different microbial elicitors is distinct in requiring either PI-PLC or PLD or both. Moreover, treatment of *Vicia sativa* with symbiotic Rhizobium-secreted nodulation factors (Nod factors), which are lipo-chitooligosaccharides, induced the accumulation of PA via the PI-PLC/DGK pathway and only partially via PLD (170). PLD activity was also suggested to play a negative role in defense during symbiosis, allowing colonization of the host by the symbiotic bacteria (171). Treatment of suspension-cultured *Medicago sativa* (Alfalfa) with Nod factors, chito-tetraose or xylanase, induced the formation of PA by the activation of the PI-PLC/DGK pathway while treatment with Nod factor activated both PI-PLC and PLD (171). This demonstrates that the crosstalk between PI-PLC/DGK- and PLD signaling pathways is important for differentiating between responses of plants in relation to defense and symbiosis.

Exogenous application of PA, in the form of a synthetic short-chain, and thus water-soluble PA analogue, was found to mimic ETI in the Cf-4/Avr4 interaction and induces an oxidative burst (52) and triggers the activity of PAL and the accumulation of phytoalexins (172). On the other hand, the Avr4-induced accumulation of PA in transgenic tobacco cells expressing the Cf-4 resistance protein was specifically blocked by the PI-PLC inhibitor U73122, but not by the NADPH oxidase inhibitor DPI. Similarly, the PI-PLC inhibitor blocked the Avr4-induced accumulation of ROS (52). These findings indicate that PA generated from the PI-PLC/DGK pathway during defense is upstream of the oxidative burst. A similar treatment with different PA species, containing either long or short acyl chains, was found to induce the expression of the pathogenesis-related gene *PR1* and to cause chlorosis in Arabidopsis leaves (82). Moreover, both exogenously applied PA and DAG were found to induce ROS accumulation in the absence of the elicitor (153) and therefore each of them may

act as a signaling molecule during defense. DAG and PA also induced the expression of elicitor-responsive genes in the absence of the elicitor. Although the compounds were not able to induce phytoalexin biosynthesis by themselves, they significantly enhanced the elicitor-induced phytoalexin accumulation, indicating that a state of priming takes place (173). In conclusion, both DAG and PA seem to have a signaling function in plant defense and immunity and two distinct PA signaling pools with diverse functions appear to be generated either via the PLD- or PI-PLC/DGK pathways during recognition of microbial molecules by immune receptors and subsequent defense responses and resistance.

5.2.4 Mode of action of DAG and PA in defense-related signaling

The mode of action of PA and DAG in defense-related signaling roughly involves two mechanisms. The first relies on the differential modulation of membrane curvature and packing due to differences in the physical properties and charges between PA and DAG molecules and between these compounds and their hydrolyzed precursors (154). The second mechanism involves recruiting and binding of cytosolic signaling proteins to certain membrane subdomains, leading to either their activation or inactivation (51,174). Similar to the effect of DAG in activating PKC in animals, several PA-binding targets were identified in animals (175) and plants (51,174,176). For example, PA, in addition to the phospholipids PS and PI, activates a calcium-dependent protein kinase (CDPK) in carrot (*Daucus carota*) (177). PA also binds to Arabidopsis phosphoinositide-dependent protein kinase 1 (AtPDK1) (178), which is the ortholog of the animal central kinase PDK1 that regulates growth, cell division and apoptosis (179,180). PA binding stimulates the phosphorylation and activation of the AGC2-1/OXI1 protein kinase, which is required for root hair growth and oxidative burst-mediated signaling in Arabidopsis (178,181) and essentially regulates basal disease resistance in rice (182). Furthermore, PA can be deacetylated by PLA enzymes to form free fatty acids and lysoPA, and both are known to have signaling functions (45,183).

5.2.5 DGPP as a signaling molecule

PA can be phosphorylated by PA-kinase (PAK) to produce diacylglycerol pyrophosphate (DGPP) (184). DGPP is found only in plants, yeast and bacteria but not in animals (185,186). Similar to PA, DGPP also accumulates during responses to biotic (71,170) and abiotic (187,188) stresses. In addition to the aforementioned lipid kinases DGK and PAK, lipid phosphatases co-participate in the regulation of the levels of DGPP, PA and DAG. However, it is unclear whether DGPP acts as a signaling molecule on its own, or whether its formation is a means to lower the amount of PA, thereby suppressing PA-signaling (184,189). The accumulation of DGPP often occurs together with that of PA, as was for example observed during the activation of ETI (52), and PTI using general elicitors in suspension-cultured tomato cells (71). PA and DGPP also accumulated in response to treatment of *Vicia sativa* with Nod factors (170), mastoparan (170,184), hyperosmotic stress and salinity (187,188).

6. Putative regulation mechanisms of PI-PLC activity during defense

Receptor-mediated activation of PI-PLC is evident in animals since the discovery of the rapid turnover of phosphoinositides after receptor activation (4,5). The factors which regulate the enzymatic activity of PI-PLC are better understood in animals than in plants. In animals, class-specific regulatory domains define which enzyme activators, e.g. heterotrimeric guanine nucleotide–binding protein (G protein) subunits, protein tyrosine kinases or small G proteins, activate members of each PI-PLC class. The situation is different in plants due to the absence of these regulatory domains, which makes it difficult to predict whether similar activation mechanisms are involved. Interestingly, PI-PLC enzymes from both plants and animals share their requirement of the divalent cations Ca^{2+} and Mg^{2+} for their activity and act on the same type of substrates under similar reaction conditions (28,30,34,35,190-192). Furthermore, recent studies have shown the existence of an intra-molecular activation mechanism, which involves the X/Y-linker region and regulates enzyme activity in animal PI-PLCs (27). Sequence comparisons suggest that such a regulatory mechanism might also hold for plant PI-PLCs (34). Here, the established and putative regulatory mechanisms of PI-PLC enzyme activity in plants, based on similarities with animal counterparts, will be discussed.

6.1. Levels- and mechanisms of PI-PLC regulation

Little is known about the mechanisms behind the regulation of PI-PLC activity during defense responses in plants. However, recent studies showed that upon imposing biotic or abiotic stress regulation occurs at both the transcriptional (33,36,158) and protein level (158,193,194). Activation of PI-PLC is evident in tobacco suspension-cultured cells expressing Cf-4 after the addition of the *C. fulvum* effector Avr4 and leads to a rapid, within minutes, accumulation of PA via the PLC/DGK pathway (52). The short time required for PI-PLC activation indicates the activation of pre-existing PI-PLC enzymes, rather than transcriptional regulation, which requires relatively much longer time. The short time span required for PA synthesis from DAG suggests that DGK activation shares a similar spatiotemporal regulation as PI-PLC in response to immune receptor stimulation. This could be either by the existence of one protein complex containing both PI-PLC and DGK, possibly in close proximity of the activated receptor and/or possible co-receptors. Alternatively, a rapid recruitment mechanism sequesters these two enzymes from the cytoplasm and positions them in the vicinity of their substrates in the PM to eventually synthesize PA.

Tomato *PI-PLC* genes were also found to be differentially expressed after inoculation of tomato resistant to *C. fulvum* (36). Moreover, tomato hybrid seedlings mounting a defense reaction due to expressing both the Cf-4 receptor and the Avr4 effector protein, show differential expression patterns for distinct *PI-PLC* gene isoforms (76). Interestingly, the expression of *SlPLC3* and *SlPLC6* was upregulated in response to both defense activation and an elevated temperature (76). This indicates that transcriptional regulation of the *PI-PLCs* exists and might be responsible for regulating later responses. It also suggests that distinct plant PI-PLC isoforms are required for responses to different types of stresses.

6.1.1 Protein phosphorylation as a means to activate PI-PLC

The swiftness of PI-PLC activation during defense (52) suggests the involvement of post translational modifications (PTMs) and/or the rapid recruitment of the enzyme to the sub-cellular location where the substrate is present. Indeed, earlier studies have shown that PTM is required for the regulation of the activity of different PI-PLC enzymes in animals (Özdener et al., 2002; Katan et al., 2003). Interestingly, in this case the phosphorylated residues were located within the X/Y-linker. The effect of the phosphorylation on the enzymatic activity varied according to the class of the PI-PLC enzyme and leads to either activation or deactivation of the enzyme (23,195). Phosphorylation of both serine and tyrosine residues was also detected in the EF-hand-like and Y domains of *At*PLC2 and in the catalytic X domain of *At*PLC7 (158). *At*PLC2 was also found to be phosphorylated at a serine residue in the X/Y-linker, shortly after treating suspension-cultured cells with the bacterial elicitor flagellin (193). Sequence alignments between *At*PLC2 and the tomato PI-PLC isoforms predicted a putative phosphorylation motif (EPSS) in tomato *Sl*PLC4 (36). Sequence similarity searches, using Pattern Hit Initiated BLAST (PHI, NCBI; www.ncbi.nlm.nih.gov), identified phosphorylation motifs similar to that of *At*PLC2 and *Sl*PLC4 in PI-PLCs of several plant species within the *Solanaceae*, and also in *Torenia fournieri* and *Vitis vinifera* (see supplemental information, Figure S1). A similar phosphorylation motif was previously identified in PI-PLCβ1 from mouse, which is phosphorylated by a signal-regulated kinase and leads to the activation of PI cycle in the nucleus. Nuclear PI cycle is a signaling pathway activated by insulin-like growth factor I (IGF-I) and requires the hydrolysis of PIs in a manner that has both similarity and differences compared to the one occurred in the membrane (196). Furthermore, studies on lemon (*Citrus limon*) seedlings using protein tyrosine kinase inhibitors showed suppression of the PI-PLC-mediated increase in $InsP_3$ in response to inoculation with conidia of the fungus *Alternaria alternata*, suggesting that phosphorylation of PI-PLC is vital for its activation (197,198). Homology analysis and prediction of phosphorylation of the tomato PI-PLC isoforms shows enrichment in the number of putatively phosphorylated residues within the X/Y-linker when compared with the full-length protein sequence (34). This all suggests that phosphorylation of plant PI-PLC within the X/Y-linker may be a means to regulate its activity after stimulation of an immune receptor.

6.1.2. Regulation of PI-PLC activity by other PTMs

In addition to phosphorylation, other PTMs, like sumoylation and nitrosylation were recently shown to be involved in the regulation of specific isoforms of plant PI-PLCs. For example, *At*PLC8 was found to be a specific target for sumoylation as a result of subjecting Arabidopsis seedlings to a 30 min heat-shock treatment at 39°C (194). Sumoylation implies the conjugation of a small ubiquitin-like modifier (SUMO), thereby affecting protein stability and nuclear-cytosolic transport. However, the exact effect of sumoylation on *At*PLC8-mediated signaling is unknown.

Protein nitrosylation and nitration are additional PTMs, which involve the covalent incorporation of a NO moiety into thiol groups of specific cysteine residues of proteins

(199,200) and the introduction of a nitro group (-NO_2), often into a tyrosine residue, respectively (200). NO treatment of *Vicia faba* epidermal peels was found to induce stomatal closure and PA accumulation via the PI-PLC/DGK and PLD pathways, whereas the PI-PLC inhibitor U73122 suppressed NO-induced PA accumulation and stomatal closure (201). This indicates that NO production is upstream of PI-PLC activation during stomatal closure. However it is yet to be determined whether these observations are the result of NO acting as an upstream signaling molecule leading to the activation of PI-PLC and/or DGK or that nitrosylation is directly involved in their activation.

6.2. The requirement of PI-PLC for Ca^{2+} and Mg^{2+} ions

One of the important characteristics of eukaryotic PI-PLC enzymes is that they require Ca^{2+} ions as essential co-factors for their activity. It is suggested that Ca^{2+} facilitates a nucleophilic attack of the catalytic center of the enzyme on the 1-phosphate of the inositol moiety of the substrate. This suggestion is supported by the finding that some of the residues present in the active center contain basic residues in prokaryotic PI-PLC, which does not require Ca^{2+} for its activity. The basic residues thus take over the function of Ca^{2+} during the hydrolysis of the substrate (28,49). In fact, the requirement of Ca^{2+} for PI-PLC activity is within the nM to mM range in both animals and plants (35,191,202). In plants, it was shown that substrate preference and enzyme activity are affected by the Ca^{2+} concentration within that range. Accordingly, the degradation of PI(4,5)P_2 and PI4P is optimal in the presence of low, nM to µM, concentrations of Ca^{2+} ions, while PI degradation usually occurs at concentrations in the mM range (35). However, we showed recently that this trend is more complex as *in vitro* studies on the requirements for PI-PLC activity showed that different tomato PI-PLC isoforms differ in their sensitivity to Ca^{2+} and in their substrate preference under varying Ca^{2+} concentrations (34). Interestingly, depending on the tested PI-PLC isoform, varying degrees of PI hydrolysis occurred at µM concentrations of Ca^{2+}, and PI(4,5)P_2 was the preferred substrate at these Ca^{2+} concentrations. Also, efficient hydrolysis of both PI and PI(4,5)P_2 occurred at overlapping Ca^{2+} concentrations, indicating that simultaneous hydrolysis of PI and PI(4,5)P_2 might occur when PI-PLC is activated. Furthermore, a low enzyme activity was observed for tomato *Sl*PLC2 and *Sl*PLC4 in the absence of Ca^{2+}, while the addition of Ca^{2+} to the substrate solution increased enzyme activity. A similar observation was made for Arabidopsis *At*PLC4, which showed up to 18% of its *in vitro* enzyme activity without the addition of external Ca^{2+} (191). Also, a low enzyme activity was detected for recombinant potato *St*PLC1, -2 and -3 when Ca^{2+} was excluded from the substrate solution (35). Comparison of the enzyme activity data of tomato and potato shows that *Sl*PLC2 and *St*PLC2 are orthologs having up to 95 percent of sequence homology (36). At very low Ca^{2+} concentrations, *St*PLC2 showed exclusive preference for PI(4,5)P_2, rather than for PI (35). Interestingly, tomato *Sl*PLC2 showed the same behavior indicating that the high homology in amino acid sequence has preserved their substrate preference (34). The observed activity in the absence of externally added Ca^{2+} may be explained by the presence of residual Ca^{2+} ions that are strongly bound to the enzyme. Nevertheless, the differences between plant PI-PLC isoforms in their sensitivity to Ca^{2+} and the control of substrate preference by the actual Ca^{2+}

concentration, suggest differential optimal activation conditions for each PI-PLC isoform. This may lead to the generation of different PI-PLC-induced Ca^{2+} signatures in the cell (191).

In contrast to Ca^{2+}, Mg^{2+} is not required for the activity of eukaryotic PI-PLC when it is assayed *in vitro* using heterologously-expressed recombinant enzyme (34,35). However, Mg^{2+} appears to enhance the activity of PI-PLC purified from both plant (14,202-204) and animal sources (205), when $Ca^{2+}$is already present. In fact, there is conflicting data concerning the requirement of Mg^{2+} for the activity of plant PI-PLC, as was shown for PI-PLC purified from rice (192) and lily (*Lilium longiflorum*) (14). This might be explained by the differences between the various PI-PLC isoforms and the species from which the enzyme fraction was isolated. It is therefore important to evaluate the requirement for Mg^{2+} when studying plant PI-PLC activity originating from different species or the activity of different isoforms present in one species.

6.3. Regulation of PI-PLC activity and substrate preference by the pH

The cytoplasm of plant cells has a pH which is slightly alkaline, near 7.5 (206-208) but swiftly changes during various cellular responses, including the immune response (109,209). This change may be the result of regulating proton-pump activities in cellular membranes (210,211), which activates distinct signaling cascades (209). Cytoplasmic acidification occurs during the immune response and this is thought to be the result of the rapid migration of protons from the surrounding media and trapping them into the cytoplasm, which leads to medium alkalization (210,211). Similar to the effect of Ca^{2+}, the pH also affects the activity of recombinant tomato PI-PLC *in vitro*. Effective substrate binding by PI-PLC is thought to require protonation of specific residues in the substrate binding domains (135,212). Semi-quantitative assessment of the activity of three recombinant tomato PI-PLC isoforms shows that ,together, they are active at a broad pH range, between pH 4 and pH 9 (34). This is similar to what was previously reported for PI-PLC purified from lily pollen tubes (14) and rice suspension-cultured cells (192). The pH appears to differentially regulate tomato PI-PLC activity, as different PI-PLC isoforms showed a different pH optimum (34). Moreover, the pH optimum for PI hydrolysis by tomato PI-PLC had the tendency to be more acidic, whereas it was more neutral-to-alkaline for the hydrolysis of $PI(4,5)P_2$. This questions the notion that $PI(4,5)P_2$ is the prime substrate in plant cells undergoing an immune response during which the cytoplasm becomes acidic. It also remains to be shown whether this regulation of substrate preference actually occurs in the cell during the immune reaction where the substrates are presented as a mixture with other dynamically changing membrane phospholipids (72). Remarkably, pretreatment of tobacco suspension-cultured cells with the PI-PLC inhibitor U73122 blocks the medium alkalization response induced by both PTI and ETI types of immunity, suggesting that PI-PLC activity is upstream of proton-pump regulation (34). Although the optimum pH for PI-PLC is acidic, the enzyme is active at neutral and/or alkaline pH as well (14,34). This points to the participation of PI-PLC in lipid metabolism in the cell in the resting state, at which the pH is slightly alkaline. Interestingly, tomato *Sl*PLC4, which was previously found to be required for the HR (36), had an optimum activity at pH 5 for the hydrolysis of PI and at pH 8 for PIP2 hydrolysis (34). Accordingly, it is tempting to speculate that the activity of this PI-PLC isoform is regulated by the pH to

utilize different substrates and thereby transmit distinct signals during different cellular responses. It is therefore important to determine the pH range and correlating substrate specificity for newly studied PI-PLC enzymes, although it may be difficult to predict the pH in compartments *in planta*.

6.4. Regulation of PI-PLC activity by its X/Y-linker

Because of the involvement of PI-PLC in cellular defense signaling and lipid metabolism, its activity needs to be tightly regulated, especially during the resting state. The highly divergent X/Y-linker region seems to play an important regulatory role for the activity of most classes of animal PI-PLC (190,213,214). Recent structural and biochemical studies on human PLC-β2 showed that the X/Y-linker region acts as an auto-inhibitory module which occludes the catalytic core, thereby inhibiting undesired contact with membrane phospholipid substrates in the resting state. The same was found for animal PI-PLCδ1 and PI-PLCε (27). The highly divergent X/Y-linkers of these three PI-PLC classes share a conserved feature, as they are all enriched in negatively charged amino acid residues (27). Accordingly, auto-inhibition is suggested to be released by electrostatic repulsion of the X/Y-linker by negatively charged phospholipid membranes (27,190). This is thought to expose the catalytic core and allows the hydrolysis of the substrate. Interestingly, phosphorylation of PI-PLCγ at a specific tyrosine residue of the X/Y-linker was found to induce a conformational change which displaces the X/Y-linker and releases auto-inhibition (215-217).

Because PI-PLCδ1 activity is regulated by its X/Y-linker, enriched with negatively-charged residues, and a similar linker is present in a splice variant of PI-PLCζ, which in its turn structurally resembles the plant PI-PLC variants, we screened the tomato PI-PLC family members for similar features which can indicate a similar regulation mechanism. All enzyme isoforms showed an enrichment in negatively charged residues in their X/Y-linkers, when compared to overall amino acid composition over the full-length protein (34). This was relatively the highest in *Sl*PLC2 and lowest in *Sl*PLC1. This enrichment for negatively charged residues indicates that the activity of the tomato enzymes may be regulated by the X/Y-linker, similar to their animal counterparts although biochemical studies should be performed to test this hypothesis. Interestingly, results from phospho-proteomic analyses in Arabidopsis (PhosPhAt: http://phosphat.mpimp-golm.mpg.de/db.html) show that exclusive phosphorylation of serine residues was frequently detected within the X/Y linkers of *At*PI-PLC1, -2, -4 and -7 (see supplemental information, Table S1). In addition to this, an increase of up to 50 percent in putative phosphorylated residues in the X/Y-linker of all tomato PI-PLC isoforms, when compared to the full-length proteins, except for *Sl*PLC6, was shown (34) Accordingly, phosphorylation at the X/Y-linker could be a means to regulate the activity of these enzymes, possibly by facilitating its interaction with signaling partners, promoting interaction with substrates located in the membrane as in animal PI-PLCγ2 (218) or inducing conformational changes within the enzyme, as suggested for PI-PLCγ1 (217). Alternatively, phosphorylation is expected to increase the negative charge, thereby enhancing the release of the auto-inhibitory X/Y-linker .

6.5. The nature of the substrate pool determines its specific function in cellular signaling

Both the subcellular location and the saturation state of the fatty acid chains present in the PI-PLC substrates play an important role in generating a downstream signal. Several observations suggest that the PI-PLC substrates containing unsaturated acyl chains form a distinct signaling pool. The type of fatty acid chains present in the DAG molecule differentially affects PI-PLC and PI4P5K activities (219). Accordingly, it was proposed that the fatty acids that make up phosphoinositides function as intracellular modulators of the activity of these enzymes. Recently, it was reported that several recombinant tomato PI-PLC enzymes are unable to hydrolyze synthetic PI(4,5)P_2 containing saturated acyl, whereas naturally extracted PI, PI4P and PI(4,5)P_2, usually containing one saturated and one unsaturated acyl chain, are efficiently hydrolyzed (34,36). Additional phosphoinositide metabolizing-enzymes were also found to convert substrates selectively based on the saturation state of the acyl chain (220,221). Arabidopsis PtdIns synthase genes *PIS1* and *PIS2* encode enzymes located at the ER and both synthesize PI from the substrate cytidinediphospho-diacylglycerol (CDP-DAG). However, *in vitro* PIS1 and PIS2 enzymes utilized different CDP-DAG species with a different fatty acid composition, as PIS2 showed a preference for unsaturated substrates, whereas PIS1 did not (221). Furthermore, overexpression of PIS2, but not PIS1, increased the levels of PI4P and PI(4,5)P_2 containing unsaturated fatty acids, while overexpression of PIS1 increased the levels of saturated DAG and PE. This indicates that the PI that is synthesized by the two different isoforms of the PIS enzymes enters different metabolic pathways. Accordingly, immune signaling via PI-PLC after the activation of immune receptors most likely requires the hydrolysis of a specific signaling pool of the substrate.

6.6. Regulation of PI-PLC activity by the availability of the substrate

Substrate availability also seems to regulate the activity of PI-PLC. It was shown in animals that a protein kinase, referred to as “with no lysine 1” (WNK1), induces the synthesis of PI(4,5)P_2 by stimulating the activity of PI4K, thereby promoting signaling via PI-PLC (222). This finding and similar additional observations regarding the regulation of PI-PLCβ activity in animals by modifying the levels of the substrate, suggest that PI(4,5)P_2 production might be regulated as an independent signaling mechanism (223,224). In the same way, accumulation of PI(4,5)P_2 in response to osmotic- and salt stresses (187,225), stimulates PI-PLC activity and the accumulation of InsP_3 (225,226). Availability of substrates as a mechanism to regulate PI-PLC activity is suggested to be more relevant in higher plants, as in this case much lower amounts of cellular PI(4,5)P_2 are present (73).

Also concealing the substrate is a newly emerging mechanism by which the availability of PI(4,5)P_2 or other PIs, for PI-PLC and other PI-metabolizing enzymes can be regulated. Sequestration of PI(4,5)P_2 from the PM into discrete micro-domains was shown to occur by the action of a class of abundant proteins known as pipmodulins, which include GAP43, MARCKS and CAP23. It is hypothesized that these proteins shield PI(4,5)P_2 until they dissociate from the substrate in response to their stimulation (227,228). However, functional homologues have not yet been identified in plants (73).

7. PI-PLC controls different aspects of plant immunity

The interaction between plants and pathogenic microbes involves two main elements. The first one is the ability of plants to recognize invading microbes and trigger the appropriate defense response. The second one is the ability of successful microbes to suppress or neutralize this response. The first interface at which this interaction takes place is the PM, where receptor proteins of the host plant are being tuned to recognize either conserved or specific microbial compounds. The gap between recognizing a certain microbe by a surface immune receptor (e.g. Cf-4 and FLS2) and executing the final response, is bridged by a non-linear pathway composed of several layers either directly downstream of the receptor or located further away and closer to the final defense response (229,230). Microbes that are able to deliver their effectors inside the host cell can also face recognition by an array of specific immune receptors from the NB-LRR-type located in the cell cytoplasm. NB-LRR-mediated recognition of a matching effector and the induction of downstream defense imply that the microbe already has been interacting with the host to be able to deliver the effector to the cytoplasm. It is therefore important to realize that the readout from this interaction is likely to be influenced by the earlier interaction between the microbe and the host, before delivering the cytoplasmic effector. It is commonly accepted that plant resistance to certain microbes is determined very early and is triggered within minutes from receptor activation during the interaction. This means that the very early steps in defense, following receptor activation, are crucial for the outcome of the resistance reaction. They include reversible protein phosphorylation, leading to the activation or deactivation of substrate proteins, modulation of the composition of membrane phospholipids, increase in cytoplasmic Ca^{2+}, either by the release from intracellular stores or as external influx, and the production of NO and ROS (230).

Other processes related to the defense response are activated relatively late and involve the synthesis of antimicrobial compounds like phytoalexins, the expression of chitinases and glucanases, the induction of the phenylpropanoid metabolic pathway and the accumulation of phenolic compounds, which eventually culminates in the execution of the HR and resistance. All previous events are key measures to protect plants from being colonized by pathogenic microbes. PI-PLC activity was found to be required for both early- and late defense signaling events. Studies on the requirement of plant PI-PLC in defense against microbes clearly show a central role for this type of enzyme as a key regulator of immunity upon activation of immune receptors (Table 1). This central role is likely because PI-PLC activity controls intracellular Ca^{2+} release and both the consumption and generation of important phospholipid signaling molecules, influencing plant resistance. Different means of elicitation were used to uncover the role of PI-PLC in receptor-mediated immunity in different plant species and different plant tissues (Table 1). The applied biotic stresses included inoculation with pathogens, treatment with general and specific microbial effectors and elicitor preparations from microbial- cell wall hydrolysates or growth medium and defense-activating hormones. The majority of these studies resulted in the observation that PI-PLC plays a positive role in defense initiated by the activation of different types of receptors. It was initially found that PI-PLC activity is associated with the activation of cell

surface immune receptors (Table 1). Unexpectedly, two cytoplasmic NB-LRR immune receptors also appeared to partially require PI-PLC signaling to exert their function in immunity (82). It is however unclear how these cytoplasmic receptors activate PI-PLC and direct the enzymes towards their substrates in the PM to generate second messenger molecules amplifying defense signaling. In contrast with these findings, it was reported that both the inoculation of soybean suspension-cultured cells with virulent *P. syringae* pv. *glycinea* (*Psg*) or inhibition of PI-PLC activity induces the expression of defense-related genes that are known to be induced during compatible- and incompatible plant-pathogen interactions (231). The same group previously used the same plant system and reported a decrease in the levels of $InsP_3$ in response to *P. syringae* inoculation (232). A possible explanation for these observations could be the specific suppression of PI-PLC activity by secreted bacterial effectors in order to compromise defense.

Table 1. Various examples of experimental systems showing the involvement of the PI-PLC signaling-pathway in plant innate immunity.

Plant	tissue/cell type	Stimulation/elicitor/ treatment	Involved receptor (type)	Additional (pre)treatment	Observation	Reference
Arabidopsis	Epidermis from cotyledons of transgenic plants expressing FLS2-GFP	flg22 peptide	FLS2 (RLK)	PI-PLC inhibitor U73122 and U73343 as control	Suppression of flg22-mediated defense and internalization of the FLS2 receptor by the PLC inhibitor U73122	(34)
Arabidopsis	Leaf discs	Synchronized expression of AvrRpm1 or AvrRpt2 in transgenic plants expressing RPM1 or RPS2, or mutants lacking these resistance proteins.	RPM1 and RPS2 (NB-LRR)	Differential isotopic labeling, PI-PLC inhibitor U73122, U73343 as control or neomycin	Biphasic accumulation of PA, first by PI-PLC/DGK activity and then by PLD activity, precedes defense activation.	(82)
Arabidopsis	Detached leaves and protoplasts of IP5-ptase-aeq plants	flg22 peptide	FLS2 (RLK)	None	Inhibition of cytosolic Ca^{2+} increase in response to flg22	(233)
Arabidopsis	Suspension-cultured cells	flg22 peptide	FLS2 (RLK)	Isotopic labeling of peptides and quantification of protein phosphorylation	Phosphorylation of PI-PLC2	(193)
Brassica napus	Whole plants	SAR inducers: SA, BTH and ISR inducer: MJ		None	Increase in PI-PLC enzyme activity, without increasing protein levels	(234)

Capsicum chinense	Cell suspension	SA and MJ	NPR1 and COI1-JAZ	None	$InsP_3$ accumulation	(235)
Citrus limon	Seedlings	Conidia of *Alternaria alternata*	Multiple	Neomycine or PTK inhibitors lavendustin A and DHMC	Suppression of PI-PLC-mediated increase in $InsP_3$, PAL activity, synthesis of the phytoalexin scoparone and HR	(197,198)
Glycine max	Suspension-cultured cells	*Pseudomonas syringae* (*Psg*) or PI-PLC inhibitor U73122 and its analog U73343	Multiple	None	Induced expression of defense-related genes	(231)
G. max	Suspension-cultured cells	*Pseudomonas syringae* pv. *glycinea* (*Psg*)	Multiple	None	Decrease in $InsP_3$ levels in both compatible and incompatible interactions	(232)
G. max	Suspension-cultured cells	Polygalacturonic acid elicitor	WAK1 (RLK)	Isotopic labeling and neomycine	Increase in $InsP_3$ levels and decrease in PI4P and $PI(4,5)P_2$ levels after elicitation	(83)
Medicago sativa	Suspension-cultured cells	Glycoprotein elicitor from *Verticillium albo-atrum*	Unknown	Isotopic labeling	Elicitor activation of PI-PLC, leading to an increase in $InsP_3$ and simultaneous decrease in $PI(4,5)P_2$ levels, increase in PAL activity and accumulation of phytoalexins.	(236)
Nicotiana benthamiana	Transgenic *N. benthamiana* leaves expressing Cf-4	Transient expression of *Cladosporium fulvum* Avr4 effector	Cf-4 (RLP)	Transient expression of *SlPLC1* and *SlPLC3 genes*	Enhancement of the Cf-4/Avr4-triggered HR	(34)
Nicotiana tabacum	Transgenic tobacco suspension-cultured cells expressing the resistance protein Cf-4	*C. fulvum* Avr4 effector, flg22 peptide, chitin fragments	Cf-4 (RLP)	PI-PLC inhibitor U73122 or U73343 as control	Suppression of defense-mediated medium alkalization	(34)

N. tabacum	Suspension-cultured cells	DAG analog, phorbol ester	PKC and C1 domain-containing proteins in plants	None	Production of ROS and elevated expression of the defense-related gene *hsr203J*	(237)
N. tabacum	Transgenic tobacco suspension-cultured cells expressing the resistance protein Cf-4	Avr4, exogenously added PA	Cf-4 (RLP)	Differential isotopic labeling, PI-PLC inhibitor U73122 and U73343 as control, neomycin, PLD trans-phosphatidylation assay and exogenously added PA	Accumulation of PA and DGPP. Most PA produced via the PI-PLC/DGK pathway and partially via PLD activity. Addition of PA simulates Avr4 perception and induces oxidative burst	(52)
N. tabacum	Suspension-cultured cells	Cryptogein from the pathogenic fungus *Phytophthora cryptogea*	Unknown	Neomycin or PI-PLC inhibitor U73122	Inhibition of the second peak of cryptogein-induced Ca^{2+} increase in the cytoplasm	(238)
N. tabacum	Suspension-cultured cells	Riboflavin	Unknown in plants	PI-PLC inhibitor U73122 and U73343 as control or PLD inhibitor and exogenous application of short-chain PA	Defense activated by riboflavin and suppressed by PI-PLC and PLD inhibitors. Defense activation induced transcription of *PI-PLC*, *PLD*, *PAL* and *PR-1a* and *PR-1b* genes and production of H_2O_2 and accumulation of the phytoalexin scopoletin.	(18)
N. tabacum	Whole plants, leaves	Inoculation with TMV and *Phytophthora parasitica*		Transgenic plants expressing *OsBIDK1*	Overexpression of rice *OsBIDK1* enhanced resistance	(168)

Oryza sativa	Suspension-cultured cells	Chitin fragments	CEBiP and CERK1 (LysM proteins)	Isotopic labeling, exogenous application of synthetic short-chain PA and DAG. PI-PLC inhibitor U73122, DGK inhibitor R59022, PLD inhibitor 1-butanol and PAPH inhibitor propranolol	Elicitor treatment activated PI-PLC and PLD pathways. R59022 suppressed ROS generation in response to chitin treatment. Inhibition of PAPH activity significantly enhanced ROS generation.	(153)
O. sativa	Suspension-cultured cells	Chitin fragments	CEBiP and CERK1 (LysM proteins)	None	Chitin causes a biphasic generation of ROS, the first phase correlated with the activation of both PI-PLC and PLD and the second with PLD activation. Exogenously applied PA and DAG induced ROS generation and induced expression of elicitor-responsive genes in absence of the elicitor and enhanced elicitor-mediated accumulation of the phytoalexin momilactone-A.	(173)
Phaseolus vulgaris	Whole plants	Inoculation with *Pseudomonas syringae* pv. *lachrymans* and screening for angular leaf spot phenotype	Multiple	Exploration of QTL ALS10.1, resistant and susceptible plants from RILs selected for BSA	QTL ALS10.1 contained PI-PLC	(239)
Pisum sativum	Epicotyl tissues	Elicitor preparation isolated from germination fluid of *Mycosphaerella pinodes*	Multiple	R59022 and neomycine	Inhibition of DGK prevents the conversion of PI-PLC-generated DAG to PA and thereby enhances elicitor-mediated accumulation of the phtoalexin pisatin and PAL activity.	(122,165)
Rubia tinctorum	Suspension-cultured cells	A fraction of *Botrytis cinerea* hyphae hydrolysate, mainly containing oligosaccharides as elicitor	Multiple	Neomycine or $InsP_3$ receptor-blocker 2-APB	Reduction in maximum H_2O_2 concentrations in response to the elicitor	(240)

R. tinctorum	Suspension-cultured cells	Chitosan and low concentration phorbol ester	CEBiP and CERK1 (LysM proteins)	Neomycin or PI-PLC inhibitor U73122, PKC inhibitors and the $InsP_3$ receptor-blocker 2-APB	Chitosan- or phorbol ester induced the accumulation of the phytoalexin anthraquinone and this was suppressed by the inhibitors of PI-PLC, PKC and $InsP_3$-R	(160)
R. tinctorum	Suspension-cultured cells	Chitosan	CEBiP and CERK1 (LysM proteins)	Neomycin or PI-PLC inhibitor U73122 or blockers of Ca^{2+} release from intracellular stores	Chitosan increases PI-PLC activity and the accumulation of the phytoalexin anthraquinone and this is blocked by inhibitors of intracellular Ca^{2+} mobilization	(241)
Solanum lycopersicum	Cf-4/Avr4 hybrid seedlings	Interaction between tomato Cf-4 and *C. fulvum* Avr proteins.	Cf-4 (RLP)	None	Differential expression of PI-PLC genes	(76)
S. lycopersicum	Suspension-cultured cells	Chitosan (deacetylated derivative of chitin)	CEBiP and CERK1 (LysM proteins)	Neomycin or PI-PLC inhibitor U73122	Inhibition of chitosan-induced ROS production	(169)
S. lycopersicum	Suspension-cultured cells	Chitin fragments, xylanase and flg22	CEBiP and CERK1 (LysM proteins)	Differential isotopic labeling	Accumulation of PA and DGPP. Most PA produced via the PI-PLC/DGK pathway. Only xylanase induced the accumulation of PA via both PI-PLC/DGK and PLD pathways.	(71)

S. lycopersicum & N. benthamiana	Whole plants and detached leaves	Cf-4/Avr4 interaction, *Verticillium dahliae* and *Pseudomonas syringae* expressing *AvrPto*	Cf-4 (RLP), AvrPto/Pto (kinase), Multiple	Virus-induced gene silencing of specific *PI-PLC* gene isoforms.	Differential regulation of *PI-PLC* isoforms during infection. *Sl*PLC4 was mainly required for Cf-4/Avr4-mediated HR while *Sl*PLC6 was required for resistance to multiple types of pathogens.	(36)
S. lycopersicum	Suspension-cultured cells and whole plants, leaves	Xylanase	LeEix1 and LeEix2 (RLP)	Virus-induced gene silencing of *SlPLC2* and *SlPLC5* genes	*Sl*PLC2 is required for xylanase-induced expression of the defense-related genes PR1 and HSR203J	(242)

Abbreviations: PM, plasma membrane; MJ, methyl jasmonate; SA, salicylic acid; BTH, benzothiadiazole; PAL, phenylalanine ammonia-lyase; PAPH, PA-phospho hydrolase; ROS, reactive oxygen species; SAR, systemic acquired resistance; ISR, induced systemic resistance; PTK, protein tyrosine kinase. Multiple, different receptors involved; RK, receptor kinase; RLK, receptor-like kinase; LysM proteins, proteins containing LysM domains; QTL, quantitative trait locus; RILs, recombinant inbred lines; BSA, bulked segregant analysis; DHMC, 2,5-dihidroxy methyl cinnamate; 2-APB, 2-aminoethyldiphenyl borinate.

8. Concluding remarks

Based on what has been discussed, a current view for the implication of PI-PLC in plant immune receptor signaling is presented in Figure 4. A continuous, dynamic flux of PI4P and $PI(4,5)P_2$ is tightly regulated in the PM and nucleus, whereas PI and PI4P are also present in the membrane of other organelles. Triggering the Cf-4 or FLS2 receptor activates PI-PLC at the PM by a yet unknown mechanism, leading to the hydrolysis of $PI(4,5)P_2$ and PI4P to produce $InsP_3$ and DAG or $InsP_2$ and DAG, respectively. At a particular pH and Ca^{2+} concentration, PI might also be used as a PI-PLC substrate to produce InsP and DAG in other subcellular membranes like those of the ER and Golgi. Initial PI-PLC activation at the protein level occurs rapidly after perception of a microbe, while transcriptional activation also occurs later and probably lasts until the stimulation is terminated. The flux of PI4P produced from the phosphorylation of PI by PI4K is required for the production of $PI(4,5)P_2$ by the activity of PI4P5K. The depletion of $PI(4,5)P_2$ releases its inhibition of a K^+ outward rectifying channel, causing a drop in the concentration of K^+ in the cell. In guard cells, this stimulates the loss of turgor pressure and causes deflation of the guard cells leading to stomatal closure, which is besides of being a drought response, a common innate immune-response of many plants after bacterial infection (243). The cytoplasmic drop in K^+ ions is interpreted differently by other cell types, leading to cell cycle arrest and the execution of the HR in order to restrict the spread of the microbial attacker.

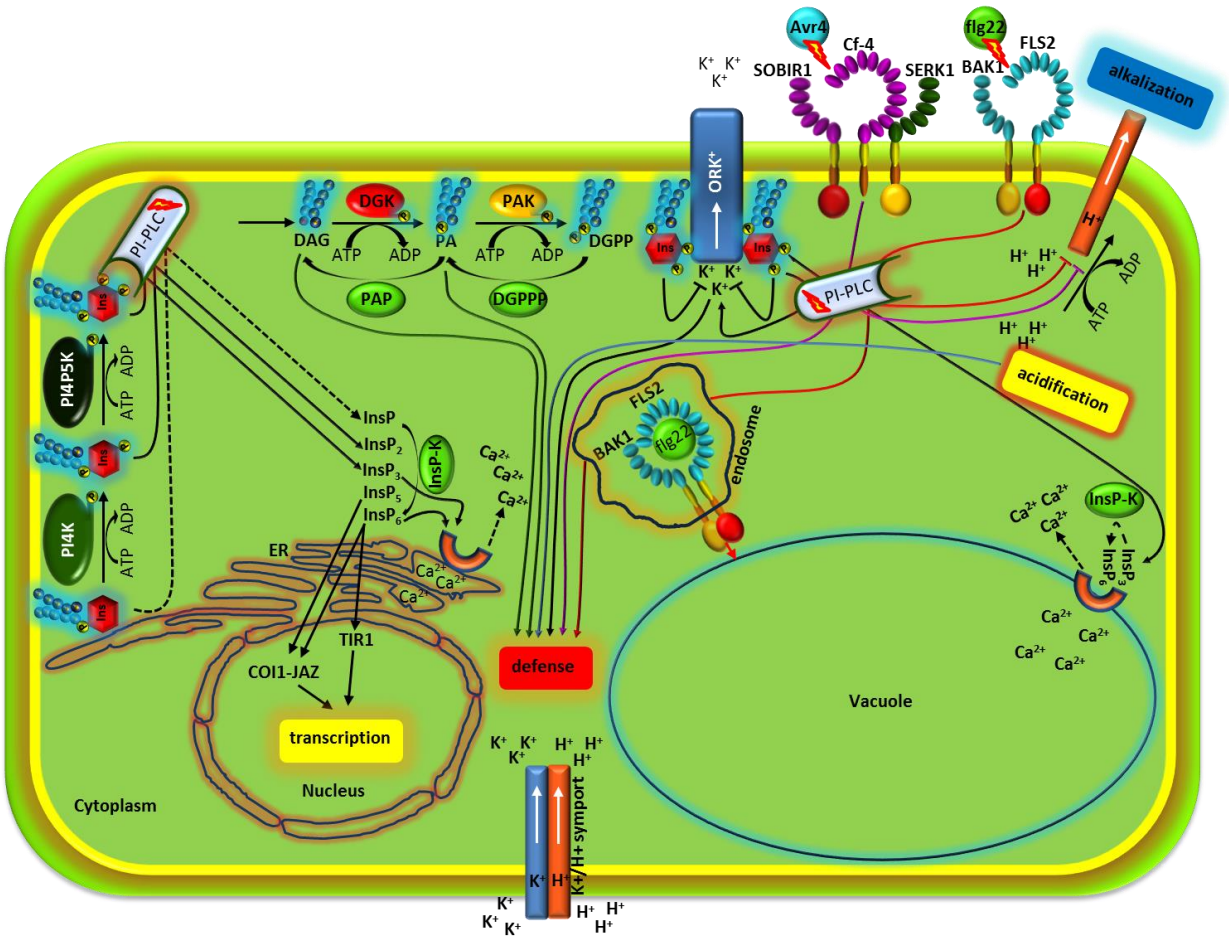

FIGURE 4. **Current view on the role of PI-PLC-mediated signaling in plant cells during receptor-mediated immunity**. For details see text.

The formation of DAG as a result of PI-PLC activity in the target membrane may result in sequestering and activation of downstream signaling proteins containing a C1-domain, leading to the activation of downstream defense responses. A large fraction of these DAG molecules is rapidly phosphorylated to PA by DGK. PA can in turn also be phosphorylated to generate DGPP. The produced DAG, PA and possibly also DGPP might modulate downstream defense responses like the generation of ROS, the accumulation of phytoalexins and the expression of defense-related genes (see Table 1). A highly dynamic inter-conversion between the different PI-PLC substrates and downstream reaction products is known to occur by the action of lipid phosphatases and lipid kinases. The generated $InsP_3$, or its phosphorylation derivative $InsP_6$, diffuses around the site of production at the membrane to release Ca^{2+} from the vacuolar- and ER membranes by a yet unknown mechanism. Intracellular Ca^{2+} oscillations triggered by PI-PLC activity function as a stimulus-specific signal, known as Ca^{2+}-signature, which either directly or indirectly via Ca^{2+}-signal sensors, regulates the activity of many enzymes and proteins in the cell (11). Inhibition of PI-PLC activity blocks the medium alkalization response, indicating that PI-PLC activity suppresses the activity of PM H^+-ATPase channels that transport protons across the PM out of the cell. Trapping protons inside the cell leads to cytoplasmic acidification and modulates the function of many cellular enzymes, including those involved in defense. The stimulation of the proton efflux by inhibiting PI-PLC activity is accompanied with a suppression of effector-mediated receptor endocytosis. This leads to the impairing the immune receptor function as observed for the FLS2 receptor (34). In summary, the previous discussion emphasizes a central role for PI-PLC in early defense signaling. Future work on PI-PLC signaling in relation to plant defense and disease resistance should help us to better understand plant immunity and to identify methods to produce plants that respond even more effectively to microbial infection and are consequently more resistant.

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Supplemental information

FIGURE S1

BLAST

Basic Local Alignment Search Tool

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Edit and Resubmit Save Search Strategies Formatting options Download

PSI blast Iteration 1

spacer_SlPLC4

Results for: lcl|49454 spacer_SlPLC4(69aa)
Your BLAST job specified more than one input sequence. This box lets you choose which input sequence to show BLAST results for.

Query ID
lcl|49454
lcl|49454
Description
spacer_SlPLC4
Molecule type
amino acid
Query Length
69

Database Name
nr
Description
All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program
BLASTP 2.2.24+ Citation

Reference
Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.
Other reports: Search Summary [Taxonomy reports] [Distance tree of results] [Multiple alignment]

Search Parameters

Search parameter name	Search parameter value
Program	blastp
Word size	3
Expect value	10
Hitlist size	500
Gapcosts	11,1
Matrix	BLOSUM62
Filter string	F
Genetic Code	1
Phi pattern	E-[KPVI]-S-[ESD]-[IL]-x-[AQ]
Window Size	40
Threshold	11

Database

Database parameter name	Database parameter value
Posted date	Aug 22, 2010 5:43 PM
Number of letters	4,000,359,389
Number of sequences	11,712,007
Entrez query	none

Karlin-Altschul statistics

Params	Ungapped	Gapped
Lambda	0.27	0.27
K	0.047	0.047
H	1	1

Results Statistics

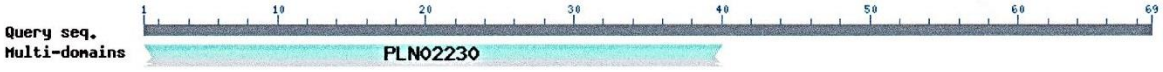
Results Statistics parameter name	Results Statistics parameter value
Length adjustment	7
Effective length of query	62
Effective length of database	3918277827
Effective search space	3918277827

Effective search space used 3918277827

Graphic Summary

Show Conserved Domains

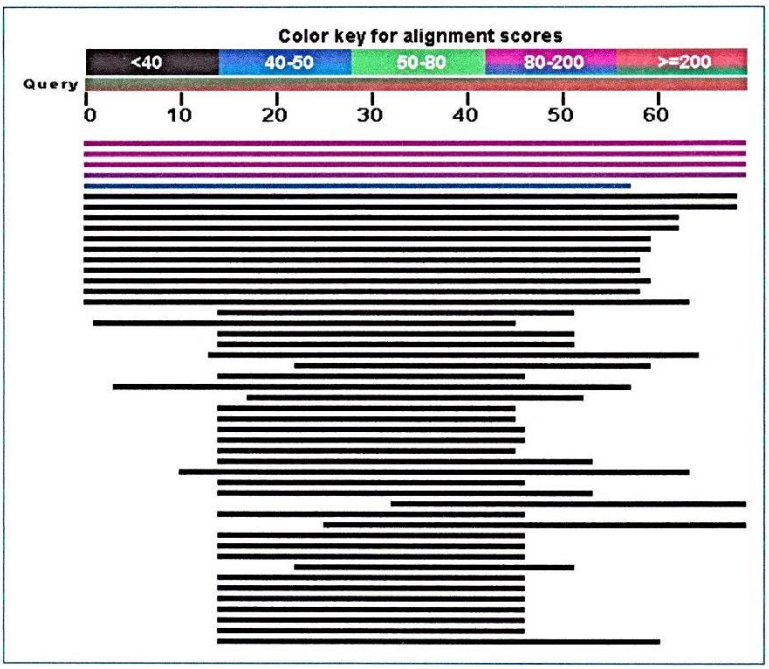
Putative conserved domains have been detected, click on the image below for detailed results.



Distribution of 48 Blast Hits on the Query Sequence

[?]

An overview of the database sequences aligned to the query sequence is shown. The score of each alignment is indicated by one of five different colors, which divides the range of scores into five groups. Multiple alignments on the same database sequence are connected by a striped line. Mousing over a hit sequence causes the definition and score to be shown in the window at the top, clicking on a hit sequence takes the user to the associated alignments. New: This graphic is an overview of database sequences aligned to the query sequence. Alignments are color-coded by score, within one of five score ranges. Multiple alignments on the same database sequence are connected by a dashed line. Mousing over an alignment shows the alignment definition and score in the box at the top. Clicking an alignment displays the alignment detail.



Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer PubChem BioAssay

Images legend

NEW - alignment score below the threshold on the previous iteration

- alignment was checked on the previous iteration

Run PSI-Blast iteration 2 with max

Sequences producing significant alignments with pattern at position 34 and E-value BETTER than threshold

Sequences producing significant alignments with pattern at position 34 and E-value BETTER than threshold

Accession	Description	Max score	Total score	Query coverage	E value	Links
NEW ABW81000.1	PI-phospholipase C PLC4 [Solanum lycopersicum]	122	122	100%	1e-33	G
NEW CAA72681.1	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase [Nicotiana rustica]	83.5	83.5	100%	9e-22	
NEW ABP57375.1	phosphoinositide-specific phospholipase C [Nicotiana tabacum]	83.1	83.1	100%	1e-21	
NEW CAA65127.1	phosphoinositide-specific phospholipase C [Nicotiana rustica]	83.1	83.1	100%	1e-21	
NEW ABU53666.1	phospholipase C [Torenia fournieri]	43.8	43.8	82%	8e-10	
NEW ABW80999.1	PI-phospholipase C PLC5 [Solanum lycopersicum]	31.4	31.4	98%	4e-06	G
NEW AAW22879.1	putative phospholipase C [Solanum lycopersicum]	31.4	31.4	98%	4e-06	
NEW ABW81003.1	PI-phospholipase C PLC1 [Solanum lycopersicum]	29.9	29.9	89%	1e-05	G
NEW CAA63777.1	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase; phosphoinositide-specific phospholipase C [Solanum tuberosum]	29.5	29.5	89%	2e-05	
NEW ABW81001.1	PI-phospholipase C PLC3 [Solanum lycopersicum]	27.7	27.7	85%	6e-05	G
NEW ABC50164.1	phospholipase C [Petunia integrifolia subsp. inflata]	27.7	27.7	85%	6e-05	
NEW ABJ99758.1	phospholipase C [Nicotiana tabacum]	27.3	27.3	84%	8e-05	
NEW AAF33824.1	phospholipase C2 [Nicotiana tabacum]	26.9	26.9	84%	1e-04	
NEW CAA63954.1	phosphoinositide-specific phospholipase C [Solanum tuberosum]	25.1	25.1	85%	4e-04	
NEW AAF33823.1	phospholipase C1 [Nicotiana tabacum]	22.1	22.1	84%	0.003	
NEW XP_002271986.1	PREDICTED: hypothetical protein [Vitis vinifera] >emb|CBI25478.3| unnamed protein product [Vitis vinifera]	21.4	21.4	91%	0.005	U G

Run PSI-Blast iteration 2 with max

Sequences with pattern at position 34 and E-value WORSE than threshold

Sequences with pattern at position 34 and E-value WORSE than threshold

Accession	Description	Max score	Total score	Query coverage	E value	Links
NEW ADE59015.1	retrotransposon-like 1 [Ovis aries]	13.4	13.4	53%	1.2	
NEW XP_001749539.1	hypothetical protein [Monosiga brevicollis MX1] >gb|EDQ85590.1| predicted protein [Monosiga brevicollis MX1]	13.4	13.4	63%	1.2	G
NEW NP_001181949.1	retrotransposon-like protein 1 [Bos taurus] >sp|Q52QI2.2|RTL1_BOVIN RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived protein PEG11; AltName: Full=Paternally expressed gene 11 protein homolog >gb|DAA17373.1| retrotransposon-like 1 [Bos taurus]	13.4	13.4	53%	1.2	G
NEW AAX84834.1	PEG11 [Bos taurus]	13.4	13.4	53%	1.2	G
NEW YP_002455528.1	hypothetical protein BafACA1_G14 [Borrelia afzelii ACA-1] >gb|ACJ73442.1| hypothetical protein BafACA1_G14 [Borrelia afzelii ACA-1]	13.0	13.0	73%	1.5	G
NEW XP_003055888.1	predicted protein [Micromonas pusilla CCMP1545] >gb|EEH59264.1| predicted protein [Micromonas pusilla CCMP1545]	12.7	12.7	53%	1.9	G
NEW NP_001127830.4	retrotransposon-like protein 1 [Sus scrofa] >gb|ACF47570.4| retrotransposon-like 1 [Sus scrofa]	12.3	12.3	46%	2.5	U G
NEW XP_002636257.1	Hypothetical protein CBG08539 [Caenorhabditis briggsae] >emb|CAP28473.1| Hypothetical protein CBG08539 [Caenorhabditis briggsae]	12.3	12.3	78%	2.5	G
NEW NP_735809.1	hypothetical protein gbs1372 [Streptococcus agalactiae NEM316] >emb|CAD47031.1| unknown [Streptococcus agalactiae NEM316]	12.0	12.0	50%	3.2	G
NEW XP_243381.5	PREDICTED: retrotransposon-like 1 [Rattus norvegicus]	11.6	11.6	44%	4.0	U G
NEW XP_001070232.2	PREDICTED: retrotransposon-like 1 [Rattus norvegicus]	11.6	11.6	44%	4.0	G
NEW XP_002723802.1	PREDICTED: retrotransposon-like 1 [Oryctolagus cuniculus]	11.6	11.6	46%	4.0	U G
NEW ACF47571.3	retrotransposon-like 1 [Sus scrofa]	11.6	11.6	46%	4.0	G
NEW EDL97526.1	rCG27781 [Rattus norvegicus]	11.6	11.6	44%	4.0	
NEW EDL18690.1	mCG1047978 [Mus musculus]	11.6	11.6	56%	4.0	

NEW	YP_877622.1	transglutaminase/protease [Clostridium novyi NT] >gb|ABK61279.1| predicted transglutaminase/protease [Clostridium novyi NT]	11.6	11.6	76%	4.0	G
NEW	XP_001110319.1	PREDICTED: retrotransposon-like protein 1-like [Macaca mulatta]	11.6	11.6	46%	4.0	UG
NEW	NP_908998.1	retrotransposon-like protein 1 [Mus musculus] >sp|Q7M732.1|RTL1_MOUSE RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived protein PEG11; AltName: Full=Paternally expressed gene 11 protein; AltName: Full=Mammalian retrotransposon derived protein 1 >tpg|DAA01153.1| TPA_exp: RTI1 [Mus musculus] >gb|ACF20046.1| retrotransposon-like 1 [Mus musculus] >gb|ACF20051.1| retrotransposon-like 1 [Mus musculus]	11.6	11.6	56%	4.0	UG
NEW	NP_982727.1	hypothetical protein [Ashbya gossypii ATCC 10895] >gb|AAS50551.1| AAR184Wp [Ashbya gossypii ATCC 10895]	11.2	11.2	53%	5.1	G
NEW	XP_002917512.1	PREDICTED: retrotransposon-like protein 1-like [Ailuropoda melanoleuca]	10.9	10.9	46%	6.6	G
NEW	CBN79655.1	hypothetical protein [Ectocarpus siliculosus]	10.9	10.9	63%	6.6	
NEW	XP_002825163.1	PREDICTED: retrotransposon-like protein 1-like [Pongo abelii]	10.9	10.9	46%	6.6	G
NEW	XP_002754334.1	PREDICTED: retrotransposon-like protein 1 [Callithrix jacchus]	10.9	10.9	46%	6.6	G
NEW	EFB14203.1	hypothetical protein PANDA_005814 [Ailuropoda melanoleuca]	10.9	10.9	46%	6.6	
NEW	EEH05867.1	GYF domain-containing protein [Ajellomyces capsulatus G186AR]	10.9	10.9	42%	6.6	
NEW	AAI50618.1	RTL1 protein [Homo sapiens]	10.9	10.9	46%	6.6	G
NEW	XP_001490006.1	PREDICTED: similar to RTI1 [Equus caballus]	10.9	10.9	46%	6.6	UG
NEW	XP_520846.2	PREDICTED: similar to PEG11 [Pan troglodytes]	10.9	10.9	46%	6.6	UG
NEW	NP_001128360.1	retrotransposon-like protein 1 [Homo sapiens]	10.9	10.9	46%	6.6	UG
NEW	A6NKG5.2	RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived protein PEG11; AltName: Full=Paternally expressed gene 11 protein; AltName: Full=Mammalian retrotransposon derived protein 1	10.9	10.9	46%	6.6	G
NEW	XP_547983.2	PREDICTED: similar to retrotransposon-like 1 [Canis familiaris]	10.9	10.9	46%	6.6	UG
NEW	XP_002066126.1	GK22102 [Drosophila willistoni] >gb|EDW77112.1| GK22102 [Drosophila willistoni]	10.5	10.5	66%	8.4	G

Run PSI-Blast iteration 2 with max

Alignments

Select All Get selected sequences Distance tree of results Multiple alignment

Significant alignments for pattern occurrence 1 at position 34

```
>gb|ABW81000.1| G PI-phospholipase C PLC4 [Solanum lycopersicum]
Length=587

 GENE ID: 100301920 LOC100301920 | PI-phospholipase C PLC4
[Solanum lycopersicum] (10 or fewer PubMed links)

 Score =  122 bits (329),  Expect = 1e-33
 Identities = 69/69 (100%), Positives = 69/69 (100%), Gaps = 0/69 (0%)

Pattern                                        *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDDA  60
              PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDDA
Sbjct    253  PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDDA  312

Query    61   THRGHVASA  69
              THRGHVASA
Sbjct    313  THRGHVASA  321


>emb|CAA72681.1|  1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase [Nicotiana
rustica]
Length=588

 Score = 83.5 bits (227),  Expect = 9e-22
 Identities = 55/71 (77%), Positives = 60/71 (84%), Gaps = 3/71 (4%)

Pattern                                          *******
Query    1    PPKEYLEASAS--VCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYLEASAS  V K+RRNSSQRS  SEDDVWG+EPSSLTADQEENEKSDSD ++ED+D
Sbjct    253  PPKEYLEASASTTVSKERRNSSQRSNCSEDDVWGTEPSSLTADQEENEKSDSD-NFEDED  311

Query    59   DATHRGHVASA  69
              D  HR   ASA
Sbjct    312  DCNHRPQFASA  322


>gb|ABP57375.1|  phosphoinositide-specific phospholipase C [Nicotiana tabacum]
Length=588

 Score = 83.1 bits (226),  Expect = 1e-21
```

```
 Identities = 54/71 (76%), Positives = 61/71 (85%), Gaps = 3/71 (4%)

Pattern                                          *******
Query    1    PPKEYLEASASVC--KDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYLEASAS    K+RRNSSQRS  SEDDVWG+EPSSLTADQEENEKSDSD ++ED+D
Sbjct    253  PPKEYLEASASTTASKERRNSSQRSNCSEDDVWGTEPSSLTADQEENEKSDSD-NFEDED  311

Query    59   DATHRGHVASA  69
              D+ HR  +ASA
Sbjct    312  DSNHRPQLASA  322


>emb|CAA65127.1|  phosphoinositide-specific phospholipase C [Nicotiana rustica]
Length=588

 Score = 83.1 bits (226),  Expect = 1e-21
 Identities = 54/71 (76%), Positives = 62/71 (87%), Gaps = 3/71 (4%)

Pattern                                          *******
Query    1    PPKEYLEASASVC--KDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYLEASAS    K+RRNSSQRS  SEDDVWG+EPSSLTA+QEENEKSDSD ++EDDD
Sbjct    253  PPKEYLEASASTTASKERRNSSQRSNCSEDDVWGAEPSSLTANQEENEKSDSD-NFEDDD  311

Query    59   DATHRGHVASA  69
              D++HR  +ASA
Sbjct    312  DSSHRPQLASA  322


>gb|ABU53666.1|  phospholipase C [Torenia fournieri]
Length=583

 Score = 43.8 bits (123),  Expect = 8e-10
 Identities = 31/57 (54%), Positives = 43/57 (75%), Gaps = 2/57 (3%)

Pattern                                       *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDD  57
              PPKEYLEA  S+  D+ N+SQ+ KDS++DVWG EPSSLTA +E+ +K D + +  D+
Sbjct    255  PPKEYLEAQNSL--DKGNNSQKEKDSDEDVWGKEPSSLTAYEEDEDKIDVEVTDPDN  309


>gb|ABW80999.1|  PI-phospholipase C PLC5 [Solanum lycopersicum]
Length=584

 GENE ID: 100301919 LOC100301919 | PI-phospholipase C PLC5
[Solanum lycopersicum]

 Score = 31.4 bits (90),  Expect = 4e-06
 Identities = 28/70 (40%), Positives = 42/70 (60%), Gaps = 7/70 (10%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDS-EDDVWGSEPSSLTA-DQEENEKSDSDKSYEDDD  58
              PPKEYLE+     K++R++S   KDS  +D+   E S + A D + +E+SDSD+  ED D
Sbjct    248  PPKEYLES-----KNQRDTSPVGKDSFREDLLKKEKSEIGAEDHDTDERSDSDQDDEDGD  302

Query    59   DATHRGHVAS  68
               +T     +S
Sbjct    303  TSTSNDQQSS  312


>gb|AAW22879.1|  putative phospholipase C [Solanum lycopersicum]
Length=574

 Score = 31.4 bits (90),  Expect = 4e-06
 Identities = 28/70 (40%), Positives = 42/70 (60%), Gaps = 7/70 (10%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDS-EDDVWGSEPSSLTA-DQEENEKSDSDKSYEDDD  58
              PPKEYLE+     K++R++S   KDS  +D+   E S + A D + +E+SDSD+  ED D
Sbjct    237  PPKEYLES-----KNQRDTSPVGKDSFREDLLKKEKSEIGAEDHDTDERSDSDQDDEDGD  291

Query    59   DATHRGHVAS  68
               +T     +S
Sbjct    292  TSTSNDQQSS  301


>gb|ABW81003.1|  PI-phospholipase C PLC1 [Solanum lycopersicum]
Length=601

 GENE ID: 100301923 LOC100301923 | PI-phospholipase C PLC1
[Solanum lycopersicum]

 Score = 29.9 bits (86),  Expect = 1e-05
```

```
 Identities = 23/64 (35%), Positives = 37/64 (57%), Gaps = 4/64 (6%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSK-DSEDDVWGSEPSSLTADQEE-NEKSDSDKSYEDDD  58
              PPKEYLE+  +   ++ N SQ+ K  SE+  WG+E S L+      +E  D+ +  +D+
Sbjct    256  PPKEYLESKKT--SEKENGSQKGKKSSEEKAWGAEISDLSQKMMAFSENKDNGECQDDEA  313

Query    59   DATH  62
              D+ H
Sbjct    314  DSHH  317


>emb|CAA63777.1|  1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase; phosphoinositide-specific
phospholipase C [Solanum tuberosum]
Length=596

 Score = 29.5 bits (85),  Expect = 2e-05
 Identities = 25/64 (39%), Positives = 36/64 (56%), Gaps = 4/64 (6%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSK-DSEDDVWGSEPSSLTADQ-EENEKSDSDKSYEDDD  58
              PPKEYLE+     KD  N SQ+ K  SE+  WG+E S L+      +E  D+ +  +D+
Sbjct    256  PPKEYLESKKPSEKD--NGSQKGKKSSEEKAWGAEISDLSQKMIAYSENKDNGECQDDEA  313

Query    59   DATH  62
              D+ H
Sbjct    314  DSHH  317


>gb|ABW81001.1|  PI-phospholipase C PLC3 [Solanum lycopersicum]
Length=583

 GENE ID: 100301921 LOC100301921 | PI-phospholipase C PLC3
[Solanum lycopersicum]

 Score = 27.7 bits (80),  Expect = 6e-05
 Identities = 22/59 (37%), Positives = 31/59 (52%), Gaps = 7/59 (11%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDD  59
              PPKEYL+A     K+ + +       + + WG E S + A    N+K DSD+   DDDD
Sbjct    253  PPKEYLQA-----KEVKETGATKGTDDTEAWGREVSDIKA--RYNDKYDSDEGEADDDD  304


>gb|ABC50164.1|  phospholipase C [Petunia integrifolia subsp. inflata]
Length=588

 Score = 27.7 bits (80),  Expect = 6e-05
 Identities = 21/59 (35%), Positives = 31/59 (52%), Gaps = 4/59 (6%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDD  59
              PPKEYL+A     KD +N  +    ++ + WG E S L A   + + SD     EDD++
Sbjct    256  PPKEYLQAKEVKEKDSKNGPE----ADAEAWGREVSDLKARYNDKDDSDEGDGGEDDEN  310


>gb|ABJ99758.1|  phospholipase C [Nicotiana tabacum]
Length=588

 Score = 27.3 bits (79),  Expect = 8e-05
 Identities = 23/58 (39%), Positives = 31/58 (53%), Gaps = 3/58 (5%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYL+A     KD +  ++ S D+E   WG E S L A   + + SD     EDD+
Sbjct    253  PPKEYLQAKEVKEKDSKKGTE-SPDTE--AWGREVSDLKARYNDKDDSDDGAGVEDDE  307


>gb|AAF33824.1|AF223573_1  phospholipase C2 [Nicotiana tabacum]
Length=605

 Score = 26.9 bits (78),  Expect = 1e-04
 Identities = 23/59 (38%), Positives = 31/59 (52%), Gaps = 5/59 (8%)

Pattern                                          *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSED-DVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYL+A     KD    S++  D+ D + WG E S L A   + + SD     EDD+
Sbjct    253  PPKEYLQAKEVKEKD----SKKGTDAPDTEAWGREVSDLKARYNDKDDSDDGAGVEDDE  307


>emb|CAA63954.1|  phosphoinositide-specific phospholipase C [Solanum tuberosum]
Length=585
```

```
 Score = 25.1 bits (73),  Expect = 4e-04
 Identities = 20/59 (33%), Positives = 31/59 (52%), Gaps = 5/59 (8%)

Pattern                                      *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDD  59
              PPKEYL+A      +   + + +  ++ + WG E S + A    N+K DSD+   DD D
Sbjct    253  PPKEYLQAKEV---NETGAMKGTDQTDTEAWGREVSDIKA--RYNDKDDSDEGEADDSD  306


>gb|AAF33823.1|AF223351_1  phospholipase C1 [Nicotiana tabacum]
Length=586

 Score = 22.1 bits (65),  Expect = 0.003
 Identities = 22/58 (37%), Positives = 30/58 (51%), Gaps = 3/58 (5%)

Pattern                                      *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDD  58
              PPKEYL+A     KD +  ++ S D+E    G E S L A   + + SD     EDD+
Sbjct    252  PPKEYLQAKEVKEKDSKKGTE-SPDTE--ARGREVSDLKARYNDKDDSDDGAGVEDDE  306


>ref|XP_002271986.1|  PREDICTED: hypothetical protein [Vitis vinifera]
 emb|CBI25478.3|  unnamed protein product [Vitis vinifera]
Length=592

 GENE ID: 100248420 LOC100248420 | hypothetical protein LOC100248420
[Vitis vinifera] (10 or fewer PubMed links)

 Score = 21.4 bits (63),  Expect = 0.005
 Identities = 21/63 (33%), Positives = 31/63 (49%), Gaps = 2/63 (3%)

Pattern                                      *******
Query    1    PPKEYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDDA  60
              PPKE +EA     K + NSS R +D  ++    E S L A+ E  E+      YE++  +
Sbjct    258  PPKEDVEAKR--IKGKENSSPRERDICEESSQKEVSDLLAELEAAERESESYEYEENSTS  315

Query    61   THR  63
                R
Sbjct    316  DGR  318


>gb|ADE59015.1|  retrotransposon-like 1 [Ovis aries]
Length=1333

 Score = 13.4 bits (41),  Expect = 1.2
 Identities = 15/39 (38%), Positives = 21/39 (53%), Gaps = 2/39 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENEKSDSD  51
              D   S Q S D  DD+  SEPS L    D +++E++  D
Sbjct    559  DEETSDQPSSDGSDDLSESEPSELQQAGDSDQSEETFYD  597


>ref|XP_001749539.1|  hypothetical protein [Monosiga brevicollis MX1]
 gb|EDQ85590.1|  predicted protein [Monosiga brevicollis MX1]
Length=437

 GENE ID: 5894788 MONBRDRAFT_29080 | hypothetical protein
[Monosiga brevicollis MX1]

 Score = 13.4 bits (41),  Expect = 1.2
 Identities = 16/46 (34%), Positives = 24/46 (52%), Gaps = 2/46 (4%)

Pattern                                        *******
Query    2    PKEYLEASASVCKDRRNSSQRSKDSEDDVWG--SEPSSLTADQEEN  45
              P+  L   +SV  D  NSS R+K  +D++    +E S L A  + N
Sbjct    386  PRMQLRRRSSVAADPVNSSSRTKQRDDELEADLAEVSDLLAQLKTN  431


>ref|NP_001181949.1|  retrotransposon-like protein 1 [Bos taurus]
 sp|Q52QI2.2|RTL1_BOVIN  RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived
protein PEG11; AltName: Full=Paternally
expressed gene 11 protein homolog
 gb|DAA17373.1|  retrotransposon-like 1 [Bos taurus]
Length=1331

 GENE ID: 606737 RTL1 | retrotransposon-like 1 [Bos taurus]
(10 or fewer PubMed links)

 Score = 13.4 bits (41),  Expect = 1.2
```

```
 Identities = 15/39 (38%), Positives = 21/39 (53%), Gaps = 2/39 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENEKSDSD  51
              D   S Q S D  DD+  SEPS L    D +++E++  D
Sbjct    558  DEETSDQPSSDGSDDLSESEPSELQQAGDSDQSEETFYD  596


>qb|AAX84834.1| G PEG11 [Bos taurus]
Length=770

 GENE ID: 606737 RTL1 | retrotransposon-like 1 [Bos taurus]
(10 or fewer PubMed links)

 Score = 13.4 bits (41),  Expect = 1.2
 Identities = 15/39 (38%), Positives = 21/39 (53%), Gaps = 2/39 (5%)

Pattern                          *******
Query    15  DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENEKSDSD  51
             D   S Q S D  DD+  SEPS L    D +++E++  D
Sbjct    48  DEETSDQPSSDGSDDLSESEPSELQQAGDSDQSEETFYD  86


>ref|YP_002455528.1| G hypothetical protein BafACA1_G14 [Borrelia afzelii ACA-1]
 qb|ACJ73442.1| G hypothetical protein BafACA1_G14 [Borrelia afzelii ACA-1]
Length=893

 GENE ID: 7205170 BafACA1_G14 | hypothetical protein [Borrelia afzelii ACA-1]

 Score = 13.0 bits (40),  Expect = 1.5
 Identities = 17/55 (30%), Positives = 30/55 (54%), Gaps = 4/55 (7%)

Pattern                              *******
Query    14   KDRRNSSQRSKDSEDDVW--GSEPSSLTADQEENEKSDSDKSYEDD--DDATHRG  64
              +D+ NSSQ + D   +     SE SSL+   E++ +++   S ++D  +D T  G
Sbjct    68   EDQDNSSQETADLSQETTEKNSEISSLSQKAEKDNETEEISSAKEDTTEDCTSLG  122


>ref|XP_003055888.1| G predicted protein [Micromonas pusilla CCMP1545]
 qb|EEH59264.1| G predicted protein [Micromonas pusilla CCMP1545]
Length=743

 GENE ID: 9681897 MICPUCDRAFT_46500 | hypothetical protein
[Micromonas pusilla CCMP1545]

 Score = 12.7 bits (39),  Expect = 1.9
 Identities = 14/37 (37%), Positives = 21/37 (56%), Gaps = 0/37 (0%)

Pattern                    *******
Query    23   SKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDD  59
              S+++E D +  EPS L A   E  + D D   E+D+D
Sbjct    414  SQETELDAFDEEPSELDAALAELGERDEDLDAEEDED  450


>ref|NP_001127830.4| UG retrotransposon-like protein 1 [Sus scrofa]
 qb|ACF47570.4| G retrotransposon-like 1 [Sus scrofa]
Length=1357

 GENE ID: 100187562 RTL1 | retrotransposon-like 1 [Sus scrofa]

 Score = 12.3 bits (38),  Expect = 2.5
 Identities = 14/34 (41%), Positives = 18/34 (52%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D +++E
Sbjct    563  DEETSDQPSSDGSDDLSESEPSELQQAGDSDQSE  596


>ref|XP_002636257.1| G Hypothetical protein CBG08539 [Caenorhabditis briggsae]
 emb|CAP28473.1| G Hypothetical protein CBG08539 [Caenorhabditis briggsae]
Length=881

 GENE ID: 8578252 CBG08539 | Hypothetical protein CBG08539
[Caenorhabditis briggsae] (10 or fewer PubMed links)

 Score = 12.3 bits (38),  Expect = 2.5
 Identities = 16/54 (29%), Positives = 25/54 (46%), Gaps = 9/54 (16%)

Pattern                                    *******
```

```
Query    4    EYLEASASVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDD  57
              E L+ + S+ +D+ N     +K+        SE S +  D E+   S     SY DD
Sbjct    318  EGLQDNMSLVQDQLNQHTMAKEQ-----ASEISEINQDNEDKGNS----SYSDD  362


>ref|NP_735809.1| G hypothetical protein gbs1372 [Streptococcus agalactiae NEM316]
 emb|CAD47031.1| G unknown [Streptococcus agalactiae NEM316]
Length=271

 GENE ID: 1030415 gbs1372 | hypothetical protein
[Streptococcus agalactiae NEM316] (10 or fewer PubMed links)

 Score = 12.0 bits (37),  Expect = 3.2
 Identities = 12/36 (33%), Positives = 23/36 (63%), Gaps = 1/36 (2%)

Pattern                       *******
Query    18   NSSQRSKDSEDDVWGSEPSSLTA-DQEENEKSDSDK  52
              ++   +++SE  +  +E S L A D+EENE+ + +K
Sbjct    148  SAPSETEESETYISETEKSDLIAEDEEENEREEQEK  183


>ref|XP_243381.5| UG PREDICTED: retrotransposon-like 1 [Rattus norvegicus]
Length=1587

 GENE ID: 314581 Rtl1 | retrotransposon-like 1 [Rattus norvegicus]

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/33 (42%), Positives = 17/33 (51%), Gaps = 2/33 (6%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEEN  45
              D   S Q S D  DD+  SEPS L    D ++N
Sbjct    798  DDETSDQPSSDGSDDLSESEPSELQQAGDSDQN  830


>ref|XP_001070232.2| G PREDICTED: retrotransposon-like 1 [Rattus norvegicus]
Length=1484

 GENE ID: 314581 Rtl1 | retrotransposon-like 1 [Rattus norvegicus]

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/33 (42%), Positives = 17/33 (51%), Gaps = 2/33 (6%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEEN  45
              D   S Q S D  DD+  SEPS L    D ++N
Sbjct    695  DDETSDQPSSDGSDDLSESEPSELQQAGDSDQN  727


>ref|XP_002723802.1| UG PREDICTED: retrotransposon-like 1 [Oryctolagus cuniculus]
Length=1228

 GENE ID: 100357459 LOC100357459 | retrotransposon-like 1
[Oryctolagus cuniculus]

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    652  DEETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  685


>gb|ACF47571.3| G retrotransposon-like 1 [Sus scrofa]
Length=837

 GENE ID: 100187562 RTL1 | retrotransposon-like 1 [Sus scrofa]

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/34 (41%), Positives = 18/34 (52%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D +++E
Sbjct    206  DDETSDQPSSDGSDDLSESEPSELQQAGDSDQSE  239


>gb|EDL97526.1|  rCG27781 [Rattus norvegicus]
Length=1133
```

```
 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/33 (42%), Positives = 17/33 (51%), Gaps = 2/33 (6%)

Pattern                         *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEEN  45
              D   S Q S D  DD+  SEPS L    D ++N
Sbjct    678  DDETSDQPSSDGSDDLSESEPSELQQAGDSDQN  710


>gb|EDL18690.1|  mCG1047978 [Mus musculus]
Length=1189

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 16/39 (41%), Positives = 18/39 (46%), Gaps = 6/39 (15%)

Pattern                         *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKS  53
              D   S Q S D  DD+  SEPS L          DSD+S
Sbjct    726  DDETSDQPSSDGSDDLSESEPSELQQ------AGDSDQS  758


>ref|YP_877622.1| G transglutaminase/protease [Clostridium novyi NT]
 gb|ABK61279.1| G predicted transglutaminase/protease [Clostridium novyi NT]
Length=868

 GENE ID: 4541049 NT01CX_1541 | transglutaminase/protease [Clostridium novyi NT]
(10 or fewer PubMed links)

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 13/53 (24%), Positives = 29/53 (54%), Gaps = 0/53 (0%)

Pattern                                *******
Query    11   SVCKDRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKSYEDDDDATHR  63
              +V +D+ NSS +  + E     +E SS+ A++  + K  + K ++   ++ +R
Sbjct    158  NVLEDKDNSSVKDNNIEKIDENNEKSSILAEENGSHKYTATKRHKKKHNSRNR  210


>ref|XP_001110319.1| UG PREDICTED: retrotransposon-like protein 1-like [Macaca mulatta]
Length=1359

 GENE ID: 718079 LOC718079 | similar to retrotransposon-like 1 [Macaca mulatta]

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                         *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    573  DEETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  606


>ref|NP_908998.1| UG retrotransposon-like protein 1 [Mus musculus]
 sp|Q7M732.1|RTL1_MOUSE G RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived
protein PEG11; AltName: Full=Paternally
expressed gene 11 protein; AltName: Full=Mammalian retrotransposon
derived protein 1
 tpg|DAA01153.1| G TPA_exp: RTL1 [Mus musculus]
 gb|ACF20046.1| G retrotransposon-like 1 [Mus musculus]
 gb|ACF20051.1| G retrotransposon-like 1 [Mus musculus]
Length=1744

 GENE ID: 353326 Rtl1 | retrotransposon-like 1 [Mus musculus]
(Over 10 PubMed links)

 Score = 11.6 bits (36),  Expect = 4.0
 Identities = 16/39 (41%), Positives = 18/39 (46%), Gaps = 6/39 (15%)

Pattern                         *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTADQEENEKSDSDKS  53
              D   S Q S D  DD+  SEPS L          DSD+S
Sbjct    826  DDETSDQPSSDGSDDLSESEPSELQQ------AGDSDQS  858


>ref|NP_982727.1| G hypothetical protein [Ashbya gossypii ATCC 10895]
 gb|AAS50551.1| G AAR184Wp [Ashbya gossypii ATCC 10895]
Length=836

 GENE ID: 4618686 AGOS_AAR184W | hypothetical protein
[Ashbya gossypii ATCC 10895] (10 or fewer PubMed links)
```

```
 Score = 11.2 bits (35),  Expect = 5.1
 Identities = 17/48 (35%), Positives = 28/48 (58%), Gaps = 11/48 (22%)

Pattern         *******
Query    33   SEPSSLTA----------DQEENEKSDSDKSYEDD-DDATHRGHVASA  69
             SE SSL+A          + EE+EKSD +++ ED+ +D   R  ++S+
Sbjct    174  SEVSSLSASDEEAIEDSEEDEEDEKSDGNRTQEDEFEDDDERDVISSS  221


>ref|XP_002917512.1| G PREDICTED: retrotransposon-like protein 1-like [Ailuropoda melanoleuca]
Length=1255

 GENE ID: 100472923 RTL1 | retrotransposon-like 1 [Ailuropoda melanoleuca]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
             D   S Q S D  DD+  SEPS L    D + +E
Sbjct    557  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  590


>emb|CBN79655.1|  hypothetical protein [Ectocarpus siliculosus]
Length=1196

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 15/46 (32%), Positives = 25/46 (54%), Gaps = 2/46 (4%)

Pattern                 *******
Query    26   SEDDVWG--SEPSSLTADQEENEKSDSDKSYEDDDDATHRGHVASA  69
             +E  VW   SE SSL+A ++   + D  +  E++  +  R  VA+A
Sbjct    818  AEKSVWSLKSEVSSLSARRDNASRRDRQREREEEALSARREAVAAA  863


>ref|XP_002825163.1| G PREDICTED: retrotransposon-like protein 1-like [Pongo abelii]
Length=1359

 GENE ID: 100450895 LOC100450895 | retrotransposon-like protein 1-like
[Pongo abelii]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
             D   S Q S D  DD+  SEPS L    D + +E
Sbjct    573  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  606


>ref|XP_002754334.1| G PREDICTED: retrotransposon-like protein 1 [Callithrix jacchus]
Length=1323

 GENE ID: 100414312 RTL1 | retrotransposon-like 1 [Callithrix jacchus]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
             D   S Q S D  DD+  SEPS L    D + +E
Sbjct    594  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  627


>gb|EFB14203.1|  hypothetical protein PANDA_005814 [Ailuropoda melanoleuca]
Length=591

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15  DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
             D   S Q S D  DD+  SEPS L    D + +E
Sbjct    48  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  81


>gb|EEH05867.1|  GYF domain-containing protein [Ajellomyces capsulatus G186AR]
Length=1591

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 16/31 (51%), Positives = 19/31 (61%), Gaps = 2/31 (6%)
```

```
Pattern                    *******
Query    23   SKDSEDDVWGS--EPSSLTADQEENEKSDSD  51
              SKDS DDV  S  E SSL A +  +E  DS+
Sbjct    377  SKDSSDDVGSSMKEKSSLAALERLSEVEDSN  407


>gb|AAI50618.1|  RTL1 protein [Homo sapiens]
Length=1358

 GENE ID: 388015 RTL1 | retrotransposon-like 1 [Homo sapiens]
(10 or fewer PubMed links)

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    572  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  605


>ref|XP_001490006.1|  PREDICTED: similar to RTL1 [Equus caballus]
Length=1349

 GENE ID: 100056048 LOC100056048 | similar to RTL1 [Equus caballus]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    559  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  592


>ref|XP_520846.2|  PREDICTED: similar to PEG11 [Pan troglodytes]
Length=968

 GENE ID: 465395 RTL1 | retrotransposon-like 1 [Pan troglodytes]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    182  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  215


>ref|NP_001128360.1|  retrotransposon-like protein 1 [Homo sapiens]
Length=1358

 GENE ID: 388015 RTL1 | retrotransposon-like 1 [Homo sapiens]
(10 or fewer PubMed links)

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    572  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  605


>sp|A6NKG5.2|RTL1_HUMAN  RecName: Full=Retrotransposon-like protein 1; AltName: Full=Retrotransposon-derived
protein PEG11; AltName: Full=Paternally
expressed gene 11 protein; AltName: Full=Mammalian retrotransposon
derived protein 1
Length=1359

 GENE ID: 388015 RTL1 | retrotransposon-like 1 [Homo sapiens]
(10 or fewer PubMed links)

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                          *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    573  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  606
```

```
>ref|XP_547983.2| UG PREDICTED: similar to retrotransposon-like 1 [Canis familiaris]
Length=1347

 GENE ID: 490861 RTL1 | retrotransposon-like 1 [Canis lupus familiaris]

 Score = 10.9 bits (34),  Expect = 6.6
 Identities = 14/34 (41%), Positives = 17/34 (50%), Gaps = 2/34 (5%)

Pattern                         *******
Query    15   DRRNSSQRSKDSEDDVWGSEPSSLTA--DQEENE  46
              D   S Q S D  DD+  SEPS L    D + +E
Sbjct    558  DDETSDQPSSDGSDDLSESEPSELQQAGDSDHSE  591


>ref|XP_002066126.1| G GK22102 [Drosophila willistoni]
 gb|EDW77112.1| G GK22102 [Drosophila willistoni]
Length=5295

 GENE ID: 6643432 Dwil\GK22102 | GK22102 gene product from transcript GK22102-RA
[Drosophila willistoni] (10 or fewer PubMed links)

 Score = 10.5 bits (33),  Expect = 8.4
 Identities = 13/47 (27%), Positives = 21/47 (44%), Gaps = 1/47 (2%)

Pattern                         *******
Query    15     DRRNSSQRSKDSEDDVWGSEPSSLTADQEENEK-SDSDKSYEDDDDA  60
                D +   Q  KD+E      E S + A++   EK  ++ K    D +A
Sbjct    3098   DAKKQKQAEKDAEKKAQQGEVSEIVAEKITQEKVEETQKPSVKDSEA  3144
```

FIGURE S1. **Sequence similarity searches, using Pattern Hit Initiated BLAST (PHI, NCBI; www.ncbi.nlm.nih.gov), identifies phosphorylation motifs similar to that of *At*PLC2 and *Sl*PLC4 in PI-PLCs of several plant species.** PHI blast using the amino acid sequence of the X/Y-linker region of tomato SlPLC4 and the PHI pattern E-[KPVI]-S-[ESD]-[IL]-x-[AQ], which represent all encountered amino acids in tomato PI-PLCs at this position, identifies similar phosphorylation motifs (indicated with asterisks) in PI-PLCs of several plant species within the *Solanaceae* and in *Torenia fournieri*. Search results also show homology at the putative phosphorylation region of SlPLC4 with transposon-like elements from animals.

TABLE S1**. PI-PLC phosphorylation results obtained from phospho-proteomic analyses in Arabidopsis (PhosPhAt: http://phosphat.mpimp-golm.mpg.de/db.html).** Among the nine *A. thaliana* PI-PLC isoforms, AtPLC1, AtPLC2, AtPLC4 and AtPLC7 (red rectangle) were found to be phosphorylated. Interestingly, all phosphorylation events were found to take place in the X/Y-linker region, which is present between the catalytic X and Y domains of the various PI-PLCs.

Phosphat Arabidopsis phosphorylation database					*http://phosphat.mpimp-golm.mpg.de/db.html*															
gene	**Code**	**Name**	**protein**	**species**	**peptide**	**modifiedpeptide**	**precursor**	**charge**	**no_pSTY**	**bins**	**PubMed**	**Search engine**	**Score**	**ppm**	**Treatment**	**CellCompartment**	**Instrument**	**Enrichment**	**Tissue**	**Genotype**
AtPLC1	At5g58670	**AtPLC2**	AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.251801	2	1	30.4.4		Mascot	36		nitrogen starvation / nitrate resupply	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
AtPLC2	At3g08510		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / sucrose resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744A/sirk1
AtPLC3	At4g38530		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / sucrose resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	col-0
AtPLC4	At5g58700		AT3G08510.1	*A. thaliana*	NKSEAKDDLDGND DDDDDDDEDK	NK(pS)EAKDDLDGND DDDDDDDEDK	893.31884	3	1	30.4.4	21768351	Mascot	104.74		none	membranes	LTQ-Orbitrap	IMAC / TiO2	rosette	stn8
AtPLC5	At5g58690		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.241	2	1	30.4.4	15308754	Mascot	42	-17.44928		plasma membrane	Q-TOF	Fe-IMAC	cell culture	col-0
AtPLC6	At2g40116		AT3G08510.1	*A. thaliana*	EIKTIFEK	EIK(pT)IFEK	544.273556	2	1	30.4.4		Mascot	20		phosphate starvation / phosphate resupply	plasma membrane	LTQ-Orbitrap	ZrO2	seedlings	col-0
AtPLC7	At3g55940		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	sirk1
AtPLC8	At3g47290		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / mannitol resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744D/sirk1
AtPLC9	At3g47220		AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / mannitol resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.249446	2	1	30.4.4		Mascot	21		isoxabene	plasma membrane	LTQ-Orbitrap	TiO2	seedlings	ixr
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744A/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / sucrose resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744D/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24188	2	1	30.4.4	17586839	Other		-2.29801	sucrose starvation / mannitol resupply	plasma membrane	LTQ	Fe-IMAC	seedlings	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24188	2	1	30.4.4	17586839	Mascot	28	-2.29801	sucrose starvation / mannitol resupply	plasma membrane	LTQ	Fe-IMAC	seedlings	col-0
			AT3G08510.1	*A. thaliana*	NKSEAKDDLDGND DDDDDDDEDK	NK(pS)EAKDDLDGND DDDDDDDEDK	893.32	3	1	30.4.4	20374526	Mascot	63		Abscisic acid / gibberellic acid / jasmonic acid / indole-3-acetic acid / kinetin	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.2494	2	1	30.4.4	18463617	Mascot	41	-0.47		total	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / sucrose resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / mannitol resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.251642	2	1	30.4.4		Mascot	22		full nutrition	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.251153	2	1	30.4.4		Mascot	32		isoxabene	plasma membrane	LTQ-Orbitrap	TiO2	seedlings	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	600.29	2	1	30.4.4	17651370	Mascot	36	0		plasma membrane	Q-TOF	Fe-IMAC	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / mannitol resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744A/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.251323	2	1	30.4.4		Mascot	28		isoxabene	plasma membrane	LTQ-Orbitrap	TiO2	seedlings	ixr
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / sucrose resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.251801	2	1	30.4.4		Mascot	99		nitrogen starvation / nitrate resupply	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.25	2	1	30.4.4	20466843	Mascot	41				LTQ-Orbitrap	HAMMOC / IMAC	cell culture	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1/sirk1
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation / mannitol resupply	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	sirk1
			AT3G08510.1	*A. thaliana*	RLSLSEEQLEK	RLS(pS)L(pS)EEQLEK	657.57	2	1	30.4.4	18686298	Mascot	56	319.95		low density membrane fraction (tonoplast enriched)	LTQ	Ga3+	leaf	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24097	2	1	30.4.4		Mascot	34		nitrate starvation / nitrate resupply	plasma membrane	LTQ-Orbitrap	ZrO2	seedlings	col-0
			AT3G08510.1	*A. thaliana*	EVPSFIQR	EVP(pS)FIQR	528.24971	2	1	30.4.4		Other			sucrose starvation	microsomal fraction	LTQ-Orbitrap	TiO2	seedlings	35S::SIRK1S744D/sirk1
		AtPLC7	AT3G55940.1	*A. thaliana*	YRHTVSVAPAEIK	YRH(pT)V(pS)VAPAEIK	775.886784	2	1	30.4.4		Mascot	99		nitrogen starvation / nitrate resupply	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G55940.1	*A. thaliana*	YRHTVSVAPAEIK	YRH(pT)V(pS)VAPAEIK	775.886784	2	1	30.4.4		Mascot	99		nitrogen starvation	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G55940.1	*A. thaliana*	YRHTVSVAPAEIK	YRH(pT)V(pS)VAPAEIK	775.886784	2	1	30.4.4		Mascot	34		nitrogen starvation / nitrate resupply	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G55940.1	*A. thaliana*	YRHTVSVAPAEIK	YRH(pT)V(pS)VAPAEIK	775.886784	2	1	30.4.4		Mascot	99		full nutrition	plasma membrane	LTQ-Orbitrap	TiO2	cell culture	col-0
			AT3G55940.1	*A. thaliana*	TLTSPVELIK	TL(pT)(pS)PVELIK	630.813137	2	2	30.4.4		Mascot	19		isoxabene	plasma membrane	LTQ-Orbitrap	TiO2	seedlings	col-0
			AT3G55940.1	*A. thaliana*	SVDKNDSNGDDDD DDDDDDDDDGD DK	(pS)VDKND(pS)NGDDD DDDDDDDDDDGDD K	1014.343	3	1	30.4.4	15308754	Mascot	63	30.95483		plasma membrane	Q-TOF	Fe-IMAC	cell culture	col-0
		AtPLC1	AT5G58670.1	*A. thaliana*	EYLQTQISKGSTTDE STR	EYLQTQI(pS)KGSTTDE STR	1062.75	2	1	30.4.4	18686298	Mascot	24	254.36		low density membrane fraction (tonoplast enriched)	LTQ	Ga3+	leaf	col-0
		AtPLC4	AT5G58700.1	*A. thaliana*	GKDSDEDVWGKEP EDLISTQSDLDK	GKD(pS)DEDVWGKEP EDLISTQSDLDK	962.7569817	3	1	30.4.4	23111157	Seaquest	5.802		nitrate starvation / nitrate resupply	total protein	LTQ	Ti-IMAC	seedlings	col-0
			AT5G58700.1	*A. thaliana*	GKDSDEDVWGKEP EDLISTQSDLDK	GKD(pS)DEDVWGKEP EDLISTQSDLDK	962.76	3	1	30.4.4	20466843	Mascot	67				LTQ-Orbitrap	HAMMOC / IMAC	cell culture	col-0

SUMMARY

Innate immunity is crucial for plants to defend themselves against a plethora of pathogens. Plants lack an adaptive immune system, but have a weak memory system that is known as systemic acquired resistance, effective against above-ground pathogens, and induced systemic resistance, effective against root pathogens. In both cases, plants do respond more quickly and to a higher level to a second infection. This phenomenon is also known as priming. On the other hand, swift immune reactions mediated by cell surface- and cytoplasmic immune receptors are vital for plant immunity and lead to the execution of downstream defense responses to prevent disease. Conserved molecules from pathogens are known as Pathogen-Associated Molecular Patterns (PAMPs), such as lipopolysaccharides (LPS) and flagellin (and its epitope, flg22) from bacteria, and chitin and glycans from fungi. PAMPs are recognized by cell surface immune receptors, known as Pattern Recognition Receptors (PRRs). The activation of PRRs mediates what is commonly known as PAMP-Triggered Immunity (PTI). Conversely, effectors are specialized molecules secreted by pathogens that can suppress PTI, but can also be recognized by Receptor-Like proteins (RLPs) on the cell surface of the host or in the cytoplasm by Nucleotide-Binding Leucine-Rich Repeat proteins (NB-LRRs). The response initiated after effector recognition either at the cell surface by RLPs or in the cytoplasm by NB-LRRs is referred to as Effector-Triggered Immunity (ETI).

Phospholipid signaling via Phosphatidylinositol-Specific Phospholipase-C (PI-PLC, or in short PLC) has long been suggested to play a role in immune receptor-mediated defense signaling in plants, as has been reported before in animals. However, phospholipid signaling in plants has, by far, not received the same attention as in animals. This is partly due to the presence of multiple PLC isoforms within one plant species and differences in signaling mechanisms compared to animals. In this study, the role of the tomato *PLC* gene family in signaling during plant defense is investigated.

Chapter 1 provides a general introduction in which the types of plant resistance and immune reactions are briefly discussed, with an emphasis on phospholipids as defense signaling molecules. The differences between plant- and animal PLC-mediated signaling are discussed and the *Cladosporium fulvum* – tomato system, which is the main model system used in this study, is introduced. Finally, an outline of the thesis is provided.

In **Chapter 2**, the identification and partial characterization of the tomato *PLC* gene family is reported. Based on expression profiling of this gene family during the onset of the resistance response, *PLC4* and *PLC6* were selected for their possible role in resistance to multiple pathogens. The effects of down-regulation (by Virus-Induced Gene Silencing) and transient over-expression (by agroinfiltration) of both genes on the Hypersensitive Response (HR) and resistance against multiple pathogens were studied. Tomato PLC4 was found to be required for the Cf-4/Avr4-induced HR, whereas PLC6 appeared to play a role in resistance of tomato, not only to *C. fulvum*, but also to the vascular fungal pathogen *Verticillium dahliae* and the bacterial pathogen *Pseudomonas syringae*. Heterologous expression, combined with activity assays of the encoded enzymes, was used to prove that the studied *PLC4* and *PLC6*

genes encode active enzymes, suggesting that the observed phenotypes depend on their activity.

Chapter 3 describes the transcriptional regulation of the various tomato *PLC* genes upon initiation of the HR. *PLC* gene regulation was studied in transgenic tomato seedlings expressing both the Cf-4 and Avr4 proteins that resulted from crossing plants carrying *Cf-4* with plants carrying the *Avr4* gene. In these *Cf-4*/*Avr4* F1 plants, the HR was activated in a synchronized way by reducing both the ambient temperature and humidity, as the Cf-4-mediated HR is both temperature- and humidity-sensitive, being suppressed at elevated temperatures and 100% relative humidity. *PLC3* and, to a lesser extent, *PLC6* were transcriptionally up-regulated in response to elevated temperatures in all seedlings, including the control parental lines only expressing Cf-4 or Avr4. This up-regulation of *PLC* gene expression was accompanied by a rapid increase in the levels of phosphatidic acid and a decrease in the levels of PI and PI-phosphate, suggesting the involvement of the enzymes encoded by *PLC3* and *PLC6* in the response of the seedlings to elevated temperatures. The levels of structural phospholipids, such as phosphatidylcholine and phosphatidylglycerol, decreased during recovery of the seedlings from the elevated temperature, indicating the involvement of phospholipase D activity and a change in the composition of phospholipids in the plasma membrane of plant cells.

In **Chapter 4**, the roles of tomato *PLC* genes are re-evaluated, based on the recently published tomato genome sequence. Interestingly, yet another tomato *PLC* gene (*SlPLC7*) was identified. The tomato *PLC* gene sequences were used as a query to identify all related *PLC* sequences in other plant genomic sequences available to date in the public sequence database at NCBI. Subsequently, a phylogenetic analysis was performed in order to relate the tomato *PLC* genes to their orthologues in other plant species. Based on recent observations described in the literature, it is proposed that the X/Y-linker region of the plant PI-PLC enzymes plays a role in their activation. In this chapter, the enzymatic activity of recombinant proteins encoded by all tomato *PLC* genes, except for PLC7, was studied. The optimum PLC activity requirements, with regard to the pH and Ca^{2+} concentration of the incubation medium and substrate-preference, were determined for three PLC isoforms. This was achieved by performing *in vitro* PLC enzyme assays using non-radioactive techniques to visualize the substrates and reaction products. It is demonstrated that the PLC inhibitor U73122 blocks activity of these three PLC enzymes *in vitro*. The same inhibitor was subsequently used to study the requirement of PLC activity in ETI and PTI mediated by different cell surface immune receptors, using medium alkalization as a read-out. The effect of the inhibition of PLC activity on the internalization of the receptor-like kinase Flagellin Sensing 2 (FLS2), in response to treatment with the PAMP flg22 was also monitored.

Chapter 5 provides an overview and discussion of the current status of what is known about PLC defense signaling in plants. It starts by looking back to the discovery of the PLC pathway in animals and plants and discusses the main differences and similarities of the PLC enzymes in the two kingdoms. Subsequently, the differences between PI-PLCs and other types of PLC enzymes in eukaryotes and prokaryotes are discussed. From this overview it becomes clear that the essential role of PLC enzymes in phospholipid signaling relates on the one hand to depletion of specific substrates and on the other hand to the generation of the respective reaction products, which are all involved in signaling. Accordingly, the findings

described in this thesis, as well as those reported by others in the literature, are placed in a broader perspective and provide clues for the involvement of phospholipid molecules in different defense response pathways. Based on the available data, the different activation mechanisms of plant PLC enzymes, following the triggering of immune receptors, are described. Finally, a model is put forward that summarizes all the information that has been discussed in this chapter. It proposes a central role for PLC signaling downstream of plant immune receptors.

SAMENVATTING

Intrinsieke immuniteit is van cruciaal belang voor planten om zich te verdedigen tegen een overvloed aan ziekteverwekkers. Planten hebben geen adaptief afweersysteem zoals zoogdieren en de mens, maar ze beschikken wel over een geheugensysteem dat bekend staat als systemisch verworven resistentie ("systemic acquired resistance"), dat effectief is tegen bovengrondse pathogenen en geïnduceerde systemische resistentie ("induced systemic resistance"), dat effectief is tegen ondergrondse pathogenen. In beide gevallen zal een plant sneller en sterker reageren op een volgende infectie. Dit fenomeen staat ook bekend als "priming". Anderzijds zijn snelle lokale afweerreacties, geactiveerd door immuunreceptoren aanwezig aan het celoppervlak of in het cytoplasma, van cruciaal belang voor de afweer van planten tegen ziekteverwekkers. Geconserveerde verbindingen afkomstig van pathogenen staan bekend als pathogeen-geassocieerde moleculaire patronen ("Pathogen-Associated Molecular Patterns"; PAMPs). Voorbeelden van PAMPs zijn lipopolysacchariden (LPS) en het eiwit flagelline (en zijn epitoop, flg22) van bacteriën en chitine en glucanen van schimmels. PAMPs worden herkend door immuunreceptoren die aanwezig zijn op het celoppervlak en "Pattern Recognition Receptors" (PRRs) worden genoemd. Activering van PRRs, welke meestal receptor-achtige kinases ("Receptor-Like Kinases"; RLKs) zijn, door PAMPs leidt tot "PAMP-Triggered Immunity" (PTI). Effectoren, ook wel bekend als virulentiefactoren, daarentegen zijn meestal soort-specifieke moleculen die door pathogenen worden uitgescheiden en PTI kunnen onderdrukken of te niet doen. Effectoren kunnen enerzijds herkend worden door RLKs of receptor-achtige eiwitten ("Receptor-Like Proteins"; RLPs) op het celoppervlak van de gastheerplant, en anderzijds door "Nucleotide-Binding Leucine-Rich Repeat" eiwitten (NB-LRRs) die zich in het cytoplasm of de kern van de waardplant bevinden. Herkenning van een effector door een RLK, RLP of NB-LRR leidt tot "Effector-Triggered Immunity" (ETI).

Er werd reeds lang aangenomen dat, net als bij dieren, signaal transductie via Phosphatidylinositol-specifieke Phospholipase-C (PI-PLC, of kortweg PLC) in planten een rol speelt bij de verdedigingsreactie die geactiveerd wordt door immuunreceptoren. Fosfolipide signalering heeft bij planten echter veel minder aandacht gekregen dan bij dieren. Dit is deels te wijten aan de aanwezigheid van meerdere, verglijkbare, PLC isovormen in een bepaalde plantensoort en een intrinsiek verschil in de signaleringsmechanismen tussen dieren en planten. In het in dit proefschrift beschreven onderzoek wordt de rol van de *PLC* genfamilie van tomaat (*Solanum lycopersicon*) in de signaal transductie tijdens de afweerreactie van de plant tegen ziekteverwekkers bestudeerd.

Hoofdstuk 1 bestaat uit een algemene inleiding waarin de verschillende soorten van resistentie van planten en de bijbehorende immuunreacties in het kort worden besproken, met de nadruk op specifieke fosfolipiden als signaalmoleculen. De verschillen tussen signalering geactiveerd door PLCs in planten en in dieren worden besproken en vervolgens wordt de interactie tussen de ziekteverwekkende schimmel *Cladosporium fulvum* en tomaat, het meest gebruikte modelsysteem in deze studie, geïntroduceerd. In deze interactie herkennen Cf resistentie eiwitten van tomaat de door *C. fulvum* uitgescheiden avirulentie (Avr) eiwitten,

waardoor resistentie optreedt. Tot slot wordt er een kort overzicht van de inhoud van het proefschrift gegeven.

In **Hoofdstuk 2** wordt de identificatie en gedeeltelijke karakterisering van de tomaat *PLC* genfamilie beschreven. Op basis van de verkregen expressieprofielen van deze genfamilie tijdens het begin van de resistentiereactie, werden *PLC4* en *PLC6* geselecteerd voor verder onderzoek vanwege hun mogelijke rol in de resistentie van de tomatenplant tegen meerdere pathogenen. In de testplant *Nicotiana benthamiana* werd het effect van een veranderde expressie van beide genen bestudeerd. Dat werd gedaan door middel van "knock-down", met behulp van virus-geïnduceerde gen silencing en overexpressie, met behulp van transgene *Agrobacterium tumefaciens*; agroinfiltratie, van beide genen, waarna het effect op de overgevoeligheidsreactie ("Hypersensitive Response"; HR) en resistentie tegen meerdere ziekteverwekkers werd bestudeerd. Tomaat PLC4 bleek vereist voor de door de Cf-4/Avr4 combinatie geïnduceerde HR, terwijl PLC6 een rol bleek te spelen bij de resistentie van tomaat, niet alleen tegen het bladpathogeen *C. fulvum* maar ook tegen het vaatpathogeen *Verticillium dahliae* en de bacterie *Pseudomonas syringae*. Heterologe expressie, gecombineerd met activiteitstoetsen van de gecodeerde enzymen, werd gebruikt om aan te tonen dat de bestudeerde *PLC4* en *PLC6* genen coderen voor actieve enzymen, wat suggereert dat de waargenomen fenotypes afhankelijk zijn van hun enzym activiteit.

Hoofdstuk 3 beschrijft de transcriptionele regulatie van de verschillende *PLC* genen van tomaat bij de aanvang van de HR. *PLC* genregulatie werd onderzocht in transgene zaailingen van tomaat die zowel het Cf-4 als het Avr4 eiwit produceren. Deze zaailingen zijn verkregen uit een kruising tussen planten die het *Cf-4* gen bevatten en planten die het *Avr4* gen van *C. fulvum* bevatten. In deze *Cf-4/Avr4* F1 planten kan de HR synchroon worden geactiveerd door zowel de omgevingstemperatuur als de luchtvochtigheid te manipuleren. Dit komt doordat de door Cf-4/Avr4 geactiveerde HR zowel gevoelig is voor de temperatuur als de luchtvochtigheid en kan worden onderdrukt bij een relatief hoge temperatuur en 100% relatieve luchtvochtigheid. Verlaging van de temperatuur en luchtvochtigheid leidt vervolgens to het activeren van een systemische HR. De expressie van *PLC3* en in mindere mate die van *PLC6* was verhoogd als reactie op de verhoogde temperaturen in alle zaailingen, inclusief de ouderlijnen die *Cf-4* of *Avr4* bevatten. Deze verhoging van de *PLC* genexpressie ging gepaard met een snelle toename van de niveaus van het fosfolipide "phosphatidic acid" (PA) (een mogelijk product van PLC activiteit) en een daling van de niveaus van PI en PI-fosfaat (het substraat van PLCs). Mogelijk spelen de enzymen gecodeerd door *PLC3* en *PLC6* dus een rol in de reactie van de zaailingen op verhoogde temperaturen. De niveaus van diverse structurele fosfolipiden, zoals phosphatidylcholine en phosphatidylglycerol, namen af gedurende het herstel van de zaailingen na een verlaging van de temperatuur, wat wijst op de betrokkenheid van phospholipase D activiteit en een hiermee gepaard gaande verandering in de samenstelling van de fosfolipiden in het plasmamembraan van de plantencellen.

In **Hoofdstuk 4** wordt de rol van de diverse tomaat *PLC* genen in ziekteresistentie opnieuw geëvalueerd op basis van de recent gepubliceerde genoomsequentie van tomaat. Interessant was dat er nog een extra tomaat *PLC* gen (*SlPLC7*) werd geïdentificeerd. De diverse tomaat *PLC*-gensequenties werden gebruikt voor een zoekopdracht in de publieke sequentie-databanken van NCBI om daarmee alle gerelateerde *PLC* sequenties in andere planten te identificeren. Vervolgens werd een fylogenetische analyse uitgevoerd om de

relaties te bekijken tussen de tomaat *PLC* genen en hun orthologen in andere plantensoorten. Op basis van recent gepubliceerde data wordt voorgesteld dat het X/Y-linkergebied, dat aanwezig is in alle plant PI-PLC enzymen, een rol speelt bij hun activatie. In dit hoofdstuk wordt de enzymactiviteit van de recombinante eiwitten die gecodeerd worden door alle tomaat *PLC* genen, behalve *PLC7*, beschreven. De optimale PLC activiteit, in relatie tot de pH en de Ca^{2+} concentratie in het incubatiemedium en de substraat specificiteit, werden bepaald voor drie verschillende PLC isovormen. Dit werd gedaan door middel van *in vitro* PLC enzymtoetsen, gecombineerd met niet-radioactieve technieken om de substraten en de bijbehorende reactieproducten te visualiseren. Er werd aangetoond dat de PLC remmer U73122 de activiteit van deze drie PLC enzymen *in vitro* blokkeert. Dezelfde remmer werd vervolgens ook gebruikt om het belang van PLC activiteit in ETI en PTI te bestuderen; hierbij werd met name gekeken naar de rol van PLCs op medium alkalisatie, een van de vele responsen die optreden bij PTI en ETI. Daarnaast werd het effect van het remmen van de PLC activiteit op de internalisatie (endocytose) van de RLK Flagellin Sensing 2 (FLS2) bestudeerd na behandeling met de PAMP flg22.

Hoofdstuk 5 geeft een overzicht, in de vorm van een algemene discussie, van de beschikbare informatie in de literatuur met betrekking tot de rol van PLC signalering in de verdediging van planten tegen ziekteverwekkers. Het overzicht begint met het beschrijven van de ontdekking van de PLC signaleringsroute in dieren en planten en bespreekt de belangrijkste verschillen en overeenkomsten tussen de PLC-enzymen in deze twee koninkrijken. Vervolgens worden de verschillen tussen PI-PLCs en andere soorten PLC enzymen in eukaryoten en prokaryoten besproken. Uit dit overzicht wordt duidelijk dat de essentiële rol van PLC enzymen in fosfolipide signalering enerzijds het uitputten van specifieke substraten betreft en anderzijds het genereren van de respectievelijke reactieproducten die allen betrokken zijn bij de signalering in de cel. Vervolgens worden de in dit proefschrift beschreven bevindingen, evenals de waarnemingen die beschreven zijn door anderen, in een breder perspectief geplaatst, wat aanwijzingen geeft voor de betrokkenheid van fosfolipiden in verschillende afweerroutes. Op basis van de beschikbare gegevens worden de verschillende mechanismen van activatie van de PLC enzymen in planten, na de activatie van immuunreceptoren, beschreven. Ten slotte wordt een model gepresenteerd waarin alle informatie die in dit hoofdstuk werd besproken wordt samengevat en geïntegreerd. In dit model speelt in planten de PLC signalering na de activatie van immuunreceptoren een centrale rol.

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Ali, Grardy, Henriek, Rob, Ester; thank you all for supporting research at the Laboratory of Phytopathology, including that of me. I am also grateful for the assistance that I received from Florian and Arjan during their MSc project with me; I appreciated your contribution greatly. Last but not least, I am grateful for the outstanding plant care performed by Henk and Bert from Unifarm. Henk, thank you very much for all the plants and materials

that you have arranged for me. Bert, I appreciate your accuracy and understanding of the critical requirements of some experiments, thanks for your support. Finally, I wish all my colleagues at the Laboratory of Phytopathology lots of success and satisfaction in their research projects and in their personal lives.

Ahmed

Curriculum Vitae

Ahmed Mohamed Abd-El-Haliem was born on May 16th, 1977 in Ismailia, Egypt. After finishing his high school in Ismailia, he moved to El-Arish in the North of the Sinai Peninsula where he spent four years studying at the faculty of environmental agricultural Sciences of Suez Canal University. After obtaining his bachelor degree in 1998, he went to his beloved South Sinai where he "shifted gears" and worked for two years as a front office manager in different resorts in Dahab. This allowed him to do lots of snorkeling, diving, fishing and exploration of the mountains in the area. March 2000 marks his first experience with the Netherlands, the country from which his wife originates. He started his journey in the Netherlands by following intensive Dutch courses at the ROC Aventus in Deventer, which he combined with several part time jobs via work agencies. One and half year later, he obtained his diploma of the "Staatsexamens Nederlands als tweede taal" (NTII), program II. This gave him the option to study in the Netherlands in Dutch. He took the challenge and enrolled into the Dutch HLO program at Larenstein University for Applied Sciences in Velp. During his study at Larenstein, he did his BSc internship in 2003 at the biotech company Keygene in Wageningen where he worked on the generation of RNAi silencing lines to study newly identified proteins interacting with the Mi resistance protein. In 2004, he carried out his major BSc thesis at Plant Research International in Wageningen, where he worked on the analysis of stress-inducible gene expression in tomato seeds as a result of seed priming.

Although studying molecular biology in Dutch seemed at the beginning to be more difficult than anticipated, he managed to earn his HLO degree in Plant Biotechnology in 2004. Directly after his graduation, he joined the Laboratory of Phytopathology of Wageningen University where he worked for one year as a research assistant on the functional analysis of genes required for Cf-4-mediated resistance to *Cladosporium fulvum*. During that period he obtained the possibility to do a PhD by applying for a "Mozaïek" grant at the Netherlands Organization for Scientific Research (NWO). In parallel with his participation in the "Mozaïek" competition procedures, he obtained his MSc diploma Plant Biotechnology in 2006 from Wageningen University and obtained a "Mozaïek" grant at the end of the same year. Ahmed started his PhD project in 2007 in which he investigated the role of tomato phospholipase C in defense against pathogens under the supervision of Dr. Matthieu Joosten and Professor Pierre de Wit. In 2011 he started to work as a pre-postdoc at the Laboratory of Plant Breeding of Wageningen University, working on the identification and functional analysis of *Phytophthora infestans* effector targets in plants.

List of Publications

Abd-El-Haliem A and Joosten MHAJ (2014). Plant Phosphatidylinositol-Specific Phospholipase C (PI-PLC): Activation, Regulation and Function in Receptor-Mediated Defense Signaling Against Microbes (manuscript in preparation).

Abd-El-Haliem A, Vossen JH, van Zeijl A, Dezhsetan S, Testerink C, Martine, Robatzek S, Joosten MHAJ (2014). Biochemical characterization of the tomato phosphatidylinositol-specific Phospholipase C (PI-PLC) family and its role in plant immunity (manuscript submitted).

Gonorazky G, Ramirez L, **Abd-El-Haliem A**, Vossen JH, Lamattina L, ten Have A, Joosten MHAJ, Laxalt AM (2014). The tomato phosphatidylinositol-phospholipase C2 (SlPLC2) is required for defense gene induction by the fungal elicitor xylanase. Journal of Plant Physiology, 171:959–965.

Abd-El-Haliem A, Meijer HJG, Tameling WIL, Vossen JH, Joosten MHAJ (2012). Defense activation triggers differential expression of phospholipase-C (PLC) genes and elevated temperature induces phosphatidic acid (PA) accumulation in tomato. Plant Signaling & Behavior, 7:9, 1073-1078.

Abd-El-Haliem A (2012). An Unbiased Method for the Quantitation of Disease Phenotypes Using a Custom-Built Macro Plugin for the Program ImageJ. Book chapter in Plant Fungal Pathogens, Methods in Molecular Biology, 835:635-644.

Liebrand TWH, Smit P, **Abd-El-Haliem A**, de Jonge R, Cordewener JHG, America AHP, Sklenar J, Jones AME, Robatzek S, Thomma BPHJ, Tameling WIL, Joosten MHAJ (2012). Endoplasmic reticulum-quality control chaperones facilitate the biogenesis of Cf receptor-like proteins involved in pathogen resistance of tomato. Plant Physiology, 159:1819-1833.

Fradin EF, **Abd-El-Haliem A**, Masini L, van den Berg GCM, Joosten MHAJ, Thomma BPHJ (2011). Interfamily transfer of tomato Ve1 mediates Verticillium resistance in Arabidopsis. Plant Physiology, 156:2255-2265.

Vossen JH*, **Abd-El-Haliem A***, Fradin EF, van den Berg GCM, Ekengren SK, Meijer HJG, Seifi Abdolabad AR, Bai Y, Have A ten, Munnik T, Thomma BPHJ, Joosten MHAJ (2010). Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family members and the role of PLC4 and PLC6 in HR and disease resistance. The Plant Journal, 62:224-239, **(*)** shared first authorship.

Gabriëls SHEJ; Vossen JH, Ekengren SK, van Ooijen G, **Abd-El-Haliem A,** van den Berg GCM, Rainey DY, Martin GB, Takken FLW, de Wit PJGM, Joosten MHAJ (2007). An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. The Plant Journal 50:14-28.

Education Statement of the Graduate School

Experimental Plant Sciences

Date: **23 October 2014**

Group: **Phytopathology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
"The Role of Phospholipid Signalling in the Defence of Plants Against Pathogens'	Apr 17, 2007
► **Writing or rewriting a project proposal**	
"The role of phospholipid signalling in the defense of plants against pathogens", NWO Mozaiek	2005-2006
► **Writing a review or book chapter**	
"An unbiased method for the quantitation of disease phenotypes using a custom-built macro plugin for the program", Image J, 10.1007/978-1-61779-501-5_41.	2012
"Plant Phosphatidylinositol-Specific Phospholipase C (PI-PLC): Activation, Regulation and Function in Receptor-Mediated Defense Signaling Against Microbes" (review, in submission)	2014
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days, Wageningen University	Sep 13, 2007
1st EPS Joint Retreat of the PhD Students, Wageningen	Oct 02-03 2008
EPS PhD student days, University of Leiden	Feb 26, 2009
► **EPS theme symposia**	
EPS theme 2 symposium: 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
EPS theme 2 Symposium & WCS: 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
EPS theme 2 symposium: 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
► **NWO Lunteren days and other National Platforms**	
NWO-CW – Lipids and Biomembranes, Lunteren.	Mar 05-06, 2007
ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 02-03, 2007
PR-Proteins & Induced Resistance Against Pathogens & insects, Doorn, The Netherlands	May 10-11, 2007
ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 07- 08, 2008
ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 19-20, 2010
CBSG Summit, Wageningen, The Netherlands	Feb 29, 2012
ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 02-03, 2012
Next Generation Plant Breeding Conference, Ede, The Netherlands	Nov 11-14, 2012
► **Seminars (series), workshops and symposia**	
Flying Seminar Carrington, 'Diversification of Small RNA Pathways in Plants'	Mar 26, 2007
EPS Seminar Andrew Bent, 'Active site discovery in LRR domains: FLS2/flagellin perception and other examples'	June 18, 2007

European Flying Seminar Scott Poethig 'Regulation of phase change in plants by miRNAs and trans-acting siRNAs'	Sep 24, 2007
EPS Seminar T. Nürnberger, 'Patterns and receptors in plant immunity'	Dec 17, 2008
EPS Seminar Cyril Zipfel, 'Innate immunity'	Feb 06, 2009
EPS Seminar Series Plant Sciences: Plant Physiology and Molecular Biology chair groups	Sep 08, 2009
EPS Seminar Series Plant Sciences: Horticulture and Bioinformatics chair groups	Oct 13, 2009
Mini Symposium: How to write a world-class paper	Oct 26, 2010
EPS Seminar Brande Wulff, 'Isolation of novel resistance genes for stem rust race Ug99 from diploid wheat relatives'	Jun 09, 2010
EPS Seminar Paul Birch, 'Trying to understand susceptibility and exploit resistance in potato-Phytophthora infestans' interactions'	May 20, 2010
Plant Sciences Seminar Paul Struik, 'CSA: bridging the scales in crop production' and Holger Meinke 'Bridging the genotype-phenotype gap' and 'Bridging disciplines to improve rice-based systems'	Apr 13, 2010
Plant Sciences Seminar Jaap Molenaar, 'Math@WURk' and Gerco Angenent,'Communication between genes and proteins for plant development'	Feb 09, 2010
Seminars Frontiers in Plant-Microbe interactions Christiane Gebhardt, 'The molecular basis of quantitative traits in potato' and Peter Moffett, 'Constitutive and R gene-induced defences against plant viruses'	Feb 05, 2010
EPS Seminar Nick Panopoulos, 'Playing the HRP: Evolution of Our Understanding of HRP Gene'	Jan 11, 2010
Plant Sciences Seminar Ken Giller, 'Systems analysis for integrated assessment of trade-offs within agricultural systems, and Richard Visser, 'From bridging to closing the gap between phenotype and genotype'	Dec 08, 2009
Mini-symposium 'Plant Breeding in the Genomics Era', Wageningen, The Netherlands	Nov 25, 2011
Seminar Ralph Panstruga, 'Comparative pathogenomics of powdery mildew fungi: chasing the molecular secrets of obligate biotrophy and fungal pathogenesis'	Dec 04, 2012
EPS workshop "Plant Endomembranes", Vrije Universiteit, Amsterdam	Jul 02, 2010
► **Seminar plus**	
► **International symposia and congresses**	
Molecular Plant Microbe Interactions (XIII), Sorrento, Italy	Jul 21-27, 2007
Keystone symposium: 'Receptors and Signaling in Plant Development and Biotic Interactions', Tahoe city, California, USA.	Mar 14-19, 2010
Molecular Plant Microbe Interactions (XV), Kyoto, Japan	Jul 29- Aug 02, 2012
► **Presentations**	
Poster: Molecular Plant Microbe Interactions (XIII), Sorrento (Italy)	Jul 21-27, 2007
Oral: Joint EPS theme 2 Symposium & WCS, Utrecht University.	Jan 15, 2010
Poster: Keystone symposium: Receptors and Signaling in Plant Development and Biotic Interactions	Mar 14-19, 2010
Oral: ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 19, 2010
Poster: ALW meeting 'Experimental Plant Sciences (EPS)', Lunteren, The Netherlands	Apr 02-03, 2012
Oral: Molecular Plant Microbe Interactions (XV), Kyoto, Japan	Jul 29- Aug 02, 2012
Poster: CBSG Summit, Wageningen, The Netherlands	Feb 29, 2012
Oral (Flash): CBSG Summit, Wageningen, The Netherlands	Feb 29, 2012
Oral:Hogeschool van Arnhem en Nijmegen (HAN)	Sep 30, 2009
Poster: 1st EPS Joint Retreat, Wageningen, The Netherlands.	Oct 02-03 2008
► **IAB interview**	Dec 04, 2009
► **Excursions**	
CBSG match making event, visiting two breeding companies	Oct 18, 2012

Subtotal Scientific Exposure	*28.3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS Summer School, BioExploit, "Evolution of Plant Pathogen Interactions: From Principles to Practice", Wageningen, The Netherlands	Jun18-20, 2008
EPS Autumn School 'Host-Microbe-Interactomics', Wageningen, The Netherlands	Nov 01-03, 2011
► **Journal club**	
member of PhD discussion group Phytopathology	2007-2010
► **Individual research training**	
Subtotal In-Depth Studies	*4.8 credits**

4) Personal development	*date*
► **Skill training courses**	
Techniques for Writing and Presenting Scientific Papers	Apr 14-17, 2009
EndNote X advanced	Mar 06, 2008
Postdoc Career Development Initiative (PCDI), Netherlands Genomics Initiative, Heeze, The Netherlands	Apr 21-23, 2010
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Membership of EPS-PhD council	2008-2010
Membership EPS-Education Committee	2009-2010
Subtotal Personal Development	*4.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**51.1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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