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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 to quickly respond to biotic and abiotic stresses. The rapid activation of phosphatidylinositol-specific phospholipase C (PLC) enzymes occurs early after the stimulation of plant immune-receptors. Genomes of different plant species encode multiple PLC homologs belonging to one class, PLCζ. Here we determined whether all tomato homologs encode active enzymes and whether they can generate signals that are distinct from one another. We searched the recently completed tomato (*Solanum lycopersicum*) genome sequence and identified a total of seven *PLCs*. Recombinant proteins were produced for all tomato PLCs, except for *Sl*PLC7. The purified proteins showed typical PLC activity, as different PLC substrates were hydrolysed to produce diacylglycerol. We studied *Sl*PLC2, *Sl*PLC4 and *Sl*PLC5 enzymes in more detail and observed distinct requirements for Ca^{2+} ions and pH, for both their optimum activity and substrate preference. This indicates that each enzyme could be differentially and specifically regulated *in vivo*, leading to the generation of PLC homolog-specific signals in response to different stimuli. PLC overexpression and specific inhibition of PLC activity revealed that PLC is required for both specific effector- and more general "pattern"-triggered immunity. For the latter, we found that both the flagellin-triggered response and the internalization of the corresponding receptor, Flagellin Sensing 2 (FLS2) of *Arabidopsis thaliana*, are suppressed by inhibition of PLC activity. Altogether, our data support an important role for PLC enzymes in plant defence signalling downstream of immune receptors.
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1. Introduction

Eukaryotic phosphatidylinositol-phospholipase C (PI-PLC or PLC) enzymes exist in six different classes in animals and they play important roles in intracellular signalling by hydrolysing inositol lipids in cellular membranes [1–3]. A typical PLC enzyme contains an N-terminal EF hand domain which is involved in calcium binding. This domain is followed by the catalytic X (PLCXc) and Y (PLCYc) domains which are known to form together a distorted triose phosphate isomerase (TIM) barrel structure containing the active-site residues [4]. The X and Y domains are separated by what is often described as a highly variable linker (X/Y-linker). The X/Y-linker of mammalian PLCs is enriched with dense clusters of negatively charged amino acid residues, which are involved in auto-inhibition of the enzyme at the resting state or activation by electrostatic repulsion when the PLC enzyme approaches the negatively charged membrane [4]. In addition, PLCs have a C2 domain that is located at the C-terminal end [1,2]. This domain was found to be crucial for the enzymatic activity of PLCδ1 from rat [5,6] and is required for the binding of Ca^{2+} ions [7]. In plants, it was recently

Abbreviations: At, Arabidopsis thaliana; DAG, diacylglycerol; ETI, effector-triggered immunity; HR, hypersensitive response; $InsP_3$, inositol 1,4,5-triphosphate; PA, phosphatidic acid; PAMPs, pathogen-associated molecular patterns; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PTI, PAMP-triggered immunity; PKC, protein kinase C; *Sl*, *Solanum lycopersicum*; TLC, thin layer chromatography.

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demonstrated that the C2 domain of a PLC from rice is responsible for targeting the enzyme to the plasma-membrane in response to Ca^{2+} [3].

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolysed by PLC 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 [8], while DAG is retained in the membrane where it activates protein kinase C (PKC) [9] or serves as a substrate for other enzymes [1,10,11]. The activation of PKC is responsible for triggering a cascade of other downstream targets, mainly by phosphorylation [12–14], while triggering $InsP_3$-receptors is responsible for the generation of different Ca^{2+} signatures. The generated Ca^{2+} signals are thought to differentially coordinate the biochemical activity of several Ca^{2+}-dependent targets [13,15,16]. Moreover, 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, especially in response to external stimulation. An example for this is the involvement of PLC activity in innate [17–20] and adaptive immunity [21].

Plants possess an immune system that responds to both general and specific stimuli [22,23]. Accordingly, general structural components of microbes are recognized as non-self. These components are referred to as Pathogen- or Microbe-Associated Molecular Patterns, PAMPs or MAMPs respectively, which activate PAMP- or MAMP-triggered immunity (PTI or MTI, respectively). The response to the more specific pathogen-derived components is referred to as effector-triggered immunity (ETI) [22,23]. In both PTI/MTI and ETI, a cellular signal transduction cascade is activated upon pathogen perception by the corresponding immune receptors, which eventually leads to an effective defence. Evidence for the involvement of PLC activity in plant defence is emerging [24–31]. For example, PLC is activated early during elicitation of the oxidative burst in cultured soybean [31] or tomato cells [29] in response to PAMPs. PLC is also required for the benzothiadiazole-induced oxidative burst and hypersensitive cell death in rice suspension-cultured cells [26]. Moreover, a tobacco (*Nicotiana tabacum*) cell culture expressing the *Cf-4* gene, providing resistance to the fungal tomato pathogen *Cladosporium fulvum* [32], shows PLC activation and rapid accumulation of phosphatidic acid (PA) upon treatment with the matching *C. fulvum* effector Avr4 [25]. In *Arabidopsis thaliana*, PLC was found to be activated during disease resistance responses triggered upon recognition of the effectors AvrRpm1 and AvrRpt2 from the bacteria *Pseudomonas syringae* [30]. Moreover, we demonstrated previously that tomato *PLC4* and *PLC6* genes encode active PLC enzymes and play a role in resistance to different microbial pathogens [24]. Altogether, these observations underpin a key role of PLC signalling in plant defences against microbes. Remarkably, all plant PLCs reported so far are related to the PLCζ class [33]. PLCζ enzymes are known to act as important signalling components by controlling calcium oscillations [34]. However, it is unclear why plants do possess so many similar PLC homologs. Moreover, it remains unknown how plant PLCs are activated and whether these different homologs are able to generate stimulus-specific signals. It is also still unrevealed how the signals that are generated by the activation of plant PLCs are relayed to induce the final downstream responses. This is particularly unclear in plants, as they lack homologs of the downstream targets of mammalian PLC, such as PKCs and the $InsP_3$-receptors [1].

Previously we have identified several genes of the PLC family and showed that their expression is differentially regulated during plant immunity. We also observed differential requirement of PLC homologs for plant immune responses towards different types of microbial pathogens [26]. Here, we further studied the tomato PLC protein family to gather information on their biochemical activity and link this to the *in vivo* responses occurring after triggering plant immune receptors. By performing *in vitro* studies of three plant PLC homologs we found that their activity and substrate preference are controlled by the concentration of Ca^{2+} and the pH, suggesting a role for these two components in the generation of homolog-specific signals *in vivo*. Furthermore, we demonstrate that PLC activity is required for the internalization of the Flagellin Sensing 2 (FLS2) protein and thus its function in immune signalling.

2. Materials and methods

2.1. Phylogenetic reconstruction of plant PI-PLCs

Protein sequences used for the phylogenetic reconstruction of plant PI-PLCs were identified through BLAST searches of predicted proteomes of selected species using *A. thaliana* PI-PLC amino acid sequences as a query. Species were selected based on their phylogenetic position and availability of a sequenced genome. The predicted proteome sequences of *Amborella trichopoda* v1.0 [35], *Aquilegia coerulea* v1.1, *Aquilegia coerulea* Genome Sequencing Project; http://www.phytozome.net, *Medicago truncatula* Mt4.0v1; [36], *Mimulus guttatus* v2.0; [37], *Oryza sativa* v7.0; [38], *Physcomitrella patens* v3.0; [39], *Selaginella moellendorffii* v1.0; [40], *Solanum lycopersicum* iTAG2.3; [41], *Solanum tuberosum* v3.4; [42], *Vitis vinifera* (Genoscope.12X; [43]) and *Zea mays* v6a; [44] were obtained through Phytozome 10 (http://phytozome.jgi.doe.gov). Full-length (predicted) protein sequences were aligned using MAFFT v7.017 [45], implemented in Geneious R8 (Biomatters, Auckland, New Zealand), using default parameter settings. After manual inspection, alignments were used for tree building using MrBayes 3.2.2 [46] implemented in Geneious R8, using default parameter settings, with the exception of the rate matrix, which was set to "wag". Phylogeny was rooted according to species phylogeny.

2.2. Computational analysis of the protein sequence of the X/Y-linker present in the PI-PLCs

Domain prediction of the *Sl*PLC proteins was performed using the SMART server (http://smart.embl-heidelberg.de/), in combination with HMM (http://hmmer.janelia.org/), COILS (http://www.ch.embnet.org/software/COILS_form.html) and Pfam searches (http://pfam.sanger.ac.uk/). The X/Y-linker regions were delineated as the regions located between the PLC X and PLC Y catalytic domains in each PLC. The number of negatively charged aspartic acid (D) and glutamic acid (E) residues present in the full-length PLC protein and in its X/Y-linker region was determined using Vector NTI (Invitrogen). Putative phosphorylation sites in the PLC protein and its X/Y-linker were predicted using the NetPhos 2.0 server [47], 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.

2.3. Cloning and heterologous expression of recombinant tomato PLC enzymes

The open reading frames (ORFs) of the previously isolated tomato *PLC* genes were amplified from plasmids [24] and N-terminally tagged with GST by cloning them in the pGEX-KG vector [48]. Accordingly, the following primers (forward, F) 5′-TTCTAGATATGTCTAAACAAACATACAGAATCTG-3′ and (reverse, R) 5′-TCTCGAGCTATACATGTAACATTATTTTTACAAAT-3′ were used to add *XbaI*/*XhoI* restriction sites (corresponding restriction sites underlined respectively) by PCR amplification, and subsequently insert the ORF of *SlPLC1* in pGEX-KG. Similarly, 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 using *SalI*/*XhoI* restriction sites after amplification with the primers (F) 5′-TGTCGACTAGATATGTCCAAACAGACGTACAGAGTC-3′ and (R) 5′-TCTCGAGTTAGATAAATTCGAAACGCATAAGTAG-3′. The ORF of *SlPLC5* was cloned using *NcoI*/*XhoI* restriction sites after amplification using the primers (F) 5′-TCCATGGTTATGTTTGGGTGTTTCAACCGTAAAT-3′ and (R) 5′-TCTCGAGTCAAAGAAATTGAAATCGCATGAG