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# A transmembrane phospholipase D in *Phytophthora*; a novel PLD subfamily

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**Abstract**

Phospholipase D (PLD) is a ubiquitous enzyme in eukaryotes that participates in various cellular processes. Its catalytic domain is characterized by two HKD motifs in the C-terminal part. Until now, two subfamilies were recognized based on their N-terminal domain structure. The first has a PX domain in combination with a PH domain and is designated as PXPH-PLD. Members of the second subfamily, named C2-PLD, have a C2 domain and have, so far, only been found in plants. Here we describe a novel PLD subfamily that we identified in *Phytophthora*, a genus belonging to the class oomycetes and comprising many important plant pathogens. We cloned *Pipld1* from *Phytophthora infestans* and retrieved full-length sequences of its homologues from *Phytophthora sojae* and *Phytophthora ramorum* genome databases. Their promoters contain two putative regulatory elements, one of which is highly conserved in all three genes. The three *Phytophthora pld1* genes encode nearly identical proteins of around 1807 amino acids, with the two characteristic HKD motifs in the C-terminal part. Homology of the predicted proteins with known PLDs however is restricted to the two catalytic HKD motifs and adjacent domains. In the N-terminal part *Phytophthora* PLD1 has a PX-like domain, but it lacks a PH domain. Instead the N-terminal region contains five putative membrane spanning domains suggesting that *Phytophthora* PLD1 is a transmembrane protein. Since *Phytophthora PLD1* cannot be categorized in one of the two existing subfamilies we propose to create a novel subfamily named PXTM-PLD.


*Keywords:* Signal transduction; Transmembrane domain; Oomycetes

## 1. Introduction

Phospholipase D (PLD; EX 3.1.4.4.) is a ubiquitous enzyme in eukaryotes that hydrolyses the phosphodiester bond of glycerophospholipids generating phosphatidic acid (PA) and a free headgroup. The activities of PLD and other phospholipases not only affect the structure and stability of cellular membranes but they also regulate many cellular functions. PLD participates in various cellular processes in mammals, yeast and plants, including receptor signaling, intracellular membrane transport and actin cytoskeleton reorganization (reviewed by McDermott et al., 2004; Meijer and Munnik, 2003), and its product PA acts as an important second messenger in plants and mammals (Munnik, 2001). In the yeast *Saccharomyces cerevisiae* PLD activity is essential for sporulation (Rudge et al., 2004). In plants, PLD and its product PA are involved in various processes including senescence, fruit ripening, wounding, osmotic stress and responses induced by elicitors (for references, see Meijer and Munnik, 2003). Also for virulence of pathogens PLD might be important. In *Candida albicans* deletion of the *CaPLD1* gene resulted in a major loss of virulence on

*Abbreviations:* PA, phosphatidic acid; PLD, phospholipase D; PH, Pleckstrin homology; PX, PHOX homology; PIP, phosphatidylinositol-phosphate; PIP2, phosphatidylinositol-biphosphate; TM, Transmembrane.

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mouse whereas no effects were observed during normal growth (Hube et al., 2001).

Genes encoding PLD have been cloned from many organisms, including mammals, yeast and plants. Characteristic for all known PLDs are two invariant HKD motifs located in the C-terminal part. They constitute the active sites essential for catalytic PLD activity. Also the N-terminal part contains specific domains; they vary and based on that PLDs are categorized into two main subfamilies: C2-PLDs and PXPH-PLDs. The C2 domain is a $Ca^{2+}$/phospolipid binding fold that is, so far, only found in plant PLDs and hence, the C2-PLD subfamily is considered plant-specific. *Arabidopsis thaliana* has 10 C2-PLDs genes that are divided in four groups based on their sequence and $Ca^{2+}$ and $PI(4,5)P_2$ dependency (Wang, 2004). PXPH-PLDs contain at their N-terminus a combination of the phosphoinositide-binding domain PX (Phox homology) and a PH (Pleckstrin homology) domain. This subfamily occurs throughout the eukaryotic kingdom. *A. thaliana* and mammals have two genes encoding PXPH-PLDs whereas yeast, nematodes and *Drosophila* contain only one copy (McDermott et al., 2004). PX domains are characterized as phosphoinositide-binding modules that bind, among others, phosphatidylinositol 3-phosphate (PI3P) (Lemmon, 2003). The compounds that bind to PH domains are more diverse and include $PI(4,5)P_2$, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (Lemmon, 2003).

The subject of our studies is *P. infestans*, a fungal-like heterokont classified as an oomycete (Baldauf, 2003) and causing the devastating late blight disease in potato and tomato (Erwin and Ribeiro, 1996). Our aim is to unravel signaling pathways involved in virulence and pathogenesis of *P. infestans* and to generate knowledge that may help to design novel strategies for controlling oomycete pathogens. Crucial steps in infection cycles of fungi and fungal-like organisms include spore production, spore dispersal and spore differentiation. Previously we demonstrated that the G-protein subunits α and β in *P. infestans* are essential for virulence and sporulation, respectively (Latijnhouwers et al., 2004; Latijnhouwers and Govers, 2003). Furthermore we showed that the G-protein activator mastoparan triggers encystment of *P. infestans* zoospores and stimulates PLD activity resulting in the formation of PA (Latijnhouwers et al., 2002). As has been reported for plants, PLD activity was also stimulated by primary and secondary alcohols in *P. infestans*. The finding that addition of exogenous PA and primary and secondary alcohols to zoospores also results in encystment points to a central role for PLD and its product PA in zoospore differentiation. This is supported by studies of Zhang et al. (1992) on another *Phytophthora* species, *Phytophthora palmivora*. They demonstrated that differentiation of zoospores was accompanied with an increase of PA probably generated via PLD activity and that, as in *P. infestans*, addition of exogenous PA resulted in encystment (Zhang et al., 1992).

To further investigate the role of PLD in *P. infestans*, we set out to clone genes encoding PLDs. In this study, we describe the identification of a novel PLD found in at least three *Phytophthora* species, *P. infestans*, *P. sojae* and *P. ramorum*. The overall domain structure of this PLD is different from the two known PLD subfamilies described above, C2-PLDs and PXPH-PLDs. This novel PLD possesses a PX domain but lacks the PH domain. Instead, the N-terminal region contains five putative membrane spanning domains suggesting that it is a transmembrane protein. We propose to name this novel class PXTM-PLD.

## 2. Materials and methods

### 2.1. Cloning and sequencing of a P. infestans PLD gene

Mining the SPC database comprising 75,000 *P. infestans* ESTs (Randall et al., 2005) for PLD catalytic domains resulted in the identification of a cDNA clone (PF058G05) encoding a putative PLD. The cDNA insert was used as probe to screen a *P. infestans* BAC library with a 10× genome coverage (Whisson et al., 2001). Colony hybridization was performed according to standard procedures and the $^{32}$P-labelled probe was prepared by the random hexamer method with a random primer labelling kit (Prime-a-Gene®, Promega). DNA from hybridizing BACs was isolated as described (Jiang et al., in press) and the presence of the putative PLD sequence confirmed by a PCR based analysis using primers PLD-F1 5′-cgtgggatgtggtcagcggg-3′ and PLD-R1 5′-cgattgcgtacgattccgttgccg-3′. From positive BACs *Bam*HI and *Eco*RI, fragments were subcloned into pBluescript and transformed to *E. coli*, strain DH5α. PLD subclones were identified by colony-PCR using primers PLD-F1 en PLD-R1. The full-length sequence was assembled using standard DNA analysis programs.

### 2.2. Sequence alignments and database mining

GenBank (nonredundant, dbEST, and Trace DB), the *Phytophthora* Genome Consortium database (PGC; http://www.pfgd.org/), and the SPC database were searched for homologous of PiPLD1 using the BLAST algorithm. The SPC database is a proprietary database of Syngenta Inc. containing ca. 75,000 ESTs from *P. infestans* (courtesy of the Syngenta *Phytophthora* Consortium, Research Triangle Park, NC) (Randall et al., 2005). The genome sequences of *P. sojae* and *P. ramorum* were generated at the DOE Joint Genome Institute and are available at http://www.jgi.doe.gov. Multiple sequence alignments were conducted using the program ClustalW on the BCM Search Launcher (http://dot.imgen.bcm.tmc.edu/multi-align/Options/clustalw.html) and formatted manually. The following PLDs were used for alignments: *A. thaliana*; PLDζ1 (NP_188302), PLDζ2 (NP_187214), PLDα1 (NP_188194), PLDβ1 (NP_565963), and PLDγ1 (NP_192922), *Oryza sativa* PLD1 (AAT38042) and PLD2 (NP_908573), *S. cerevisiae* SPO14 (NP_012956), *Homo*