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Functional analysis of a lipolytic protein encoded in phytoplasma phage based genomic island

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ABSTRACT

Wall-less bacteria known as phytoplasmas are obligate transkingdom parasites and pathogens of plants and insect vectors. These unusual bacteria possess some of the smallest genomes known among pathogenic bacteria, and have never been successfully isolated in artificial culture. Disease symptoms induced by phytoplasmas in infected plants include abnormal growth and often severe yellowing of leaves, but mechanisms involved in phytoplasma parasitism and pathogenicity are little understood. A phage based genomic island (sequence variable mosaic, SVM) in the genome of Malaysian periwinkle yellows (MPY) phytoplasma harbors a gene encoding membrane-targeted proteins, including a putative phospholipase (PL), potentially important in pathogen–host interactions. Since some phytoplasmal disease symptoms could possibly be accounted for, at least in part, by damage and/or degradation of host cell membranes, we hypothesize that the MPY phytoplasma putative PL is an active enzyme. To test this hypothesis, functional analysis of the MPY putative *pl* gene-encoded protein was carried out *in vitro* after its expression in bacterial and yeast hosts. The results demonstrated that the heterologously expressed phytoplasmal putative PL is an active lipolytic enzyme and could possibly act as a pathogenicity factor in the plant, and/or insect, host.

1. Introduction

Phytoplasmas are unculturable plant pathogenic bacteria that infect both gymnosperm and angiosperm plants, and are capable of obligate transkingdom parasitism and pathogenicity in both plants and insect vectors. In spite of numerous attempts for over 40 years, it has not been possible to isolate and cultivate phytoplasmas in cell-free media. Plants infected by phytoplasmas develop unusual symptoms, suggesting relation of the disease with disturbances in the balance of phytohormones or plant growth regulators. Symptoms include yellowing of shoots and leaves, development of green flowers and loss of normal flower pigments (virescence), development of floral parts into leafy structures (phyllody), proliferation of axillary shoots resulting in a witches'-broom appearance, and eventual death of tissues and whole plants (Lee et al. 2000). Phytoplasmal diseases worldwide bring large economic losses for growers of vegetables, fruit and nut trees, and ornamentals and cause significant damage to natural ecosystems. Mechanisms involved in their parasitism and pathogenicity remain poorly understood.

The genomes of phytoplasmas, ranging from 0.5 to 1.2 Mbp, are some of the smallest known among pathogenic bacteria (Marcone et al. 1999). Yet, the genomes are characterized by chromosomal regions of repeated sequences that termed sequence-variable mosaics (SVMs) (Jomantiene and Davis 2006; Jomantiene et al. 2007), formed by repetitive and targeted insertions of ancient phage genomes (Wei et al. 2008). The SVMs harbor genes encoding diverse, putative membrane-targeted proteins potentially important in pathogen–host interactions (Bai et al. 2006; Jomantiene and Davis 2006; Jomantiene et al. 2007; Toruno et al. 2010), but with the single exception of thymidylate kinase (Li et al. 2008; Miyata et al. 2003; Miyata et al. 2004), no direct *in vitro* expression and functional analysis of such proteins has been reported.

Malaysian periwinkle yellows (MPY) phytoplasma is a '*Candidatus Phytoplasma asteris*'-related strain and member of 16S rDNA RFLP subgroup 16SrI-B. This pathogen induces severe yellowing of leaves in plants of Madagascar periwinkle (*Catharanthus roseus* (L.) G Don). Since such symptoms of disease could be accounted for, at least in part, by damage and/or degradation of host cell membranes, we hypothesize that a putative phospholipase (PL) gene, that is present in the genomic SVM region of strain MPY (Jomantiene and

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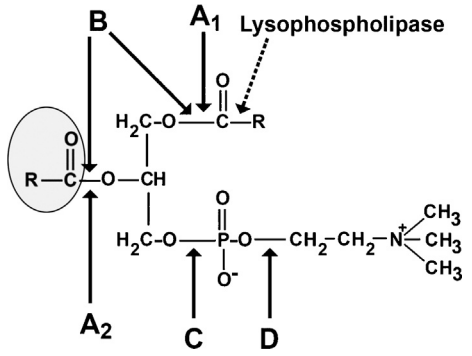

Fig. 1. The sites of action of phospholipases on phospholipids. Truncated line arrow shows the specific bond targeted by lysophospholipase in the phosphoslipid molecule only after its hydrolysis of either phospholipase A2 or phospholipase B (gray oval).

Davis 2006), could be involved in the development of yellowing symptoms. Since the MPY genome encodes a putative preprotein phophoslipase with a signal peptide (pPL), it is possible that the PL protein could act as a secreted or membrane-localized phytoplasma virulence factor.

Phospholipases (PL) are lipolytic enzymes that act upon phospholipids, one of the major constituents of eukaryotic and prokaryotic cell membranes. Depending on the site of action, phospholipases are classified, and qualifying letters (*e.g.*, A, B, C and D) are used to distinguish differentially acting phospholipases and to indicate the specific bond targeted in the phospholipid molecule (Fig. 1) (Ghannoum 2000; Istivan and Coloe 2006; Ramrakhiani and Chand 2011). PLAs are divided into two subgroups based on position specificity as follows: phospholipases A1 and A2 cleave fatty acids from the sn-1 or sn-2 position, respectively. Group B phospholipases (PLBs) display both phospholipase A1 and A2 activities that can result in total deacylation of phospholipids. Group C phospholipases (PLCs) cleave within the phosphate group, and group D phospholipases (PLDs) cleave the nitrogen-containing group (Fig. 1). Lysophospholipases also represent acyl hydrolases and release long-chain fatty acids after their action, but only when the substrate phosphatidylcholine is degraded to lysophosphatidylcholine by PLA enzymes (Banerji et al. 2008). Bacterial PL participate in numerous processes related to early stages of disease, such as damage to membrane integrity and changes in cellular signal transduction pathways, and formation of new membrane pores, generating inflammatory responses (Banerji et al. 2008; Istivan and Coloe 2006; Lucas et al. 2010; Ghannoum 2000). It has been shown that bacterial phospholipases are able to induce a high degree of membrane destruction, as in the case of *Pseudomonas aeruginosa* phospholipase A2(PLA2 named SlaA) and *Clostridium perfrigens* β-toxin, a phospholipase C (Jepson and Titball 2000; Sitkiewicz et al. 2006). Interesting, that in the case of pathogen *Pseudomonas aeruginosa* additionally to phospholipase A2 activity, a degradation of lung surfactants (phospholipids) *via* the joint action of a PLC and a lipase has been described which reflects about a synergy between phospholipases and lipases in some host cell lyses cases (Konig et al. 1994). Thus, it is reasonable to hypothesize that a putatively secreted or surface membrane-exposed PL protein alone or in joint action with other lipases/phospholipases encoded by a phytoplasma genome could act as a pathogenicity factor in the plant, and/or perhaps insect, host.

In this study, we provide the first direct, *in vitro* functional analysis of a putative virulence factor protein encoded by a phytoplasma genome. We employed two gene expression/protein synthesis systems, eukaryotic (yeast) and prokaryotic (bacterial), and assayed enzymatic activity of the proteins synthesized in both systems. The results revealed that the protein encoded by the MPY putative PL gene is an enzymatically active enzyme exhibiting several catalytic activities on phospholipids, consistent with the concept that it may play an important role in pathogen–host interactions and fitness of phytoplasmas, as well as other bacteria.

2. Materials and methods

2.1. Generation of putative PL expression plasmids

All DNA manipulations were performed according to standard procedures (Sambrook and Russell 2001). Enzymes and kits for DNA manipulations were purchased from Thermo Fisher Scientific Baltics (Vilnius, Lithuania). The DNA ORF2 (open reading frame 2) of the DNA fragment MPY-5 (GenBank no. EF200534) of the Malaysian periwinkle yellows phytoplasma, containing a putative phospholipase (PL) gene, was amplified by Polymerase Chain Reaction (PCR) from the plasmid containing PCR DNA fragments from sequence-variable mosaics (SVMs) (Jomantiene et al. 2007). The full putative PL sequence was analyzed using SMART (simple modular architecture research tool) program, which identified a putative signal sequence. A truncated form of putative PL without the signal sequence (starting from the Tyr33 residue) was also produced. The primers used contained the *Xba*I site sequences at the one end of the putative PL coding sequence and 6xHis coding sequence at the 3′-end of some constructs. The nucleotide sequences of the primers were as follows: MPY-PL-Dir 5′gctctagatatgtatcctttgttaaacaaagttacaac; MPY-PL-Rev 5′gctctagatcaaatattaaattgttctaaccattc; MPY-PLsigP-Dir 5′gctctagatatgtttaaattaaaaaagaatttattat; and MPY-PL6His-Rev 5′gctctagatcaatggtgatggtgatgatgaatattaaattgttctaaccattc.

The PCR products were cloned into the *Escherichia. coli* expression vector pET-21a (Merck, Darmstadt, Germany) yielding recombinant (r) plasmids named pET-21a-rPL, pET-21a-rPL-1, pET-21a-rPL-2, and pET-21a-rPL-3, and into the yeast *Saccharomyces cerevisiae* expression vector pFX7 (Sasnauskas et al. 1999) yielding recombinant plasmids named pFX7-rPL, pFX7-rPL-1, pFX7-rPL-2, and pFX7-rPL-3, the latter allowing the selection of yeast transformants resistant to formaldehyde (Sasnauskas et al. 1992). The DNA sequences encoding the recombinant rPL constructs were confirmed by DNA sequencing. The signal sequence substitution was performed by cloning the DNA sequence encoding PL, without the native signal peptide-encoding sequence, in frame with *S. cerevisiae* α factor secretion signal sequence (αF) into the expression vector pFX7αF under the control of the Gal10/PYK1 promoter. The DNA sequence encoding PL fused with αF was confirmed by DNA sequencing. The deduced amino acid sequence of native PL protein with signal peptide and without the 6xHis tail (represented by rPL-1) is composed of 303 residues, and that without signal sequence (represented by rPL-3) is composed of 271 residues; the two proteins have calculated molecular masses of 35,383 and 31,507 Da, correspondingly.

2.2. Expression and purification of recombinant MPY PL

rPL proteins were expressed in *E. coli* BL21 (DE3) and *S. cerevisiae* strain AH22-214p (*a, leu2 his4 ura3 pep4*). *E. coli* BL 21 (DE3) cells transformed with the pET-21a-rPL plasmids were grown overnight in LB medium with 50 μg/mL ampicillin at 37 °C. On the following day, the culture medium was diluted 20 times with Terrific Broth medium containing antibiotics and grown at 37 °C. Protein expression was induced with 1 mM IPTG when the optical density (OD) at 600 nm had reached 1 and cells were grown for additional 4 h. Then cells were harvested at 4 °C by centrifugation at 6500 g for 15 min and the cell pellets were resuspended in 50 mM NaH_2PO_4 (pH 8.0), 600 mM NaCl, 10 mM imidazole, and EDTA-free complete