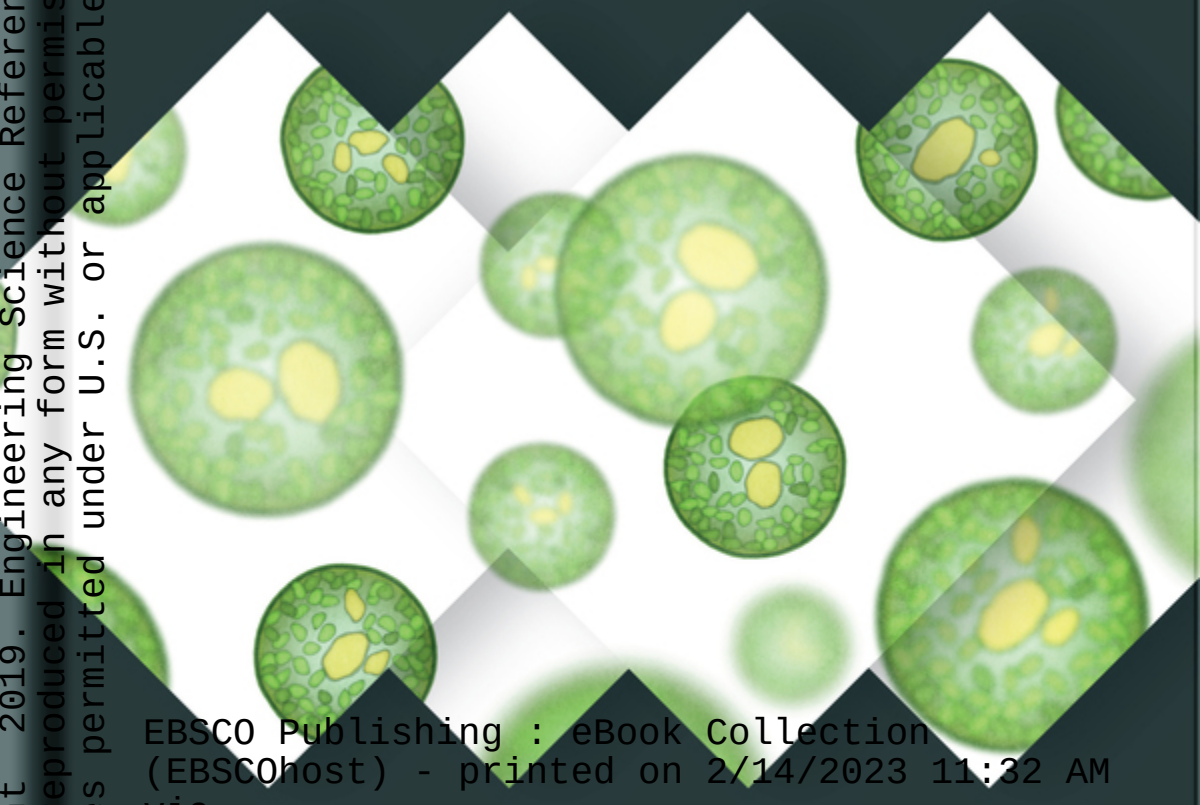
Research Insights

Unique Sequence Signatures in Plant Lipolytic Enzymes

Emerging Research and Opportunities



Nihed Ben Halima

Unique Sequence Signatures in Plant Lipolytic Enzymes:

Emerging Research and Opportunities

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Preface

Lipids are biomolecules with complicated structural variability and they constitute a significant amount of biomass in the earth. There are many classes of lipids based on polarity or on chemical structure. The polar lipids are, for instance, phospholipids, while the nonpolar (neutral) lipids are, for instance, triacylglycerol. Besides, lipids could be classified based on their chemical structure that could be either abundant lipids or degraded lipid metabolites such as Fatty acids (FA) and Mono/Di-glyceride (MG, DG). Thus, there are so many classes of lipids with precise nomenclature. Plants constitute a large part of vivant organisms in earth. Lipids and in particular plant lipids are of increasing interest because of their multiple roles such as in food technology and medicine as they could be potential sources of dietary essential polyunsaturated fatty acids (FUFAs) in significant amounts. They could be involved in many processes such as in nutrition and storage of energy and are of relevance for signal transduction processes, cell differentiation and phagocytosis (Yeung, Ozdamar, Paroutis, & Grinstein, 2006).

In general, the ability of any organism (e.g., plants) to make any particular product (lipids in our context) is predicated on its capability for the secretion of a particular set of enzymes (lipolytic enzymes).

Lipolytic enzymes play essential roles dealing with the turnover of the large amount of lipids in our planet. These enzymes are without doubt, biologically significant proteins, besides; they found many applications such as in the fields of lipid metabolism, biochemistry, medicine, nutraceutical and food technology.

Studying lipolytic enzymes from plants would be very promising as these enzymes; in particular, phospholipases might play important roles in plant growth, development and stress response. Many reports have focalized in studying the mechanism of action of such enzymes and their diverse functions (Matlashewski, Urquhart, Sahasra-dudhe, & Altosaar, 1982; Pokotylo, Pejchar, Potocky, Kocourkova, Krckova, Ruelland, Kravets, & Martinec,

2013; Pokotylo, Kolesnikov, Kravets, Zachowski, & Ruelland, 2014; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

Within the last decades, the interest in lipolytic enzymes, e.g. lipases and phospholipases, has increased considerably and due to the importance of such enzymes, this interest would probably be gradually increased so far and in the future.

Recent advances in protein chemistry, biochemistry, and enzymology have promoted research on lipolytic enzymes. Novel and potent tools for related disciplines like crystallography, NMR technology and molecular modelling would endorse revealing new sequences of amino acids and three-dimensional structures of lipases and phospholipases.

The investigation and understanding of relationship of proteomes and lipidomes would be an outstanding feature in recent development in biotechnology. The mechanism of action of major lipolytic enzymes and their roles in lipid signaling in particular in plants remain to be elucidated. High performance analytical techniques as well as bioinformatics analysis would be very interesting research tools to accelerate discovering of novel proteins (enzymes) as well as lipids using the *de novo* sequencing approaches.

We would like to try to cover major aspects of plant lipolytic enzymes research from genomics, proteomics and phylogenies to related annotated functions.

The book entitled *Unique Sequence Signatures in Plant Lipolytic Enzymes: Emerging Research and Opportunities* addresses the importance of lipases and phospholipases from plants. The major focus of this compendium is on the structural aspects of these enzymes. Insights into their functions and their phylogenetic evolution would also be discussed in the current book via previous publications that seemed to offer helpful information regarding the biochemistry of lipolytic enzymes.

Lipases are lipolytic enzymes that catalyze mainly hydrolysis of oil, i.e. triglycerides. Plant lipases received scrutiny of investigation especially in seeds since 1890, for instance, lipase from castor bean (*Ricinus communis*), which was first described in the seed (Green, 1890).

Phospholipases are grouped in plants into phospholipase C (PLC), phospholipase D (PLD) and phospholipases A (PLA1 and PLA2) according to the site of glycerophospholipid hydrolysis (Wang, Ryu, & Wang, 2012; Wang, 2001). Moreover, within each group, there are different kinds of enzyme clustered into families or subfamilies that differ in their structures, substrate selectivities and reaction conditions (Wang, 2001; Wang, 2004; Wang, Ryu, & Wang, 2012). Plant phospholipases play critical roles in growth and

development of plants as well as their response to abiotic and biotic stresses and thus, many researchers have paid attention in recent years toward the characterization of such enzymes (Wang, Ryu, & Wang, 2012; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

Phospholipase C (PLC) hydrolyzes the phosphodiester bond on the glycerol side of phospholipids to produce diacylglycerol (DAG) and a phosphorylated head group. PLCs in plants can be divided into three groups according to substrate specificity and cellular functions: Non-specific PLC (NPC) that acts on the common phospholipids, e.g. PC and PE, Phosphoinositide-specific PLC (PI-PLC) that hydrolyzes phosphoinositides, and the Glycosylphosphatidylinositol (GPI)-PLC that hydrolyzes GPI-anchored proteins (Wang, 2001; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of the phosphodiester bond of phospholipids (PLs) to produce phosphatidic acid (PA) and an alcohol moiety. PLD can also catalyze the interconversion of polar head groups of PLs by transphosphatidylation reaction. This latter reaction leads to synthesis naturally less abundant PLs such as phosphatidylglycerol (PG), phosphatidylserine (PS), or phosphatidylethanolamine (PE) from highly abundant ones such as lecithin or phosphatidylcholine (PC) (Damnjanovic & Iwasaki, 2013). The activity of PLD was first demonstrated in plants in the report of Hanahan and Chaikoff (1947), and since then this enzyme has been investigated for its role in membrane lipid metabolism. Besides, PLD could be implicated in many plant processes such as in signal transduction (Cockcroft, 1984; Bocckino, Blackmore, Wilson, & Exton, 1987).

Plant phospholipases C and D have been given particularly much interest in the report of Hong et al. (2016) not only because they are the most thoroughly investigated plant lipolytic enzymes, but also because they might be of great importance to plant growth and have diverse functions especially in stress responses.

We hope that this book will be a convincing valuable compendium to pertinent researchers and motivate us to pursue such research.

This book, as such targets a rich array of readers that includes academics from medical and biochemical disciplines as well as professionals from biotechnological industries.

The current book would likely be of particular interest to nutraceutical and biotechnology companies as the lipidomics, i.e., lipids and related enzymes are widely used in industrial interests.

Therefore, *Unique Sequence Signatures in Plant Lipolytic Enzymes: Emerging Research and Opportunities* focuses on the biochemistry of lipolytic enzymes, particularly, plant lipases and phospholipases as well as their structures and catalytic mechanisms. The book explores the conserved domains and motifs of plant lipolytic enzymes by identifying the main residues involved in catalysis in such type of enzymes, the phylogeny of some important plant lipolytic enzymes and calculates the evolutionary distance in those enzymes. Organized into six chapters, this book begins with an overview of general aspects of plant lipolytic enzymes. It then proceeds with a discussion on the importance of plant lipases and phospholipases, including their essential functions in plant growth as well as their external applications. The next three chapters look at the characterization of plant lipases, phospholipases C and phospholipases D. Moreover, the book talks about the identification of the main conserved domains/motifs, phylogenetic analysis and evolutionary distance calculation in these kinds of enzymes. A chapter discussing the evolutionary history of lipolytic enzymes among plant kingdom and the other kingdoms of life concludes this book. Such book would be a valuable resource aiding scientists and researchers working in the field of lipolytic enzymology.

ORGANIZATION OF THE BOOK

The book is organized into six chapters. A brief description of each of the chapters follows:

Chapter 1 reviews the general features of lipolytic enzymes from plants. The chapter aim to implement the proteomic and lipidomic techniques to identify proteins and lipids associated with the growth of plants including, for instance, the presence of lipolytic enzymes such as phospholipases. Besides, oils, enzymes and other related biomolecules of nutraceutic or dietary usage from plants would be valorized for this purpose. Here we will target academics, researchers and industrial applications by the valorization of such plant biomolecules for use, for instance, in food, pharmaceutical and biotechnology companies.

Chapter 2 presents an overview of the diverse functions of lipases and phospholipases from plants. The chapter addresses the issue of the importance regarding the functions of plant lipolytic enzymes not only for plants but also for external applications of such enzymes especially in industrial applications such as in food, pharmaceutical and biotechnology companies.

Chapter 3 describes the main authored plant lipases. The structural characterization into these lipolytic enzymes as well as their importance would be the focus of this chapter.

Chapter 4 analyses the main features of plant phospholipases C (PLCs). In fact, three groups of PLCs in plants were existed according to the specificity of substrate and the cellular functions. These groups are non-specific PLC (NPC), phosphoinositide-specific PLC (PI-PLC) and glycosylphosphatidylinositol (GPI)-PLC. The chapter will highlight the progress made on such enzymes.

Chapter 5 analyses structural insights into plant phospholipases D and their importance in internal functions of plants and in external applications.

Chapter 6 concludes on the importance of overviewing the potential evolutionary history of lipolytic enzymes. This chapter aims to identify the zonal distribution of lipases and phospholipases from plant kingdom and related organisms. It should reveal some interesting findings especially between such plant enzymes and those from algae.

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Chapter 1
Plant Lipolytic Enzymes: Generalities

ABSTRACT

The main plant lipolytic enzymes discussed in this chapter are hydrolases that catalyze neutral lipids (non-polar lipids), that is, lipases or those hydrolyzing polar lipids, for example, phospholipases (A, C, and D) as well as those of related field like diacylglycerol kinases, lipoprotein lipases, lipid phosphate phosphatases, and lysophospholipases. This chapter provides generalities regarding some important aspects of plant lipolytic enzymes from either higher plants or algae, such as sources, reactions catalyzed, and specificities. Major issues for purification of plant enzymes with lipolytic activities are highlighted in this overview. New insights into implementation of relevant proteomic and lipidomic techniques to identify and characterize plant lipolytic enzymes with the aid of internet resources are also reviewed in this chapter.

INTRODUCTION

Lipolytic enzymes are biocatalysts of all kinds of lipids, i.e., neutral or polar lipids; and these substrates constitute a large quantity of biomass content in earth, found in all vivant organisms. Plants are amazing sources of lipids and there lipolytic enzymes would play important roles in either the biological turnover of their proper materials or the implication in external applications such of industrial uses, e.g., food technology, pharmacies and medicine, etc. Therefore, lipolytic enzymes are indispensable for intracellular lipids

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trafficking and are found in cells where their importance are obvious, e.g., digestive cells, but also found in less obvious cells importance such in the venoms of reptiles and among extracellular microbial enzymes. Some definition could be assigned to a lipolytic enzyme as an esterase capable of hydrolyzing long-chain fatty acid esters, e.g., esters of oleic acid; and lipases would be assigned when a glycerol is the alcohol moiety of the ester and phospholipases or lysophospholipases or galactolipases when it is a glycerol derivative and cholesterol esterases when it is a sterol. In addition, it is worth noting to indicate that these different kinds of esters are found in general as natural of physiological substrates for the lipolytic enzymes, but these enzymes are also endowed to use artificial esters.

Lipolytic enzymes could be designed as hydrolyzing esters of different natures and known as, for example, several phosphoric ester hydrolases, amide hydrolases, phospholipases, sphingomyelinases and ceramidases. Other related enzymes to the catalysis of substrates containing lipids could be found like the glycosidases that split various carbohydrates from glycolipids and conjugated sterols or triphosphoinositide phosphatases.

The study of lipolytic enzymes is very interesting because, in one hand, of their obvious biological important roles, and on the other hand, of their applications in particular medical research as, for instance, cholesterol esterases for cholesterol esters absorption, hormone-sensitive lipase for fat mobilization from adipose tissue and intracellular phospholipases for the absorption and metabolism of polyunsaturated fatty acids.

The numerous types of lipolytic activities that exist in nature either in prokaryotic or eukaryotic vivant organism make them outstanding enzymes to be studied. Lipolytic enzymes from plants are the focus of the current chapter. These enzymes could be used in many sectors, e.g., medicine, food, chemistry and biochemistry, etc.

Biochemical research on lipolytic enzymes focus on their structure and catalytic mechanism as well as on the property that distinguishes them from other enzymes, e.g., amylases and proteases, which consists on the ability of lipolytic enzymes to act at interfaces on insoluble water substrates, i.e., lipids and related substrates, e.g., phospholipids.

Understanding of how these lipolytic enzymes function under this condition is the investigation of many researchers who emphasize on better understanding of general intracellular metabolism that also takes place, in many cases, at interfaces, namely at the biological membranes. Therefore, studying lipolytic enzymes, especially their physico-chemical characters, their biochemical characteristics and their kinetics, is very important in better elucidating the

intracellular metabolism of any vivant organisms. The fascinating aspect of these enzymes, which are water-soluble proteins, but their substrates are not, resides in their reactions which have to take place at interfaces, i.e., oil-water or micelle-water interfaces.

An attempt to summarize some knowledge of lipolytic enzymes especially from plants (higher plants or algae) is the focus of the current chapter.

BACKGROUND

Enzymes with lipolytic activities are indispensable biocatalysts for the biological turnover of lipids and are of great importance to be used in many applications such as medicine, food and nutraceuticals. Lipolytic enzymes are required for digestion, that is, biocatalysts in the biochemical reaction of the transfer of lipid from one vivant organism to another especially from plant to animal or from animal to animal. These enzymes are, thus, essential in the metabolism of lipids of different origins, in particular in the intracellular lipids' metabolism within the organism, as well as in the functioning of biological membranes and they play key roles in the deposition and mobilization of lipids, oils and fats, which are used as energy reservoirs.

All enzymes with lipolytic activities could be hydrolases belonging to enzyme class 3 according to enzyme classification recommended by the Enzyme Commission of the International Union of Biochemistry (EC nomenclature) (Florkin & Stotz, 1965). Any enzymes that act on substrates of lipids, either simple lipids (also described as neutral lipids that are generally nonpolar lipids) or complex lipids (that may contain polar lipids), are designed as lipolytic enzymes. Glycosidases could be lipolytic enzymes because they split the carbohydrate residues of glycolipids that are complex lipids. Also, each enzyme could be lipolytic enzyme when it act as remote from the lipid part of a complex lipid such as triphosphoinositide phosphatase. Nevertheless, majority of lipolytic enzymes are ester hydrolases, enzyme group EC 3.1, with exception of the ceramidases that are C-N hydrolyzing enzymes (EC 3.5). When lipolytic enzymes hydrolyze esters of fatty acids, they are designed as carboxyl ester hydrolases (EC 3.1.1). In addition, phospholipases C and D (also they were named phospholipases 3 and 4, respectively) are included in the EC classification and are, indeed, phosphoric diester hydrolases (EC 3.1.4). However, phosphatidate phosphohydrolases are phosphoric monoester hydrolases (EC 3.1.3) (Brockerhoff & Jensen, 1974).

Example of nomenclature of some lipolytic enzymes with the numbering recommended by the Enzyme Commission is given in Table 1.

In fact, hydrolases could be classified according to the criterion of the type of bond they hydrolyze and that according to the substrates on which they act. Glycerol is the well-documented molecule used in this second criterion of classification, as it is considered as the molecular backbone of most of the lipid substrate occurring in nature for the majority of lipolytic enzymes, which act on esters of glycerol. For instance, one nomenclature example from the six possible isomers of a triacylglycerol containing one residue each of palmitic, oleic and stearic acid may be described as l-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol, or *sn*-glycerol-1-palmitate-2-oleate-3-stearate, being borne in mind the glycerol stereospecifically numbered. The book of Brockerhoff and Jensen (1974) unveiled more details concerning the nomenclature, kinetics of lipolysis, nature, and some specific properties of most kinds of lipolytic enzymes.

Many other books have reported some other general information on lipids and lipolytic enzymes such as the report of Doolittle and Reue (1999), Müller and Petry (2004), Murphy (2005), Sandoval (2012), Plaxton and Lambers (2015), and Barh et al. (2015).

In general, the classification of any kind of hydrolases could be commonly based on substrate specificity, mode of action as well as amino acid sequence similarities (Henrissat & Davies, 1997; Henrissat, 1991). Therefore,

Table 1. Nomenclature of lipolytic enzymes according to Enzymatic Commission classification (EC)

EC	Systematic Name	Trivial Name
3.1.1.3	Glycerol-ester hydrolase	Lipase
3.1.1.13	Sterol-ester hydrolase	Cholesterol esterase
3.1.1.4	Phosphoglyceride 2-acyl-hydrolase	Phospholipase 2 (PLA2)
	Phosphoglyceride 1-acyl-hydrolase	Phospholipase 1 (PLA1)
3.1.1.5	Lysophosphoglyceride acyl-hydrolase	Lysophospholipase
3.1.3.4.	Phosphatidate phospho-hydrolase	Phosphatidic acid phosphatase
3.1.4.3	Phosphoglyceride diglyceride-hydrolase	Phospholipase 3 (PLC)
3.1.4.-	Sphingomyelin *N*-acylsphingosine-hydrolase	Sphingomyelinase
3.1.4.4	Phosphoglyceride phosphatidate-hydrolase	Phospholipase 4 (PLD)
3.1.5.-	*N*-Acylsphingosine acyl-hydrolase	Ceramidase

Source: Brockerhoff and Jensen, 1974.

many hydrolases were classified in the basis of substrate specificities as recommended by the International Union of Biochemistry and Molecular Biology (IUBMB) and were expressed in the EC number with given the code, for instance, EC 3.1.1.x, where x represents generally the substrate specificity. Metabolism reactions would involve many kinds of carbohydrate active enzymes (CAZymes), which are grouped into sequence-based families on the CAZy database (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014), and the structural fold as well as the catalytic mechanism are highly conserved within these families. Lipolytic enzymes that catalyze carbohydrate residues of complex lipids, e.g., glycolipids and galactolipids, could belong to the CAZymes.

Therefore, lipolytic enzymes include several families of enzymes that catalyze substrates of lipids. Apart lipases, there are many other lipolytic enzymes that catalyze complex lipids such as phospholipases, glycosidases, galactolipases and numerous other related metabolic enzymes. For this end, lipolytic enzymes can be used for either lipids hydrolyzing or lipids synthesis. For example, the biosynthesis of galactolipids that are the main lipids from plants (Amara, Lafont, Fiorentino, Boullanger, Carrière, & De Caro, 2009), implies three types of enzymes that are monogalagtosyldiacyglycerol (MGDG) synthases, digalactosyldiacyglycerol (DGDG) synthases and desaturases (Bastien, Botella, Chevalier, Block, Jouhet, Breton, Girard-Egrot, & Maréchal, 2016).

LARGE SPECTRUM OF LIPOLYTIC ENZYMES

Esterases (EC 3.1.1.1) are carboxylester hydrolases, which consist of a large spectrum of enzymes that are able to catalyze the hydrolysis of carboxylic ester bonds. Lipases are serine hydrolases defined as triacylglycerol acylhydrolases (E.C. 3.1.1.3), which should be differentiated from esterases. For more information regarding such differentiation, please refer to the report of Ben Ali et al. (2012).

Lipases (Triacylglycerol hydrolases) are biocatalysts of the hydrolysis of triacylglycerols to diacylglycerols, monoaclyglycerol, fatty acids and glycerol. Lipases are lipolytic enzymes that are produced by several organisms e.g., animal, microorganisms and in particular (the focus of this volume) they are widely distributed in plants, which constitute a special class of carboxylic esterases capable of releasing long-chain fatty acids from natural water-insoluble carboxylic esters.

Lipolytic enzymes also catalyze synthesis reactions such as alcoholysis, aminolysis and interesterification (Villeneuve, Muderhwa & Graille, 2000). As well, lipolytic enzymes are capable of expressing other annex activities such as cholesterol esterase, phospholipase, lysophopholipase, amidase or cutinase activities (Svendsen, 2000).

Cutinases, for instance, usually catalyze the hydrolysis of ester bonds in cutine polymers, are considered as intermediates between lipases and esterases because they are also capable of hydrolyzing long-chain and short-chain triglycerides without requirement of interfacial activation.

Phospholipases are other important lipolytic enzymes widely distributed in biological membrane cells. In fact, phospholipids are building blocks of cell membranes and the most abundant phospholipid in plants are phosphatidylcholine (PC), which is involved in fatty acid modifications and storage lipid synthesis.

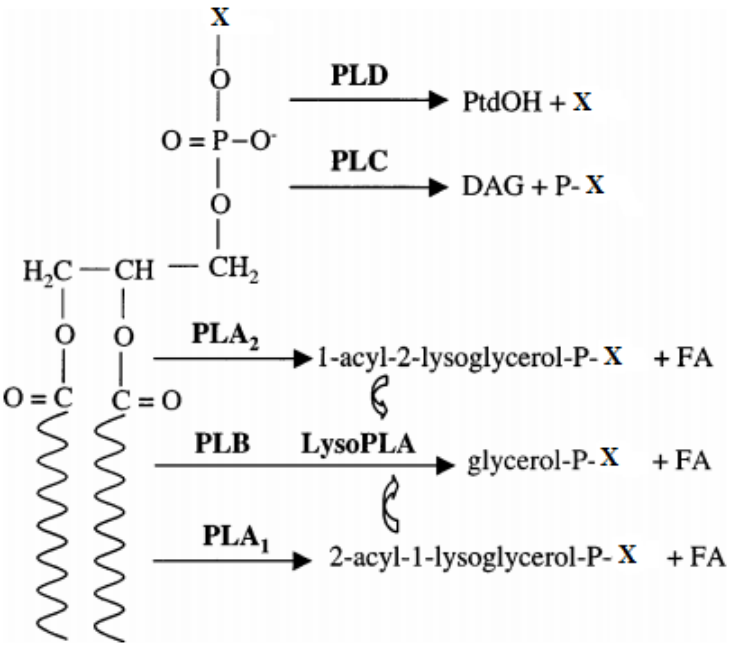
Phospholipases are important enzymes in cell regulation and metabolism of plants (Wang, Ryu, & Wang, 2012; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016), which contribute to the hydrolysis of plant phospholipids in various products that include phosphatidic acid, diacylglycerol, free fatty acids and lysophopholipids.

In fact, different classes of phospholipases were found in plants, which cleave acyl groups of phospholipids such as phospholipases A1, A2, B; lysophospholipases and acyl hydrolases. In addition, there are other classes of phospholipases, which catalyze the cleavage of the head group of phospholipids such as phospholipases C and D. For example, PLCs hydrolyze the ester bond between the glycerol and the phosphate group of phospholipids, thus releasing diacylglycerol (DAG) and the respective phosphorylated head group (Figure 1).

It is important to note that there are different families or subfamilies of phospholipases, which could differ in structure, substrate selectivity, cofactor requirements and reaction conditions (Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016). Moreover, many studies have highlighted that such enzymes play diverse and significant roles in plant growth, development and responses to either abiotic or biotic challenges (Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

- Glycolipase and glycosidase activities, which are involved in galactolipid and sulfolipid breakdown have been described in plant extracts on a biochemical level. A large number of lipase-like sequences were identified in the *Arabidopsis* genome whose function still is not

Figure 1. Hydrolysis of a phospholipid (such as phosphatidylcholine: PtdCho) by PLD, PLC, PLA2, PLA1, PLB, and lysoPLA and the respective reaction products. Note that the arrow lines for PLD, PLC, and PLA2 indicate their site of hydrolysis, but those for PLB, lysoPLA, and PLA1 do not. PLA1 hydrolyzes the sn-1 acylester bond, whereas lysoPLA removes the last fatty acid from lysophospholipids that can be produced by PLA2 and PLA1, as marked by the curved arrows. PLB sequentially removes two fatty acids from phospholipids, and its final reaction products are the same as those of lysoPLA. X, may be choline; P-X, phospho-choline; FA, fatty acid.



fully elucidated. The chapter of Dörmann (2005) summarises the lipid hydrolysing activities, following a classification system, which was also presented by Wang (2001) and Beisson et al. (2003).

The report of Weselake (2005) could be of interest to readers to disclose lipid storage and the relevant lipolytic enzymes.

- Fatty acids biosynthesis and manipulation involve many kinds of relevant lipolytic enzymes such as Acetyl-CoA carboxylase (ACCase: acetyl-coenzyme A: bicarbonate ligase (ATP), EC 6.4.1.2), which catalyses the first committed step in fatty acid synthesis.

Together with ACCase, fatty acid synthase is the second major enzyme complex involved in de novo fatty acid formation. For more information on fatty acids biosynthesis, please refer to the report of Harwood (2005).

Desaturases are other examples of enzymes related to fatty acid manipulation. In fact, Plants have two main types of fatty acid desaturases, which use glycerolipids and acyl carrier proteins (ACP) as substrates unlike most animal and fungal desaturases.

Plant stearoyl-ACP desaturase is unusual enzyme defined as soluble Δ9 desaturase. For more information on plant desaturases and fatty acid manipulation, please refer to the report of Hildebrand et al. (2005).

- Lipid oxidation leads to structural changes in biological membranes and lipoproteins and later cause various chronic diseases such as arteriosclerosis (Morel, Hessler, & Chisolm, 1983), diabetes (Nishisaki, 1981) and other nervous diseases (Braughler & Hall, 1989).

Various enzymes (lipolytic enzymes) are known to participate in the enzymatic oxidation of lipids. Among these enzymes, lipoxygenases selectively oxidize polyunsaturated fatty acids containing one or more *cis,cis*-1,4-pentadiene system and yield *cis-trans*-conjugated hydroperoxy fatty acids (Siedow, 1991; Yamamoto, 1992).

One example of plant lipoxygenase-activity was reported in soybean (Abousalham & Verger, 2006).

On the other hand, Ben Ali et al. (2005) have reported cholesterol oleate (CO), hormone-sensitive lipase (HSL) and other cholesterol esterases. In fact, they have developed a specific cholesterol esterase assay using CO dispersed in phosphatidylcholine and gum Arabic by sonication. HSL is in particular an important enzyme contributing to the hydrolysis of cholesteryl ester in steroidogenic tissues, releasing the cholesterol required for adrenal steroidogenesis (Ben Ali, Carrière, Verger, Petry, Muller, & Abousalham, 2005).

BIOINFORMATIC RESOURCES FOR PLANT LIPOLYTIC ENZYMES AND RELATED AREA

In recent years, cDNA and genomic DNA clones have been isolated for many of the plant lipolytic enzymes described in this chapter.

These DNA clones were beneficial to analyze in more detail lipase expression, structure and function. As well, DNA sequence analysis has provided important information about lipase protein and gene structure, which include nucleotide and amino acid sequence, exon/intron organization, chromosome localization and phylogenetic relationships among lipolytic enzymes.

Much of the data derived from the molecular and genetic characterization of genes as well as proteins (with lipolytic activity) have been deposited into a wide array of electronic databanks and databases that are continuously updated to include the most recent findings. Thus, researchers throughout the globe can contribute to and/or retrieve information from these online databases.

The goal of this chapter section is to provide some information regarding bioinformatics resources for plant lipolytic enzymes, which include lists addresses and key features of some important databases relevant to the general interest and utility of plant lipidomics and related enzymes.

The widespread of the Internet is in favor to boost access to several online databases such as the established databases, which provide access to nucleic acid and amino acid sequences, gene mapping data and phenotype information.

For example, a list of selected World Wide Web sites and resources for biologists is as following.

- **GrainGenes** (https://wheat.pw.usda.gov/GG3/): An online database for Triticeae and Avena.
- **TriFLDB** (https://integbio.jp/dbcatalog/en/record/nbdc01047): A database for Triticeae Full-Length CDS. For more detail of this database, please see the report of Mochida et al. (2009).
- **Integbio Database Catalog** (https://integbio.jp/dbcatalog/?lang=en): An online catalog of life science databases.
- **ExPASy Bioinformatics Resource Portal** (https://www.expasy.org/): ExPASy is the SIB (Swiss Institute of Bioinformatics) Bioinformatics Resource Portal that provides access to scientific databases and software tools in different area of life sciences including proteomics, genomics, phylogeny, systems biology, population genetics, transcriptomics, etc.
- **ExpressionView** (https://www2.unil.ch/cbg/index.php?title=ExpressionView): ExpressionView is an R package that provides an interactive environment to explore biclusters identified in gene expression data. A sophisticated ordering algorithm is used to present the biclusters in a visually appealing layout. From this overview, the user can select individual biclusters and access all the biologically relevant data

associated with it. The package is aimed to facilitate the collaboration between bioinformaticians and life scientists who are not familiar with the R language.

- **MLTreeMap** (http://mltreemap.org/): MLTreeMap is an online site to phylogenetic analysis of metagenomics sequence data, i.e., is a tool to characterize phylogenetic and functional properties of metagenomes.
- **STRING** (https://string-db.org/): Database of known and predicted protein-protein interactions.
- **GenBank** (https://www.ncbi.nlm.nih.gov/genbank/): GenBank is the NIH (National Institutes of Health) genetic sequence database, an annotated collection of all publicly available DNA sequences (Benson, Cavanaugh, Clark, Karsch-Mizrachi, Lipman, Ostell, & Sayers, 2013). GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at NCBI. These three organizations exchange data on a daily basis.
- **National Center for Biotechnology Information (NCBI)** (https://www.ncbi.nlm.nih.gov/): The NCBI is a well-established network site for sequence searches. It advances science and health by providing access to biomedical and genomic information. NCBI provides an array of searchable nucleotide and protein sequence databases, which are updated daily.

Many reports deal with providing additional information to internet resources for the molecular biologist (Harper, 1995; Swmdell, Miller, & Myers, 1996) as well as for molecular and genetic analysis of lipases (Reue, 1999). The report of Murphy (2005) has highlighted additional information on links to databases relative to plant lipidomics (Table 2).

PURIFICATION OF PLANT LIPOLYTIC ENZYMES

Lipases and phospholipases are the widely used lipolytic enzymes in many applications ranging e.g., from the food industries to manufacturing as catalysts of high specificity and selectivity as well as friendly environments. Research on the isolation, purification and characterization of lipolytic enzymes, in particular lipases and phospholipases, from diverse sources, have been the subject of many reports such as those published by of Aires-Barros et al. (1994), Palekar et al. (2000) and Vasudevan (2004).

Table 2. Some links to databases, books and other resources for plant lipids and related area

Lipidomics-Related Web Sites
http://www.ksu.edu/lipid/lipidomics http://hcc.musc.edu/research/shared_resources/lipidomics.cfm http://medschool.mc.vanderbilt.edu/brownlab/comlip.html http://www.wissenschaft-online.de/gbm/homepage/abstract_detail.php?artikel_id=265 http://www1.elsevier.com/gej-ng/29/50/lipids/119/47/26/article.pdf
General Lipids
• **Cyberlipid**: A web site containing much useful information on lipid chemistry, biochemistry and analysis (http://www.cyberlipid.org/). • **Lipid Nomenclature**: This is the IUPAC guide (http://www.chem.qmw.ac.uk/iupac/lipid). • **Conjugated Linoleic Acid**: Wisconsin Food Research Institute (http://www.wisc.edu/fri/clarefs.htm). • Compilation of trivial names of fatty acids (by R.O. Adlof and F.D. Gunstone) (http://www.aocs.org/member/division/analytic/fanames.htm). • **Lipidat:** A relational database of thermodynamic and associated information on lipid mesophase and crystal polymorphic transitions, including lipid molecular structures (glyceroand sphingolipids) (http://www.lipidat.chemistry.ohio-state.edu/). • **Lipid Bank for Web:** A database of information on lipid structures and properties with thousands of references (http://lipid.bio.m.u-tokyo.ac.jp/). • **Lipid Analysis Unit**: This site is a general information resource on lipids supported by the Lipid Analysis Unit at the Scottish Crop Research Institute (http://www.lipid.co.uk).
Plant Lipids
• On-line chemical database for new seed crops produced by the New Crop Research Unit at NCAUR, Peoria, IL, USA (http://www.ncaur.usda.gov/nc/ncdb/search.html-ssi/). • Similar database to the previous provided by BAGKF (Institute for Chemistry and Physics • of Lipids), Munster, Germany – SOFA (Seed Oil Fatty Acids) (www.bagkf.de/sofa). • A catalogue of genes for plant lipid biosynthesis at Michigan State University • (http://www.canr.msu.edu/lgc/index.html). • NPLC (National Plant Lipid Cooperative) (http://www.msu.edu/user/ohlrogge/). • A further source of links to web-based lipid information, includes: NPLC Directory of Plant • Lipid Scientists: o The NPLC Electronic Mailing List o The NPLC Database of Plant Lipid Literature o The Plant Lipid Home Page – (http://blue.butler.edu/~kschmid/lipids.html) • The Malaysian Palm Oil Board (MPOB) – web site devoted to all aspects of palm oil-biology, technology, food and non-food uses, and commercial matters (http://www.mpob.gov.my).
Books on Plant Lipids
• Thompson,W.W., Mudd, J.B. and Gibbs, M. (Eds.). (1983). *Biosynthesis and Function of Plant Lipids*, American Society of Plant Physiologist, Baltimore, USA (6th Ann. Symp. Bot. Riverside, California, 1983). • Murata, N. and Somerville, C. (Eds.). (1993). *Biochemistry and Molecular Biology and Storage of Plant Lipids*, American Society of Plant Physiologist, Rockville, USA (US/Japan Binational Seminar, Kona, Hawaii, 1992). • Harwood, J.L. (Ed.). (1998) *Plant Lipid Biosynthesis, Fundamentals and Agricultural Applications*, • Cambridge University Press, Cambridge (Soc. Exp. Biol. conference, Canterbury, UK, 1997). • Gunstone, F.D., Harwood, J.L. and Padley, F.B., eds (1994) *The Lipid Handbook*, 2nd edn, • Chapman and Hall, London (This is one of the premiere reference works dealing with lipid composition and analysis). • Gurr, M.I. and James, A.T. (1971) *Lipid Biochemistry: An Introduction*, Chapman and Hall, • London (Early editions contain many descriptions of experimental techniques and examples applied to plant lipids. The latest is the 5th edition and is written by Gurr, M.I., Harwood, J.L. and Frayn, K.N. (2002) Blackwell Science, Oxford). • Harwood, J.L. and Russell, N.J. (1984) *Lipids in Plants and Microbes*, George Allen and Unwin, London. • Hitchcock, C. and Nichols, B.W. (1971) Plant Lipid Biochemistry: The Biochemistry of Fatty Acids and Acyl Lipids with Particular Reference to Higher Plants and Algae. • Stumpf, P.K. (Ed.). (1980). *Lipids: Structure and Function*, Vol 4, The biochemistry of plants, a comprehensive treatise, Stumpf PK and Conn eds in chief, Academic Press, New York. • Moore, T.S. (1993). *Lipid Metabolism in Plants*, CRC Press, Boca Raton.

continued on following page

Table 2. Continued

Other Resources
• Wintermans, J.F.G.M. and Kuiper, P.J.C. (1982) *Biochemistry and Metabolism of Plant* • *Lipids*, Elsevier, Amsterdam. Published proceeding from the International Plant Lipid Congresses • Siegenthaler, P.A. and Eichenberger, W., eds (1984) *Structure, Function and Metabolism of Plant Lipids*, Elsevier, Amsterdam. Published proceeding from the International Plant Lipid Congresses • Stumpf, P.K., Mudd, J.B. and Nes, W.D., eds (1987) *Metabolism, Structure and Function of Plant Lipids*, Plenum, New York. Published proceeding from the International Plant Lipid Congresses • *Loders Croklaan* is part of Unilever Ltd. The site covers science, technology, and nutrition related to lipids in general and to Unilever's products in particular (www.croklaan.com) • *ITERG*. French Research Institute dealing with oils and fats research and technology (www.iterg.com) • *Natural of Norway*. Manufacturers of conjugated linoleic acid for health food and other applications (www.natural.no). • The web site of the *British Nutrition Foundation* carries information on lipids in addition to other food components (www.nutrition.org.uk). • ***CTVO***: A European group devoted to the chemical and technological utilization of vegetable oils (www.danet.de/fnr/ctvo). • ***IENICA***: An Interactive European Network for industrial crops and their applications (www.csl.gov.uk/ienica/). • ***ACTIN***: A UK group devoted to non-food uses of oils and fats (www.actin.co.uk). • European web site for the *American Soybean Association* (www.asa-europe.org). • **International Food Science and Technology**: Contains information on various food problems, including those involving lipids (www.ifst.org). • **Oil World:** Is a German company producing data on a weekly basis for oilseeds, oils and fats and oilmeals and covers production, imports, exports and disappearance. Information is based on different commodity oils and fats and is presented on the basis of individual countries (www.oilworld.de). • **FFA Sciences**: Is a company manufacturing probes to measure free fatty acid levels in oils and clinical samples (www.ffasciences.com). • **BritanniaFoods***:* Has some articles 'By Invitation Only' of interest to lipid technologists mainly (www.britanniafood.com). • **Peter Lapinskas:** Consultant to the Oils and Fats Industry – some interesting data on unusual seed oils (www.lapinskas.com).

Source: Murphy, 2005.

In fact, many steps would be involved to purify lipolytic enzymes. Generally, they include, firstly, pre-purification steps such as precipitation by saturation with an ammonium sulfate [$(NH_4)_2SO_4$] solution, concentration with ultrafiltration and/or combined with acid or organic solvent, e.g., acetone precipitation. Normally, chromatographic steps are secondly used in the purification process. Indeed, almost all purification protocols would use chromatographic steps after the pre-purification steps, which consist of chromatographic columns, for instance, gel filtration, and ion exchange and affinity chromatography.

The chapter of Vasudevan (2004) have examined in some detail the different techniques as well as strategies to be used for lipase purification.

Purification of Lipases from Higher Plants

Some studies have dealt with purification of lipases from plant sources especially from the higher plants (Hammer & Murphy, 1993; Ncube,

Adlercreutz, Read, & Matiasson, 1993; Tani, Ohishi, & Watanabe, 1994; Fuchs Vine, & Hills, 1996).

These studies would reflect such purification in regards to the plant lipase stability and the number of chromatographic steps required. For example, Fuchs et al. (1996) has reported purification and characterization of an acid lipase from castor oil seeds (*Ricinus communis* cv. *zanzibarenis*) and highlighted protein instability. Indeed, the purification route followed in this study involved the solubilization of this castor acid lipase from delipidated oil bodies using a detergent, CHAPS (3-cholamidopropyl-dimethylammoni1o-1-propanesulfate). The lipase was purified to apparent homogeneity by the aid of anion exchange followed in series gel filtration and adsorption chromatography, and the observed purification factors were low because of the instability of the protein when solubilized in the detergent (Fuchs Vine, & Hills, 1996).

The report of Hammer and Murphy (1993) on the properties of a lipid body lipase from pinyon (*Pinus edulis*) revealed that they faced problems to its purification and all their attempts to this purification were reported to be unsuccessful despite electrophoretic purification of 64 kDa subunit of this lipid body lipase. Nevertheless, it is important to notify that electrophoretic step is used, in normal purification circumstances, as a finishing analytical step and not as the basic step (Palekar, Vasudevan, & Yan, 2000).

The study of Ncube et al. (1993) divulged the protocol followed for the purification of a seedling lipase from rape (*Brassica napus*). Indeed, the purification scheme involved many steps that include homogenization, centrifugation, and three columns types of chromatography (DEAE-Sephadex, Octyl-Sepharose, and Sephacryl S-300). The purification steps are summarized in Table 3.

Table 3. Purification data for rape (Brassica napus) seedling lipase

Purification Step	Nature of Chromatography	Specific Activity (U/mg)	% Activity Recovery	Purification Fold
100 000 g supernatant	-	6.7	100	1
Acetone precipitate	-	47.4	80	7.1
DEAE-Sephadex	Ion exchange	94.8	78	14.2
Octyl-Sepharose	Hydrophobic interaction	1900	31	283
Sephacryl S–300	Gel filtration	2140	16	321

Source: Ncube et al. (1993)

From this Table 3, the most crucial step of the purification protocol followed is clearly the hydrophobic interaction chromatography using the Octyl-Sepharose, which allowed an important boost of the level of purification showing its extremely high level in comparison with the previous purification step, i.e., the ion exchange chromatography using DEAE-sephadex. Indeed, a 20-fold purification is obtained over this ion exchange chromatography step (DEAE-Sephadex), which is not matched by any of the other steps of purification. For this end, hydrophobic interaction chromatography would be suggested as one of the finishing steps of purification.

Some Issues

The difficulty to extract and obtain a sufficient quantity of enzymes with lipolytic activity from plant material hampers the widespread reports on plant lipolytic enzymes as well as their applications especially at industrial scale (Villeneuve, 2003; de Sousa, Cavalcanti-Oliveira Ed, & Aranda, 2010; Rivera, Mateos-Díaz, & Sandoval, 2012). In addition, enzymes with lipolytic activities especially from plants sources, in particular phospholipases, have difficulty to be purified at perfect homogeneity with high level of activity recovery due to their existence in multiple forms with distinctively different biochemical properties and cellular functions (Wang, Ryu, & Wang, 2012). Other reason of such difficulty, the association of plant phospholipases with other proteins. In fact, phospholipases, commonly, interact *in vivo* with other proteins, e.g., cytoskeleton, and their purification is difficult due to their association with the interacted proteins. In fact, there was an evidence of co-purification of actin and actin-binding proteins with phospholipase C (PLC) explaining as to why no plant PLC was ever purified to homogeneity despite intensive study by several researchers around the world (Huang & Crain, 2009).

On the other hand, it is demonstrated that the extraction of lipolytic activity from the latex polymer matrix has not been easy (Rivera, Mateos-Díaz, & Sandoval, 2012). Indeed, classically, the first step for the enrichment of lipolytic activity present in the latex is the removal of proteins and lipids contaminants. For this end, the soluble proteins could be removed in the mixture and a mix of water and a solvent could achieve the elimination of lipids present in the latex sample in order to realize the enzyme extraction (Moulin, Giordani, & Teissere, 1992; Moulin, Teissère & Bernard, 1994; Fiorillo, Palocci, & Soro, 2007). Nevertheless, other employed strategy used for this purpose consist on the use of some nonionic and zwitterionic detergents such as CHAPS

and Triton TX-100. For example, Fiorillo et al. (2007) have reported study of *Euphorbia characias* lipase in which they studied various detergents for the enzyme extraction and they combined them with various physical and chemical process, including in particular the use of a zwitterionic detergent (CHAPS) that improve the extraction yield.

Moreover, the detection of lipolytic activity in the papaya latex (Abdelkafi, 2009a; Abdelkafi, Ogata, Barouh, Fouquet, Lebrun, Pina, Scheirlinckx, Villeneuve, & Carrière; Abdelkafi, Barouh, & Fouquet, 2011; Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet, Scheirlinckx, Villeneuve, & Carrière, 2012; Giordani, Moulin, & Verger, 1991) has triggered a novel interest in this latex due, in particular, to the fact that these enzymes with lipolytic activity are not soluble and thus regarded as "naturally immobilized" biocatalysts (Azarkan, El Moussaoui, Van Wuytswinkel, Dehon, & Looze, 2003; Giordani, Moulin, & Verger, 1991).

In fact, most of the latex lipolytic activity is proven to be associated to the latex-insoluble fraction (Rivera, Mateos-Díaz, & Sandoval, 2012). And up to now, most of the several attempts to solubilize the active enzymes from particulate fractions of the latex were unfortunately unsuccessful (Giordani, Moulin, & Verger, 1991; Moulin, Teissère & Bernard, 1994). Some trials to isolate the latex-associate lipolytic enzymes were however successful. For example, Abdelkafi et al. (2009b) have reported the extraction of 10% lipase activity that corresponds to a GDSL-motif carboxylester hydrolase. The report of Dhouib et al. (2010) determined the first sequence of triacylglycerol lipase from *C. papaya* latex by the mean of functional proteomics. Indeed, their reported enzyme shows 35% identity and 51% similarity to the castor bean acid lipase, suggesting the important role of this enzyme as responsible to great extent to the lipolytic activity present in the latex.

It is worthy to note that it is unclear, at this moment, which of the lipolytic enzymes present in the latex are responsible for the catalytic activity assigned to the many applications of CPL (*Carica papaya* lipase). The report of Rivera et al. (2012) give some guidelines of practical protocol for a partial purification of the latex-associated lipolytic activity from *Carica papaya*. Such guidelines could better characterize such enzyme. As well, it is becoming necessary to isolate, purify, clone and express the individual lipolytic enzyme present in the C. papaya latex in order to advance the characterization of such enzyme (Rivera, Mateos-Díaz, & Sandoval, 2012).

Indeed, despite the identification of the GDSL esterase (CpEst) reported by Abdelkafi et al. (2009b) as well as the lipase homologous to castor bean lipase reported by Dhouib et al. (2010), these proteins were not purified to

homogeneity. One explanation for this failure to obtain pure enzymes might be that the latex lipolytic enzymes are covalently linked to the polyisoprenoid polymers present in the latex and that classical techniques for protein separation and purification are not well adapted (Tsai, Chen, Yang, Ng, & Chen, 2006).

Solutions and Recommendations

In order to overcome the problem of obtaining sufficient quantity of plant lipolytic enzymes for exploiting them in either laboratory scale for more characterization or industrial applications, cloning and expressing the plant genes coding to enzymes with lipolytic activity in an adequate host is one alternative to be explored (Rivera, Mateos-Díaz, & Sandoval, 2012). *De novo* sequencing of the desired plant lipolytic enzymes would be an outstanding other alternative to be studied with the aid of functional genomics, functional proteomics using high performance of analytical techniques such as liquid chromatography coupled with mass spectrometry in tandem as well as bioinformatics analysis and *in silico* approaches. For this end, one example of this latter solution is demonstrated in the reports of Abdelkafi et al. (2009b, 2012). Indeed, although the proteins from *Carica papaya* latex could not be purified to complete homogeneity due to their presence in high molecular mass aggregates, the sequencing of their peptides resulting from tryptic digestion allowed identifying EST sequences and then the full length genes of an esterase (CpEst) and a phospholipase D (CpPLD1) (Abdelkafi, Ogata, Barouh, Fouquet, Lebrun, Pina, Scheirlinckx, Villeneuve, & Carrière, 2009b; Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet, Scheirlinckx, Villeneuve, & Carrière, 2012).

OTHER GENERALITIES RELATED TO LIPOLYTIC ENZYMES

- Lipids from eukaryotic and prokaryotic sources could be regrouped into eight categories that are: fatty acyls, glycerolipids, glycerophospholipids, sterol lipids, sphingolipids, saccharolipids, prenol lipids and polyketides, and each category of lipid could contain distinct classes, subclasses, subgroups and subsets (Lipid Maps

Consotium, 2009). Indeed, a comprehensive classification system of lipids, for instance, plant lipids, could support the emerging field of plant lipidomics, plant lipid metabolism and relevant plant lipolytic enzymes, in particular those related to plant signaling (Namasivayam, Kowsalya, Padarthi, Manigandan, Jayaraj, Johnravindar, & Jagatheesh, 2015).

- Many reports highlighted the importance of lipidomics (Brown, 2007), proteomics (Samaj & Thelen, 2007) as well as functional genomics (Brownstein & Khodursky, 2003) as potential tools to depict new pathway in plant cell signaling including enzymes with lipolytic activities.
- Lipid metabolism (catabolism and anabolism) implies, for instance, in eukaryotic organism, many biochemical reactions that involve lipases, enzymes of glycerophospholipid metabolism (biosynthesis and hydrolysis and related biocatalysts for ether lipids) and enzymes of sphingolipid metabolism (biosynthesis, hydrolysis, and related activators of sphingolipid hydrolases). As well, it is important to unravel the roles of membranes and cells in the lipid metabolism to unveil how does lipids transfer and transport carry out in biological membranes. Moreover, the study of cellular aspects of lipid metabolism with the development and maturation of relevant numerous enzymes such as lipoprotein enzymes and phospholipases as well as the genetic study for the regulation of lipid metabolism could empower to treat diseases related to all aspects of such metabolism, e.g., lipid's material, cells and membranes, and related enzymes. For more information of lipid metabolism and related aspects, please refer to the reports of Gatt et al. (1978), Freysz et al. (1986), Schettler (1967) and Baez (2013).
- It is worth noting that lipolytic enzymes have inhibitors for their activities. Some of these lipase inhibitors could be Orlisat (tetrahydrolipstatin, THL) (Hogan, Fleury, & Hadvàry, 1987; Hadvàry, Lengsfeld, & Wolfer, 1988; Borgstrom, 1988; Gargouri, Chahinian, Moreau, Ransac, & Verger, 1991; Ransac, Gargouri, Moreau, & Verger, 1991), or Organophosphorus compounds, such as diisopropylfluorophosphate and E_{600}, or other various phosphonates (Maylié, Charles, & Desnuelle, 1972; Rouard, Sari, Nurit, Entressangles, & Desnuelle, 1978). For more information on inhibition of lipase, please refer to the report of Tiss et al. (2004).

CONCLUSION

Lipolytic enzymes, especially from plants, have become more and more prominent on the bio-catalysis due to their diversity as they are found in many families, their versatility for much kind of biochemical reactions involving either hydrolysis or synthesis, as well as their catalytic reactions often being chemo-selective, region-selective or enantio-selective (Barros, Fleuri, & Macedo, 2010). Many types of lipolytic enzymes and related enzymes exist for the metabolism of lipids such as lipoprotein lipases, lipid phosphate phosphatases, phospholipases, lysophospholipases and diacylglycerol kinases.

Lipolytic enzymes from different origins of plants (vegetables, oleaginous plants, higher plants or algae) and with different characteristics such as acid lipases would be used in many applications such as food, medicine and pharmaceutics. For this end, lipases are growing significantly in the worldwide enzyme industry market (Hasan, Shah, & Hameed, 2006). As well, as plant lipolytic enzymes would successfully be used in particular in medicine, food, chemistry and biochemistry, many social and economic issues would be resolved. Therefore, there would be less poverty in all its forms everywhere, but also hunger would be ended, and, there would be achievement in food security and improvement and promotion in the sustainable agriculture.

The use of the internet resources is an effective tool to bring about scientific communication for the use of plant lipolytic enzymes. Nevertheless, the use of the Information and Communication Technologies (ICT) in particular the modern ICT facilities like the cloud computers, the high performance computers (HPC), Big Data, Data Science and the internet of thins (IoT) would improve obtaining of the scientific information which would be developed worldwide.

This chapter has provided just an overview of plant lipolytic enzymes and their related areas.

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Chapter 2
Plant Lipases and Phospholipases and Their Diverse Functions and Applications

ABSTRACT

Lipases and phospholipases from plants have very interesting features for potential functions and applications in different fields. Plant lipases and phospholipases are ubiquitous proteins found either in basic or higher organs of plants. This chapter provides an overview of the diverse functions of these enzymes. The chapter addresses the issue of the importance regarding the functions of plant lipolytic enzymes (i.e., lipases and phospholipases) and their different forms not only for plants but also for external applications. The commercial exploitation of such plant proteins as industrial enzymes would be made as a potentially attractive alternative. The applications of plant lipases and phospholipases in biotechnology and industry such of food, detergents, effluent treatment, biodiesel production, as well as medicines and nutraceuticals are reviewed.

INTRODUCTION

Lipases and phospholipases are important enzymes in biocatalysis with unique feature; they are interfacial biocatalysts that hydrolyze hydrophobic ester linkages of triacylglycerols and phospholipids, respectively. Beside their role as esterases, lipases and phospholipases could catalyze a plethora of other

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reactions. Indeed, lipases could catalyze esterification, transesterification and interesterification reactions, and phospholipases could show acyltransferase, transacylase and transphosphatidylation activities (Borrelli & Trono, 2015). Therefore, lipases and phospholipases are considered as versatile biocatalysts, which are widely used in various applications such as for biodiesels, detergents, oil degumming, nutraceuticals and food industries; and they are used also in minor applications that include cosmetics, leather and paper industries, as well as bioremediation and agriculture (Borrelli & Trono, 2015).

Lipases and phospholipases are ubiquitous in most living organisms, i.e., bacteria, yeasts, fungi, animals and plants. Although microbial lipases and phospholipases are more preferred than those from animals and plants sources because of their greater availability and their ease of production compared to the other sources, plants source could gain much interests nowadays due to their interesting features. In fact, despite the widest use of microbial lipases and phospholipases, these enzymes could present many disadvantages such as those related to the traditional purification strategies from microbial cultures that lead to non-reproducibility and low yields, as well as some native microbial enzymes could be not always suitable for biocatalytic processes. Thus, whatever sources of enzymes, cloning and expressing them in an adequate host is one alternative to be explored to overcome the problem of the quality and productivity of enzymes. In fact, with the development of biotechnology and molecular techniques for the production of recombinant heterologous enzymes in a host system could allow high-level enzyme expression as well as production of new redesigned proteins with improved catalytic properties. This alternative can meet the requirements of specific application, e.g., industrial process, better than the native enzymes whatever their sources, e.g., from microbes or plants.

The study of lipolytic enzymes from plants in particular lipases and phospholipases is very interesting, as these enzymes would play important roles inside the plant such their implication in plant growth, development as well as in biotic and abiotic stimuli stress responses. The mechanism of action, cellular locations, post-translational modifications, regulation, cellular functions, roles in growth and differentiation, and other cellular systems, all of these features of the plant lipases and phospholipases would be studied to better characterize such enzymes. In addition, these plant proteins with lipolytic activities might found many applications outside the plant where they are expressed. These external applications are particularly in industrial uses such as in the food, biotechnology, or pharmaceutical companies. The investigation and understanding of relationship of proteomes and lipidomes

(i.e., enzymes with lipolytic activities) would be an outstanding feature in recent development in biotechnology.

An overview of some distinctive features unique to plant lipases and phospholipases will be highlighted in the current chapter focusing on discussing some major challenges in the field of their important physiological functions and their potential important applications.

BACKGROUND

In plants, enzymes with lipolytic activity are of great importance in plant metabolism, growth and development. Lipases and phospholipases from plants, which are the focus of the current chapter have crucial cellular and physiological functions to maintain the vital signs of the plants.

The membrane lipid of plants in particular glycerolipids, are subject to constant turnover by the relevant lipid catalyzing enzymes, phospholipases and galactolipases. In fact, different lipase activities could be distinguished based on the substrate specificity and the position of the cleavage site within the plant lipid molecule (Dörmann, 2005). Yet, the plant mechanism underlying the regulation of membrane lipid degradation, synthesis and homeostasis are only poorly understood.

Phospholipases are the focus of intense studies because they are important enzymes to generate lipid-derived signal molecules (Drøbak, 2005; Rosahl & Feussner, 2005). Indeed, there are different classes of phospholipases that were characterized in plants, which cleave either acyl groups of phospholipids (PLA1, PLA2, PLB, lysophospholipases and acyl hydrolases) or head group of phospholipids (PLC and PLD).

According to Wang et al. (2012), plant phospholipases are classified into four major types, phospholipase A1 (PLA1), phospholipase A2 (PLA2), phospholipase C (PLC) and phospholipase D (PLD). Moreover, within each type, different families or subfamilies of enzymes could be grouped that can differ in substrate specificity, cofactor requirement and/or reaction conditions. These differences could be of great importance to determine the cellular function of each specific phospholipase in plants.

Many kinds of enzymes including lipolytic enzymes are continually used as key components in many applications as either industrial enzymes such in nutraceutical, food and beverages, or specialty enzymes such in pharmaceuticals, research and biotechnology. The global enzymes market (https://www.grandviewresearch.com/industry-analysis/enzymes-industry)

is expected to reach USD 17.50 billion by 2024, according to a new report by Grand View Research, Inc. (https://www.grandviewresearch.com/press-release/global-enzymes-market). Industrial enzymes in particular lipases and phospholipases are gaining importance due to the increase in their applications.

In fact, many reports have indicated the growth of lipases and phospholipases in the worldwide enzymes market. The following online links register increase expectation on the demand of enzymes, which include lipolytic enzymes, on the global enzymes market:

- https://www.futuremarketinsights.com/reports/phospholipase-enzyme-market
- https://www.mordorintelligence.com/industry-reports/lipase-market
- https://www.marketsandmarkets.com/PressReleases/lipase.asp
- https://www.futuremarketinsights.com/reports/lipase-market
- https://www.futuremarketinsights.com/press-release/lipase-market
- https://www.freedoniagroup.com/brochure/28xx/2824smwe.pdf
- https://www.mordorintelligence.com/industry-reports/global-industrial-enzymes-market-industry

It is important to note that the source of the enzymes in the worldwide market could varied from prokaryotes to eukaryotes, for instance, plant source of lipase are used in the region of Europe, according to the Future Market Insights (2017) (https://www.futuremarketinsights.com/reports/lipase-market).

On the other hand, the enzymes could be commercialized in many forms such as liquid, powder or gel.

The lipolytic enzymes (lipases) are the third largest group of commercialized enzymes, after carbohydrases and proteases (https://www.grandviewresearch.com/press-release/global-enzymes-market). Lipases and phospholipases are of great importance as specialty enzymes and especially as industrial enzymes due to their multiple interesting features such as their stability in organic solvents, their selectivity and their wide variety of substrates.

Lipases from plants sources (e.g., oleaginous, seeds, higher plants, algae) have continually much potential applications at laboratory scale (Villeneuve, 2003; Paques & Macedo, 2006), but also for further industrial scale (Rivera, Mateos-Díaz, & Sandoval, 2012). At the same, plant phospholipases (PLD, PLC and PLA) have a large amount of potential applications at either laboratory scale or industrial one (Wang, Ryu, & Wang, 2012).

Lipases and phospholipases are mostly obtained from microorganisms or mammals origins. Therefore, these kinds of enzymes have been mostly studied and used in various industrial applications, like medical, pharmaceutical, and food industries (Hasan, Shah, & Hameed, 2006; Ramrakhiani & Chand, 2011). Nevertheless, lipases and phospholipases from plant origins have emerged various applications, for instance, plant lipases are highly specific for fatty acids of the plant (Mala Pahoja & Ali, 2002); and this latter feature could be exploited in biotechnological applications such as the production of biodiesel and tailored lipids (Mukherjee, 1994; de Sousa, Cavalcanti-Oliveira, & Aranda, 2010). Although, plant lipases could be used in different industrial applications, there are some difficulties to extract and obtain a sufficient plant lipase quantity (Villeneuve, 2003; de Sousa, Cavalcanti-Oliveira, & Aranda, 2010), thus hampering the emerging of such enzymes in the industrial applications. Nevertheless, some solutions could be used such as the *de novo* sequencing of the desired plant lipolytic enzymes (Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet, Scheirlinckx, Villeneuve, & Carrière, 2009, 2012) or cloning and expressing them in an adequate host (Rivera, Mateos-Díaz, & Sandoval, 2012).

SOME MAJOR FUNCTIONS OF PLANT LIPASES AND PHOSPHOLIPASES

Classification of Plant Lipases

In plants, "true" lipases have been found especially in cereals and bean seeds, and they are abundant particularly in the oil bodies of oleaginous seeds (Villeneuve, 2003; Barros, Fleuri, & Macedo, 2010; Moussavou Mounguengui, Brunschwig, Baréa, Villeneuve, & Blin, 2013). In fact, lipolytic enzymes in seeds are needed for the mobilization of the stored fatty acids, which need to be released to provide the necessary energy and carbon for the seedling growth (Quettier & Eastmond, 2009). Lipase activity is, thus, generally absent in un-germinated seeds; nevertheless, it rapidly appears alongside germination (Moussavou Mounguengui, Brunschwig, Baréa, Villeneuve, & Blin, 2013). On the other hand, the study of Padham, et al. (2007) reported the identification of a putative lipase from *Arabidopsis* in the plastoglobuli of chloroplasts, where it is probably involved in the mobilization of fatty

acids. In addition, latex, the milky sap that is produced by rubber trees, also contains lipolytic activity (Paques & Macedo, 2006).

Plant Sources of Lipases and Phospholipases for Biocatalysis

Lipases from plant sources especially from oilseeds have interesting features for biocatalysis. One of such features is their very activity in environment with very low water activity (a_w), which make these plant lipases suitable to act as biocatalysts in various organic solvents (Barros, Fleuri, & Macedo, 2010; Moussavou Mounguengui, Brunschwig, Baréa, Villeneuve, & Blin, 2013).

Cereal seeds are important sources of plant lipases. Lipases from rice, barley, wheat, oat and maize are some reported examples of cereal seeds lipases that have been tested for their use as biocatalysts, and most of these lipases could be resistant to rigorous environments especially high temperatures and alkali conditions (Mohamed, Mohamed, Mohamed, & Fahmy, 2000; Bhardwaj, Raju, & Rajasekharan, 2001). The resistance of the cereal lipases to such extreme reactions conditions make them an excellent alternative to chemical catalysts in the industrial processes that need these extreme environments of high temperatures and alkali conditions.

Interestingly, lipase from rice bran could be used as biocatalyst in the esterification of palm oil fatty acids and glycerol for the biosynthesis of acylglycerols (Chong, Tey, Dom, Cheong, Satiawihardja, Ibrahim, Rahman, Biak, & Ling, 2007).

Moreover, lipases from latex of different rubber trees have gained much interest in recent years as promising enzymes in potential industrial applications (Paques & Macedo, 2006). In particular, lipase from *Carica papaya* has been emerged as a versatile biocatalyst in many biotechnological applications. This enzyme could be used either as a crude extract or in a partially purified form, in many applications such as in the esterification and inter-esterification reactions in organic media, the fats and oils modifications, and the asymmetric resolution of different chiral acids (De María, Sinisterra, Tsai, & Alcántara, 2006).

For the phospholipases, Table 1 gives classification of phospholipases from some plants with their localization. On the other hand, among the classified phospholipases, PLD was reported to be the most important plant phospholipase with potential applications especially in the biocatalysis of

Table 1. Classification of plant phospholipases

Type	Organism	Name	Localization	References
PLA1	*Arabidopsis thaliana*	DAD1	Chloroplastic (anther)	Ishiguro, et al. (2001)
PLA2	*Arabidopsis thaliana*, carnation, castor bean, citrus, durum wheat, elm, rice, soybean, tobacco	sPLA2 (Groups XIA and XIB)	Secreted, cytosolic (various tissues)	Verlotta, et al. (2013)
	Arabidopsis thaliana, rape, broad bean, cucumber, pepper, potato, tobacco, tomato	Patatin-like phospholipase	Cytosolic (various tissues)	- Matos and Pham-Thi (2009) - Scherer, et al. (2010)
PLB	Broad bean, ricin bean	PLB-like	Cytosolic (leaves)	- Kim, et al. (1994) - Helmy, et al. (1999)
PLC	*Arabidopsis thaliana*, bean, cedar, cereals, *Lilium*, moss, potato, soybean, tobacco, tomato	PI-PLC	Cytosolic (various tissues)	Pokotylo, et al. (2014)
	Arabidopsis thaliana, avocado, peanut, rape, rice, soybean, tomato, wheat	NPC	Cytosolic (various tissues)	Pokotylo, et al. (2013)
PLD	*Arabidopsis thaliana,* cabbage, castor bean, grape, *Jatropha curcas*, mustard, peanut, poplar, poppy, strawberry, rice, sunflower, tobacco, tomato	C2-PLD (PLDα, PLDβ, PLDγ, PLDδ, PLDε), PXPH-PLD (PLDζ1, PLDζ2)	Secreted, cytosolic, membrane-bound (various tissues)	- Selvy, et al. (2011) - Khatoon, et al. (2015)

Source: Borrelli and Trono (2015)

transphosphatidylation reactions (Ulbrich-Hofmann, Lerchner, Oblozinsky, & Bezakova, 2005).

Among plant phospholipases, cabbage (*Savoy cabbage*) is the most traditional source of plant PLD. In fact, cabbage PLD has been successfully used for the modification of polar head-groups of phospholipids, to produce synthetic or low-abundance phospholipids for use in nutraceutical and pharmacological applications (Mukherjee, 2005).

General Features of Plant Phospholipases

Phospholipases in plants play important roles in various cellular and physiological processes (Wang, 2004). Generally, the roles of plant phospholipases could be grouped into three major categories. The first role could be the cellular regulation such as signaling messengers and mediators in vesicular trafficking, secretion and cytoskeletal rearrangements. The two other roles of plant phospholipases could be their involvement in the membrane lipid remodeling, as well as the lipid degradation. Besides, some plant phospholipases could be involved in the storage of lipid biosynthesis,

for instance, releasing unusual fatty acids from phospholipids to generate triacylglycerols.

On the other hand, the different families or subfamilies of plant phospholipases that can differ in substrate specificity, products and reaction conditions could be explored for different applications. Some of the major applications of plant phospholipases are those related to industrial uses including oil refinery, dairy, cosmetics and health food manufacturing (Wang, Ryu, & Wang, 2012).

For functional features of plant phospholipases, we will delineate in particular the functions of the C and D phospholipases, which cleave the first and the second phosphodiesteric bond of head groups of phospholipids, respectively.

Cellular Functions of Plant PLDs

Plant PLD members have been involved in a wide variety of cellular and physiological processes. Some of these cellular functions of plant PLDs are as following: the involvement in the regulating of stomatal opening and closure; signaling plant responses to abiotic and biotic stresses; organizing the structures of microtubule and actin cytoskeletal; promoting the growth of the pollen tube; cycling phosphorus; signaling nitrogen availability; and remodeling plant membrane phospholipids in response to phosphate deprivation and during and after freezing (Wang, Ryu, & Wang, 2012).

In fact, PLDα family members were proven to be involved in maintaining proper water balance, which include response to abscisic acid, drought (Sang, Zheng, & Li, 2001), salt (Hong, Pan, & Welti, 2008) and freezing stresses (Li, Wang, & Li, 2008), but also in response to pathogens (Laxalt, ter Riet, Verdonk, 2001; de Torres, Fernandez-Delmond, & Niittyla, 2002). Nevertheless, both PLDβ and PLDγ members have been proven to be implicated in early responses to pathogens (de Torres, Fernandez-Delmond, & Niittyla, 2002). PLDδ, however, could increase freezing tolerance (Li, Wang, & Li, 2008; Li, Li, & Zhang, 2004; Chen, Xiao, & Chye, 2008), regulate cytoskeletal organization (Gardiner et al., 2001), help plants to cope with drought stress (Katagiri, Takahashi, & Shinozaki, 2001) and improve plant stress tolerance by dampening H_2O_2-induced apoptosis (Zhang, Wang, & Qin, 2003). The overexpression of PLDε could lead to an increase in nitrate uptake, biomass accumulation and root elongation (Hong, Devaiah, & Bahn, 2009). PLDζs could be involved in the initiation of the remodeling pathway that converts

phospholipids into galactolipids to conserve and recycle phosphorus under phosphorus-limited growth conditions (Li, Welti, & Wang, 2006; Cruz-Ramirez, Oropeza-Aburto, & Razo-Hernandez, 2006). As well, PLDζs play a role in vesicular trafficking and auxin response (Li & Xue, 2007).

In plants, PLDs play important roles in response to wounding and other stresses through mediating actions and production of stress hormones (Wang, 2000). Indeed, different stresses in plants lead to different expression patterns for PLD isozymes. PLDα could be specifically involved in the actions of abscisic acid and ethylene in plant senescence as well as in the control of water loss (Wang, 2000; Wang, 2001). For more general information regarding the roles of PLD (roles in growth and differentiation, in vesicle trafficking, in exocytosis and endocytosis), please refer to the report of Exton (2004).

CELLULAR FUNCTIONS OF PLANT PLCS

PI-Specific PLC

The expression of PI-PLCs in plants is induced in response to various stimuli including the plant hormone abscisic acid, salt, cold, nutrients and dehydration (Tasma, Brendel, & Whitham, 2008; Hirayama, Ohto, & Mizoguchi, 1995; Shi, Gonzales, & Bhattacharyya, 1995; Yamamoto, Conkling, & Sussex, 1995; Hirayama, Mitsukawa, & Shibata, 1997; Kopka, Pical, & Gray, 1998). Moreover, PI-PLCs are reported to be involved in the pollen tube growth as well as the root hair growth (Dowd, Coursol, & Skirpan, 2006; Helling, Possart, & Cottier, 2006; Thole, Vermeer, & Zhang, 2008). It is important to note that the functional studies of PI-PLCs in plants have mostly been related to the production of inositol 1,4,5-triphosphate (IP_3) that is a potent Ca^{2+} mobilizer (Mueller-Roeber & Pical, 2002).

Nonspecific PLC

Non-specific PLCs could be involved in phospholipid degradation and could be induced by phosphate limitation (Nambara & Marion-Poll, 2005; Gaude, Nakamura, & Scheible, 2008). For example, NPC5 was proven to be involved in phospholipid degradation and digalactosyldiacylglycerol accumulation in the leaves of *Arabidopsis* and not in its roots (Gaude, Nakamura, & Scheible, 2008); whereas, *NPC4* was proven to be induced greatly by phosphate

limitation in the roots and leaves of *Arabidopsis* (Nambara & Marion-Poll, 2005; Gaude, Nakamura, & Scheible, 2008).

For information regarding the functional role in lipid signaling of plant PI-PLC and NPC, please refers to the review of Singh et al. (2015).

For more information regarding the cellular and physiological functions of plant PLD and PLC, please refers to the review of Hong et al. (2016).

SOME MAJOR APPLICATIONS OF PLANT LIPASES AND PHOSPHOLIPASES

Lipases catalyze several reactions under different conditions. Indeed, lipases catalyze the cleavage of carboxyl ester bonds and this reaction is designed as hydrolysis in the presence of water as substrate, but when water is the product of lipase reaction, this latter reaction is designed as esterification. Actually, lipase act at the organic-aqueous interface to catalyze the hydrolysis of carboxylate ester bonds and release free fatty acids and organic alcohols under natural conditions and especially in the presence of excess of water.

In fact, the equilibrium between the forward and reverse reactions of lipase is controlled by their water activity (a_w). Thus, under limiting water conditions, esterification, the reverse reaction, can occur. Besides, under low a_w, lipases could catalyze different transesterification reactions. The term transesterification refers to the exchange of groups between an ester and an alcohol (alcoholysis), between an ester and an amine (aminolysis), between an ester and an acid (acidolysis), or between two esters (interesterification) (Rahaman, Salleh, & Basri, 2006).

Plant lipases could find applications in many fields such as in food, oils and fats, medicines and fine chemistry, detergents, effluent treatment, biodiesel production and in the cellulose pulp industry (Barros, Fleuri, & Macedo, 2010).

Table 2 presents a summary of the applications of some plant lipolytic enzymes from different origins (oleaginous plants, seeds, cereals and laticifers).

Many reports have highlighted the importance of enzymes with lipolytic activities for used in industrial applications (Ramnath, Sithole, & Govinden, 2017; Guerrand, 2017; Fan, Niehus, & Sandoval, 2012; Li, Yang, Yang, Zhu, & Wang, 2012; Hudlicky & Reed, 2009; Haki & Rakshit, 2003; Jaeger & Eggert, 2002).

Table 2. Some plant lipolytic enzymes (oleaginous plants, seeds, cereals and laticifers) and their applications

Lipolytic Enzymes Source	Application	Authors
Oleaginous plants		
Castor bean	Esterification of fatty acids and glycerol Synthesis of structured lipids	Tüter (1998)
Rapeseed	Production of low molecular weight esters	Liaquat and Apent (2000)
Vernonia seed (*Vernonia galamensis*)	Hydrolysis of oils	Ncube and Read (1995)
Cereals		
Wheat seed	Hydrolysis and esterification	- Pierozan et al. (2009) - Xia et al. (2009)
Corn	TG hydrolysis	-Lin and Huang (1984) -Lin et al. (1986)
Rice (bran)	Phospholipid hydrolysis	Bhardwaj et al. (2001)
Laticifers		
Euphorbia characias	Hydrolysis of TG and synthetic Monoesters	Fiorillo et al. (2007)
Babaco (*Carica pentagona)*	Alcoholysis of sunflower oil Naproxen resolution	- Cambon et al. (2008) - Cambon et al. (2009) - Chen et al. (2005)
Carica papaya	Lipids modification Asymmetric resolutions	-Abdelkafi et al. (2009) -Abdelkafi et al. (2011) - Domínguez de María et al. (2006) - Dhouib et al. (2010)

- Enzymes in particular those with lipolytic activity, are currently being applied in multiple industries. The flexibility of enzyme properties found in lipases and phospholipases enables application in a number of degradation and synthesizing processes, such as the detergent, food, baking, pulp and paper, organic synthesis, fats and oils, and leather industries (Table 3). Therefore, basing on Table 3, lipases have enormous potential in the lipid bio-industry due to their availability and stability in organic media (Sharma & Kanwar 2014).

Recently, lipases have gained much interest as biocatalysts in biodiesel production (Fan, Niehus, & Sandoval, 2012; Soumanou, Djenontin, Tchobo, Sohounhloue, & Bornscheuer, 2012; Mazou, Djossou, Tchobo, Villeneuve & Soumanou, 2016).

Table 3. Lipolytic enzymes used in various industrial processes and their application

Industry	Lipolytic Enzyme	Application
Detergent	Lipase	Lipid stain removal
Starch and fuel	Lipase	Synthesis of lipase-catalyzed biodiesel
Food	Lipase	Improvement of food texture
Baking	Lipase	-Dough stability and conditioning
	Phospholipase	Dough stability and conditioning
	Lipoxygenase	Dough strengthening, bread whitening
Pulp and paper	Lipase	Pitch control, contaminant control
Fats and oils	Lipase	Transesterification
	Phospholipase	Degumming, lysolecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
Leather	Lipase	Depickling
Environmental application	Lipase	Degradation of lipid wastes
		Removal of solid and water pollution by hydrocarbons, oils, and lipids

Source: Ramnath et al. (2017)

In fact, the majority of plant lipases have problems of the implementation at large scale, i.e., industrial applications because in particular to the low content of enzyme including lipase in the post-germination seeds. Laticifers such as plants of the family of *Caricaceae* or *Euphorbiceae* overcome such disadvantage as their enzymes in particular those with lipolytic activity are available in large amounts stored in their latex (Paques & Macedo, 2006).

Some Important Implications of Plant Phospholipases

Phospholipases, mostly studied and obtained from microorganism and animal sources, could be used in various applications, like in the oil refining, baking, dairy and health food industries. Actually, PLD and PLC are the most useful phospholipases in the pharmaceutical, medical and analytical purposes (Ramrakhiani & Chand, 2011). Nevertheless, PLA are more suitable than the other types of phospholipases for enzymatic degumming of edible oils as well as for the synthesis of triglycerides with important quantity of polyunsaturated fatty acids (Gunstone, 1999).

In plants, there is recent progress on the biochemical properties and functions of phospholipases, which improve the use of plant phospholipases in various applications. As reported by Ulbrich-Hofmann et al. (2005), plant PLD could be involved in the synthesis of chemicals by transphosphatidylation, which could serve enzymatically to the modification of phospholipids as well as the synthesis of phospholipid conjugates used in pharmaceutical, cosmetics and food industries. Indeed, PLD is able to exchange the alcohol moiety attached to the phosphatidyl moiety of a given substrate of phospholipid by its ability to catalyze the transphosphatidylation reaction (Ulbrich-Hofmann, Lerchner, Oblozinsky, & Bezakova, 2005).

There are multiple forms of secretory phospholipase A2 (PLA2) in plants, which show distinctive differences from that originated of animal sources in term of substrate selectivities with respect to the head group and acyl chains of given glycerophospholipids (Lee, Bahn, & Shin, 2005; Mansfeld, 2009).

Obviously, plant phospholipases are promising enzymes due to their unique properties among the other sources of phospholipases, which open new horizons to engineer such biocatalysts with improved performance to be used in various applications. Plant phospholipases might be of great importance particularly in enzymatic degumming in the vegetable oil refinement as well as in the production of biodiesel because of the substrate specificities adaptation to phospholipids of the plants (Wang, Ryu, & Wang, 2012).

The plant sPLAs, for instance, are expected to have more advantages than those from animal sources with respect in particular to the yield and performance in polyunsaturated fatty acids incorporation, e.g., α-linolenic acid into egg-yolk phosphatidyl-choline for the purpose of producing functional food with a high impact on human health. Thus, plant phospholipases are beneficial in the feasibility of the processes of production of phospholipids with uncommon fatty acids that match special functional requirements (Wang, Ryu, & Wang, 2012).

Plant phospholipases have also a helpful aspect in applications, which consists in their lower extent of interfacial activation that make these enzymes further promising in relevant applications (Kim, Chung, & Ok, 1999).

Moreover, plant phospholipases are expected to be exploited in crop improvement by genetic manipulations. Indeed, the overexpression or suppression of genes coding to phospholipases was reported to enhance crop yield due to an increase in resistance of such genetically modified plants to a variety of phyto-pathogen infections as well as to abiotic stresses (Yang, Devaiah, & Pan, 2007; Peng, Zhang, & Cao, 2010; Vossen, Abd-El-Haliem,

& Fradin, 2010; Yamaguchi, Kuroda, & Yamakawa, 2009; Ryu, Lee, & Hwang, 2009).

The report of Casado et al. (2012) reviews some important uses of phospholipases in food industry including edible oils, baking products, dairy, emulsifying agents. In fact, phospholipase could be used as an emulsifier in aqueous food products, like in the case of sauces, bakery products and dressings. Increase in this end use application of phospholipase is expected to drive the market of this enzyme.

Individual PLDs have been reported to have distinct function; indeed, the overexpression of *PLDα* gene from *Setaria italica* in *Arabidopsis*, for instance, enhanced the sensitivity of *Arabidopsis* to abscisic acid and improved its drought tolerance (Peng, Zhang, & Cao, 2010). As well, it was demonstrated that attenuation of *PLDα1* expression in *Arabidopsis* lead to improve oil stability, and seed quality and viability (Devaiah, Pan, Hong, & Roth, 2007). On the other hand, it was reported that suppression of a PLD gene in rice, *OsPLDβ1*, has activated defense responses and increased disease resistance in rice (Yamaguchi, Kuroda, & Yamakawa, 2009). However, the overexpression of *PLDε* has enhanced nitrogen signaling and growth in *Arabidopsis* (Hong, Devaiah, & Bahn, 2009). In addition, the overexpression of *PLDδ* increased freezing tolerance in *Arabidopsis* (Li, Wang, & Li, 2008 ; Li, Li, & Zhang, 2004; Chen, Xiao, & Chye, 2008).

On the other hand, plant PLC in particular plant PI-PLC is implicated in response to a variety of environmental stress (Tasma, Brendel, & Whitham, 2008). It was reported that enhanced expression of the gene *ZmPLC1* that codes to PLC, improved drought tolerance in maize (Wang, Yang, & Yue, 2008). It is important to note that there are other reported plant lipases, which have been implicated in various physiological as well as biochemical processes in plants (Wang, Ryu, & Wang, 2012).

CONCLUSION

Plant enzymes with lipolytic activity, in particular lipases and phospholipases, constitute multi-gene families, which are implicated in plant growth and development as well as in various cellular and physiological functions. Although, tremendous progress has been made in various aspects of plants lipases and phospholipases and especially of the C- and D-types of phospholipases, our understanding of these enzymes is far from complete.

Plant lipases and phospholipases, although less studied than microbial or animal ones, should be considered as potential biocatalysts for many applications in particular for oils and fats restructuring. Interestingly, when a lipolytic activity is found in raw plant material that is already widely used in industry particularly in food industry, it is a very promising alternative to microbial lipases. To date, the latex lipolytic activity constitute the most promising plant lipase that must be more exploited as a competitive industrial enzyme than other sources due in particular to its large availability, its ease of extraction and its cheap cost.

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Chapter 3
Plant Lipases

ABSTRACT

Most searches in plant lipases have been devoted to seed lipases, but other sources of plant lipases are being exploited. The current chapter is undertaken to show readily available sources of lipases from plants as well as for their biochemical properties and some of their other eminent characteristics. The sequence and structural characterization into these lipolytic enzymes as well as their importance for biotechnological applications would also be the focus of this chapter. In fact, plant lipases have interesting features, particularly biochemical properties (e.g., pH and temperature), and with respect to their specific hydrolytic properties would make these enzymes alternative remedies for treatment of many diseases of the gastrointestinal tract and of pancreatic insufficiency with exogenous plant acid stable lipases.

INTRODUCTION

Most of the efforts in plant lipases have been devoted to seed lipases. In fact, intact seeds contain sources of energy in particular lipids in their components, e.g. germ and endosperm. The main type of lipids in seeds are triacylglycerols (TAG) in which lipases would act during germination of the seeds resulting in progressive disappearance of their stores of TAG. During seed germination, lipases act on TAG to release fatty acids (FAs) and have a relatively high activity due to the usual rapidity of the germination. These enzymes are fort probable rate controlling to assume the required FAs, which cannot be solely

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oxidized to provide energy during the process of germination. Thus, plant lipases, in particular from seeds, are interesting biocatalysts indispensable to the perfect development of germination.

Dormant lipases in seeds could be activated even without germination when seeds are crushed or long time stored. In these cases, free FAs (FFAs) will be accumulated and cause an unacceptable seed oil. This issue is addressed in particular to the highly food fat oil seeds varieties industrially utilized such as maize, corn, soybean and safflower. To this end, additional refining processes would be required in food oil industries to remove the acidity of the resulting accumulation of FFAs.

Yet, very few reports were studied plant lipases even though those from seeds. Nota bene that most outstanding reports in this area have been focused on plant sources of exotic properties lipases, for instance, acid stable lipases for potential therapies. Plant could have lipases with interesting biochemical properties, in particular, stable acid or alkali lipases, as well as thermo-stable lipase activities. Plant lipases could also present many other advantages, even over microbial and animal lipases, in view of some of their awesome features such as availability, specificity and low cost. These characteristics would make plant lipases as an important alternative for industrial applications. In addition, plant lipases, in particular from latex, could be naturally immobilized enzymes, an outstanding feature that cannot be exist in other sources of enzymes.

The emphasized parts of the current chapter would be as follows. Firstly, the chapter begins with recapitulating a brief definition of lipases with their reaction catalysis that describe hydrolysis and synthesis reactions. Then, the main sources of lipases are the second part of the current chapter. The important part of this review is deviated to structural insights of lipase, an amazing enzyme. For this end, some title/subtitles of the related theme are as following: Structure and catalytic mechanism including: The α/β Hydrolase Fold, The Catalytic Triad, The Oxyanion Hole, The Lipases α-Helical Loop, the Lid and The Catalytic Mechanism. The following part of the chapter reviews the notion of selectivity in plant lipase. In fact, selectivity is related to lipases' reactions and any source of lipase could acquires three distinguishable selectivity, which are: Type-selectivity, Regioselectivity and Enantioselectivity. The basis of such types of selectivity is also discussed in the current manuscript. Finally, plant lipases roles, e.g., physiological roles and potential applications outside plants are present in this chapter.

Therefore, the main objectives of the chapter are to give a global idea of plant lipases by highlighting their most important relevant characteristics including in particular their sources, structures and selectivity.

BACKGROUND

Plant lipases have generally been studied less than those from animal or microbial sources. These latter forms of lipases have extensively been used in industry to a much higher degree since the past than the plants form (Macrae & Hammond, 1985; Seitz, 1974). Nevertheless, it has been shown that lipases from plants can exhibit many advantages over other sources of enzymes such as high biocatalytic activities, availability, low cost and easy purification (Villeneuve, 2003; Mukherjee, 1996).

Before entering in deep discussion and details view of points concerning plant lipases, let us begin with refreshing our minds with a brief general definition of lipase and its reactions that catalyze. Lipases are hydrolases that act on triacylglycerols (E.C. 3.1.1.3) to produce diacylglycerols, monoacylglycerol, fatty acids, as well as glycerol. In addition to hydrolysis reactions, lipases catalyze also synthesis reactions, i.e., esterification and transesterification. Lipases are different from esterases (E.C.3.1.1.1) especially by the nature of their substrates either carboxyl esters of long-chain acylglycerol ($\geq$10 carbon atoms) for true lipases or carboxyl esters of short-chain acylglycerol ($\leq$10 carbon atoms) for esterases. Structurally, the consensus motifs of lipases and esterases, which are described by ProSite database (Hofmann, Bucher, & Falquet, 1999) are very close.

The plant lipases, our focus in this chapter, are mostly reported in either oilseeds or laticifers. The later plant's constituent consist on secretory cells present in the leaves and/or stems of the plant that produce latex and rubber. Plant lipases are essential enzymes contributing to provide energy to the seedling during hydrolysis of the oils stored in the seeds (Mala Pahoja & Ali, 2002). The plant lipases could be presented as anti-phytopathogen agents, e.g., antifungal agents, providing protection to the plants such in the case of lipases from laticifers as well as some lipases reported from *Arabidopsis* (Kwon, Jin, & Lee, 2009; Lee, Kim, & Kwon, 2009).

Moreover, plant lipases could remain active at a range of pH and temperature, which could be extended from 4 to 9, and 37°C to 80°C, respectively (Mala Pahoja & Ali, 2002; Bhardwaj, Raju, & Rajasekharan, 2001). Beside these

interesting biochemical properties, the isolation and purification of pant lipases could be carried out with easiness and relatively simple techniques. But, the purification to homogeneity for such enzymes are generally a complex process, thus, few plant lipases are available at a complete homogeneity. Nevertheless, plant lipases, purified in either complete homogeneity or not, have potential interesting applications such as in food, energy, detergents and pharmaceutical industries.

REACTION CATALYZED BY PLANT LIPASES

Reaction of Hydrolysis

Lipases, generally and naturally, are important active catalysts on a broad range of substrates and hydrolyze in particular the ester bond substrate of tri-,di-, and mono-glycerides into fatty acids and glycerol. The lipase reaction of hydrolysis is carried out, in all cases, at the interface of a biphasic system reaction. This is an exception feature that characterize lipases from the other types of enzymes, e.g., amylases and proteases, because the substrate of lipase is always hydrophobic and thus the biphasic system reaction is the result of lipase hydrolysis reaction that occurs in an immiscible phases between aqueous phase (lipases) and organic phase containing the hydrophobic substrate.

Reactions of Synthesis

In particular thermodynamic favorable conditions especially in a medium with low thermodynamic water activity, lipases are able to catalyze another type of reaction termed as synthesis reactions. There is a large variety of the lipase synthesis reactions. These latter reactions can be classified into two main types of reactions named Esterification and Transesterification (Reis, Holmberg, & Watzke, 2009). The reaction of Esterification occurs when a fatty acid is linked to an alcohol with a covalent bond by the action of the enzyme, producing an ester and a water molecule released. Similar reactions are Thio-esterification and Amidation but with a thiol and an amine are as substrates, respectively, in place of an alcohol. On the other hand, Transesterification groups various types of reactions, i.e., alcoholysis, aminolysis, acidolysis, and interesterification reactions (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

SOURCES OF PLANT LIPASES

The most authored plant lipases are those found in oleaginous plants, cereals and laticifers. Discussion will be limited to lipases with exotic and interesting properties, e.g., acid lipases, such as lipases from castor beans (oleaginous plants), oats (cereals) and *Carica papaya* latex (laticifers).

Oleaginous Plants

It is evidence that all oleaginous plants contain lipases because of the high content of triacylglycerol in such plant compartments, especially in their seeds. Some of the most cited lipase examples of these seeds are those from plants like castor bean, almond, linseed, coconut, sunflower, sesame, oat, rapeseed and corn.

Triacylglycerols (TAGs), the most present kind of lipids in oilseeds, function as energy reserve source in these seeds. Indeed, these neutral lipids (TAGs) are the proper structure of an oil and they are stored in oil bodies, the form of TAGs store, evenly used to produce energy. TAGs are quickly hydrolyzed during the seed germination process. In fact, different lipolytic enzymes are involved during the steps of seed germination, for instance, they control first step of seeds fatty acids mobilization (Eastmond, 2004; Barros, Fleuri, & Macedo, 2010). And, seed lipases could be present in different compartments of the seed, they can be present in subcellular compartments, free or associated with organelles (Eastmond, 2004).

Castor Bean (Ricinus Communis)

The lipase in the seed of castor bean (*Ricinus communis*) has been investigated since 19th century in 1890 by Green (1980) who was the first author describing this enzyme and since then, this lipase has received much attention for further scrutiny. Many publications have reviewed and studied castor bean lipases especially those from its seed due to some peculiar characteristics of such enzyme (Ory, 1969; Yamada, 1957; Ory & St. Angelo, 1971; Thanki, Patel, & Patel, 1970; Ory, Yatsu, & Kircher, 1968; Tüter, 1998; Eastmond, 2004). In fact, dormant seed of castor bean is known to contain an acid lipase showing an optimum activity at pH of 4.2-4.5 (Eastmond, 2004, Brockerhoff & Jensen, 1974). However, the germinated seed of castor bean has been reported to contain a neutral lipase with a pH optimum of 6.8 (Yamada, 1957). Some

researchers could not succeed to confirm the presence of this enzyme (Ory & St. Angelo, 1971), but other researchers have reaffirmed the presence of a neutral lipase from castor bean (Thanki, Patel, & Patel, 1970). Meanwhile, the acid lipase from castor bean remains the most important enzyme for potential applications either for industrial use, for instance, as a biocatalyst for esterification reactions (Tüter, 1998) or for medical uses, for instance, for potential therapy for cystic fibrosis (Tursi, Phair, & Barnes, 1994).

Ory et al. (1968) have demonstrated that the acidic lipase of castor bean is localized in the spherosomes of the bean, which are the oil-bearing organelles of the seed. Indeed, this enzyme is associated with the spherosomes, possibly as part of, or near to, the membrane (Ory, Yatsu, & Kircher, 1968).

The acid lipase of the castor bean seed that exhibited an optimum activity at a pH near to 4.5, was inactivated at pH values above 6.0 and at a temperature of 30°C. The same lipase showed specificity for short and medium chain fatty acids as well as for non-saturated ones and it exhibited some regioselectivity for fatty acids at the positions *sn*-1 and *sn*-2 (Eastmond, 2004).

Cereals

Cereals are plants members of the *Gramineae* or *Poaceae* plant families. Cereals constitute the main source of food; they are also nutraceutical and therapeutical agents as they contain various types of beneficial elements such as starch, proteins, fibers, lipids and other medicinal compounds. Moreover, they have also been found to contain a wide range of chemical substances with a high antioxidant activity. Beside these important features of cereals that make them an important food staple source, they are also a source of lipases due to the considerable content of lipids in their compartments. Grains of cereals could contain from 2 to 18% of lipids, depending on the species and varieties, and about 85% of the triacylglycerol fatty acids are oleic and linoleic acids (Peterson & Wood, 1997; Hilditch & Williams, 1964). Lipids in cereals are predominantly located in the embryo (germ) and aleurone layer (bran) (Barros, Fleuri, & Macedo, 2010), while the lipolytic activity could be present in different parts of the cereal grain, for example, the majority of lipolytic activity of wheat (75-80%) is found in the bran and 20-25% in the germ (O'Connor, Perry, & Harwood, 1992). Most attention has been paid to cereal seed lipases due to their relevant relation to the process of rancidification, which can occur during the grain storage. Besides, the exact

location and physiological function of these enzymes in the different cereal tissues remain important aspects to be discovered and investigated (Borgston & Brockman, 1984).

Oats

A genuine lipase is that which hydrolyzes fatty acids bonded to their respective triacylglycerols. In oat grains, a genuine lipase was detected that hydrolyzed both tributyrin and triolein (Martin & Peers, 1953). These authors have denoted that this lipase did not digest di- and monobutyrins, suggesting the specificity of this oat lipase for the triglyceride. Indeed, the increased solubility in water of these two substrates relative to tributyrin made them not very suitable as substrates to the lipase. Also, the purity of such substrates was doubtful. Besides, other authors (Berner & Hammond, 1970) were focused on further examination of the specificity of oat lipase. They noticed that this lipase hydrolyzed acids of lard and cocoa butter from both 1- and 2-position. However, the lipase preferentially hydrolyzed 18:2 (22.7%) from the lard and 18:1 (52.0%) from the cocoa butter, knowing that original contents of such acids are 8.9% and 35.6%, respectively.

Lipase activity (glycerol tri[1-^{14}C]oleate hydrolysis) was essayed in three cultivars (Hinoat, Sentinel and OA 424-1) of developing oat (*Avena sativa* L.) grains from 2 to 44 days postanthesis (Urquhart, Brumell, Altosaar, Matlashewski, & Sahasrabudhe, 1984). This work provides an attempt to detail new information on the changes in oat lipase activity during grain development, maturation and germination. In fact, lipase activity was detected throughout the oat kernel development and during germination. Therefore, the presence of active oat lipase throughout grain development and at increased levels during germination is in favor to the presence of lipolytic activity and its products in untreated oat groats, meal or groat fractions and, thus, if these materials are utilized, lipolytic activity and its products must be considered.

Mohamed et al. (2000) studied lipase from oat (*Avena fatua*) that has been demonstrated to contain four isoenzymes (EI, EII, EIII and EIV), which were separated by chromatography. Indeed, the oat lipase was partially purified by ion change chromatography according to the same author. Some characteristics of these oat isoenzymes are as follows. EII and EIII presented Km values of 0.52 and 0.38 mM, respectively, a pH optimum of 9.0 and optimum activities at 75°C and 65°C, respectively. Nevertheless, heat stability is noticed for both

of these two isoenzymes; they were stable at 90°C with 39% and 23% activity retained, for EII and EIII, respectively. These enzymes were stimulated in the presence of Ca^{2+} and Ba^{2+} ions, and inhibited by Zn^{2+} and Mn^{2+} ions. These properties, especially the high temperature stability and optimum activity at alkaline condition, make oat lipases (EII and EIII) as potential alternatives for industrial applications.

Oat lipolytic enzymes were identified and measured by several other authors (Kaukovirta-Norja, Wilhelmson, & Poutanen, 2004; Sahasrabudhe, 1982; Fendri, Ben Saad, Khemakhem, Ben Halima, Gdoura, & Abdelkafi, 2013; Peterson, 1999; Piazza, Bilyk, Brower, & Haas, 1992). Interestingly, Tursi et al. (1994) tested the presence of acid stable lipases in many plant sources and they concluded that only two of the tested plants, dehulled oat and castor bean, showed significant lipolytic activity at pH 5.6. However, the same authors noted that oat may be more interesting as practical commercial source of such type of lipase due to their abundance than castor bean. Therefore, oat may be a potential useful source of acid stable lipase for the treatment and therapy of patients with pancreatic insufficiency in particular those who have cystic fibrosis (Tursi, Phair, & Barnes, 1994).

Widhe and Onselius (1949) recognized that *Avena sativa* L. lipase is potentially more active than other cereal grains such as barley, wheat, and rye.

Oats are unique among the common cereal grains since oats have a high lipid content and their lipolytic enzymes are 10–15 times more active than those of wheat (Matlashewski, Urquhart, Sahasra-dudhe, & Altosaar, 1982). Lipids from oats are a heterogeneous mixture of acyl lipids and unsaponifiable components. Neutral lipids are mainly made up of triacylglycerols and account for 50-60% of total oat lipids (Montealegre, Verardo, Gomez-Caravaca, García-Ruiz, Marina, & Caboni, 2012). Kaimainen et al. (2012) indicated that *Avena sativa* seeds oil is also rich in polar lipids (glycolipids and phospholipids) which can amount to 34%.

In fact, oats grain has the highest capacity to accumulate an important amount of oil in the endosperm compared with other cereals; it is also important to note that Maize is the only cereal having similar high grain oil content to that of oats, but it accumulates oil mostly in the embryo (Leng, 1961). In view of this fact, among all cereals, oat oil in the endosperm remains an interesting lipid to be further studied. Ben Halima et al. (2014, 2015) reported some characteristics of oat lipids and their importance in many applications such as in food, medicine and nutraceuticals.

Other Characteristics Of Oat Lipids

Lipid deposition and fatty acid composition were characterized in different parts of two cultivars of oat grain tissues, named cultivar Freja (6% oil) and cultivar Matilda (10% oil), according to the report of Banas et al. (2007). These authors found that up to 84% of the grain lipids were deposited in the oat endosperm during the first half of oat grain development when seeds were still green with a milky endosperm. In fact, 86-90% of the oat lipids were stored in the endosperm using chemical and microscopical analyses (Banas, Dexbski, Banas Heneen, Dahlqvist, Bafor, Gummeson, Marttila, Ekman, Carlsson, & Stymne, 2007; Heneen, Banas, Leonova, Carlsson, Marttila, Debski, & Stymne, 2009).

The mobilization of lipid reserves in different tissues of oat (*Avena sativa* L.) during germination was studied in the report of Leonova et al. (2010) who proved that the oat oil droplets appeared near the scutellar epithelium and the aleurone of the grain were oil bodies surrounded by oleosins. In fact, oleosins are proteins that stabilize and prevent oil bodies fusion and they contribute to facilitate the activity of lipase to rapidly mobilize fatty acids during the germination of oil seeds (Hsieh & Huang, 2004; Siloto, Findlay, Lopez-Villalobos, Yeung, Nykiforuk, & Moloney, 2006).

In general, triacylglycerol (TAG) is a form of energy of the oil seeds storage and TAG could be mobilized by lipases releasing free fatty acids (FFAs) as well as it could be degraded under β-oxidation and glyoxylate cycles that could subsequently be converted into sugars (Leonova, Grimberg, Marttila, Stymne, & Carlsson, 2010; Clarke, Wilkinson, & Laidman, 1983; Graham, 2008).

As the endosperm of cereal may be considered to be a dead tissue in the mature grain, the β-oxidation could not be occurred there. For this end, Leonova et al. (2010) have tried to determine the fate of the endosperm lipids in cereal grains during germination with the use of oat as a model of cereal grain.

The results of their report, as observed microscopically, suggested that TAG of oat endosperm lipid could not be a dead-end product because it was absorbed by the scutellum either as a form of FFA or as an intact form of TAG that would be immediately degraded to FFAs. These lipids could be transported from the scutellum to the embryo (Leonova, Grimberg, Marttila, Stymne, & Carlsson, 2010).

Indeed, many seed dicotyledonous plants, during germination, exhibited the presence of β-oxidation for energy production from the large amount of lipids accumulated in their endosperm; and a programmed cell death takes place in this endosperm when its reserves are exhausted (Schmid, Simpson, & Gietl, 1999). Nevertheless, this death takes place in the endosperm of mature grains of monocotyledons (Young & Gallie, 2000); thus, β-oxidation would not be involved during their germination (Banas, Dexbski, Banas et al., 2007).

On the other hand, there are important reports on starch and protein degradation in the endosperm of cereals during germination due to their implication in malting and food industries (Fincher, 1989; Ziegler, 1995; Beck, & Ziegler, 1989). Nevertheless, cereal lipid degradation during germination have been less reported, for instance, study on oat lipid processing was reported with how it could interfere in food industry (Kaukovirta-Norja, Wilhelmson, & Poutanen, 2004), and the report of Lehtinen et al. (2002) have demonstrated that some fractions of oat lipid could be susceptible to be normally used at high temperature. The report of Leonova et al. (2010) have described lipid mobilization during the germination of oat (*Avena sativa* L.). Indeed, there is a negative correlation between starch and the increased lipid content in the oat grains (Frey & Holland, 1999). Therefore, the concept of the mobilization of lipid during oat germination would require further research and interest for breeding high-oil cereal cultivars ((Leonova, Grimberg, Marttila, Stymne, & Carlsson, 2010).

For more information of oat lipids, please see the report of Ben Halima et al. (2015).

Laticifers

Beside seeds (oleaginous seeds or oilseeds and other cereal seeds), laticifers are other source of plant lipases. Laticifers produce latex, which is a milky fluid containing different compounds dispersed in an aqueous medium such as proteins, sugars, resin, alkaloids, oils, etc. The latex contains also lipolytic activity in which, in most cases, is related to the insoluble fraction of the latex. Besides, latex lipolytic enzymes are considered as naturally immobilized enzymes because they are, in most cases, trapped in the insoluble fraction (Abdelkafi, Barouh & Fouquet, 2011). It is worth to note that lipolytic activity is present in the latex of several plant families, including *Caricacea*, *Euphorbaceae*, *Bromeliaceae*, *Asclepciadaceae*, *Moraceae* and *Apocynaceae*;

and the presence of this activity in such plant families was reported as early as 1935 (Paques & Macedo, 2006).

Many researchers have focused on studying lipase activity of latex from different plants, for instance, Cambon et al. (2006) have reported comparison of such activity in hydrolysis and acyl transfer reactions from two latex plant extracts (*Vasconcellea* × *Heilbornii* Cv. and *Plumeria rubra*). Moulin et al. (1994) have reported the structure-function relationships of a lipase from *Euphorbia characias* latex and that from ricin (*Ricinus communis*) and such relationships may be proven with the fact that the studied plants (*Ricinus communis* and *Euphorbia characias*) belong to the same family of *Euphorbiaceae*.

Although, it is clear that latex lipases are involved in plant defense against external agents, e.g., phyto-pathogens, as well as in the metabolism of terpenes (Rivera, 2012), the whole function of these enzymes has not yet been elucidated.

Carica Papaya Latex

Lipolytic activity in papaya latex has been reported since 1935 and is known as *Carica papay* lipase (CPL) (Rivera, 2012). Villeneuve et al. (1995) have reported an *sn*-3 stereoselectivity of CPL that was observed in interesterification reactions. Whereas, Mukherjee and Kiewitt (1998) have reported an *sn*-1,3 regioselectivity of commercial lipases that was observed in the production of structured lipids resembling human milk fat, and, interestingly, CPL had been observed to have similar activity to those of the tested commercial lipases.

Steinke et al., 2001 mentioned that papaya latex containing lipases is able to esterify long-chain fatty acids for the production of wax esters by the esterification reaction with oleyl alcohol. On the other hand, CPL has been applied in the sitostanol esterification to catalyze this reaction with the use of canola oil and oleic acid as acyl donor group (Villeneuve, Turon & Caro, 2005; Weber, Weitkamp & Mukherjee, 2001). Moreover, CPL has been used as biocatalyst to resolve naproxen and 2-aryl propionic esters, ketoprofen and fenoprofen (Cheng & Tsai, 2004; Ng & Tsai, 2005; Chen, Tsai, & Villeneuve, 2005). Thus, CPL is an interesting enzyme able to accept catalyzing a wide variety of substrates (Abdelkafi, Ogata, Barouh, Fouquet, Lebrun, Pina, Scheirlinckx, Villeneuve, & Carrière, 2009; Abdelkafi, Barouh, & Fouquet, 2011; Domínguez de María, Sinisterra, & Tsai, 2006).

STRUCTURE AND CATALYTIC MECHANISM OF PLANT LIPASES

An important list of lipases sequences and structures could be found in databanks/databases, and the lipase three-dimensional structures are available in the Protein Data Bank (http://www.rcsb.org/pdb/home/). Additionally, the same lipase could be presented with several structures in different conformations and/or with different substrates. In fact, a common α/β hydrolase fold as well as a conserved catalytic triad characterize any lipase. In addition, most lipases possess the consensus motif Gly-X1-Ser-X2-Gly (where X1 and X2 are any amino acid). It is possible to classify lipases into different superfamilies and homologous families according to their structures and the amino acid residues forming the lipase catalytic triad as well as those forming the lipase active site that stabilize the reaction intermediate, known as residues of the oxyanion hole.

Structurally speaking, the study of proteins three-dimensional structures, which design their spatial conformation, aid to depict the different organizations of residues forming the proteins studied. The important lipase structural elements are discussed below.

The α/β Hydrolase Fold

The alpha/beta-hydrolase fold is among the widely identified conserved lipase 3-D structure; such structure is also widely present in various hydrolytic enzymes of different origins, e.g., proteases, acetylcholinesterases, serine carboxypeptidases, haloalkane dehalogenases and dienelactone hyrolases (Jaeger, Dijkstra, & Reetz, 1999). The α/β-hydrolase fold, the conserved lipase structure, is generally composed of β-sheet strands and α-helices (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). Some variations especially in the amount of these secondary structures (β-sheet and α-helices) as well as the length of the loops and the architecture of the substrate binding sites could be found in some α/β-hydrolases fold (Pleiss, Fischer, & Schmid, 1998; Jaeger, Dijkstra, & Reetz, 1999; van Pouderoyen, Eggert, & Jaeger, 2001). It is worthy to note that the structure of lipases contains also disulfide bridges, which are often important for the stability of the enzymes as well as for their catalytic activity.

The Catalytic Triad

The catalytic triad that consists in a serine (Ser) as nucleophile, an aspartate/glutamate (Asp/Glu) as the acidic residue, and a histidine (His), is conserved structural element that characterize lipases (Brady, Brzozowski, & Derewenda, 1990; Winkler, Darcy, & Hunziker, 1990). However, similar catalytic triad could be found in other hydrolases in particular in serine proteases with a different order in the sequence (Ollis, Cheah, & Cygler, 1992). Interestingly, the consensus sequence Gly-X1-Ser-X2-Gly that contains the catalytic serine could be replaced by a GDSL sequence that is located nearer to the N-terminus (Akoh, Lee, & Liaw, 2004).

The Oxyanion Hole

The lipase oxyanion hole is composed of active site amino acids that stabilize the reaction intermediate. In fact, two amino acids stabilize the tetrahedral intermediate produced during the lipase catalytic mechanism through the presence of hydrogen bonds between their backbone amide proton and the oxygen of the substrate carbonyl group, all of which form the so-called lipase oxyanion hole (Pleiss, Fischer, & Peiker, 2000; Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Indeed, the first residue of the lipase oxyanion hole is located in the loop between the β3-strand and the αA-helix, which is located in the N-terminal part of the lipase. Interestingly, Pleiss et al. (2000) have identified two types of oxyanion holes: *Gly*X and *GlyGly*Gly*X*, with regard to the sequence surrounding this first residue. However, the second residue of the lipase oxyanion hole is the X2 amino acid residue of the consensus sequence Gly-X1-Ser-X2-Gly, which is located after strand β5 in the nucleophilic elbow, a conserved structure common to all lipases. Noting that the oxyanion hole could be present either in the closed conformation, without geometrical modification that can be produced during the opening of the lid, or just in the opening of the lid (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). A third type of lipase oxyanion hole: type Y, has been identified by Fischer et al. (2006). In fact, the hydroxyl group of a strictly conserved tyrosine side chain forms the oxyanion hole of type Y (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). This type can be found in some esterases such as cocaine esterases (Pleiss, 2009).

The type of oxyanion hole may play an important role in the specificities of lipases toward their substrates. For instance, lipases with the *GlyX* type usually hydrolyze substrates with medium and long carbon chain length, while the *GlyGlyGlyX* type is found in short length specific lipases and carboxylesterases (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

It is worthy to note that GDSL lipases do not have the so-called nucleophilic elbow. And, their oxyanion hole has, somewhat, a particular structure: the catalytic Serine (Ser) serves as a proton donor in the oxyanion hole, together with highly conserved Glycine (Gly) and Asparagine (Asn). This organization of such residues constituting this oxyanion hole was proposed to compensate for the lack of hydrogen bond of the intermediate with the catalytic histidine (His) (Akoh, Lee, & Liaw, 2004; Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Lipases α–Helical Loop, the Lid

Remembering that the highly conserved sequences of a protein in particular the consensus sequence, the motifs and the domains are the main criteria to define the type of its sub/family, e.g., lipases independently of their origins (plants, animals, microbes, etc.). Thus, even though the resolution of the first 3-D structures of lipases from *R. miehei* and human pancreatic lipase were established (Brady, Brzozowski, & Derewenda, 1990; Winkler, Darcy, & Hunziker, 1990), these structures enable to overcome the structural generalities of a given lipase whatever its origins, i.e., plants. Indeed, these two first lipases resolved 3-D structures allowed the identification of a lid over the active site, another amazing element structure of a lipase. The lipase lid is composed of one or more α-helices. It joins the main structure of lipase by a flexible structure. In fact, the lid is a mobile element that uncovers the active site of a lipase in the presence of a lipid-water interface, thus, generating a conformational change, which enables the access of the substrate to this active site (Brzozowski, Savage, & Verma, 2000; Derewenda & Derewenda, 1991; Grochulski, Li, & Schrag, 1993, 1994). This latter described mechanism, which is known as the interfacial activation of lipase, could explain the non Michaelis-Menton behavior observed in most lipases. Indeed, the activity of a lipase increases intensely when the concentration of its substrate is high enough to form micelles and emulsions (Reis, Holmberg, & Watzke, 2009; Fickers, Destain, & Thonart, 2008), and thus sigmoid curves are obtained when

the lipase reaction initial rate is plotted against the substrate concentration (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). Nevertheless, when the interface is absent, the entrance to the lipase active site will be blocked and so the lipase will be inactive (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Substrate Binding Site

The substrate-binding site is another important element structure of a given lipase. In fact, the lipase active site is located inside of a pocket on the top of central β-sheet of the lipase structure. Moreover, the surface of the pocket's border of a lipase is mainly composed of hydrophobic residues in order to interact with the hydrophobic substrate (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). In addition, in many lipases, the active sites are different with regard to their deepness of the pocket, size, shape and physicochemical properties of their amino acids. To this end, Pleiss et al. (1998) have classified lipases in three groups according to the geometry of their binding site. Indeed, the first group is characterized by a hydrophobic and crevice-like binding site, which is located in the proximity of the lipase surface. The second group is characterized by a funnel-like binding site. And, the third group by a tunnel-like binding site (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Catalytic Mechanism

The lipase catalytic mechanism is important to be talking about. In fact, such mechanism would start by an acylation that consist in a proton transfer between three lipase amino acid residues of Aspartate (Asp), Histidine (His) and Serine (Ser), all of which cause the activation of the hydroxyl group of the catalytic Ser. As a consequence for this step, the hydroxyl residue of the Ser attacks the carbonyl group of the substrate, with subsequently increased nucleophilicity (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). Actually, the first tetrahedral intermediate is formed with a negative charge on the oxygen of the carbonyl group (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). On the other hand, the oxyanion hole would stabilize the charge distribution and also reduce the state energy of the tetrahedral intermediate by forming at least two hydrogen bonds (Casas-

Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). Then, a deacylation step would take place, where a nucleophile attacks the lipase, releasing the product and regeneration the lipase. This nucleophile can be water in the case of the reaction of hydrolysis or an alcohol in the case of the reaction of alcoholysis (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

SELECTIVITY

The selectivity of lipase is important to describe the product of catalysis. Indeed, the selectivity is related to the preference of lipase to perform given reactions. There are three distinguishable types of lipase selectivity, which are as follows: Type-selectivity, Regioselectivity and Enantioselectivity. Some features of these three types of lipase selectivity are discussed below.

Type-Selectivity

The Type-selectivity for a lipase is associated to its preference for a given substrate such as tri-, di-, or mono-glycerides. Moreover, such selectivity is also referred to the lipase preference toward length of substrates, e.g., short-, medium-, or long-chain fatty acids, as well as to the degree of unsaturation and potential substrate substitutions. The preference of a lipase for acyl groups of different sizes is, somewhat, directly influenced by the shape of its binding site as well as the nature of the amino acids composing this binding sites. Nevertheless, lipases could show chemo-selectivity, which is the specificity of lipases toward a specific chemical group (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Regioselectivity

The lipase Regioselectivity is defined as its preferential attack toward a given ester bond in the glycerol backbone of triglycerides, i.e., the primary or secondary ester bond. Indeed, lipase regioselectivity could be *sn*-1(3) or *sn*-2 (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). On the other hand, there are some lipases, which are nonspecific lipases, i.e., that act randomly on the triglycerides (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012).

Enantioselectivity

The lipase Enantioselectivity refers to its preference toward a particular enantiomer of a chiral molecule in a racemate chemical reaction mixture of both enantiomers (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012). In fact, a chiral molecule is a molecule with an asymmetric center that can adopt two enantiomeric forms, designed as, *R* and *S*. Indeed, enantiomers *R* and *S* cannot be superimposable mirror images of each other and their chemical properties like solubility, reactivity and melting point are very similar. Nevertheless, these two enantiomers often have different biological properties; for instance, one of them might show therapeutic activity, while the other enantiomer might be inactive or even toxic (Soykova Pachnerova, 1963). Therefore, the lipase enantioselectivity would be of great interest in medicine and pharmaceutical uses.

SOME ROLES OF PLANT LIPASES

The most studied plant lipases are those located in seeds (from either oleaginous plants (oilseeds) or cereal seeds). They are part of the energy reserve tissues in seeds with triacylglycerols and oil bodies. In seeds, lipases hydrolyze the reserve of triacylglycerols, which is fundamental for the germination of the seed and further growth of the seedling and the plant (Adlercreutz, Gitlesen & Ncube, 1997). Also, lipases in plants have essential roles in their metabolism, rearrangement and chlorophyll degradation as well as fruits ripening (Tsuchiya, Ohta, & Okawa, 1999). Besides, plant lipases have been found to play an important defense role, as anti-phytopathogen, for instance, since it has been demonstrated that their production was simultaneously been induced in the presence of phyto-pathogens (Stintzi, Heitz, & Prasad, 1993).

Some main features and biochemical properties of some plant lipases from different origins (oleaginous plants, cereals and laticifers) are given in Table 1. On the other hand, Table 2 shows the hydrolysis of several triacylglycerols and of synthetic substrates (acyl 4-methyl-umbelliphenyl) by lipases from some seed sources. In fact, Tables 1 and 2 just give a small idea about the diversity of action in plant lipases including their quite interesting features, e.g., specificity and biochemical characteristics, which contribute to make plant lipases as potential industrial enzymes.

Table 1. Main features and biochemical characteristics of some plant lipases

Source	Biochemical Characteristics			Activator	Inhibitor	Application
	Optimum pH	**Optimum Temperature**	**Selectivity [a]**			
Oleaginous plants						
Jatropha curcas	pH 7.5	37°C		Ca^{2+} Mg^{2+}	Fe^{2+}	Biodiesel synthesis
Castor bean	pH 4.5	30°C	R: *sn* -1 and *sn* -2, CL: short and medium, SD: unsaturated	Ca^{2+}	p-Chloro-mercuribenzoic acid	Esterification of fatty acids and glycerol
Coconut	pH 8.5	30–40°C	R: *sn* -1,3			
Cereals						
Rice (bran)	pH 11	80°C	R: nonspecific on TG, *sn* -2 on PC			
Corn			CL: Long, SD: unsaturated			TG hydrolysis
Oat	9.0	75°C (EII isoenzyme) and 65°C (EIII isoenzyme)		Ba^{2+} Ca^{2+}	Mn^{2+} and Zn^{2+}	Oat shelf-life studies
Wheat	pH 5.5	32–37°C				Hydrolysis and esterification
Laticifers						
Euphorbia characias	pH 5	60°C	CL: short and medium			Hydrolysis of TG and synthetic monoesters
Babaco (*Carica pentagona)*	pH 8	50°C	*a* w 0.38, R: *sn* -1,3, CL: short, SD: unsaturated			Alcoholysis of sunflower oil, Naproxen resolution
Carica papaya	pH 9	50°C	R: *sn* -3			Lipids modification, asymmetric resolutions

TG triglycerides; *PC* phosphatidylcholine; [a] *R* regioselectivity; *CL* chain-length preference; *SD* fatty acid saturation degree preference

Source: (Rivera, Mateos-Díaz, & Sandoval, 2012; Barros, Fleuri, & Macedo, 2010)

CONCLUSION

Lipases can be found in different origins such as microbial, animal and vegetable, with just some variations in their catalytic properties. Although the extensive range as well as highest studies on microbial (bacteria, fungal

Table 2. Hydrolysis of several triacylglycerols and of synthetic substrates (acyl 4-methyl-umbelliphenyl) by lipases from some seed sources

Substrates		**Relative Activity (%)**		
Triacylglycerols		**Lipase Source**		
		Corn	**Elm**	**Pinus**
Tricaproin	C10:0	27	100	74
Trilaurein	C12:0	0	4	78
Trimyristin	C14:0	0	3	89
Tripalmitin	C16:0	0	0	85
Tristearin	C18:0	0	0	103
Triolein	C18:1	38	4	96
Trilinolein	C18:2	100	6	100
Triricinolenin	C18:3	0	0	114
Tribehenin	C22:0	0	0	83
Trierucin	C22:1	45	0	-
Substrates (4-Methylumbelliferyl)				
Laureate		32	169	100
Palmitate		12	29	28
Estearin		5	53	6
Oleate		100	100	81

Source: (Barros, Fleuri, & Macedo, 2010)

and yeast) or animal (pancreatic, hepatic and gastric) lipases in comparison to those from vegetable sources, the plant lipase in particular those from seeds have being the focus of much attention as biocatalysts due in some cases, to their advantages over animal and microbial lipases. For example, availability, specificity and low cost are interesting features of some plant lipases compared to microbial ones because the use of microbial lipases on an industrial scale is still accompanied with high production costs, thus plant lipases could be another searching source as potential industrial alternative for lipolytic activity.

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KEY TERMS AND DEFINITIONS

Enzyme: A substance of protein produced by a living organism that acts as a biocatalyst to bring about a specific biochemical reaction. This protein is characterized by a particular activity in a given substrate, for example, starch for amylolytic activity (amylase), or lipid for lipolytic activity (lipase).

Enzyme Specificity: Refers to the tendency for a given enzyme to catalyze a specific set of chemical reactions.

Plant: Is one of five big groups (kingdoms) of living organisms. It is autotrophic eukaryote, which means it has complex cells and makes its own food. Usually it cannot move (not counting growth). Plants include the following familiar types of organisms: trees, herbs, bushes, grasses, vines, ferns, mosses, and green algae.

Chapter 4
Plant Phospholipases C:
Case Study of Oat PI-PLC

ABSTRACT

Plant phospholipases C (PLCs) that hydrolyze the phosphodiesteric linkages of the head group of phospholipids can be grouped into three major types—non-specific PLC (NPC), phosphoinositide-specific PLC (PI-PLC), and glycosylphosphatidylinositol (GPI)-PLC—according to the specificity of substrate and the cellular functions. After an overview of the main features in these plant enzymes, a case study of oat (Avena sativa) PI-PLCs isoforms is presented in this chapter to highlight insights into structure characterization of such predicted isozymes. Although oat PI-PLCs could not be purified to homogeneity due to their association with other proteins, particularly the actin cytoskeleton, the intended enzymes could be identified, analyzed, and characterized by functional proteomics, bioinformatics, and in silico approaches. In this chapter, sequence and structure analyses, as well as phylogenetic evolution of the predicted oat PI-PLCs, were reported to show the specific motifs and the main putative catalytic residues characteristics of such plant enzymes.

INTRODUCTION

Phospholipase C (PLC) is an enzyme that hydrolyzes the phosphodiesteric bond of the glycerol side of a phospholipid (PL) head group, i.e., the glycerophosphate ester linkage of PL head group, to produce diacylglycerol (DAG) and a phosphorylated head group from the PL. Plants could present

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three different families of PLCs according, in particular, to substrate specificity and cellular functions. These families of PLC are briefly noticed as following: glycosylphosphatidylinositol PLC (GPI)-PLC that hydrolyzes GPI-anchored proteins, non-specific PLC (NPC) that acts on the common phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), and phosphoinositide-specific PLC (PI-PLC) that hydrolyzes phosphoinositides. Obviously, like so many types of enzymes, different isoforms of PLCs can be expressed in plants under either standard or stress conditions. It is important to point out that the detection of PLC activity in plants is sensitive to the relatively high activity of other enzymes especially PLD as well as the possible interconversion of PLD and PLC products by kinases and phosphatases. The *Arabidopsis* Genome Project was beneficial to reveal multiple PLCs aiming to better characterize these enzymes, while the identification and understanding of the plants PLCs properties and functions remain hindered by the lack of some information regarding their molecular structure. The importance of plant PLCs resides in the fact that they are among enzymes thought to hydrolyze the phosphodiesteric linkages of the head group of plant membrane phospholipids.

Also, it is important to study the molecular structure of these hydrolyzing lipids enzymes, which hydrolyze the phosphodiester bond on the glycerol side of membrane PLs to produce DAG and a phosphorylated head group. Here, in this chapter, we intend to describe especially PI-PLCs from a *gramineae* plant, oat (*Avena sativa*).

In fact, the structure and function of *Avena sativa* PI-PLCs remain poorly understood and their properties need to be further elucidated.

For this end, in the present study, bioinformatic approaches were used on isoforms of identified oat PLC. In fact, although oat phospholipases could not be purified to homogeneity due to their association with other proteins, the intended enzymes bands could be separated by SDS-PAGE from the oat seedling extract. These materials were digested with trypsin and the amino acid sequences of the tryptic peptides were determined by LC/ESI/MS/MS and database searches. These sequences were used to identify cDNAs from expressed sequence tags (ESTs) and Transcripts Shotgun Assembly (TSAs) of *Avena sativa*. Based upon EST and TSA sequences, predicted full-length protein would be identified and assigned as AsPiPLC for phosphoinositide phospholipase C (PI-PLC). Insights into structures characterization of such predicted isozymes were analyzed using *in silico* approaches. Primary, secondary and tertiary structures were analyzed. Phylogeny analysis, protein motif/domain identification and 3D homology modeling structure were thus

presented. Primary structure prediction analysis of these enzymes showed the specific motifs characteristic of such enzyme. A multiple sequence alignment of the putative oat PLC revealed consensus sequences with the well-known important putative catalytic residues for especially PI-PLC.

BACKGROUND

Plant PLCs are important biocatalysts that hydrolyze phospholipids (PLs) to produce diacylglycerol (DAG) as well as the respective phosphorylated head group of the substrate. They could be classified into three distinguished families of phospholipases according to substrate specificity and cellular function, i.e., glycosylphosphatidylinositol (GPI)-PLC, non-specific PLC (NPC), and phosphoinositide-specific PLC (PI-PLC). In fact, the (GPI)-PLCs hydrolyze GPI-anchors on proteins; while NPCs, also known as PC-PLCs, could catalyze common membrane PLs especially PC but other PLs such as PE; whereas PI-PLCs, also known as phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$)-PLCs, are recognized to catalyze primarily phosphoinositides (PIs) (Nakamura, Awai, & Masuda, 2005; Peters, Li, & Narasimhan, 2010, Wang, Ryu, & Wang, 2012).

Indeed, the chapter of Dörmann in 2005 has reported plant membrane lipids and the related enzymes in particular phospholipases that hydrolyze either the phospholipid head groups (PLD and PLC) or the acyl groups from membrane lipids (such as PLA1, PLA2 and lysophospholipases). The same author confirm the presence of three classes of PLCs in plants, which are, as described by Wang in 2001 or by Drøbak in 2005, the non-specific PLCs (NPCs) that are mostly active with PC, the PI-specific PLCs (PI-PLCs) that are involved in phosphoinositide signaling and the GPI-specific PLCs. It is worthy to note that the expression of different isozymes of PLC is also induced under Pi starvation (Siebers, Dörmann, & Hölzl, 2015). In addition, PLCs are proven to be implicated in phospholipid turnover in leaves (Andersson, Larsson, & Tjellström, 2005; Gaude, Nakamura, & Scheible, 2008; Nakamura, Awai, & Masuda, 2005).

The current chapter would focus on PI-PLCs from the common oat (*Avena sativa*), as this latter crop is among the most cultivated crops worldwide belonging to the *Poaceae* family (*Gramineae*) and is a promising plant thanks to its beneficial lipid fraction and the relevant enzymes, which could be valorized in industrial applications (Ben Halima, Ben Saad, Khemakhem, Fendri, & Abdelkafi, 2015a).

Below are some enzymatic properties of Plant PLCs ((GPI)-PLCs, NPCs and PI-PLCs).

Glycosylphosphatidylinositol PLCs: (GPI)-PLCs

The glycosylphosphatidylinositol (GPI)-PLCs in plants are known to hydrolyze GPI-anchors on proteins. In fact, they cleave the terminal plant lipid that links GPI-anchored proteins to the plasma membrane (Wang, 2001). Precisely, GPI-specific PLCs catalyze the hydrolysis of the lipid moiety of glycosylated PI (GPI) anchored proteins. It is important to note that GPI-anchored proteins are found as extracellular proteins and they could function as enzymes such as phosphatases or nitrate reductases, to recruit nutrients. As well, GPI-anchored proteins could function as receptors to interact with extracellular ligands and they could also function as matrix proteins such as arabinogalactan proteins (Thompson & Okuyama, 2000). Thus, the localization of the GPI anchored proteins is often in the outer membrane of plant cells where they act, in most cases, as enzymes or receptors.

Although GPI-PLCs have been more characterized in microorganisms and animals than in plants (Roberts, 1996), a GPI-PLC was found in plant, in particular in peanut, where it was partially purified from the seeds. Indeed, this GPI-PLC was proven to cleave solubilized GPI-anchor, but did not act on membrane-bound GPI (Butikofer & Brodbeck, 1993).

Moreover, several genes with sequence similarities to GPI-specific PLCs are found in *Arabidopsis*. However, the respective activity of such genes has only been characterized in crude plant extracts (Butikofer & Brodbeck, 1993).

On the other hand, structural analysis of the lipid moiety of a GPI-anchored arabinogalactan has been in favor to raise the possibility of the presence of a GPI-PLD in plants (Oxley and Bacic, 1999).

The report of Roberts (1996) highlighted that extracellular GPI-PLD occurs in animal blood serum, but no GPI-PLD activity has been characterized in plants (Wang, 2001).

Nonspecific PLCs: NPCs

The nonspecific-PLC (also can be referred as phosphatidyl-choline-PLC: PC-PLC) is a biocatalyst that acts on common membrane phospholipids such as PC and PE as substrates. PLCs that hydrolyze phosphatidyl-choline have been found in particulate or soluble preparations of various plant

species and tissues (Kates, 1955; Chrastil & Parrish, 1987; Rouet-Mayer, Valentova, SimondCote, Daussan & Thevenot, 1995). Indeed, plant NPCs are implicated in breakdown of broader substrate ranges of PLs such as PE, PG and especially PC, and were discovered in 1847 as "lecithin" molecules by Theodore Nicolas Gobley, a French chemist. Besides, these phospholipases were first discovered under the name of "lecithinase C" in bacteria (Macfarlane and Knight, 1941). As well, PC-PLCs were identified in animal cells (Billah & Anthes, 1990; Exton, 1994) and in fungi (Morelle, Bernard, Debeaupuis, Buitrago, Tabouret & Latge, 2005).

In plants, although putative PC-PLC was observed as early as 1955 (Kates, 1955), its roles and functions in plants have long remained elusive and vague. Obviously, the current lack of molecular and genetic PC-PLCs characterization in either animal or plant sources, hinders the progress of research for these enzymes.

PC-specific PLCs were detected in several plant species as well as different genes were predicted to encode these enzymes especially in the *Arabidopsis* genome (Dörmann, 2005). Nevertheless, NPCs have not been well characterized and analyzed in plants at the molecular level (Dörmann, 2005). Recently, Pokotylo et al. (2013) have reviewed summarized information concerning the plant NPC and highlighted its biochemical properties, sequence analysis, cellular and tissue distribution and physiological functions.

The *Arabidopsis* genome was revealed to contain six putative NPCs (NPC1-NPC6) with sequence homology and similarities to bacterial NPC (Wang, 2001). NPCs could be involved under Pi starvation. Indeed, the expression of the two Arabidopsis NPCs (NPC4 and NPC5) was proven to be induced under Pi starvation (Gaude, Nakamura, & Scheible, 2008; Nakamura, Awai, & Masuda, 2005). In general, NPCs are proteins with approximately 60 kDa molecular mass consisting of 514-538 amino acid residues. The multiple sequence alignments between *Arabidopsis* and *M. tuberculosis* NPCs has revealed the presence of three highly conserved domains among plants and bacteria (Nambara & Marion-Poll, 2005). Nevertheless, none of these three domains is corresponded to any known motifs or domains in related phospholipases, e.g., PI-PLCs or PLDs, thus, suggesting that NPCs are biochemically and structurally distinct from other phospholipases (Wang, Ryu, & Wang, 2012). Still remain the *Arabidopsis* NPCs as a good model to elucidate and analyze the enzymatic properties of plant NPCs (Nakamura, Awai, & Masuda, 2005; Peters, Li & Narasimhan, 2010). In fact, the NPC4 displayed a high activity toward PE and PC, and also toward PG and phosphatidylserine (PS), but with lower activity than the substrates PC or PE (Peters, Li & Narasimhan, 2010).

Also, some activity of NPCs was displayed toward the substrate PI(4,5)P_2, but it was tenfold less than that on PC (Nakamura, Awai, & Masuda, 2005).

Most recently, the report of Hong et al. (2016) has reviewed the NPCs as hydrolyzing enzymes on common membrane phospholipids, and focused on their classification and domain structures, substrate preference and regulation of activities, expression patterns and subcellular localization, as well as cellular and physiological functions and mechanisms of NPC actions.

Phosphoinositide-Specific PLCs: PI-PLCs

The class of PI-specific PLCs that hydrolyze phosphoinositides has been characterized in greatest detail.

Phosphoinositide-specific phospholipases C (PI-PLCs) are essential enzymes that hydrolyze, in a Ca^{2+}-dependent manner, membrane phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2/ PI(4,5)P_2/PIP_2) to generate two second messengers: 1) a lipid, DAG that remains bound to the membrane, and; 2) a soluble molecule, inositol 1,4,5-triphosphate (IP_3) that is released as a soluble mediator into the cytosol (Wang, Ryu, & Wang, 2012; Pokotylo, Kolesnikov, Kravets, Zachowski & Ruelland, 2014; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

In fact, these enzymes catalyze the cleavage of phosphoinositides, in particular PIP_2 and release IP_3 and DAG, which are the two molecules serving as second messengers in the plant cell. Indeed, whereas PIP_2 diffuses to intracellular organelles where it stimulates calcium release, DAG remains in the membrane where it activates protein kinase C (Mueller-Roeber & Pical, 2002; Meijer & Munnik, 2003).

Hirayama et al. (1995) isolated the first cDNA encoding a PI-PLC from higher plant (*Arabidopsis thaliana*). Several additional *PI-PLC* genes are present in *Arabidopsis*. Because these phospholipases are only poorly active on other phospholipids, PI-PLCs are believed to be exclusively involved in PIP_2 signaling, rather than in bulk membrane lipid turnover (Dörmann, 2005; Drøbak, 2005).

Compared with the other groups of PLCs, the group of PI-PLCs is better characterized. Multiple PLCs have been cloned from various plants such as *Arabidopsis*, soybean, tobacco, and potato (Hartweck et al., 1997; Hirayama, Mitsukawa, Shibata, & Shinozaki, 1997; Hirayama, Ohto, Mizoguchi, & Shinozaki, 1995; Kopka, Pical, Gray, & Muller-Rober, 1998).

According to the review of Munnik et al. (1998), the domains structures, molecular weight and overall sequence similarity of plant PI-PLCs are more closely related to mammalian PLCδ than to the other PI-PLCs. Plant PI-PLCs have multiple conserved structural folds. Indeed, all plant PI-PLCs contain two necessary catalytic domains for the phosphoesterase activity that form a TIM barrel-like structure, known as domain X of approximately 170 amino acids, and domain Y of approximately 260 amino acids. As well, plant PI-PLCs contain a Ca^{2+}-dependent phospholipid-binding domain, called C2 domain (protein kinase C-conserved region 2), at the C terminus, but they lack a pleckstrin homology domain (PH domain), which is found in the mammalian PI-PLCs (Wang, 2001). Moreover, EF-hand motifs are present at the N-terminus of some plant PI-PLCs reported from soybean and *Arabidopsis*, but not from potato (Wang, 2001; Kopka, Pical, Gray, & Muller-Rober, 1998).

Therefore, PI-PLCs could be designed as multidomain proteins that are structurally related to the PI-PLCζs (Pokotylo, Kolesnikov, Kravets, Zachowski, & Ruelland, 2014), the simplest animal PI-PLCs, which are only composed of EF-hand, X/Y and C2 domains. Nevertheless, plant PI-PLCs do not have a conventional EF-hand domain since they are often truncated, while some PI-PLCs have no EF-hand domain at all (Pokotylo, Kolesnikov, Kravets, Zachowski, & Ruelland, 2014).

The report of Tasma et al. (2008) highlighted that plant PI-PLCs, in particular from *Arabidopsis thaliana*, are structurally related to the mammalian PI-PLCζ that contains no PH domain. It is worth noting that the *Arabidopsis* genome has been reported to have nine putative PI-PLCs. Moreover, the AtPLC8 and AtPLC9 are thought to be inactive forms because of key amino acid substitutions in their catalytic domain (Wang, 2004; Mueller-Roeber & Pical, 2002). Overall, PI-PLCs exhibit calcium-dependent hydrolysis of $PI(4,5)P_2$ (Wang, Ryu, & Wang, 2012).

The *Arabidopsis* genome was revealed to contain nine PI-PLCs (AtPLC1-AtPLC9) with sequence homology to rice PI-PLC (Zheng, Liu, Li, Shang, Zhou, & Sun, 2012; Hirayama, Ohto, Mizoguchi, & Shinozaki, 1995; Singh, Kanwar, Pandey, Tyagi, Sopory, Kapoor, & Pandey, 2013; Tasma, Brendel, & Whitham, 2008). Beside, many other reports have studied PI-PLCs from different plants species, including wheat (Khalil, Wang, Wright, Ralevski, Donayo & Gulick, 2011; Komis, Galatis, Quader, Galanopoulou, & Apostolakos, 2008), tomato (Vossen, Abd-El-Haliem, Fradin, van den Berg, Ekengren & Meijer, 2010), soybean (Chou, Shigaki, Dammann, Liu, & Bhattacharyya, 2004; Shi, Gonzales, & Bhattacharyya, 1995), potato (Kopka,

Pical, Gray, & Muller-Rober, 1998), pea (Liu, Liu, Pan, Yang, Zhan & Huang, 2006), *Brassica napus* (Georges et al., 2009), mung bean (Kim, Kim, Lee, Lee, Jung, & Bahk, 2004), lily (Pan, Wang, Ma, & Sun, 2005), maize (Wang, Yang, Yue, Gao, Yin, & Zhang, 2008), physcomitrella (*Physcomitrella patens*) (Repp, Mikami, Mittmann & Hartmann, 2004; Mikami, Repp, Graebe-Abts, & Hartmann, 2004), and oat (Tate, Schaller, Sussman, & Crain, 1989; Huang, Tate, Crain, & Coté Huang, 1995; Huang & Crain, 2009). The structures of these studied plant PI-PLCs were resembled to animal PI-PLCζ.

For more information on plant PI-PLCs, please refer to the publication of Hong et al. (2016), which reviewed the progress made on the plant PI-PLCs: their classification and domain structures, catalytic properties and regulation, expression patterns and subcellular localization, cellular and physiological functions, and mechanisms of PI-PLC actions.

MAIN FOCUS OF THE CHAPTER: CASE STUDY OF OAT PI-PLC

The focus of this chapter is mainly on oat (*Avena sativa*) PI-PLC isozymes. In fact, the common oat (*Avena sativa* L.) is an allohexaploid ($2n = 6x = 42$) crop species from the *Poaceae* family (*Gramineae*) with an estimated 1C genome size of 13.23pg, corresponding to about 13000 Mbp (Bennett and Smith, 1976). Hence, it has a large genome that remains to be fully sequenced and assessed. Oats have beneficial components, in particular their fraction of lipids and their enzymes that could be valorized in biotechnological and industrial applications (Ben Halima, Ben Slima, Moalla, Fetoui, Pichon, Gdoura, & Abdelkafi, 2014; Ben Halima, Ben Saad, Khemakhem, Fendri, & Abdelkafi, 2015a; Ben Halima, Borchani, Fendri, Khemakhem, Gosset, Baril, Pichon, Ayadi, & Abdelkafi; Ben Halima, Khemakhem, Fendri, Ogata, Baril, Pichon, & Abdelkafi, 2016). Complex polyploidy genomes such as oats need substantial studies and contributions of the bioinformatics pipeline. Functional proteomics is one of the useful tool to identify *de novo* protein sequences from those of uncompleted sequence genome such that of oat. In this study, extracts of oat seedling were identified as potential lipolytic activities extracts through in particular the catalytic activity of PLC. The proteins in the oat seedling extracts are isolated and characterized. Aiming to understand the mechanisms of hydrolytic action of oat PLC especially PI-PLCs (AsPI-PLC) isozymes, the sequencing of peptides resulting from

tryptic digestion allowed identifying EST and TSA sequences from that the AsPI-PLCs genes were compared by computational bioinformatics analysis.

MATERIALS AND METHODS

Materials

Oat seeds (*Avena sativa* L.) were purchased from local market in Sfax, South of Tunisia. A voucher specimen was deposited at the Biological Engineering Department, National School of Engineers of Sfax, Tunisia. The oat seedlings were used as started plant material in this study and their extracts are the crude extracts of hydrolases, in particular phospholipases (PLCs), which were the subject of the current study.

All other chemicals used were of reagent grade and were supplied by Invitrogen and Sigma Chemical Co. (St Louis, France).

Extraction of Hydrolases from Oat Seedling Extract

Oat (*Avena sativa*) seeds were placed to germinate on wet tissue paper in a plastic box, grown in the dark for just 5 days at room temperature. On designed day after planting (day 10), *Avena sativa* seedlings were ground using mortar and pestle with 0.02 M sodium acetate buffer (pH 5.6), filtered through two layers of cheese cloth to remove large particles and the supernatant obtained was centrifuged at 15,000 × g for 20 min. The supernatant was used as oat crude extract of hydrolases. Then, acetone was added to the oat crude extract (2:1; v:v), sample centrifuged at 14,000 × g for 15 min and supernatant discarded. The partially delipidated acetone powder was resuspended in water. The mixture was stirred for 20 min at 4°C, sonicated for 5 min and finally centrifuged at 14,000 g for 5 min before collecting the supernatant fraction, which was used as oat fraction enriched in hydrolases activities.

Determination of Soluble Protein Concentration

The concentration of soluble protein in oat (*Avena sativa*) extracts was determined by the method of Bradford (Bradford, 1976), as well as by the BCA method following the manufacturer's instructions (Smith, Krohn, Hermanson, Mallia, & Gartner, 1988) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein electrophoresis was performed using sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) [NuPAGE Novex 4–12% (w/v) Bis–Tris Gel 1.0mm, 12well (Invitrogen) and NuPAGE MOPS SDS for Running Buffer (Invitrogen)] according to the method described by Laemmli (Laemmli, 1970). The apparent molecular weights of proteins were estimated by co-electrophoresis of marker proteins (See Blue, Invitrogen) with weights ranging from 14 to 97 kDa. The protein in the sample buffer [0.9 g glycerol, 0.1 mL 1% bromo-phenol blue, 1 mL 10% (w/v) SDS, and 0.5 µL tris(2-carboxyethyl) phosphine: reducing conditions with NuPAGE® LDS 4× LDS Sample Buffer (Invitrogen)] was heated for 5 min in boiling water and applied to the gel. The proteins separated on the SDS-PAGE were stained with Coomassie Brilliant Blue R-250 (Simply Blue™ Safe Stain (Invitrogen)).

In-Gel Tryptic Digestion and Protein Identification by Mass Spectrometry

Protein Bands of interest were manually excised from gels (SDS-PAGE) and automated tryptic digestion was conducted as previously described (Abdelkafi, Ogata, Barouh, Fouquet, Lebrun, Pina, Scheirlinckx, Villeneuve, & Carrière, 2009; Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet, Scheirlinckx, Villeneuve, & Carrière, 2012; Fendri, Tardif, Fierobe, Lignon, Valette, Page, & Perret, 2009) or manually treated as follows. Gel bands were manually excised in a sterile laminar flowhood, transferred individually to 1.5 mL microtubes and cut into cubes of roughly 1 mm3. Gel cubes were destained for 1 h and 30 min at 4 °C using a solution of 45% acetonitrile and 55 mM ammonium bicarbonate. After gel cubes washing and in-gel trypsin proteolysis of proteins, the peptides produced were extracted onto Poros beads and purified with ZipTips (Millipore, France) as previously described (Beaufour, Godin, Vallée, Cadene, & Bénédetti, 2012).

Extracted proteolytic peptides were analyzed by nanoUltraHPLC–nanoESI UHR–QTOF MS. Experiments were performed using an UltiMate™ 3000 NanoRSLC System (Dionex, Sunnyvale, CA) connected to a Bruker MaXis UHR-QTOF 2 GHz mass spectrometer equipped with an online nano-ESI ion source. The LC–MS setup was controlled by Bruker Hystar™ software version 3.2. Peptides were pre-concentrated online on a Dionex Acclaim

PepMap100 C18 reverse-phase precolumn (inner diameter 100 µm, length 2 cm, particle size 5 µm, pore size 100 Å), and separated on a nanoscale Acclaim Pepmap100 C18 column (inner diameter 75 µm, length 25 cm, particle size 2 µm, pore size 100 Å) at a flowrate of 450 nL/min using a 2–35% gradient of acetonitrile in 0.1% formic acid. Peaks with the three highest intensities and a minimum of 400 ion counts were selected for CID MS/MS fragmentation using an isolation window of 3–9 Da depending on the m/z value.

Acquired MS/MS spectra were searched against the UniProtKB/Swiss-Prot/TrEMBL (database version 51.6; 257,964 sequence entries), non-redundant NCBI (http://www.ncbi.nlm.nih.gov) and the EST *A. sativa* L. database containing 25,400 entries (AM071411-CN180783) using the Mascot identification engine (version 2.3, Matrix Science, France). The search was conducted allowing for a maximum of two missed cleavages, 5 ppm tolerance for precursor ions and 0.04 Da for fragment ions, respectively. Methionine oxidation was allowed.

Since contaminations from human (mainly keratins) origin could be present in the samples analyzed, the search in databases was restricted to plant species using UniProtKB/Swiss-Prot/TrEMBL, 49,887 sequence entries; NCBI nr, 551,056 sequence entries. In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides.

BIOINFORMATIC ANALYSIS

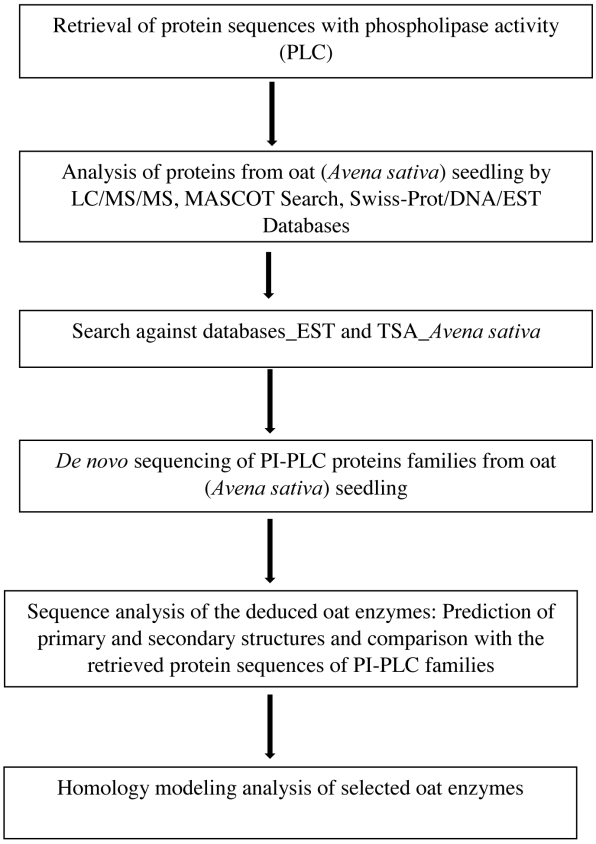
Retrieval of Protein Sequences

The amino acid sequences from plant phospholipases serving to comparison with the *de novo* sequencing of oat proteins were retrieved from protein database of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/protein/). The sequences were saved in FASTA format. An outline of the *in silico* approach steps followed in this study has been portrayed (Figure 1).

Sequence Analysis

Bioinformatic analysis of the *A. sativa* peptide sequences, ESTs, genomic sequences and deduced protein sequences was performed using the following

Figure 1. Flow chart depicting the in silico steps used in the study for the prediction and analysis of oat enzymes from PLC families



tools. Multiple sequence alignment was performed using the ClustalW algorithm (Thompson, Higgins, & Gibson, 1994). The peptide sequences were compared with the NCBI (National Center for Biotechnology Information, USA) non-redundant sequence databases, the Transcriptome Shotgun Assembly (TSA) *A. sativa* database (GAJE01000001-GAJE01050182) and the Expressed Sequence Tag (EST) *A. sativa* database that contain 25,400 entries (AM071411-CN180783) using BLAST (Altschul, Wootton, Gertz, Agarwala, Morgulis, Schaffer, & Yu, 2005).

Primary structure analysis using predicted amino acid sequences was performed using the ExPASy Proteomics tools. The Translate tool (web.expasy.org/translate/) was used to translate DNA sequences to protein sequences, whereas the Compute pI/Mw tool (web.expasy.org/compute_pi/) was used to

compute the theoretical isoelectric point (pI) and molecular mass (Bairoch, Apweiler, Wu et al., 2005; Gasteiger, Gattiker, Hoogland, Ivanyi, Appel, & Bairoch, 2003). The BioEdit software package (Hall, 1999) was used to manipulate, edit and compare DNA and amino acid sequences.

The prediction of the signal peptide sequence was performed using the signalP 4.1 application (Petersen, Brunak, von Heijne, & Nielsen, 2011).

To predict N- and O-glycosylation sites, the servers NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 (www.cbs.dtu.dk/services/NetOGlyc/) (Steentoft, Vakhrushev, Joshi et al., 2013) were used.

Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) package version 7 (Kumar, Stecher & Tamura, 2016). The program MUSCLE (Edgar, 2004), implemented in MEGA7 package, was used to perform multiple alignment of amino acid sequences of AsPiPLC and AsPLD and their homologous for phylogenetic analysis. The evolutionary history was inferred using either the Neighbor-Joining method (Saitou and Nei, 1987) or the UPGMA method (Sneath & Sokal, 1973). The evolutionary distances were computed using the JTT matrix-based method (Jones, Taylor, & Thornton, 1992) and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. The robustness of the inferred tree was evaluated by bootstrap (1000 replications) (Felsenstein, 1985).

Conserved Protein Motifs Analysis and Subcellular Location Prediction

In order to investigate conserved protein motifs in more detail, the protein sequences from oat were analyzed using Multiple Expectation maximization for Motif Elicitation (MEME)/Motif Alignment & Search Tool (MAST) (Bailey & Elkan, 1994; Bailey & Gribskov, 1998; Bailey, Johnson, Grant, & Noble, 2015) (http://meme-suite.org) with the number of different motifs as 30, motif sites distribution as zero or one occurrence per sequence, and motifs width as 6 (minimum) and 50 (maximum).

The functional annotations of these motifs were analyzed by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Finn, Bateman, Clements, Coggill, Eberhardt, Eddy, Heger, Hetherington, Holm, & Mistry, 2014). The mapping between Pfam (http://pfam.xfam.org) analysis and Gene Ontology (GO) was provided by InterPro (Sangrador-Vegas, Mitchell, Chang, Yong & Finn, 2016).

The subcellular localization prediction of oat protein was carried out using the CELLO v.2.5 server (http://cello.life.nctu.edu.tw/) (Yu, Chen, Lu, & Hwang, 2006).

Secondary Structure Prediction

The prediction of the protein secondary structures were determined using self-optimized prediction method (SOPMA) software (http://npsa-pbil.ibcp.fr/cgibin/npsa_automat.p1?page=/NPSA/npsa_sopma.html) (Geourjon & Deleage, 1995). The parameters of similarity threshold and window width were set to 8 and 17 and rest parameters were taken as default.

Molecular and Homology Modeling

A 3D structure model of oat phospholipase (AsPi-PLC) was predicated on homology modeling, which was been performed using the Swiss-Model server (http://swissmodel.expasy.org/).

Results and Discussion

Previous studies have reported PLC activity in oat (*Avena sativa*) tissues in particular PI-PLC in oat roots (Tate, Schaller, Sussman, & Crain, 1989; Huang, Tate, Crain, & Coté, 1995; Huang & Crain, 2009). Obviously, no plant phospholipases was ever purified due to their association with other proteins such as actin co-purified with an oat root PI-PLC (Huang & Crain, 2009).

Taken together these findings, we have intended in this study to the identification of phospholipase isoforms, in particular those of PLC (AsPiPLCs), from 10 days old oat seedlings extract by functional proteomics. By the mean of LC/MS/MS technique and bioinformatics tools, novel amino acid sequence isozymes of oat PI-PLCs were reconstructed and characterized.

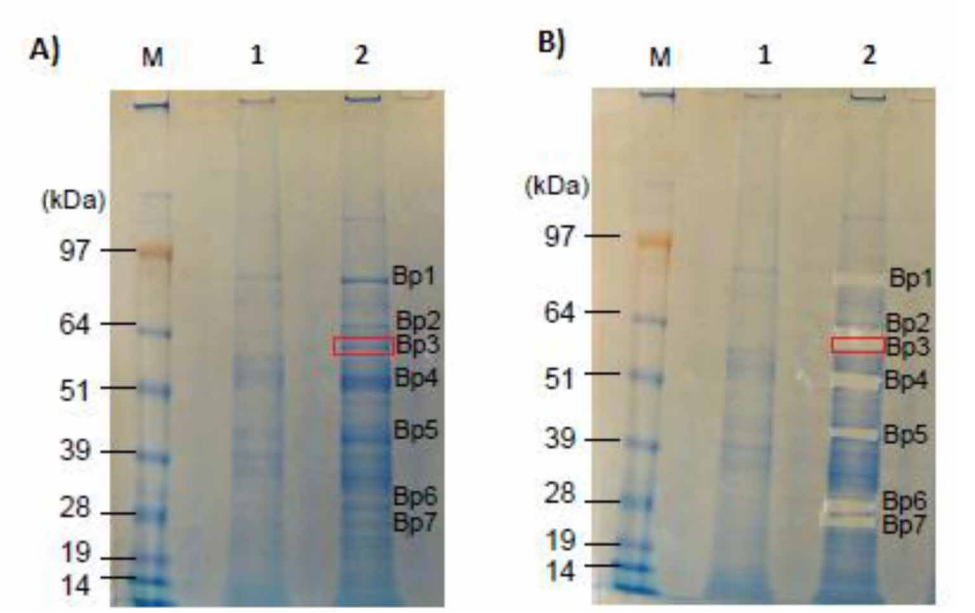
Extraction and Identification of Oat Seedlings Proteins of Hydrolytically Catalytic Activities

Oat (*Avena sativa* L.) seedlings from 10 days old were used as starting material for hydrolases extraction. An aliquot of this extract was analyzed by SDS-PAGE followed a Coomassie blue staining step and a number of protein bands were excised from the preparative gel (Figure 2). All the excised proteins

bands from the preparative gels (Figure 2) were digested with trypsin and analyzed by LC/ESI/MS/MS.

The amino acid sequences of these peptides were determined either by manual interpretation of the collision-induced spectra of the major peptide ion or by computer-aided fragment-matching algorithms. The majority of the protein bands excised from SDS-PAGE (Figure 2) were identified as glycoside hydrolases, some of these bands corresponding to several proteins. Prediction and analysis of several catalytic activities in oat seedling extract (*Avena sativa*) with structure and function insights into PI-PLCs from computational approaches would be highlighted in this study. In fact, a significant score could be obtained for the match of protein band 3 with PI-PLC, in the DNA/EST_*Avena sativa* databases.

Figure 2. SDS-PAGE analysis of the 10 days old oat (Avena sativa) seedlings extract. Lane 1, oat crude extract; lane 2, oat extract enriched proteins, in particular hydrolases after acetone precipitation. A) and B), gel with non-excised and excised proteins, respectively; Bp, Band of protein, and M, molecular mass markers. The gel was stained with Coomassie blue. Bp 3 that is highlighted in red rectangles, could be matched to PI-PLC, according to EST/DNA_Avena sativa Databases after in situ trypsic digestion, LC/MS/MS and bioinformatics analysis.



**For a more accurate representation see the electronic version.*

Therefore, *de novo* sequence peptides could be identified for band 3 corresponding to *Avena sativa* PI-PLC (AsPiPLC).

Indeed, the peptide sequences obtained could be used to screen for *A. sativa* EST/genomic sequences dataset (AM071411-CN180783; GAJE01000001-GAJE01050182). Interestingly, three genomic scaffolds (TSA_*A. sativa*: GAJE01023621.1- GAJE01023623.1) and one EST_*A. sativa* (CN815254.1) could be identified and corresponded to the peptide sequences of band 3 using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov) (Altschul, Madden, Schaffer, Zhang, Zhang, Miller, & Lipman, 1997). These genomic scaffolds are useful tools to the identification of at least two oat PI-PLCs isoforms.

We could then predict the structure of the identified genes by comparing the oat genomic scaffolds with related plant phospholipases using BLAST search analysis (Altschul, Madden, Schaffer, Zhang, Zhang, Miller, & Lipman, 1997).

Based on these analyses, the proteins isolated from *A. sativa* seedling extract that correspond to band 3 were identified as PI-PLCs and were named AsPiPLC_x (where x is the number of predicted isoforms; in this study, we reported two oat PI-PLC isoforms).

Sequence Analysis of Oat PI-PLC Proteins

At least, two predicted isozymes of PI-PLC would be found in oat seedling extract (AsPiPLC1 and AsPiPLC2). However, AsPiPLC1 (accession number: BK010346) was selected for much intense further analysis. The predicted AsPiPLC1 cDNA (1437 bp) corresponds to a 478 amino acid residue protein (Figure 3).

The predicted molecular weight of the protein is 53.92 kDa and the theoretical isoelectric point (pI) is 5.88. Moreover, the amino acid sequences of the predicted oat PI-PLCs (AsPiPLCs) were used to perform searches in the NCBI databases to choose homologous sequences and to perform multiple sequence alignments and phylogenetic evolution. For this end, AsPiPLC1 shows an important degree of homology at the protein sequence level with proteins from the plant phosphatidylinositide-specific phospholipases C family and the nearest homologous (orthologous) used for phylogenetic evolution of AsPiPLCs are found to be matched to *Poaceae* family, in particular *Aegilops tauschii* (XP_020171388.1) and *Brachypodium distachyon* (XP_003579061.1) (Figure 4).

Figure 3. Open reading frame and amino acid sequences of AsPiPLC1. Bold italics mark the start codon (atg) and an asterisk indicates the translation stop codon (tag). Amino acid sequences obtained by mass spectrometry that could be matched to oat DNA are shown in red.

atg	gta	ata	cta	gca	atg	gaa	gca	gca	aac	aga	att	tgc	ggt	cat	aat	tcc	tac	tta	act	**60**
M	V	I	L	A	M	E	A	A	N	R	I	C	G	H	N	S	Y	L	T	**20**
gga	aac	cag	ctc	aat	agt	gac	tcc	agt	gat	att	cct	atc	ata	aca	gca	ttg	cag	aga	ggc	**120**
G	N	Q	L	N	S	D	S	S	D	I	P	I	I	T	A	L	Q	R	G	**40**
gtc	aga	gtg	att	gaa	ctc	gat	atg	tgg	cca	aac	tcc	gca	aag	aac	cat	gtt	gag	att	ctg	**180**
V	R	V	I	E	L	D	M	W	P	N	S	A	K	N	H	V	E	I	L	**60**
cat	gga	ggg	aca	ttg	act	gca	cct	gta	gat	atc	ctg	aga	tgt	tta	aag	tcc	att	aaa	gaa	**240**
H	G	G	T	L	T	A	P	V	D	I	L	R	C	L	K	S	I	K	E	**80**
cac	gcc	ttc	agc	gct	tcg	aca	tat	cct	ctt	ata	att	act	ctt	gaa	gat	cac	ctc	aca	gca	**300**
H	A	F	S	A	S	T	Y	P	L	I	I	T	L	E	D	H	L	T	A	**100**
gat	ctc	caa	gcc	aaa	gta	gct	gag	atg	atc	agg	gaa	aca	ttt	ggg	gat	ctc	ctt	tac	gtc	**360**
D	L	Q	A	K	V	A	E	M	I	R	E	T	F	G	D	L	L	Y	V	**120**
cct	agt	tca	gac	aca	cta	aat	gag	ttt	cct	tct	cca	gaa	gct	cta	atg	aaa	agg	ata	atc	**420**
P	S	S	D	T	L	N	E	F	P	S	P	E	A	L	M	K	R	I	I	**140**
atc	tca	act	aaa	cca	cca	gga	gaa	ttc	aag	gaa	ttt	ctt	aaa	gct	cag	gat	aat	cag	aaa	**480**
I	S	T	K	P	P	G	E	F	K	E	F	L	K	A	Q	D	N	Q	K	**160**
aat	agt	ggc	aag	acg	gct	gaa	ttg	gca	gag	gaa	gga	aac	ttg	aga	aga	tta	gat	tca	aat	**540**
N	S	G	K	T	A	E	L	A	E	E	G	N	L	R	R	L	D	S	N	**180**
gct	gat	gat	tct	gat	ggc	aag	gat	gag	ccg	gat	gaa	gaa	gaa	gat	gat	gaa	tcg	gag	gaa	**600**
A	D	D	S	D	G	K	D	E	P	D	E	E	E	D	D	E	S	E	E	**200**
gag	gac	ccc	aaa	ttt	cag	cca	gat	act	gcc	tgt	gag	tat	agg	aaa	ctg	ata	acc	atc	cac	**660**
E	D	P	K	F	Q	P	D	T	A	C	E	Y	R	K	L	I	T	I	H	**220**
gct	gga	aaa	cca	aaa	ggc	cat	ttg	agg	gat	gcg	ctt	aag	gtc	gac	cct	gac	aaa	gtc	aga	**720**
A	G	K	P	K	G	H	L	R	D	A	L	K	V	D	P	D	K	V	R	**240**
cgc	ctt	tct	tta	agc	gag	act	cag	ttg	tct	aaa	gcg	act	gtt	tct	cat	ggt	gcc	gat	atc	**780**
R	L	S	L	S	E	T	Q	L	S	K	A	T	V	S	H	G	A	D	I	**260**
ata	agg	ttc	act	gag	aaa	aat	ata	ctg	agg	att	tat	cca	aag	ggt	act	agg	att	aat	tct	**840**
I	R	F	T	E	K	N	I	L	R	I	Y	P	K	G	T	R	I	N	S	**280**
tct	aac	tat	gat	cca	ata	aat	gcc	tgg	act	cat	ggt	gcc	cag	atg	gtt	gca	ttc	aac	atg	**900**
S	N	Y	D	P	I	N	A	W	T	H	G	A	Q	M	V	A	F	N	M	**300**
cag	ggg	cat	gac	aaa	cca	ctg	agg	tta	atg	caa	gga	ttt	ttc	aga	gca	aat	ggg	ggc	tgt	**960**
Q	G	H	D	K	P	L	R	L	M	Q	G	F	F	R	A	N	G	G	C	**320**
gga	tat	gtt	aaa	aag	ccc	gac	ttc	ttg	cta	aag	ata	ggc	cca	aac	ggc	gaa	gta	ttt	gac	**1020**
G	Y	V	K	K	P	D	F	L	L	K	I	G	P	N	G	E	V	F	D	**340**
cct	aaa	gca	att	atg	ccg	gtg	aag	aaa	acc	ctg	aag	gtc	aaa	gta	tat	atg	gga	gat	ggg	**1080**
P	K	A	I	M	P	V	K	K	T	L	K	V	K	V	Y	M	G	D	G	**360**
tgg	cgc	atg	gac	ttc	agt	aaa	act	cat	ttc	gac	gcc	ttt	tct	ccg	cca	gat	ttc	tac	gct	**1140**
W	R	M	D	F	S	K	T	H	F	D	A	F	S	P	P	D	F	Y	A	**380**
agg	gta	ggg	atc	gcg	gga	gtg	cgt	gcg	gat	tgc	gtg	atg	aag	aag	acc	cgg	acg	atc	gag	**1200**
R	V	G	I	A	G	V	R	A	D	C	V	M	K	K	T	R	T	I	E	**400**
gac	cag	tgg	gtg	ccg	gtg	tgg	gat	gag	gag	ttc	tcg	ttc	ccg	cta	acg	gtc	ccg	gag	ctg	**1260**
D	Q	W	V	P	V	W	D	E	E	F	S	F	P	L	T	V	P	E	L	**420**
gcc	ctg	ctg	cgc	gtg	gag	gtc	cag	gag	tac	gac	atg	tcg	gag	aag	cac	gac	ttt	ggc	gga	**1320**
A	L	L	R	V	E	V	Q	E	Y	D	M	S	E	K	H	D	F	G	G	**440**
cag	acg	tgc	ctg	ccg	gtg	atg	gag	ctg	aag	caa	ggg	atc	cgc	gcc	gtg	ccc	ctc	cat	gac	**1380**
Q	T	C	L	P	V	M	E	L	K	Q	G	I	R	A	V	P	L	H	D	**460**
cgc	aag	ggg	aac	agg	tac	aag	tct	gtc	agg	ctc	ctc	atg	cgc	ttc	gag	ctc	gtg	tag		**1437**
R	K	G	N	R	Y	K	S	V	R	L	L	M	R	F	E	L	V	*		**478**

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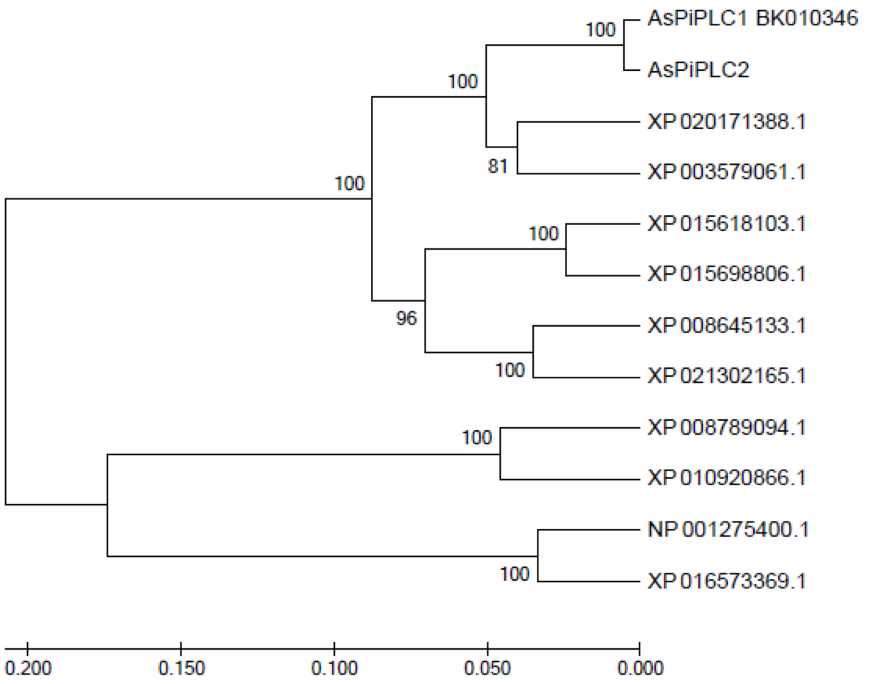
The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.98050268 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site. The analysis involved

Figure 4. Evolutionary relationships of taxa related to oat PI-PLC (AsPiPLC1 and AsPiPLC2)

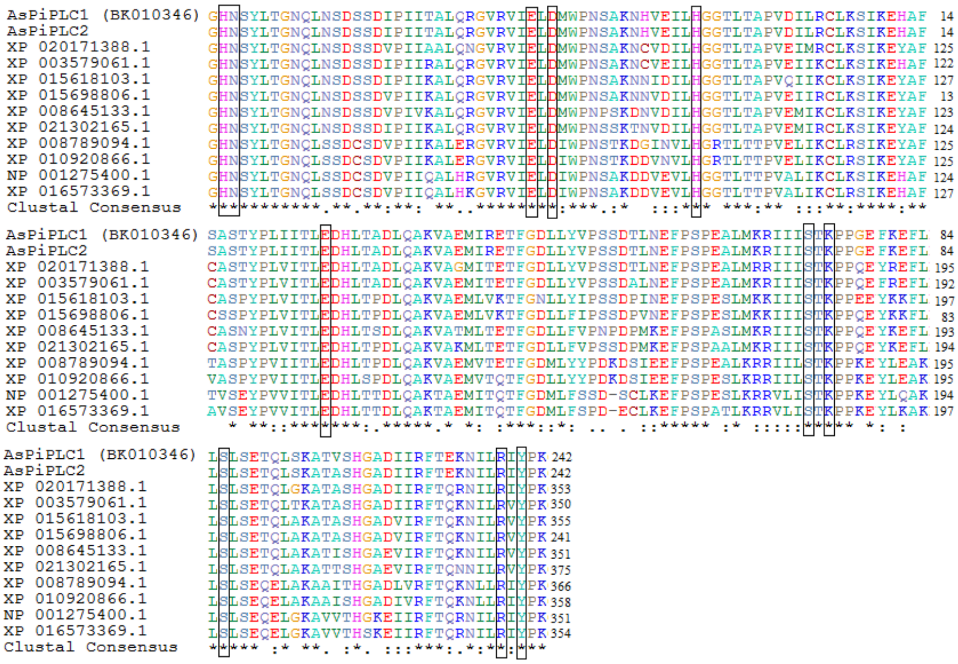


12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 468 positions in the final dataset. We have chosen homologues from monocots and eudicots groups. The Monocots PLCs homologues from Poaceae family are as following: Aegilops tauschii (XP_020171388.1), Brachypodium distachyon (XP_003579061.1), Oryza sativa (XP_015618103.1), Oryza brachyantha (XP_015698806.1), Zea mays (XP_008645133.1) and Sorghum bicolor (XP_021302165.1). While the monocots non-Poaceae used are Phoenix dactylifera (XP_008789094.1) and Elaeis guineensis (XP_010920866.1) from Arecaceae family. The eudicots used are Solanum tuberosum (NP_001275400.1) and Capsicum annuum (XP_016573369.1) from Solanaceae family.

The deduced amino acid sequence for AsPiPLC1 was aligned with several plant PI-PLCs and shares the main domains and active sites (Figure 5).

Like other plant PI-PLCs, AsPiPLC1 contains specific catalytic domains with corresponding conserved feature residues for putative active and catalytic sites as well as putative Ca binding site of plant PI-PLCs (Figure 5), which are likely homologs to mammalian PI-PLC in terms of overall sequence

Figure 5. Multiple amino acid sequences alignment of oat isozymes PI-PLC (AsPiPLC1 and AsPiPLC2) with homologous PLCs identified from NCBI databases (http://www.ncbi.nlm.nih.gov/). We have chosen homologues from monocots and eudicots groups that were used in phylogenetic evolution of Figure 2. The Monocots PLCs homologues from Poaceae family are as following: Aegilops tauschii (XP_020171388.1), Brachypodium distachyon (XP_003579061.1), Oryza sativa (XP_015618103.1), Oryza brachyantha (XP_015698806.1), Zea mays (XP_008645133.1) and Sorghum bicolor (XP_021302165.1). While the monocots non-Poaceae used are Phoenix dactylifera (XP_008789094.1) and Elaeis guineensis (XP_010920866.1) from Arecaceae family. The eudicots used are Solanum tuberosum (NP_001275400.1) and Capsicum annuum (XP_016573369.1) from Solanaceae family. The predicted residues implicated in conserved plant PI-PLC domains are highlighted.



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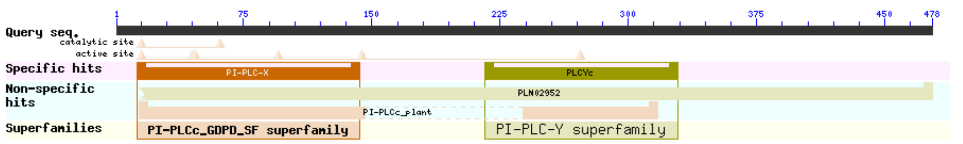
similarity and domain organization. Moreover, the domain arrangement of plant PI-PLCs, in particular AsPiPLC1, could be structurally similar to the mammalian PLC-zeta isoform, which lacks the N-terminal pleckstrin homology (PH) domain. However, it contains EF-hand like motifs (which are absent in a few plant PLCs), a PLC catalytic core domain with X-box and Y-box highly conserved regions split by a linker sequence, and a C2 (protein kinase C-conserved region 2) domain (Figure 6).

Figure 6. Putative conserved domains in oat PLC (AsPiPLC1) as detected by the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (A) and Prosite tool (http://prosite.expasy.org) (B)

A)



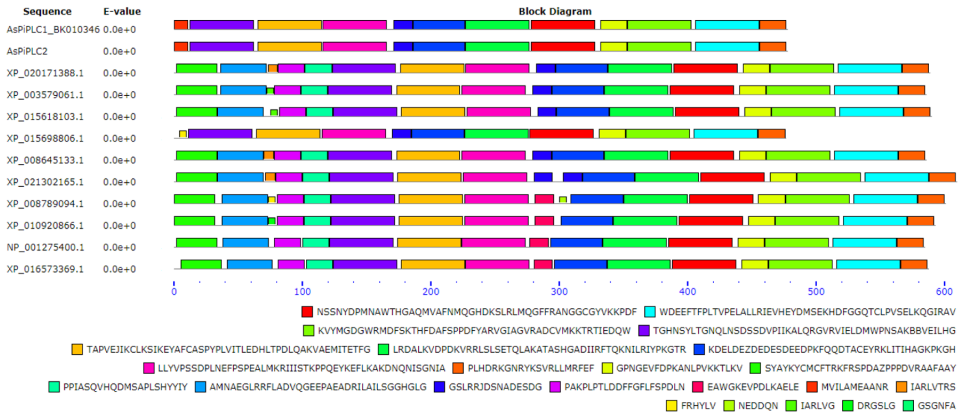
B)

A search against the Conserved Domain Database (Marchler-Bauer, 2017) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), revealed that the AsPiPLC1 amino acid sequence possesses domain conserved in PI-PLC (accession PLN02952) and specifically into plant PI-PLC (accession cd08599), all of which contain a conserved X-domain (pfam00388) and Y-domain (cl27705 and smart00149) (Figure 6 (A)).

The critical residues for enzyme activity, assigned by comparison with other plant PI-PLC proteins, include especially the two His (His 15 and His 61 for AsPiPLC1), which are involved in the active site. In fact, the important active residues involved in catalysis are highlighted in Figure 5. The Prosite tool (http://prosite.expasy.org) depicted the conservation of three domains in AsPiPLC1 characterized for PI-PLC. These domains are as follows (Figure 6 (B)): PIPLC-X-box domain (PS50007): 14 to 144 amino acids in AsPiPLC1, PIPLC-Y-box domain (PS50008): 243 to 329 amino acids extension in AsPiPLC1 and C2 domain (PS50004): 347-443 in AsPiPLC1. HMMER (https://www.ebi.ac.uk/Tools/hmmer) and Pfam (http://pfam.xfam.org) analysis also confirmed the presence of these domains in AsPiPLC1.

GO term prediction of the conserved motifs found in AsPiPLC1 (Figure 7) denoted the presence of two GO terms for molecular function: (GO:0004435) for phosphatidylinositol phospholipase C activity and (GO:0008081) for phosphoric diester hydrolase activity. Distribution of GO terms in the Biological Process category in AsPiPLC1 revealed three GO terms, which are as following: (GO:0006629) for lipid metabolic process, (GO:0007165) for signal transduction and (GO:0035556) for intracellular signal transduction.

Figure 7. Predicted structure of PLC proteins in A. sativa (AsPiPLC), Aegilops tauschii (XP_020171388.1), Brachypodium distachyon (XP_003579061.1), Oryza sativa (XP_015618103.1), Oryza brachyantha (XP_015698806.1), Zea mays (XP_008645133.1), Sorghum bicolor (XP_021302165.1), Phoenix dactylifera (XP_008789094.1), Elaeis guineensis (XP_010920866.1), Solanum tuberosum (NP_001275400.1) and Capsicum annuum (XP_016573369.1). Conserved motifs of the PLC proteins obtained by the MAST/MEME Version 4.12.0 software. The highly conserved motifs are functionally associated with phosphatidylinositol phospholipase C activity (GO:0004435) and phosphoric diester hydrolase activity (GO:0008081). Their Biological Process could be essentially as following: lipid metabolic process (GO:0006629), signal transduction (GO:0007165) and intracellular signal transduction (GO:0035556).



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Advanced structural features of the selected oat PLC could be summarized as following. The subcellular localization of AsPiPLC1 is mainly cytoplasmic with a reliability of 2.639.

No signal peptide residues could be predicted in AsPiPLC1 using the Expasy SignalP V4.1 program, suggesting that this enzyme would probably be synthesized in the cytosol as mature protein. As well, no sites of N-glycosylation was predicted in this sequence (AsPiPLC1) using the NetNGlyc 1.0 Server. Whereas, five possible O-glycosylation sites at residues 142, 162, 165, 179 and 184 using the NetOGlyc 4.0 Server.

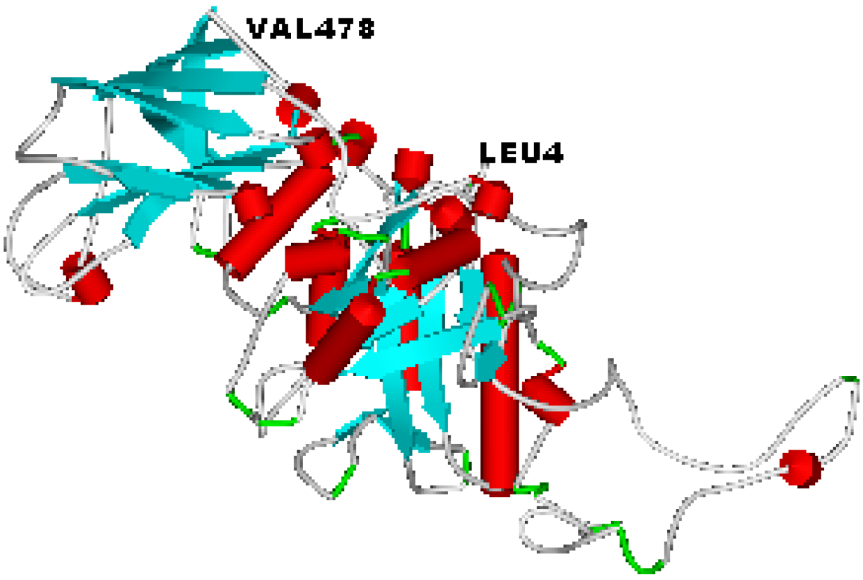
AsPiPLC1 was subjected to the prediction of protein secondary structures using the SOPMA online server software (http://npsa-pbil.ibcp.fr/cgibin/npsa_automat.p1?page=/NPSA/npsa_sopma.html). The results show that AsPiPLC1 has large proportion of random coils (39.75%), alpha helixes (29.50%) and extended strands (19.87%).

The Swiss-Model server was used to predict the 3D structure of AsPiPLC1 based on known crystal structures of homologous proteins (Figure 8). The lack of a 3D structure for the majority of *Avena sativa* proteins in PDB motivated us to construct the corresponding 3D model for AsPiPLC1 using homology modeling. Indeed, the most successful techniques for prediction of 3-D structures of proteins rely on aligning the sequence of a protein of to a homolog of known structure.

The crystal structure of Rattus norvegicus PI-PLC complexed with inositol-4,5-bisphosphate (PDB ID: 1DJZ.2.A) was used as the template for 3D structure prediction of oat PI-PLC (AsPiPLC1) with 30.44% sequence identity.

The analysis of secondary structures of the studied oat protein (AsPiPLC1) was in agreement with the related 3D-structure, which revealed abundant random coils and alpha helixes structures (Figure 8).

Figure 8. 3D structure prediction of AsPiPLC1. The N-terminal and the C-terminal of the structure prediction were designed by corresponding amino acid (red: Helix; blue: Sheet; green: Turn; grey: Coil)



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CONCLUSION

The present case study is intended to identify oat PI-PLCs by functional proteomics and genomics approaches via the Transcriptome Shotgun Assembly (TSA) or the EST databases of *Avena sativa*.

SDS-PAGE analysis of the 10 days old oat seedlings extracts allowed the identification of at least seven proteins (Figure 2), and the majority of them corresponded to hydrolase proteins family. Band 3 (60 kDa) could be identified as a PI-PLC after *de novo* peptide sequences were generated from tryptic digestion and used to screen *A. sativa* ESTs and TSAs. Based upon these genomic sequences, a full-length gene with a 1437 bp open reading frame (ORF) was identified in the draft genome of oat (Gutierrez-Gonzalez, Tu, & Garvin, 2013). The ORF encodes a 478 amino acid residue protein (AsPiPLC1) with a theoretical molecular mass of 53.92 kDa. Since the apparent molecular mass estimated from SDS-PAGE was 60 kDa (Figure 2), AsPiPLC1 might be glycosylated (five O-glycosylation sites are predicted from sequence analysis), but other post-translational modifications might also be involved and related to AsPiPLC1. As well, PI-PLC association with other proteins such as the actin cytoskeleton (Huang & Crain, 2009) could be in favor for this differentiation in molecular weight.

Although several eukaryotic and prokaryotic PI-PLCs were identified and characterized, information on the plant PI-PLC family is still limited. In plants, this enzyme [EC 3.1.4.11] is generally considered to be mainly involved in many physiological processes, in standard or stress conditions, such as seed germination, growth of seedlings, phosphatidylinositol metabolism in roots and the regulation of plant growth and development (Pokotylo, Kolesniko, Kravets, Zachowski & Ruelland, 2014; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016; Huang & Crain, 2009; Yun, Joo, Kaufman, Kim, Kirakosyan, Philosoph-Hadas, Kim & Chang, 2006). Plant PI-PLCs that act as signal transducers and generate two second messengers, IP_3 and DAG, would found multiple functions especially in food, cosmetic, medical and pharmaceutical uses.

The results obtained in this study on the identification of PI-PLC from *A. sativa* may be considered a further step in the characterization of this enzyme in plants. The physiological role of AsPiPLC1 and its interaction with actin cytoskeleton, however, to be elucidated. The complete sequencing of the *A. sativa* genome (Gutierrez-Gonzalez, Tu, & Garvin, 2013) will certainly accelerate the identification of other lipolytic enzyme from *A. sativa* seedlings extracts.

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Chapter 5
Plant Phospholipases D:
Case Study of Oat PLD

ABSTRACT

Studying phospholipases D (PLDs) from plants is very interesting as these enzymes might play important roles in many processes. PLDs are thought to cleave the phosphodiesteric bonds of the head group of phospholipids. The structure and function of oat (Avena sativa) PLD remain poorly understood, and their properties need to be further elucidated. In the chapter, functional proteomics and bioinformatic approaches were used to identify and characterize isoforms of oat PLD. Insights into structural characterization of oat predicted isozymes were analyzed using in silico approaches. Primary structures were analyzed, and a phylogeny analysis and protein motif/domain identification were presented. A multiple sequence alignment of the putative oat PLD revealed consensus sequences with the well-known important putative catalytic residues characterized by such enzymes. The results here provide a detailed view of the main residues involved in catalysis in this kind of enzyme.

INTRODUCTION

Plant phospholipase D (PLD) is an important enzyme for the physiological processes and cellular functions of the plant, which catalyzes the hydrolysis of glycerophospholipids, especially phosphatidylcholine (PC), to produce phosphatidic acid (PA) and a free head group, e.g. choline. In fact, PLD is widely distributed in nature and is produced by various living organisms, from

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eukaryotes e.g., plants and animals to bacteria and archaea. This ubiquitous enzyme cleaves the terminal phosphodiesteric bond of the substrate, i.e., phospholipid, and it could also catalyze the transphosphatidylation reaction in the presence of primary alcohol within the substrate. The bio-catalysis of such reaction consists of transferring the phosphatidyl moiety to an alcohol like ethanol, which will result in the production of phosphatidylethanol. This reaction of transphosphatidylation is unique to PLD and it has been used to identify PLD activity *in vivo*. It important to note that there are different families or subfamilies of PLD that can differ in some features such as cofactor requirement, substrate specificity and reaction conditions.

Structurally speaking, most PLDs are members of the so-called PLD superfamily, which includes PLDs, PS synthases (PSS), CL synthases (CLS), Yersinia murine toxin (Ymt), poxvirus envelope proteins, tyrosil-DNA phosphodiesterase and several nucleases. The protein members deposited in the corresponding database such as the PFAM database are affiliated according to their DNA sequence similarity and the conservation of the consensus sequences, domains and motifs rather than the function. Thus, the function for some family members is not yet known. According to the PFAM database (http://pfam.sanger.ac.uk), more than 17,500 protein sequences from more than 5000 species currently available for PLD superfamily members.

Here we will review some of the most important characterization of the plant PLDs and we will report especially PLD from oats.

In fact, the common oat (*Avena sativa* L.) is an allohexaploid (2n=6x=42) crop species and, thus, it has a large genome that remains to be fully assessed. Complex polyploidy genomes such as oats need substantial studies and contributions of the bioinformatics pipeline. There is not any cultivar of oat that has been already had completed sequence genome, hence, many oat protein sequences, for instance, PLD, need further characterization.

Functional proteomics is one of the useful tool to identify *de novo* protein sequences from those of uncompleted sequence genome such that of oat.

In this study, we have identified extracts of oat seedling as a potential lipolytic activities through the catalytic activity of PLD. The proteins in the oat seedling extracts are isolated and characterized. Aiming to understand the mechanisms of hydrolytic action of oat phospholipase D (AsPLD) isozymes, the sequencing of peptides resulting from tryptic digestion allowed identifying EST and TSA sequences from that the AsPLDs genes were compared by computational bioinformatics analysis.

Since any amino acid sequence of oat phospholipase is available in protein data bank, an attempt has been made to more characterization and identification of the catalytic amino acid residues of the enzyme by bioinformatics tools to define/predict biochemical properties of soluble oat seedling extracts. The primary aim of this work is to identify new insights into oat PLD based on mass spectrometry and bioinformatic tools and so far to get insight about the oat genome.

BACKGROUND

Phospholipids (PLs) and related enzymes, i.e. phospholipases are important components of cells that worth better characterization either as cellular/ molecular level or as external applications i.e., in food, pharmaceutical, and medicine, etc. For example, PLs could be used as emulsifiers, components of cosmetics, liposome preparations, and for medical formulations. A recent reflection done by Dowhan in 2017 highlighted some vital functions of PLs (Dowhan, 2017).

Phospholipases in particular D are among major lipid hydrolyzing enzymes, implicated in lipid mediated signaling, which are ubiquitous enzymes present in several and various living organisms from eukaryotes to prokaryotes and archaea. They play critical roles in multiple plant growth and developmental processes, as well as in hormone and stress responses and possible multivalent functionality could be assigned for these enzymes (Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016; Novotna, Valentova, Martinec, Feltl, & Nokhrina, 2000; Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet… Carrière, 2012).

Remembering that phospholipase D (PLD) [EC3.1.4.4] is an enzyme that catalyzes the hydrolysis of the phosphodiester bond of phospholipids (PLs) to produce phosphatidic acid (PA) and an alcohol moiety. PLD can also catalyze the interconversion of polar head groups of PLs by transphosphatidylation reaction. This latter reaction leads to synthesis naturally less abundant PLs such as phosphatidylglycerol (PG), phosphatidylserine (PS), or phosphatidylethanolamine (PE) from highly abundant ones such as lecithin or phosphatidylcholine (PC) (Damnjanovic & Iwasaki, 2013).

Indeed, the transphosphatidylation activity of the PLD make this enzyme as a useful protein for enzymatic synthesis of various PLs.

The report of Selvy et al. (2011) and that of Damnjanovic and Iwasaki (2013) reviewed the enzymology, functionality and potential applications of

PLD alongside with its molecular structure and with regard to its different origins.

In fact, PLD could mediate syntheses of natural and tailor-made PLs with functional head groups. These syntheses are made from the easily available substrate, i.e., lecithin or phosphatidylcholine (PC).

Enzymes of plant origin mainly performed the earliest studies on such PLD-mediated synthesis, but were later supplanted by ones from non-plant origin in particular microorganism such as actinomycetes.

Structurally speaking, many PLDs belong to the PLD superfamily and have one or two copies of a signature sequence, HxKxxxxD or HKD motif (where x is any amino acid residue), in the primary structure of the enzyme. Besides, PLD superfamily members are characterized to share a common core structure, and thereby, they share a common catalytic mechanism. The catalysis of these enzymes proceeds via two-step reaction alongside with the formation of phosphatidyl-enzyme intermediate. It is important to note that the catalytic histidine (His or H) residue is critical in the reaction course. In fact, both of the two catalytic His residues are critical for such reaction because one would act as a nucleophile and the other His would function as a general acid/base.

On the other hand, PLD could undergo protein engineering to improve its activity as a biocatalyst as well as its stability. As well, PLD is being engineered to alter the head group specificity and provide insight into further identification of the catalytically important residues. The reviews of Damnjanovic and Iwasaki (2013) and Selvy et al. (2011) focus on recent advances of PLD to further deepen the understanding of the nature of this useful enzyme.

The majority of the plant PLDs are members of HKD family enzymes, with more than 80 genes identified and several dozen cloned. The plant PLDs are more complex than those from bacteria due to their regulatory domains encoded that facilitate differential activities under various signaling environments (Wang, 1999, 2000, 2002; Munnik, 2001).

Plant PLD has been the first described PLD and thus it contributes to the rich history of the PLD superfamily. Indeed, the activity of PLD was first discovered in plants, more precisely during the research of Hanahan and Chaikoff (1947a,b; 1948) on PLD of carrots and cabbage leaves. Also, the hydrolytic and transphosphatidylation activities of PLD were originally reported from plants, in 1947 (Hanahan & Chaikoff, 1947b) and 1967 (Yang, Freer, & Benson, 1967; Dawson, 1967), respectively. As well, the first cloned PLD was that from the castor bean in 1994 (Wang, Xu, & Zheng, 1994).

PLD attracted renewed attention especially when its role in signal transduction was implicated (Cockcroft, 1984; Bocckino, Blackmore, Wilson, & Exton, 1987).

Many researchers isolated and characterized multiple PLDs from many sources, e.g., microorganisms such as actinomycetes (Damnjanovic & Iwasaki, 2013) or from the other various living organisms (Hammond, Altshuller, Sung, Rudge, Rose, & Engebrecht, 1995; Rose, Kiesau, Proft, & Entian, 1995; Pappan, Qin, Dyer, Zheng, & Wang, 1997; Wang & Wang, 2001).

Since the *Arabidopsis thaliana* genome has been sequenced, the PLD superfamily members in the model plant *Arabidopsis* has been most extensively studied in plants and the genetic manipulation of this model organism was more feasible than in other non-sequenced genome plants. All the studied plant species have a PLD family comprising more than 10 *PLD* genes (Wang, 2005). The bulk of literature on plant PLDs encompass those from *Arabidopsis*. For this end, Table 1 focuses on some few noteworthy exceptions of *Arabidopsis* PLDs from other organisms. Other details for the description of the *Arabidopsis* PLDs could be accessed from the reports of Wang et al. (2012) and Hong et al. (2016) in terms of PLDs structures, biochemical properties and cellular functions.

Although, mammalian PLDs occur as splice variants of two gene products, plant PLDs have multiple genes and isozymes. Nevertheless, all PLD isozymes are generally characterized by two conserved $HXKX_4DX_6GG/S$ sequences, which are designated as HKD motifs (Ponting & Kerr, 1996; Koonin, 1996). These motifs are required for the catalytic activity and are

Table 1. Plant PLD enzymes (Arabidopsis)

PLD	Regulatory Domain	Catalytic Requirements	Substrate	Signaling
PLDα	C2-domain	mM Ca^{2+}	PC > PE	Hormone/stress response, senescence, nutrient sensing
PLDβ	C2-domain	μM Ca^{2+}, $PI(4,5)P_2$	PC = PE = PS = NAPE	Actin polymerization
PLDγ	C2-domain	μM Ca^{2+}, PI(4,5)P2	PE = NAPE > PC	hormone/stress response (?)
PLDδ	C2-domain	μM Ca^{2+}, oleate, $PI(4,5)P_2$	PE > PC	Cell viability, ROS response, binds microtubules
PLDε	C2-domain	μM Ca^{2+}, oleate, $PI(4,5)P_2$	PE > PC	Root growth, elongation
PLDζ	PX/PH	$PI(4,5)P_2$	PC	Root growth, elongation

NAPE: N-acyl phosphatidylethanolamine

Source: (*Selvy, Lavieri, Lindsley, & Brown, 2011)*

found not only in plants PLD but are also present in other relevant proteins from microorganisms such as those found in bacterial phospholipid synthases, in bacterial endonucleases, in pox virus envelope proteins, in a murine toxin from *Yersinia* and in tyrosyl-DNA phosphodiesterase (Ponting & Kerr, 1996; Interthal, Pouliot, & Champoux, 2001). Indeed, the proteins that contain the HKD motifs are said to comprise a PLD superfamily. Moreover, proteins of the PLD superfamily exhibiting PLD activity are characterized by four conserved block sequences (I-IV) and only sequences I and IV contain the famous HKD motifs (Sung, Roper, Zhang, Rudge, Temel, Hammond, Morris, Moss, Engebrecht, & Frohman, 1997). Meanwhile the functions of the block sequences I and III are not very well depicted, it is assumed that they participate in the catalysis. On the other hand, there are two adjacent domains, named PX (Phox) and PH (Pleckstrin homology) domains that are found located in the PLDs N-terminal sequences of mammalian, yeast and nematode and also in the plant PLD ζ isozymes. However, these two domains are absent in bacterial PLD and in the most of plant enzymes (Frohman, Sung, & Morris, 1999; Qin & Wang, 2002). Interestingly, PLDs from plants that lack the PX/PH tandem domains contain a Ca^{2+}/phospholipid binding domain, called domain C2 (protein kinase C-conserved region 2), located in the N-terminus of the plant PLD sequence and they require Ca^{2+} for the activity (Qin & Wang, 2002).

It is worth noting that mammalian, yeast, *Tetrahymena* and also certain plant PLDs are depending on phosphatidylinositol 4,5-bisphosphate (PIP_2) for the activity (Qin & Wang, 2002; Brown, Gutowski, Moomaw, Slaughter, & Sternweis, 1993; Sciorra, Rudge, Prestwich, Frohman, Engebrecht, & Morris, 1999; Hodgkin, Masson, Powner, Saqib, Ponting, & Wakelam, 2000; Wang, & Wang, 2001). Besides, the PH domain is recognized to its ability to bind PIP_2 (Hodgkin, Masson, Powner, Saqib, Ponting, & Wakelam, 2000). However, there is also a particular site between the conserved block sequences II and III, which could bind PIP_2 and is required for the activity (Sciorra, Rudge, Prestwich, Frohman, Engebrecht, & Morris, 1999). Another phospholipid, the phosphatidylinositol 3,4,5-trisphosphate (PIP_3) could stimulate the PLD activity, but there may be other phospholipids that have little or no effect.

Plants have distinctive forms of PLDs based on differences in biochemical characteristics as well as cellular functions. Multiple forms of PLDs exist in either algae (Beligni, Bagnato, Prados, Bondino, Laxalt, Munnik, & Have, 2015) or higher plants such as *Arabidopsis* (Qin & Wang, 2002), cotton (Tang, Dong, & Liu, 2016), rice (Li, Lin, & Xue, 2007 ; Ueki, Morioka, Komari, &

Kumashiro, 1995), poppy (Oblozinsky, Bezakova & Mansfeld, 2011), poplar and grape (Liu, Zhang, Yang, & Hu, 2010).

As indicated above, multiple PLDs could be present in plants (at least 12 genes) and they can be grouped into distinctive types as follows: *α, β, γ, δ, ε* and *ζ*, based on their molecular and enzymatic characteristics (Qin & Wang, 2002).

The enzymatic properties of the different plant PLDs especially from *Arabidopsis* (The C2-PLD that include PLDα/PLDβ/ PLDγ/PLDδ/PLDε as well as the PX/PH-PLD that include PLDζ) were well-described in the reports of Qin and Wang (2002), Exton (2004), Selvy et al. (2011), Wang et al. (2012) and Hong et al. (2016).

In contrast to plant PLD, mammalian PLD has only two genes whose protein products named PLD1 and PLD2, could be alternatively spliced and could show approximately 50% of amino acid sequence identity (Hammond, Jenco, Nakashima, Cadwallader, Gu, Cook, Nozawa, Prestwich, Frohman, & Morris, 1997; Colley, Sung, Roll, Jenco, Hammond, Altshuller, Bar-Sagi, Morris, & Frohman, 1997). More information on mammalian PLD isozymes has been reported in the chapter of Exton (2004).

Issues of the 3-D Plant PLD Structure and Related Solutions

In this section, the idea has just turned to the neglected works for plant PLD crystal structures. In fact, there are multiple crystal structures available from bacterial PLDs, but no crystal structure exist for the more complex plant PLD. Nevertheless, there is a preliminary crystallographic study of a recombinant PLD from cowpea, which has been reported since 2001 (Abergel, Abousalham, Chenivesse, Riviere, Moustacas-Gardies, & Verger, 2001). On the other hand, the first crystal structure of a bacterial PLD was reported roughly two decades ago (Leiros, Secundo, Zambonelli, Servi, & Hough, 2000).

It is noteworthy that the current available models of the plant proposed tertiary structures (3D) of the catalytic domain as well as reaction mechanism would be based on the resolved structure and characterization of the bacterial PLDs.

The available structural analysis that were reported for the plant PLDs, which are limited, are based on non-crystallographic analytical tools. The mass spectrometry analysis is one of these such tools that was used to characterize

the sulfhydryl groups on cabbage PLD (Hwang, Park, Roh, Choi, & Kim, 2001).

Many plant PLDs, either of the C2-domain or the PX/PH domain subfamilies, have been cloned and expressed in bacteria via the recombinant expression tool (Qin, Wang, & Wang, 2002; Qin & Wang, 2002; Pappan, Austin-Brown, Chapman, & Wang, 1998; Qin, Pappan, & Wang, 1997). These studies have led to a greater understanding of biochemical properties of different plant PLD isoforms.

MAIN FOCUS OF THE CHAPTER: CASE STUDY OF OAT PLD

The focus of this chapter is mainly on oat (*Avena sativa*) PLD isozymes. In fact, *Avena sativa* are among the most cultivated crops that belong to the *Poaceae* family (*Gramineae*). *A. sativa* would be promising crop plant worldwide thanks to its beneficial components that could be valorized in industrial applications (Ben Halima, Ben Saad, Khemakhem, Fendri, & Abdelkafi, 2015).

Wild oats are diploid, but common oats (*Avena sativa*) are hexaploid with an estimated 1C genome size of 13.23pg, corresponding to about 13000 Mbp (Bennett & Smith, 1976).

MATERIALS AND METHODS

Plant Material

Seeds of oat (*Avena sativa* L.) were used in this study as started plant material in which during seedling extract, hydrolases in particular phospholipases (PLD), were the subject of this study.

Extraction of Hydrolases From Oat Seedling

Avena sativa seeds were placed to germinate on wet tissue paper in a plastic box. They were grown in the dark just for 5 days at room temperature. On day 10 after planting, *A. sativa* seedlings were ground using mortar and pestle with 0.02 M sodium acetate buffer (pH 5.6), filtered through two layers of cheese

cloth to remove large particles and the supernatant obtained was centrifuged at 15,000 × g for 20 min. The supernatant was used as oat crude extract of hydrolases. Then, acetone was added to the oat crude extract (2:1; v:v), sample centrifuged at 14,000 × g for 15 min and supernatant discarded. The partially delipidated acetone powder was resuspended in water. The mixture was stirred for 20 min at 4°C, sonicated for 5 min and finally centrifuged at 14,000 g for 5 min before collecting the supernatant fraction, which was used as oat fraction enriched in hydrolase activity.

Determination of Soluble Protein Concentration

The concentration of soluble protein in oat extract was determined by the method of Bradford (Bradford, 1976), as well as by the BCA method following the manufacturer's instructions (Smith, Krohn, Hermanson, Mallia, & Gartner, 1988) using bovine serum albumin as a standard.

SDS-PAGE

Protein electrophoresis was performed using sodiumdodecyl sulfate (SDS) polyacrylamide gel (PAGE) [NuPAGE® Novex 4–12% (w/v) Bis–Tris Gel 1.0mm, 12well (Invitrogen) and NuPAGE®MOPS SDS for Running Buffer (Invitrogen)] according to the method described by Laemmli (Laemmli, 1970). The apparent molecular weights of proteins were estimated by co-electrophoresis of marker proteins (See Blue, Invitrogen) with weights ranging from 14 to 97 kDa. The protein in the sample buffer [0.9 g glycerol, 0.1 mL 1% bromo-phenol blue, 1 mL 10% (w/v) SDS, and 0.5 μL tris(2-carboxyethyl) phosphine: reducing conditions with NuPAGE® LDS 4× LDS Sample Buffer (Invitrogen)] was heated for 5 min in boiling water and applied to the gel. The proteins separated on the SDS-PAGE were stained with Coomassie Brilliant Blue R-250 (Simply Blue™ Safe Stain (Invitrogen)).

In-Gel Tryptic Digestion and Protein Identification by Mass Spectrometry

Bands of interest were manually excised from gels and automated tryptic digestion was conducted as previously described (Abdelkafi, Ogata, Barouh, Fouquet, Lebrun, Pina, Scheirlinckx, Villeneuve, & Carrière, 2009; Abdelkafi, Abousalham, Fendri, Ogata, Barouh, Fouquet, Scheirlinckx, Villeneuve, &

Carrière, 2012; Fendri, Tardif, Fierobe, Lignon, Valette, Page, & Perret, 2009) or manually treated as follows. Gel bands were manually excised in a sterile laminar flowhood, transferred individually to 1.5 mL microtubes and cut into cubes of roughly 1 mm3. Gel cubes were destained for 1 h and 30 min at 4 °C using a solution of 45% acetonitrile and 55 mM ammonium bicarbonate. After gel cubes washing and in-gel trypsin proteolysis of proteins, the peptides produced were extracted onto Poros beads and purified with ZipTips (Millipore, France) as previously described (Beaufour, Godin, Vallée, Cadene, & Bénédetti, 2012).

Extracted proteolytic peptides were analyzed by nanoUltraHPLC–nanoESI UHR–QTOF MS. Experiments were performed using an UltiMate™ 3000 NanoRSLC System (Dionex, Sunnyvale, CA) connected to a Bruker MaXis UHR-QTOF 2 GHz mass spectrometer equipped with an online nano-ESI ion source. The LC–MS setup was controlled by Bruker Hystar™ software version 3.2. Peptides were pre-concentrated online on a Dionex Acclaim PepMap100 C18 reverse-phase precolumn (inner diameter 100 µm, length 2 cm, particle size 5 µm, pore size 100 Å), and separated on a nanoscale Acclaim Pepmap100 C18 column (inner diameter 75 µm, length 25 cm, particle size 2 µm, pore size 100 Å) at a flowrate of 450 nL/min using a 2–35% gradient of acetonitrile in 0.1% formic acid. Peaks with the three highest intensities and a minimum of 400 ion counts were selected for CID MS/MS fragmentation using an isolation window of 3–9 Da depending on the m/z value.

Acquired MS/MS spectra were searched against the UniProtKB/Swiss-Prot/TrEMBL (database version 51.6; 257,964 sequence entries), non-redundant NCBI (http://www.ncbi.nlm.nih.gov) and the EST *A. sativa* L. database containing 25,400 entries (AM071411-CN180783) using the Mascot identification engine (version 2.3, Matrix Science, France). The search was conducted allowing for a maximum of two missed cleavages, 5 ppm tolerance for precursor ions and 0.04 Da for fragment ions, respectively. Methionine oxidation was allowed.

Since contaminations from human (mainly keratins) origin could be present in the samples analyzed, the search in databases was restricted to plant species using UniProtKB/Swiss-Prot/TrEMBL, 49,887 sequence entries; NCBI nr, 551,056 sequence entries. In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides.

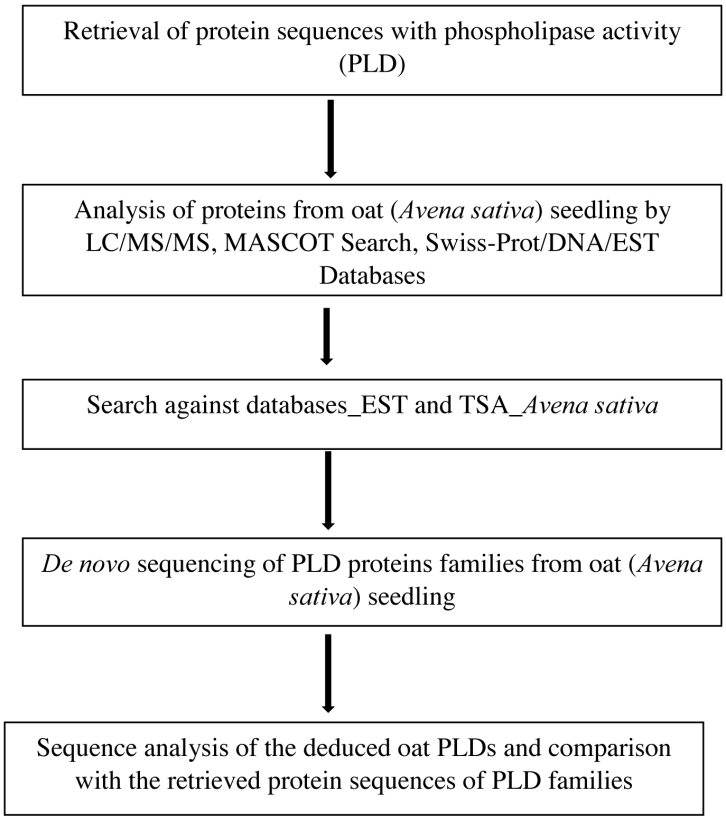
Retrieval of Protein Sequences

The amino acid sequences from plant phospholipases serving to comparison with the *de novo* sequencing of oat proteins were retrieved from protein database of National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/protein/). The sequences were saved in FASTA format. An outline of the *in silico* approach steps followed in this study has been portrayed (Figure 1).

Sequence Analysis

Bioinformatic analysis of the *A. sativa* peptide sequences, ESTs, genomic sequences and deduced protein sequences was performed using the following

Figure 1. Flow chart depicting the in silico steps used in the study for the prediction and analysis of oat enzymes from PLD families



tools. Multiple sequence alignment was performed using the ClustalW algorithm (Thompson, Higgins, & Gibson, 1994). The peptide sequences were compared with the NCBI (National Center for Biotechnology Information, USA) non-redundant sequence databases, the Transcriptome Shotgun Assembly (TSA) *A. sativa* database (GAJE01000001-GAJE01050182) and the Expressed Sequence Tag (EST) *A. sativa* database that contain 25,400 entries (AM071411-CN180783) using BLAST (Altschul, Wootton, Gertz, Agarwala, Morgulis, Schaffer, & Yu, 2005).

Primary structure analysis using predicted amino acid sequences was performed using the ExPASy Proteomics tools. The Translate tool (web.expasy.org/translate/) was used to translate DNA sequences to protein sequences, whereas the Compute pI/Mw tool (web.expasy.org/compute_pi/) was used to compute the theoretical isoelectric point (pI) and molecular mass (Bairoch, Apweiler, Wu, Barker, Boeckmann, Ferro… Yeh, 2005; Gasteiger, Gattiker, Hoogland, Ivanyi, Appel, & Bairoch, 2003). The BioEdit software package (Hall, 1999) was used to manipulate, edit and compare DNA and amino acid sequences.

Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) package version 7 (Kumar, Stecher, & Tamura, 2016). The program MUSCLE (Edgar, 2004), implemented in MEGA7 package, was used to perform multiple alignment of amino acid sequences of AsPiPLC and AsPLD and their homologous for phylogenetic analysis. The evolutionary history was inferred using either the Neighbor-Joining method (Saitou & Nei, 1987) or the UPGMA method (Sneath & Sokal, 1973). The evolutionary distances were computed using the JTT matrix-based method (Jones, Taylor, & Thornton, 1992) and were in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. The robustness of the inferred tree was evaluated by bootstrap (1000 replications) (Felsenstein, 1985).

Conserved Protein Motifs Analysis

In order to investigate conserved protein motifs in more detail, the protein sequences from oat were analyzed using Multiple Expectation maximization for Motif Elicitation (MEME)/Motif Alignment & Search Tool (MAST) (Bailey & Elkan, 1994; Bailey & Gribskov, 1998; Bailey, Johnson, Grant, & Noble, 2015) (http://meme-suite.org) with the number of different motifs

as 30, motif sites distribution as zero or one occurrence per sequence, and motifs width as 6 (minimum) and 50 (maximum).

The functional annotation of the identified motifs could be implemented by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Finn, Bateman, Clement, Coggill, Eberhardt, Eddy, Heger, Hetherington, Holm, & Mistry, 2014). The mapping between Pfam (http://pfam.xfam.org) analysis and Gene Ontology (GO) could be provided by InterPro (Sangrador-Vegas, Mitchell, Chang, Yong & Finn, 2016).

Results and Discussion

Avena sativa PLD activity has been discussed in the report of Kabachevskaya et al. (2007) with its relation to photosensitivity and photosynthesis in oat seedlings. The lipid degradation data in *Avena sativa* seedling reported by Tjellström et al. (2008) reflect a significant involvement of PLD rather than PLC. In plasma membranes from oat, a PLD-type activity and a phosphatidic acid phosphatase were the dominant lipase activities induced by phosphate deficiency (Andersson, Larsson, Tjellstrom, Liljenberg & Sandelius, 2005).

In almost all cases, no plant phospholipases was ever purified due to their association with other proteins such as actin co-purified with an oat root PI-PLC (Huang & Crain, 2009).

Taken together these findings, we have intended in this study to the identification of isoforms of PLD (AsPLDs) in 10 days old oat seedlings extract by functional proteomics. By the mean of LC/MS/MS technique and bioinformatics tools, novel amino acid sequence isozymes of oat PLD were reconstructed and characterized.

Extraction and Identification of A. Sativa Seedlings Proteins of Hydrolytically Catalytic Activities

Oat (*Avena sativa* L.) seedlings from 10 days old were used as starting material for hydrolases extraction. An aliquot of this extract was analyzed by SDS-PAGE followed a Coomassie blue staining step and a number of protein bands were excised from the preparative gel. All the excised proteins bands from the preparative gels were digested with trypsin and analyzed by LC/ESI/MS/MS.

The amino acid sequences of these peptides were determined either by manual interpretation of the collision-induced spectra of the major peptide

ion or by computer-aided fragment-matching algorithms. The majority of the protein bands excised from SDS-PAGE were identified as glycoside hydrolases, some of these bands corresponding to several proteins. A significant score could be obtained for the match of protein band (band 1) with PLD, in the DNA/EST_*Avena sativa* databases.

Therefore, *de novo* sequence peptides were identified for band 1 corresponding to oat PLD (AsPLD).

The peptide sequences obtained were then used to screen for *A. sativa* EST/genomic sequences dataset (AM071411-CN180783; GAJE01000001-GAJE01050182). Interestingly, the following genomic scaffolds (TSA_*A. sativa*: GAJE01030597-GAJE01030603) as well as (GFHU01003495 and GFHU01003496; GFHU01000532 and GFHU01000533) and ESTs_*A. sativa* (GO594938 and GO591438) that could be corresponding to the peptide sequences of band 1 were identified using BLAST (http://blast.ncbi.nlm.nih.gov) (Altschul, Madden, Schaffer, Zhang, Zhang, Miller, & Lipman, 1997). These genomic scaffolds are useful tools to the identification of at least two oat PLDs isoforms.

We could then predict the structure of the identified genes by comparing the oat genomic scaffolds with related plant phospholipases using BLAST search analysis (Altschul, Madden, Schaffer, Zhang, Zhang, Miller, & Lipman, 1997).

Based on these analyses, the proteins isolated from *A. sativa* seedling extract that correspond to band 1 were identified as PLD and were named AsPLD_x (where x is the number of predicted isoforms; in this study, we reported two oat PLD isoforms).

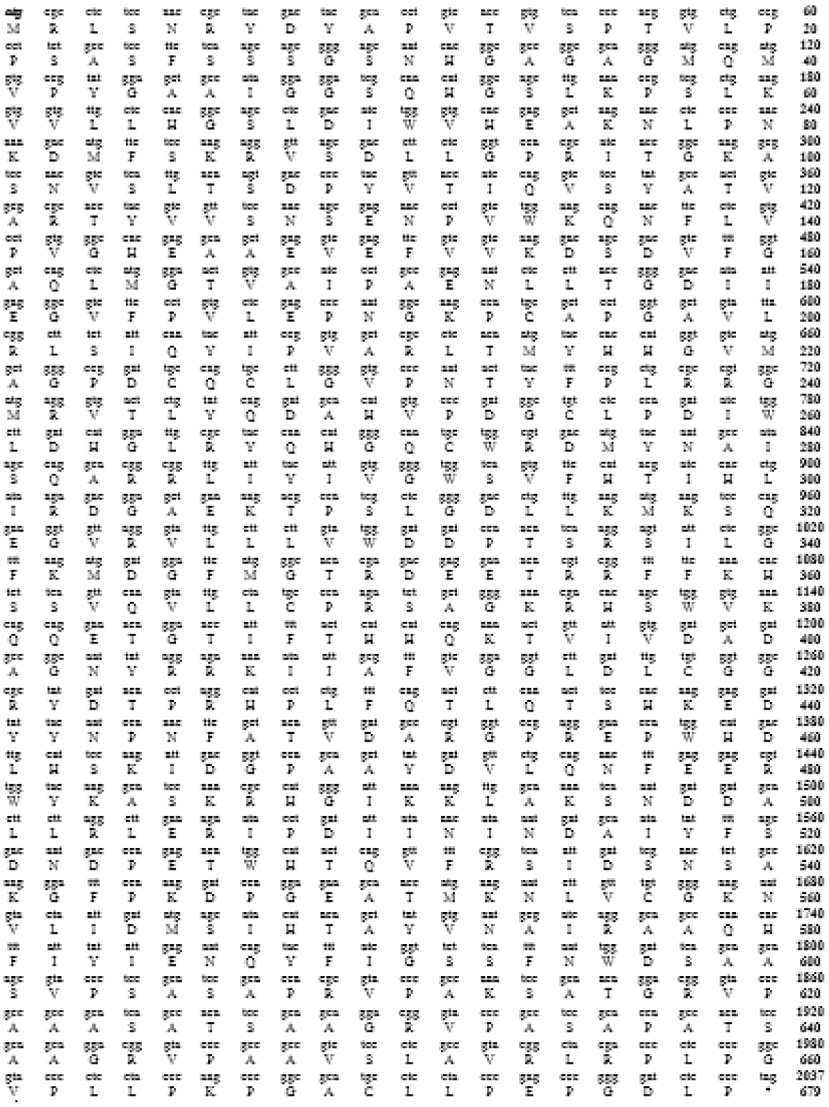
Sequence Analysis of Oat PLD Proteins

At least, two predicted isozymes of PLD would be found in oat seedling extract (AsPLD1 and AsPLD2). However, AsPLD1 was selected for much intense further analysis.

The predicted AsPLD1 cDNA (2040 bp) corresponds to a 679 amino acid residue protein (Figure 2).

The predicted molecular weight of the protein is 74.427 kDa and the theoretical isoelectric point (pI) is 8.90. Moreover, the amino acid sequences of the predicted oat PLDs (AsPLDs) were used to perform searches in the NCBI databases to choose homologous sequences and to perform multiple sequence alignments and phylogenetic evolution. For this end, AsPLD1

Figure 2. Open reading frame and amino acid sequences of AsPLD1. Bold italics mark the start codon (atg) and an asterisk indicates the translation stop codon (tag)



shows an important degree of homology at the protein sequence level with proteins from the plant phospholipases D family and the nearest homologous (orthologous) used for phylogenetic evolution of AsPLDs are found to be matched to *Poaceae* family, in particular in particular *Zea mays* (ONL99359.1) and *Oryza sativa* (CAD11899.1) (Figure 3).

Figure 3. Evolutionary relationships of taxa related to oat PLD

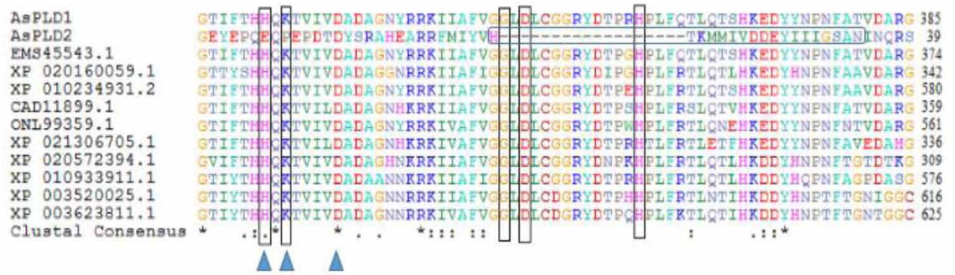
The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.13246659 is shown in Figure 3. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 12 amino acid sequences. There were a total of 147 positions in the final dataset. We have chosen homologues from monocots and eudicots groups. The Monocots PLDs homologues from Poaceae family are as following:

Triticum urartu (EMS45543.1), Aegilops tauschii (XP_020160059.1), Brachypodium distachyon (XP_010234931.2), Oryza sativa (CAD11899.1), Zea mays (ONL99359.1) and Sorghum bicolor (XP_021306705.1). While the monocots non-Poaceae used are Elaeis guineensis (XP_010933911.1) from Arecaceae family and Phalaenopsis equestris (XP_020572394.1) from Orchidaceae family. The eudicots used are Glycine max (XP_003520025.1) and Medicago truncatula (XP_003623811.1) from Fabaceae family.

The deduced amino acid sequence for AsPLD was aligned with several plant PLDs and shares the main domains and active sites (Figure 4 and 5).

A search against the Conserved Domain Database (Marchler-Bauer, 2017) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), revealed that the AsPLD1 amino acid sequence possesses domain conserved in PLD (accession PLN03008) and specifically into C2-domain plant PLD (accessions

Figure 4. Multiple amino acid sequences alignment of oat PLD (AsPLD1 and AsPLD2) with homologous PLDs identified from NCBI databases (http://www.ncbi.nlm.nih.gov/). We have chosen homologues from monocots and eudicots groups that were used in phylogenetic evolution of Figure 2. The Monocots PLDs homologues from Poaceae family are as following: Triticum urartu (EMS45543.1), Aegilops tauschii (XP_020160059.1), Brachypodium distachyon (XP_010234931.2), Oryza sativa (CAD11899.1), Zea mays (ONL99359.1) and Sorghum bicolor (XP_021306705.1). While the monocots non-Poaceae used are Elaeis guineensis (XP_010933911.1) from Arecaceae family and Phalaenopsis equestris (XP_020572394.1) from Orchidaceae family. The eudicots used are Glycine max (XP_003520025.1) and Medicago truncatula (XP_003623811.1) from Fabaceae family. The predicted residues implicated in conserved plant PLD domains are highlighted by either rectangles or triangles. The numbers of the residues relative to the first residue of each protein are shown on the right side of each sequence.



**For a more accurate representation see the electronic version.*

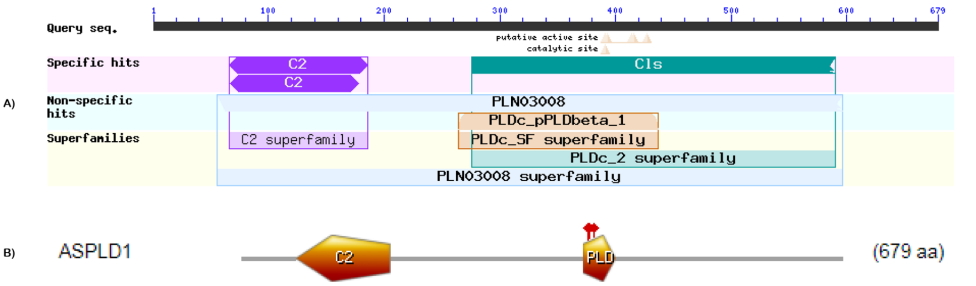
*Figure 5. Multiple amino acid sequences alignment of oat (Avena sativa) PLD (AsPLD1) with representative other PLDs (gi 20138945: ACCESSION P93733 (Arabidopsis thaliana), gi 168042337: ACCESSION XP_001773645 (Physcomitrella patens) and gi 148909361: ACCESSION ABR17779 (Picea sitchensis)). Hash marks **(#)** above the aligned sequences show the location of the conserved feature residues for putative active and catalytic sites.*

**For a more accurate representation see the electronic version.*

pfam00168 and smart00239), as well as related domains (accessions cd09198 and COG1502) (Figure 6).

The critical residues for enzyme activity, assigned by comparison with other plant PLD proteins, include especially the His residue, which is part of the HKD motif involved in the active site. In fact, the important active residues involved in catalysis are highlighted in Figures 4 and 5. The Prosite

Figure 6. Putative conserved domains in oat PLD (AsPPLD1) as detected by the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (A) and Prosite tool (http://prosite.expasy.org) (B)

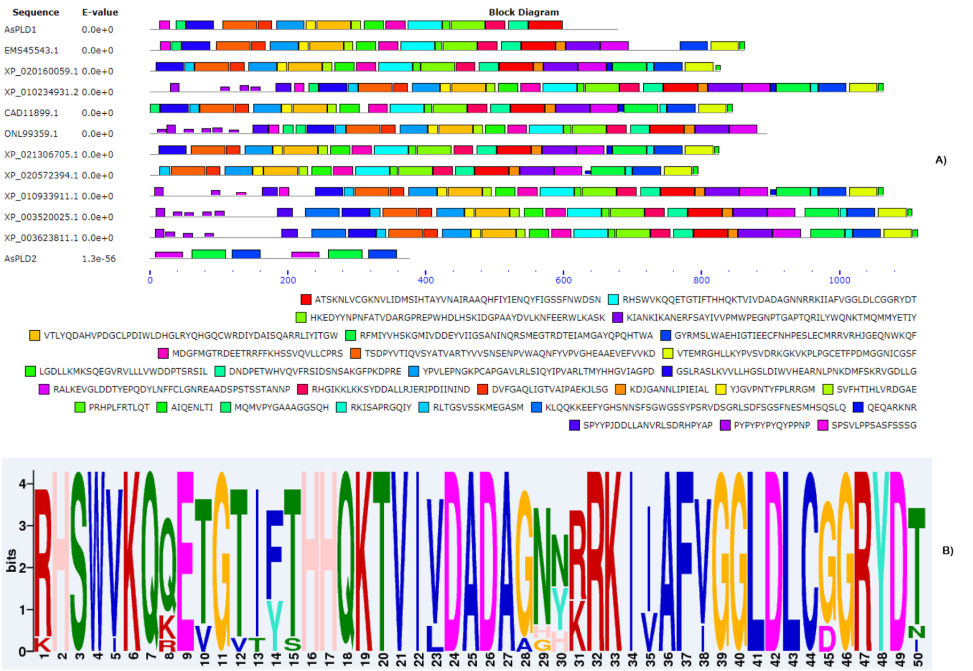


tool (http://prosite.expasy.org) depicted the C2-conserved domain for AsPLD1 (Figure 6, A'). MAST/MEME results are indicated in Figure 7 that provide the highly conserved domains in particular the conserved HKD motif found in majority of plant PLDs.

CONCLUSION

Although any oat PLD is purified in complete homogeneity due to its association with other proteins, the present study on the identification and characterization of oat PLD proteins by genomic approaches from *A. sativa* transcriptomic databases (TSA and EST) and by functional proteomics could be an outstanding tool to get insight further recognition of such type of protein.

Figure 7. Conserved motifs of the PLD proteins including AsPLD1 obtained by the MAST/MEME 4.11.4 software (A) and the sequence logo for the highly conserved motif that contain the HKD residues (B): Numbers on the x-axis represent the sequence positions in respect HKD domain. The y-axis represents the information content measured in bits.



**For a more accurate representation see the electronic version.*

The results obtained in this study on the identification of PLD from *Avena sativa* may be considered a further step in the characterization of this enzyme in plants. The physiological role of AsPLD1 and its interaction with other proteins, however, remain to be elucidated. The complete sequencing of the *A. sativa* genome (Gutierrez-Gonzalez, Tu, & Garvin, 2013) will certainly accelerate the identification of other lipolytic enzyme from *A. sativa* seedlings extracts.

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Chapter 6
Evolutionary History of Plant Lipolytic Enzymes

ABSTRACT

The discovery of enzymes with lipolytic activities in all kingdoms of life from prokaryote to eukaryote species raises the possibility of the presence of an evolutionary relationship history of these proteins among many species of various living organisms. The chapter suggests a strategy based on the phylogenetic distribution and homology conservation in plant lipolytic enzymes for possible depiction of their biological evolution. Extensive databases and online resources for lipidomics and related areas are useful tools to analyze the different lipolytic enzymes in the three major super kingdoms of life, including higher plants kingdom and confined organisms such as algae that have recently gained much interest due to their promising potential applications in lipids hydrolysis and biosynthesis. Multiple sequence alignments of the identified lipolytic enzymes from databases could serve to the identification of globally conserved residues as well as conserved sequence motifs. Estimation of evolutionary distance between the various identified lipolytic enzymes could also be carried out to better understand the pattern of evolution.

INTRODUCTION

Lipidomics, lipids and lipolytic enzymes have being gained a large interest of researchers. Lipolytic enzymes are found in multiple unicellular and pluricellular organisms. However, plants are one of the most important sources of these biocatalysts for many applications either for lipid hydrolysis or for

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lipid biosynthesis. For example, plant lipases from latex would be amazing enzymes with lipolytic activity for industrial applications such as in food, medicine, cosmetic and pharmaceutical industries.

Obviously, lipase crystallization and further its high resolution are a powerful tool to elucidate the enzyme structure. Triacylglycerol lipase, for instance, is an α/β protein, with a central β-sheet with the active site serine (Ser) placed in a loop, in which the nucleophilic residue is essential for the catalysis. In fact, the activity of lipases rely, generally, on a catalytic triad usually formed by three residues, namely Ser-Asp/Glu-His. The active site Ser is found in the consensus pentapeptide Gly-X-Ser-X-Gly (where X being any amino acid). This sole presence of this motif has identified many serine hydrolases. As well, this active site Ser could be found in other hydrolytic enzymes, not only in lipases. This conserved motif could be found in all known esterase structures, e.g., acetyl cholinesterase and cutinase. It is important to note that the conservation of the active site residues (Gly-X-Ser-X-Gly) and even though surrounded residue such as alanine or another glycine, as well as the distance between them could be critical in maintaining the structure and the activity of the lipolytic enzyme (https://www.creativebiomart.net/researcharea-lipases-proteins_1470.htm).

Lipolytic enzymes are indispensable proteins for the biological turnover of lipids. They are required as digestive biocatalysts in the transfer of lipid from one living organism to another, that is, from plant to animal and from animal to animal.

Here, we will try to show the different classes of lipolytic enzymes among different living organisms. A suggested strategy adopted by Udaya Prakash et al. (2010) would be used to hint on evolutionary history of plant lipolytic enzymes. Identification of structure and sequence homology, conserved motifs, as well as phylogenetic analysis of lipolytic enzyme from plants, namely, higher plants and close-related organisms such as algae, would reinforce the evolutionary history.

This chapter will take just a retrospective look at the importance of databases and online resources for lipidomics, lipids and lipolytic enzymes, and set current developments in a historical context of especially plant lipolytic enzymes. It is meant to be an outline description of such field, rather than a comprehensive history of such topic. Maybe the latter task is something that one of the readers of this chapter might be inspired to undertake at some time in the future.

BACKGROUND

Lipids, lipid-based derivatives and relevant enzymes are a wide range of organic molecules, which fulfil various vital roles in biological systems. Many reports have highlighted the important contributions of these molecules in many key functions such as in lipid metabolism, energy and growth and development (Baez, 2013; Freysz, Dreyfus, Massarelli, & Gatt, 1986; Gatt, Freysz, & Mandel, 1978; Schettler, 1967).

Bioinformatics for lipidomics is an emerging research and opportunity to unravel some pertinent information about lipids as well as the enzymes with lipolytic activities. There are many online resources for lipids and lipolytic enzymes. Table 1 lists few online resources for the lipids and relevant enzymes, and their databases. Three important lipid databases are reported, namely, LIPIDAT (Caffrey & Hogan, 1992), LIPIDBANK (Yasugi & Watanabe, 2002) and LMSD (Sud, Fahy, Cotter, Brown, Dennis, Glass… Subramaniam, 2007). As well, LED is a major database for lipase engineering (Fischer & Pleiss, 2003); and ESTHER is a well-recognized database for α/β-hydrolase fold superfamily of proteins (Hotelier, Renault, Cousin, Negre, Marchot, & Chatonnet, 2004; Lenfant, Hotelier, Velluet, Bourne, Marchot, & Chatonnet, 2013). Noting that, generally, lipid databases are created on the basis of their different scope and organization, unlike genes and proteins databanks, because of the lack of universal lipid classification scheme (Namasivayam, Kowsalya, Padarthi, Manigandan, Jayaraj, Johnravindar, & Jagatheesh, 2015).

Table 1. Some important online bioinformatics resources for lipidomics and related area

LIPIDAT	http://www.lipidat.ul.ie/
LipidBank	http://lipidbank.jp
LMSD	http://www.lipidmaps.org/data/structure/index.html
LIPID MAPS	http://www.lipidmaps.org/
Lipid Library	http://www.lipidlibrary.co.uk/
Cyberlipid Center	http://www.cyberlipid.org/
LMPD	http://www.lipidmaps.org/data/proteome/index.html
KEGG	http://www.genome.jp/kegg/pathway.html
ESTHER	http://bioweb.ensam.inra.fr/ESTHER/general?what=index
LED	http://www.led.uni-stuttgart.de/

Lipids and lipolytic enzymes, in whatever organisms, could be classified into several categories. On the one hand, lipids are developed and classified into eight categories, namely, glycerolipids, fatty acyls, glycerophospholipids, sphingolipids, prenol lipids, sterol lipids, saccharolipids and polyketides, according to the database LIPID MAPS, which is a US-based organization. On the other hand, lipolytic enzymes could be classified into various families.

The different classes of lipolytic enzymes, which comprise lipases (EC 3.1.1.1, triacylglycerol hydrolases) and esterases (EC 3.1.1.3, carboxyl ester hydrolases), are among the most valuable classes of hydrolases to be used in many processes especially in biotechnological applications (Casas-Godoy, Duquesne, Bordes, Sandoval, & Marty, 2012; Fan, Niehus, & Sandoval, 2012; Hudlicky & Reed 2009; Bornscheuer 2002). In fact, esterases and lipases, which are known to bio-catalyze both the hydrolysis and synthesis of lipid component, could be used in biotechnology, medicine, food, detergent, fuel and other industrial processes such as in pulp and paper and pharmaceutical manufacturing (Ramnath, Sithole, & Govinden, 2017). These enzymes are characteristically resilient biocatalysts, which could withstand harsh industrial applications with wide pH ranges, high temperatures, as well as the presence of organic solvents (Gupta, Gupta, & Rathi, 2004). As well, lipolytic enzymes exhibit characteristic chemo-, stereo-, and regio-selectivity that are of particular interest in applications, like the synthesis of optically pure compounds (Haki & Rakshit, 2003). The catalytic flexibility, robustness and high specificity of enzymes with lipolytic activities attract a lot of attention as target biocatalysts to be further insight studied.

It is known that the 3-dimensional structure of esterases and lipases displays the characteristic α/β-hydrolase fold (Ollis, Cheah, Cygler, Dijkstra, Frolow, & Franken, 1992) and a definite order of α-helices and β-sheets (Ramnath, Sithole, & Govinden, 2017).

CLASSIFICATION OF LIPOLYTIC ENZYMES OF DIFFERENT ORIGINS

Mammalian lipolytic enzymes include “pancreatic lipase gene family” and other lipases (Table 1). In fact, the pancreatic lipase gene family comprises hepatic lipase (HL), endothelial lipase (EL), lipoprotein lipase (LPL), pancreatic lipase (PL) and pancreatic lipase-related protein 1-3 (PLRP1, PLRP2 and PLRP3); as well, this family includes three PLA_1s, which

specifically catalyze phospholipids (Aoki, Inoue, Makide, Saiki, & Arai, 2007; Wong & Schotz, 2002; Saelee, Wongkham, Puapairoj, Khuntikeo, Petmitr, Chariyalertsak… Karalak, 2009; Borrelli & Trono, 2015). Indeed, there are nine identified PLA_1s in mammals with six are extracellular and three are intracellular PLA_1s enzymes, and these two groups do not share sequence homology and they apparently have distinct functions (Richmond & Smith, 2011). The other mammalian lipases that are not included in the "pancreatic lipase gene family" are the hormone-sensitive lipase (HSL) (Kraemer & Shen, 2002) and the bile salt-stimulated lipase (BSSL) (Hernell & Bläckberg, 1994) (Table 2).

In plants, lipolytic enzymes are mainly abundant in oleaginous plants and especially in the oil bodies of oleaginous seeds, but they have also been found in a significant amount in cereals and bean seeds (Tursi, Phair, & Barnes, 1994; Villeneuve, 2003; Barros, Fleuri, & Macedo, 2010; Moussavou Mounguengui, Brunschwig, Baréa, Villeneuve, & Blin, 2013). In fact, lipolytic enzymes in seeds could catalyze the mobilization of the stored fatty acids whose release could provide the carbon and energy necessary to the seedling growth (Quettier & Eastmond, 2009). In general, lipolytic activity is absent in ungerminated seeds; nevertheless, it rapidly appears after germination (Moussavou Mounguengui, Brunschwig, Baréa, Villeneuve, & Blin, 2013).

Table 2. Classification and some properties of mammalian lipolytic enzymes

Type	Lipase	Tissue	Function
Pancreatic lipase gene family	Hepatic lipase (HL, HTGL, LIPC)	Hepatocytes	Metabolism of lipoproteins
	Endothelial lipase (LIPG, EL, EDL)	Endothelial cells	Metabolism of lipoproteins
	Lipoprotein lipase (LPL)	Adipose tissue, other tissues	Metabolism of lipoproteins
	Pancreatic lipase (PL)	Pancreatic acinar cells	Intestinal absorption of FAs
	Pancreatic lipase-related protein 1 (PLRP1)	Pancreatic acinar cells	Unknown
	Pancreatic lipase-related protein 2 (PLRP2)	Pancreatic acinar cells	Intestinal absorption of FAs
	Pancreatic lipase-related protein 3 (PLRP3)	Hepatocytes	Hepatocellular carcinoma
Other lipases	Hormone-sensitive Lipase (HSL)	Adipose tissue, steroidogenic tissues	Mobilization of FAs, spermatogenesis, steroidogenesis
	Bile salt-stimulated Lipase (BSSL)	Pancreatic acinar cells, breast milk	Absorption and digestion of fats

Source: Borrelli and Trono (2015)

Lipolytic enzymes could be present in other plant tissues such as in the plastoglobuli of chloroplasts where a putative lipase has been identified that is probably involved in the mobilization of fatty acids (Padham, Hopkins, Wang, McNamara, Lo, Richardson, Smith, Taylor, & Thompson, 2007). Latex, the milky sap product from rubber trees, also contains lipase activity (Paques & Macedo, 2006). For instance, lipolytic activity from *Carica papaya* latex has been studied in many reports as seen in Abdelkafi, et al (2009a); Abdelkafi et al., (2009b); Abdelkafi et al., (2011), and; Abdelkafi et al., (2012).

In algae (macroalgae and microalgae), the almost universal presence of lipids (Ben Halima, 2016; Ben Halima, 2017) had led the researchers to study their assimilation, for instance, their lipolytic enzymes. According to the report of Davis (1915), lipolytic action was showed in several algae upon olive oil-casein emulsion, alcohol water olive oil emulsion and triacetin (Table 3).

Many other reports have studied lipolytic enzymes in algae (Hoehne-Reitan, Økland, & Reitan, 2007; Ali, Zaki, & Al-rubaie, 2015; Hubert, Poisson, Loiseau, Gauvry, Pencréac'h, Hérault & Ergan, 2017). In particular, marine microalgae have received increasing interest to study their lipids and lipolytic

Table 3. Lipolytic activity of three alga (Ulva, Mesogloea and Chondrus) upon triacetin, alcohol water olive oil emulsion and olive oil-casein emulsion

Alga	**Number cc. of N/10 NaOH to neutralize 10 cc. substrate after 10 days**			
	Triacetin			
	Triacetin + tissue	**Water + tissue**	**Triacetin alone**	**Net acidity**
Ulva Mesogloea Chondrus	.3	.025	.1	.175
	.25	.05	.1	.1
	.55	.35	.1	.1
	Alcohol water olive oil emulsion			
	Emulsion + tissue	Water + tissue	Emulsion alone	Net acidity
Ulva Mesogloea Chondrus	.9	.05	.00	.85
	.65	.075	.00	.575
	1.45	.35	.00	1.10
	Olive oil casein emulsion			
	Emulsion + tissue	Water + tissue	Emulsion	Net acidity
Ulva Mesogloea Chondrus	1.3	.1	.25	.95
	1.00	.1	.1	.8
	1.85	.05	.00	1.8

Source: Davis, A.R. (1915)

enzymes due to their ability to produce polyunsaturated fatty acids especially the omeg-3 long chain docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). For instance, *Isochrysis galbana* is among these microalgae, which was the focus of recent publication (Hubert, Poisson, Loiseau, Gauvry, Pencréac'h, Hérault & Ergan, 2017) who report expressed protein from this microalga that displays similarities with thioesterases. Actually, thioesterases would be quite interesting enzymes for engineering lipids as they could improve free fatty acid release and alter fatty acid profile with a view to obtain a particular lipid composition that could be needed for a specific application like in the case of biofuel production (Chen, Peng, & Shan, 2012; Kerviel, Hérault, Dumur, Ergan, Poisson, & Loiseau, 2014; Hubert, Poisson, Loiseau, Gauvry, Pencréac'h, Hérault & Ergan, 2017).

Various families of enzymes with lipolytic activity are produced by fungi including true lipases (EC 3.1.1.3), carboxylesterases (EC 3.1.1.3) and secretory lipases (EC 3.1.1.3). These three classes of enzymes belong to the structure-based superfamily of α/β-hydrolases, a variety of enzymes whose activities rely mainly on a catalytic triad usually formed by serine (S), histidine (H) and aspartic acid (D) residues. Fungi produce also a variety of phospholipases such as PLA1 (EC 3.1.1.32), PLA2 (EC 3.1.1.4), lysophospholipase (EC 3.1.1.5), PLC (EC 3.1.4.11) and PLD (EC 3.1.4.4) (https://www.creativebiomart.net/researcharea-lipases-proteins_1470.htm).

Bacterial lipolytic enzymes were mainly classified into eight families (families I-VIII) based on variances in amino acid sequences and biological properties according to the report of Arpigny and Jaeger (1999). In this classification, family I refers to the "true" lipases (EC 3.1.1.3) and the interfacial activation as well as the presence of a lid are characteristics of this family, which is the largest family among the eight families and is further divided into seven subfamilies (Arpigny & Jaeger, 1999; Jaeger & Eggert, 2002). The classification of the seven subfamilies (I.1-I.7) in the family I of bacterial lipases is based on structural features, types of secretion mechanism and requirement for lipase-specific foldases, and relationship to other enzyme families (Borrelli & Trono, 2015). The seven other families for bacterial lipolytic enzymes are generally carboxyl esterases along with various other lipases, e.g., family II is also called Gly-Asp-Ser-(Leu) [GDSL] (sequence motif) family and family IV is also called HSL (Hormone Sensitive Lipase) family. The classification of Arpigny and Jaeger (1999) has been revised several times; to this end, there have currently been XVI families of lipolytic enzymes, which are a part of the ESTHER database (http://bioweb.ensam.inra.fr/ESTHER/general?what=index) (Hotelier, Renault, Cousin, Negre,

Marchot, & Chatonnet, 2004; Lenfant, Hotelier, Velluet, Bourne, Marchot, & Chatonnet, 2013).

Beside, new families and subfamily of bacterial lipolytic enzymes are discovered by functional metagenomics (Fu, Hu, Xie, Guo, Ashforth, & Polyak, 2011; Lee, Lee, Oh, Song, & Yoon, 2006; Kim, Oh, Lee, Kang, Oh, & Yoon, 2009), namely, EstA, EstB, EstF, LipG, LipEH166 and EstY (Ramnath, Sithole, & Govinden, 2017). Table 4 provides a summary description for such classification of bacterial lipolytic enzymes.

Table 4. Classification of bacterial enzymes with lipolytic activity

Family	Description
I	Group of true lipases subdivided into 7 subfamilies (I.1-I.7)
II	Modified pentapeptide motif around the active serine: Gly-Asp-Ser-(Leu) [GDS(L)]; Secreted and membrane-bound esterases
III	Extracellular lipases and esterases
EstA	Related to family III but different conserved motifs (pentapeptide Gly-His-Ser-Met-Gly) [GHSMG]
IV	Many members of this family show sequence similarity to mammalian hormone-sensitive lipase (HSL); Typical motif His-Gly-Gly [HGG]; Lipolytic enzymes from psychrophilic, mesophilic, and thermophilic origins
EstB	New subfamily in family IV with second active site glutamate (conserved sequence Glu-X-Leu-Leu-Asp [EXLLD]) instead of the aspartate Asp-Pro-Leu-X-Asp (DPLXD) of the representative members of family IV
V	Conserved motif His-Gly-Gly-Gly [HGGG] upstream of the pentapeptide motif Gly-Asp-Ser-Ala-Gly [GDSAG]; Sequence similarity with nonlipolytic enzymes: epoxide hydrolases, dehalogenases, and haloperoxidases; Esterases from psychrophilic, mesophilic, and thermophilic origins
EstF	Related to family V but with a modified pentapeptide, Gly-Thr-Ser-X-Gly [GTSXG], and different flanking regions around the HG motif and their own unique conserved sequence motifs
VI	The smallest esterases known; Sequence similarity to eukaryotic lysophospholipases
VII	Large bacterial esterases; Sequence homology with eukaryotic acetylcholine esterases and intestine and (or) liver carboxylesterases
VIII	Similarity to several class C β-lactamases
LipG	Presence of an Arg-Gly sequence in oxyanion hole instead of His-Gly, a signature sequence distinctive of filamentous fungal lipases
LipEH166	Comprise newly discovered lipase LipEH166 of psychrophilic origin, and three putative open reading frames
EstY	Derived from pathogenic bacteria; First possible lipolytic virulence factors that do not belong to the GDSL family

Source: Ramnath et al. (2017)

LIPOLYTIC ENZYMES CLASSIFICATION IN THE DATABASE "LED"

It is important to note that there is a new lipolytic enzyme classification, which has more recently reported in the Lipase Engineering Database (LED) (http://www.led.uni-stuttgart.de/) (Fischer & Pleiss, 2003). Today, the classification in LED includes not only bacterial, but also yeast, fungal, algal, higher plant and mammalian lipases (http://www.led.uni-stuttgart.de/cgi-bin/LED/index.pl?page=overorg).

The lipases in such classification could be distributed into three classes based on the oxyanion hole: GX, GGGX, and Y (Fischer & Pleiss, 2003; Borrelli & Trono, 2015). Yeasts and fungal lipases, for instance, could be grouped into five different subclasses according to this classification and the amino-acid sequence similarities, two subclasses in the GX class, two subclasses in the GGGX class, and one subclass in the Y class (Gupta, Kumari, Syal, & Singh, 2015; Borrelli & Trono, 2015).

In fact, the Lipase Engineering Database (LED) (http://www.led.uni-stuttgart.de/) integrates information on sequence, structure and function of lipases, esterases and related proteins in particular those sharing the same α/β hydrolase fold to facilitate protein engineering. Please refer to the following publication for more information regarding this database: Widmann et al., (2008); Widmann et al., (2010a, 2010b); Koschorreck et al., (2005); Barth et al., (2004a, 2004b); Fischer and Pleiss, (2003); Pleiss et al., (2000a, 2000b); and Pleiss et al., (1998).

The LED could be applied to systematically analyze sequence–structure–function relationships of lipases, and it is a useful tool to identify functionally relevant residues apart from the active site residues, and to design mutants with desired substrate specificity (Fischer & Pleiss, 2003).

ALGAE AS A MODEL PROSPECTIVE OF PLANT LIPIDOMICS

Algae and especially microalgae are receiving more interest from the public and scientific communities because of their vital application in many sectors such as in biofuels, nutraceutical and agriculture. They could be exploited as a model for the study of the evolutionary pattern of plant lipids and plant

lipolytic enzymes due to the confinement of algae species to those of plant kingdom.

As mentioned in the report of Namasivayam et al. (2015) that deals with plant lipidomics, biochemical and molecular analysis of microalgae revealed differences in lipid signalling between species of algae and in comparison to plants.

These differences range from distinct acyl groups present in algal lipids, to a possible more direct role of plastids in the assembly of triacylglycerols (TAGs) during glycerolipid metabolism.

Therefore, microalgal lipidomics could be a model prospective of genetic engineering in the metabolism of plant lipid (Liu & Benning, 2013), but also in the evolutionary history of plant lipolytic enzymes.

ALLUSION TO LIPASE EVOLUTION

Lipases are serine hydrolases defined as triacylglycerol acylhydrolases (E.C. 3.1.1.3) that are ubiquitous enzymes, widespread in nature. They were first isolated from bacteria in the early nineteenth century and the associated research is continuously in rise due to the particular characteristics of these enzymes (Casas-Godoy et al., 2012). Lipases should be differentiated from esterases (E.C. 3.1.1.1) either by the so-called "interfacial activation" or by the nature of their substrates. Indeed, activation by the presence of an interface, also called "interfacial activation", which is the first criteria that were used to distinguish lipases and esterases, was found unsuitable for the classification of such two types of enzymes as some lipases did not exhibit such phenomenon. Lip4 from *Candida rugosa* (Tang, Shaw, & Sun, 2001) and *Candida antarctica B (*Uppenberg, Hansen, & Patkar, 1994) are two prominent cases of this phenomenon. Besides, lipases and esterases consensus motifs described by ProSite database (Hofmann, Bucher, & Falquet, 1999) are very close. Lipases were later defined as enzymes capable of hydrolyzing carboxyl esters of long-chain acylglycerol ($\geq$10 carbon atoms), while esterases hydrolyze carboxyl esters of short-chain acylglycerol ($\leq$10 carbon atoms). However, as both enzymes could show a broad substrate specificity, both criteria should be considered (Chahinian, Nini, & Boitard, 2002; Verger, 1997).

A novel approach has also been proposed by Fojan et al. (2000) to distinguish between lipases and esterases on the basis of the study of the amino acid composition and protein surface electrostatic distribution (Fojan,

Jonson, Petersen, & Petersen, 2000). Although, cutinases usually catalyze the hydrolysis of ester bonds in cutine polymers, they could be considered as intermediates between lipases and esterases because they are also capable of hydrolyzing long-chain and short-chain triglycerides without requirement of interfacial activation.

Another more recent report focused on a new classification to distinguish between lipases and esterases (Ben Ali, Verger, & Abousalham, 2012). From this report, the authors have confirmed that there are several unsuccessful attempts that are aimed at differentiating "lipases" from "esterases" by using various criteria based on the first substrate used chronologically, primary sequence comparisons, some kinetic parameters, or some structural features. Further, Ben Ali et al. (2012) have proposed a new classification of esterases on the basis of various criteria in particular those of physico-chemical, chemical, anatomical, or cellular nature.

Study of New Feruloyl Esterases to Understand Lipase Evolution

Our understanding of lipolytic enzymes evolution in plants is far from complete. The precise pattern of evolution of these enzymes remains to be elucidated. Further researches are needed in order to better understand the evolutionary and functional shifts, for instance, of lipases and feruloyl esterases (Sánchez-González, Blanco-Gámez, Parra-Saldívar, Mateos-Díaz, & Estrada-Alvarado, 2012).

The feruloyl esterase A from *Aspergillus niger* (AnFaeA) displays an α/β hydrolase fold and a catalytic triad similar to that found in fungal lipases and in particular it shows an overall fold similarity with *Rhizomucor miehei* and other related fungal lipases. It was suggested that there is an ancestral function (lipase), which had shifted, with molecular adaptation leading to a novel enzyme (type-A feruloyl esterase) (Levasseur, Gouret, & Lesage-Meessen, 2006).

The discovery of new feruloyl esterases from different origins could lead to get new insights into the evolutionary pathways of these enzymes and into new possibilities of directed evolution of related enzymes in particular lipases.

The study of new feruloyl esterases from a prokaryotic alkaliphile microorganism (*Bacillus flexus*) in the report of Sánchez-González et al. (2012) could be the starting point for new discoveries on feruloyl esterase and lipase evolutionary relationships.

ALLUSION TO PLANT PHOSPHOLIPASES EVOLUTION

Phospholipases C (PLCs) and in particular phosphoinositide-specific phospholipases C (PI-PLCs) have been identified in many eukaryotes from yeast to mammals (Kadamur & Ross, 2013; Yoko-o, Matsui, Yagisawa, Nojima, Uno, & Toh-e, 1993), while simplified PI-PLCs have also been present in bacteria (Wei, Zenewicz, & Goldfine, 2005). On the other hand, the *Arabidopsis* genome was revealed to contain six putative nonspecific phospholipases (NPC1-NPC6) with sequence homology and similarities to bacterial NPC (Wang, 2001). Therefore, the identification of PLCs (PI-PLCs and NPCs) in eukaryotes and prokaryotes species (Pokotylo, Pejchar, Potocky, Kocourkova, Krckova, Ruelland, Kravets, & Martinec, 2013; Pokotylo, Kolesnikov, Kravets, Zachowski, & Ruelland, 2014) with sequence homology and similarity indicates the probable common evolutionary origin of these enzymes (Pokotylo, Kolesnikov, Kravets, Zachowski, & Ruelland, 2014).

The *Arabidopsis* genome contains nine PI-PLCs, AtPLC1-AtPLC9 (Zheng, Liu, Li, Shang, Zhou, & Sun, 2012). Based on the sequence alignment and phylogenetic comparison, *Arabidopsis* and rice *PI-PLC* genes were likely derived from the single origin through duplications (Singh, Kanwar, Pandey, Tyagi, Sopory, & Kapoor, 2013; Tasma, Brendel, Whitham, & Bhattacharyya, 2008; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016). The gene pairs of *AtPLC4 /AtPLC5* and *AtPLC8 / AtPLC9* were probably duplicated in a tandem manner (Tasma, Brendel, Whitham, & Bhattacharyya, 2008; Hong, Zhao, Guo, Kim, Deng, Wang, Zhang, Li, & Wang, 2016).

SUGGESTED STRATEGY FOR EVOLUTIONARY HISTORY

In the present study, our aim is to understand the natural history of plant enzymes with lipolytic activity among those of other living organism. For this end, the following strategy could be adopted to fulfill the evolutionary history of plant lipolytic enzymes. First, we could study the distribution of lipolytic enzymes, namely their families' classification, in the major super kingdoms of life. Databases could be queried to retrieve these enzymes and multiple sequence alignments could be performed to detect highly conserved residues. Further, we could identify several motifs that are conserved within

the different families of lipolytic enzymes. Also, phylogenetic trees could be constructed to percolate evolutionary relationships. By employing the results and observations of such study, we could propose a model to elucidate the evolutionary history of plant lipolytic enzymes.

Such strategy was employed in the report of Udaya Prakash et al. (2010) for depicting evolution, and homology conservation, as well as for identifying unique sequence signatures in glycoside hydrolase 19 (GH19) family chitinases.

In fact, after they have analyzed the distribution of GH19 chitinase family proteins in the three major super kingdoms of life, multiple sequence alignment of these enzymes resulted in delineating regions of clear consensus within the chitinase family. Besides, the identified motifs would be utilized as markers to define uncharacterized chitinase candidates. Their study shed light on the phylogenetic distribution and they identified unique sequence signatures that define GH19 chitinase family of proteins.

Their study supports the horizontal gene transfer theory that states that GH19 chitinase genes are transferred from higher plants to bacteria. Indeed, the estimation of evolutionary distance between chitinase identified in plants and bacteria revealed that the flowering plants are more related to chtitinase in actinobacteria than that of identified in purple bacteria, i.e., the chitinases identified in actinobacteria are evolutionary more related to plant chitinases than that of the chitinases identified in purple bacteria. Based on their results, they proposed a model to elucidate the natural history of GH19 family chitinases (Udaya Prakash, Jayanthi, Sabarinathan, Kangueane, Mathew, & Sekar, 2010).

Other publications have reported new insights into the evolutionary history of plant enzymes, such as the report of Jia et al. (2015) for plant sorbitol dehydrogenase, and the report of Skamnioti et al. (2008) for evolutionary history of cutinase from Ascomycetes and *Magnaporthe grisea.* The recent report of Wang et al. (2018) that analyzed the *Lipase3* gene family in five plant species and its possible distinct evolutionary origins, and interestingly the report of Bowman et al. (2017) who highlighted land plant evolution garnered from the *Marchantia polymorpha* genome. On the other hand, the recent report of Fan et al. (2018) that compare chloroplast genomics of dipsacales species and insights into adaptive evolution; all of these recent reports could be useful strategies to study evolutionary history for whatever enzymes, e.g., plant lipolytic enzymes.

CONCLUSION

Although the term of evolution is a big issue in biology, there is no full evidence to confirm such evolution between all the living organisms. Each resemblance between lipolytic enzymes, for instance, in all species of the kingdoms of life do not mean the presence of common ancestor. We think that every resemblance of any biomolecule, e.g., lipolytic enzyme, from different sources of living organisms is rather an indication of the ability to survive and interact together such as in the case of plant-pathogen interaction involving lipolytic enzymes.

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