Recent Developments in Plant Biotechnology

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Progress in Lipidomics and Proteomics

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Cambridge
Scholars
Publishing

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This book first published 2019

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-3770-6
ISBN (13): 978-1-5275-3770-5

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CHAPTER ONE

PROMISING INSIGHTS INTO PROTEOMIC AND LIPIDOMIC FEATURES IN SOME CEREALS

Abstract

Cereals constitute the main source of food; they are also nutraceutical with potential therapeutic effects as they contain numerous functional nutrients including essential proteins and lipids.

Although, cereals are often exposed to abiotic stresses such as heavy metal (e.g., cadmium (Cd)) and salt (NaCl) contamination, identified stress-related genes could be explored using omic tools to mitigate such problems. Characterization of novel genes from halophyte/glycophyte cereal encoded proteins with interesting structure and function such as enzymes are among the recent advances in cereal biotechnology. Identification of these novel genes would be done by genomic approaches, i.e., global transcriptomic analysis and production of expressed sequence tags (ESTs), as well as by functional proteomics.

In addition to proteins, cereal lipids are also of increasing interest in the biotechnology of cereals due to their multiple applications such as in medicine, cosmetic and food technology. Oiled cereal seeds could be potential sources of antioxidants, dietary polyunsaturated fatty acids, and biomolecules such as eicosanoids (prostaglandins and lipoxins) indispensable for health.

The chapter covered an important emerging field of research concerning the use of cereal to produce compounds of high value, i.e., proteins and lipids with applications for both human health and agricultural purposes.

Keywords: *Avena sativa*, lipids, enzymes, proteins, stress, valorization, biotechnological applications.

1. Introduction

Cereals are among the most staple foods worldwide. They are edible and have versatile nutrient compounds that could be used as either foods or nutraceuticals. Cereals are recognized to be useful for a healthier world. The assessment of the vital functions of cereal components is important to satisfy targeted objective uses. Oils, enzymes and other biomolecules found in cereals could be valorized in many fields, e.g., agronomics, nutraceuticals, food, and biotechnological applications. To this end, oat, for instance, which is undervalued in comparison with the other staple cereals (wheat, barley, and rice), has an interesting biomolecular composition especially lipids, proteins, fibers and antioxidants indispensable for health. The valorization aspects of oat (*Avena sativa*) are highlighted in many reports of Ben Halima et al. (2014a, b; 2015a, b; 2016; 2019 and unpublished data). From these reports, oat lipids potentially exhibit protective effects against deltamethrin (a pesticide frequently used in Tunisian agricultural areas), that causes reproduction damage in animal models, i.e., male mice (Ben Halima et al., 2014a). Moreover, oat enzymes mainly amylases and chitinases were identified by functional proteomics (Ben Halima et al., 2016; Ben Halima, 2019). In addition, we found an oat fraction with an effective amylolytic activity that could be used as a potential additive in bread to ameliorate its characteristics (Ben Halima et al., 2015b). For more information on oat lipids and nutriment compounds valorization for potential uses in industrial applications, refer to Ben Halima et al. (2015a).

Oat could be valorized in agronomics as it may tolerate abiotic stresses such as from salt (NaCl) and cadmium (Cd) contamination (Ben Halima et al., 2014b; unpublished data). In fact, cereals like all the other living organisms could be affected directly or indirectly by environmental pollution and contamination.

Abiotic stresses imposed by salinity and heavy metals pose serious threats to the growth and productivity of crops worldwide. It is ideal to develop plants with salinity and heavy metals tolerance.

Identification, characterization, and isolation of novel genes responsive to environmental stresses in economically important crops such as oat would be promisingly done by genomic approaches to understand their tolerance and regulatory mechanisms. Among the promising approaches, global transcriptome analysis and the production of expressed sequence tags (ESTs) are considerable, as they are relatively cost-effective, efficient and

rapid for identifying and discovering novel genes involved in the regulation of abiotic stresses (Sreenivasulu et al., 2007).

Structural insights of uncharacterized proteins (RNA Recognition Motif, annexin, enzymes, etc.) from glycophyte/halophyte plants may be considered as a predicted model to better characterize these proteins in cereals.

An attempt to summarize some own reports on recent developments in cereal biotechnology and in particular proteomic and lipidomic feature of oat (*Avena sativa*) is the focus of the current chapter.

2. Proteomics features in some cereals

2.1 Mitigation of abiotic stresses

2.1.1. Wheat Annexins

Wheat is one of the most important cereals worldwide as it is the most widely consumed and grown crop. The annual world production of wheat is estimated at 700 million tons (USDA, 2014**)**. Environmental stresses, especially abiotic ones, negatively affect all cereal production including wheat (Lobell and Field, 2007).

Annexins are proteins with suggested functions in response to environmental stresses and signaling during plant growth and development. They can be considered as an evolutionary conserved multigene family of calcium-dependent phospholipid binding proteins that play important roles in stress resistance and plant development (Xu et al., 2016).

A recent report on the characterization of two annexin genes identified from Tunisian durum wheat varieties (*Triticum turgidum* L. *subsp. Durum*, cultivar Mahmoudi) has been achieved by *in silico* approach (Harbaoui et al., 2018). The primary and secondary structures of the resulting proteins (annexins) were analyzed, as well as their homology 3D-model structures. Multiple sequence alignments and the evolutionary relationships between the two identified annexins and their homologs were also studied. Insights into conserved protein motifs analysis and subcellular location prediction were performed to understand their eventual molecular function. In fact, some of the studied annexin motifs were found to be associated with calcium-dependent phospholipid binding/calcium ion binding function. Furthermore, the two newly annexins isolated from durum wheat might

play an active role in modulating plant cell responses to abiotic stress. These annexins could confer tolerance to various abiotic stresses when tested on yeast cells (Harbaoui et al., 2018).

2.1.2. *Aeluropus littoralis* stress-related genes/proteins

Halophytes are plants that are able to cope with high concentrations of salt so that they can complete their life cycle under at least 200 mM NaCl (Flowers et al., 2015). These plants that adapt to grow in saline environments offer important genetic resources (novel genes that code for functional proteins) involved in the salt stress adaptation. These particular novel genes can be isolated and successfully transferred y to glycophytes (plants growing in non-saline soils).

Aeluropus littoralis is a halophyte monocotyledonous plant belonging to the same family as wheat. A novel stress tolerance gene from *A. littoralis* designated as *AlSRG1* (*A. littoralis* Stress-Related Gene) has been studied by Ben Saad et al. (2018). *AlSRG1,* encoding a small RNA-binding protein could confer salt and drought tolerance in transgenic tobacco (Ben Saad et al., 2018). *AlSRG1* is a single copy gene with unknown function, isolated from an SSH (Suppression Subtractive Hybridization) cDNA library prepared using RNA extracted from the root of salt-stressed (300mM NaCl during 15 days) *A. littoralis* plants (Zouari et al., 2007). The characterization of the novel gene, *AlSRG1,* was performed based on bioinformatics analysis. In fact, phylogenetic and conserved domain prediction identified the protein *AlSRG1* as an uncharacterized protein of unknown function. The conserved domain database revealed that *AlSRG1* contains an RRM-SF superfamily domain. The RNA Recognition Motif (RRM), also known as RNA binding domain (RBD) or Ribonucleoprotein domain (RNP) was highlighted in *AlSRG1* primary, secondary and tertiary structures with the two conserved motifs RNP1 and RNP2 being part of it. These two conserved motifs contain the active site for RNA-binding. In addition, a 3D-model of the conserved domain could be built from known X-ray structures and sequence alignments. RRM is a highly abundant domain in eukaryotes found in proteins involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export, and RNA stability. The analysis with quantitative RT-PCR revealed higher *AlSRG1* transcript accumulation under salt stress and PEG-induced osmotic stress (Ben Saad et al., 2018). Taken together, the results of this study (Ben Saad et al., 2018) showed that this novel stress-tolerance gene might play an important positive modulation role in abiotic stress tolerance.

2.1.3. Effect of Cd and NaCl stresses on oat

The effects of cadmium (Cd) and salt (NaCl) stress on seed germination, seedling growth and antioxidant system of oat (*Avena sativa* L.) have been studied by Ben Halima et al. (2014b; unpublished data).

In fact, Cd is a major heavy metal pollutant, which is highly toxic to cereals. Vast agricultural areas worldwide could be contaminated with Cd from industrial wastes. In addition, abiotic stresses imposed by drought and salinity pose serious threats to the growth and productivity of crop plants worldwide.

Therefore, it is ideal to develop plants tolerant to Cd and salinity. Oat (*Avena sativa*) is an important crop in North America and northern Europe. The potential of *Avena sativa* plant may be reached as a genetic resource to improve salt and Cd tolerance in plants (Ben Halima et al., 2014b; unpublished data).

When *Avena sativa* plants were exposed to various concentrations of Cd and NaCl, they were found to express Cd and NaCl tolerance and accumulation *in planta*. Although high concentrations of Cd and NaCl could inhibit plant growth and reduce chlorophyll content, low and moderate concentration of such stresses could be tolerated by *Avena sativa* due to the efficiency of its antioxidant system. Indeed, abiotic stresses are the cause of imbalance in the oxidant/antioxidant system of plants. Measurements of the root and leaf endogenous Na^+, K^+ and Cd levels in *Avena sativa* plants stressed by Cd and NaCl compared to control conditions, showed an evident higher Na^+ and Cd accumulation in roots. Furthermore, the steady-state levels of transcripts of three-related genes (catalase, manganese superoxide dismutase and ascorbate peroxidase) were found at a significantly higher level in plants stressed by Cd or NaCl than control plants.

Taken together, these results of Ben Halima et al. (2014b; unpublished data) showed that oat (*Avena sativa*) could be a potentially useful candidate gene for engineering Cd and salt tolerance in cultivated plants.

3. Case study: Oat (*Avena sativa* L.)

Oat (*Avena sativa* L.) is a member of the *Poaceae* family (*Gramineae*), is one of the most cultivated crops, and is a promising crop plant worldwide. Oats are cultivated for grain, fodder, straw, and feed (Särkijärvi and

Saastamoinen, 2006) over more than 9 million hectares globally (FAO, 2014). Wild oats are diploid, but those cultivated (*Avena sativa*) are hexaploid with an estimated 1C genome size of 13.23pg, corresponding to about 13000 Mbp (Bennett and Smith, 1976). The common oat (*Avena sativa* L.) is an allohexaploid (2n=6x=42) crop species. Thus, oats have a large genome, which remains to be assessed. Complex polyploidy genomes such as oats need substantial contributions to the bioinformatics pipeline and drafting of the genome.

3.1. Glycoside hydrolases

3.1.1. Generalities

The abundance and the diversity of oligo- and polysaccharides provides a wide range of biological roles attributed either to these carbohydrates or to their relevant enzymes, i.e., the glycoside hydrolases (GHs). Carbohydrates could be in the form of mono-, di-, oligo- and polysaccharides and could be subjected to biocatalysis by GHs, enzymes hydrolyzing these sugars.

The biocatalysis by this family of enzymes is highly attractive for the generation of products used in potential application, e.g., pharmaceuticals and food industries. It is thus very important to extract and characterize such enzymes, particularly from plant tissues. Generally, GHs hydrolyze glycosidic bonds through the acid-base–assisted catalysis, deploying either double- or single-displacement mechanisms, leading to retention or inversion of anomeric configuration, respectively.

Obviously, starch is the most abundant grain component in most cereal cultivars such as wheat, barley, and oat, but it is also known as a storage polysaccharide present in the other major starch-containing crops worldwide like in pulses such as peas and beans, or in roots and tubers, such as cassava and potato. The starch that serves as an important reserve of energy in plants has provided dietary carbohydrates for animals and man for a long time. β-amylase catalyzes the liberation of maltose from the non-reducing ends of α-1,4-linked glucans and is, therefore, required for starch breakdown particularly during plant germination. The β-amylase isoforms conserved two Glu residues assigned as the “putative” catalytic residues, which would act as an acid and base pair in the catalytic process. A similar core $(\beta/\alpha)_8$-barrel architecture was found in the predicted oat β-amylases with a specific location of the active site in a pocket-like cavity structure made at one end of this core $(\beta/\alpha)_8$-barrel domain (Ben Halima et al., 2016). This suggests accessibility of the non-reducing end of the

substrate towards the *β*-amylase isoforms and thus confirming that they are exo-acting hydrolases (Ben Halima et al., 2016; Ben Halima, 2019).

Chitin is a polysaccharide, which is an important structural component of insects, and other invertebrates such as crustaceans, mollusks, and nematodes. Chitinases are, thus, an interesting type of glycoside hydrolytic enzymes that need to be further characterized in many organisms, particularly in cereals. Certainly, chitinases have found promising applications in diverse areas ranging from academic to industrial scales such as in medicine, agriculture and food industries. One promising feature of these applications includes growth inhibition of pathogenic fungi in industrial food processing. The catalytic domain of predicted oat chitinases adopts an *α*-helix-rich fold, stabilized by 3 disulfide bridges in accordance with other plant class I chitinases (Ben Halima, 2019; unpublished data).

3.1.2. *β*-amylases and chitinases

Since the amino acid sequence of oat *β*-amylase is not available in the protein data bank, an attempt was made to identify the catalytic amino acid residues as well as the 3-D structure of the enzyme by bioinformatic tools to define/predict biochemical properties of a soluble oat extract. The primary aim of the study by Ben Halima et al. (2016) was to identify a new sequence of exo-amylase through mass spectrometry and bioinformatic tools and to get insight into the oat genome. The characterization of glycoside hydrolases, for instance, GH14 family *β*-amylases and GH19 family chitinases, in oat seedling extract (Ben Halima et al., 2016; Ben Halima, 2019; unpublished data) may be considered as a further step in the characterization of these enzymes in plants.

A predicted *β*-amylase from oat seedling extract cDNA (1074 bp) corresponds to a 357 amino acid residue protein of a theoretical molecular weight of 41 kDa and with a theoretical isoelectric point of 4.84 (Ben Halima, 2019).

Moreover, a predicted oat chitinase cDNA (888 bp) corresponds to a 295 amino acid residue protein of a theoretical molecular weight of the protein of 31 kDa and with a theoretical isoelectric point of 9.10 (unpublished data). These two proteins are named AsBAMY1 and AsChi1, for *Avena sativa* *β*-amylase and chitinase, respectively (Ben Halima, 2019; unpublished data).

The sequencing of the *A. sativa* genome (drafting as well as complete sequencing) will certainly accelerate the identification of other catalytic enzymes with applications in biotechnology.

3.2. Lipidomics

In its large-scale concept, lipidomics refers to the study of the structure and function of the complete set of lipids in biological systems as well as their interaction with other lipids and metabolites including proteins (Wenk, 2005; Rolim et al., 2015; Gugiu, 2017). Indeed, lipidomics is a relatively new field, related to the advancement of high technologies of separation such as liquid chromatography (LC) and mass spectrometry (MS). Therefore, lipidomics (Wenk, 2005; Rolim et al., 2015) is developed later than proteomics along with the newly developed technologies (MS and other relevant technologies that allow the investigation of structural and biological diversity of lipids) (Fahy et al., 2011). There is a so-called Lipid Maps Structural Database (LMSD), which is the main database of lipids that contain the structure and annotation of biologically relevant lipids (Sud et al., 2007). In fact, lipids are biomolecules with complicated structural variability and can be classified into polar or neutral (nonpolar) lipids. The polar lipids include phospholipids, glycolipids, and sphingolipids. The nonpolar lipids include triacylglycerols and cholesterols. Lipids 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. Moreover, lipids could be classified based on their chemical structure; thus, there are so many classes of lipids with precise nomenclature (Fahy et al., 2005; Fahy et al., 2009). These lipids could be either abundant lipids or degraded lipid metabolites such as fatty acids (FA) and mono/di-glyceride (MG, DG) (Han and Gross, 2005).

Untargeted lipidomics aims to focus on measuring lipids whatever their classes maybe using omic workflows similar to proteomics (Washburn et al., 2001) and genomics (Yadav, 2007). However, it is worth to noting that targeted methods are normally used for certain lipids, mainly those of metabolites, specifically because of their low abundance (Han and Gross, 2005; Gugiu et al., 2006). Hence, targeted or untargeted MS analysis of lipids could be understood through lipidomics using either LC (Castro-Perez et al., 2010) or shotgun (Han and Gross, 2005) approaches coupled with tandem MS. Gugiu (2017) described the LC/MS methodology for lipid identification, which is similar to established omics workflows, e.g., proteomics (Washburn et al., 2001) or genomics (Yadav, 2007).

3.2.1. Characterization of oils and fats

Cereals constitute the main source of food. Cereal lipids are of increasing interest because of their multiple roles, e.g., in food technology and as potential sources of dietary polyunsaturated fatty acids (FUFAs) in significant amounts (Price and Parsons, 1974). Yet, oilseeds represent many versatile commodities due to their use essentially in food, feed, and medicine. Nevertheless, the total lipid content in cereals varies considerably. Furthermore, different parts of cereal seeds have different lipid contents. In wheat, for example, the germ contains about 25-30 % lipid while its endosperm has only 1 % lipid. However, considerable amounts of germ lipids of wheat are lost during milling (Galliard and Barnes, 1980).

3.2.2. Oat lipidomics

Compared to other cereals, the specific feature of *Avena sativa* L. grains is the high oil content, which could range from 3% to 18% of the grain weight in different cultivars (Banas et al., 2007; Frey and Holland, 1999; Peterson and Wood, 1997), and deposited mainly in the grain endosperm tissues (Price and Parsons, 1979; Youngs et al., 1977). However, most oat cultivars comprise about 5-6% of oil and 55-60% of the starch in the grain (Banas et al., 2007; Welsh, 1995; Doehlert et al., 2001), and high-lipid oat remains a potential oil crop (Heneen et al., 2009). Oats contain a wide range of active compounds, including avenanthramides, starches, hydrocolloid β-D-glucan, vitamins, saponins and other antioxidants (mostly phenolic esters) and a relatively high content of total lipids with a high percentage of UFAs (Kurtz and Wallo, 2007; Sur et al., 2008). Total lipids could reach 18% (Frey and Holland, 1999; Peterson and Wood, 1997), and about 41% of the groat lipids are triglycerides, while 5% are free fatty acids (Youngs, 1978). In fact, oats, apart from maize, comprise a high oil content used for a wide range of beneficial purposes. To this end, Ben Halima et al. (2015a) have reviewed oat lipids. On the other hand, oat lipid fraction was proven to be a protective agent against pesticide, e.g., deltamethrin causing reprotoxicity in male mice (Ben Halima et al., 2014a).

Studying lipolytic enzymes such as phospholipases from cereals would be very promising as these enzymes might play important roles in plant growth, development and stress response. In addition, these enzymes might find external applications such as in medicine and pharmaceuticals. Phospholipases D (PLD) and C (PLC) are among lipolytic enzymes that

hydrolyze the phosphodiester linkages of the head group of membrane phospholipids (Hong et al., 2016).

As mentioned above, 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 et al., 1982). Identifying and characterizing oat phospholipases are further research axis undertaken by Ben Halima et al. (unpublished data).

4. Conclusions

Proteins and lipids are important constituents of all living organisms in particular cereals. Tremendous progress has been made in recent years in various aspects of proteomes and lipidomes; however, our understanding of these proteins and lipids in cereals is far from complete. For example, oats are unique among the other cereal grains due to the high lipids content and their promising enzymatic activities, in particular, amylases, chitinases, and phospholipases. The investigation and understanding of the relationship of proteomes and lipidomes would be an outstanding feature in the recent development in cereal biotechnology. The mechanism of action of major phospholipases and their roles in lipid signaling in cereals remains to be elucidated. Further studies with bioinformatics analysis would be an interesting key to accelerate discovering novel proteins as well as lipids in cereals.

5. Acknowledgments

The author thanks the Tunisian Ministry of Higher Education and Scientific Research for facilities. The author is very grateful to Erasmus Nchuaji Tang, a researcher at the Department of Biochemistry, University of Yaounde I-Cameroon, for his help reading the manuscript.

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CHAPTER TWO

GLYCOSIDE HYDROLASES IN OAT (*AVENA SATIVA*) SEEDLING EXTRACT: NEW INSIGHTS FROM BIOINFORMATIC ANALYSIS INTO GH14 FAMILY *BETA*-AMYLASES AND GH19 FAMILY CHITINASES

Abstract

Oat (*Avena sativa* L.) seedling extract exhibited a high degree of catalytic activities. Bioinformatic tools were used to identify *β*-amylases and class I chitinases, the highly abundant enzymes in the oat seedling extract. These newly discovered oat enzymes are a member of the GH14 and GH19 family, respectively. Proteins in the *Avena sativa* seedling extract were separated by SDS-PAGE. 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, at least 6 and 4 predicted enzymes were identified and designated as *β*-amylases and chitinases, respectively. Insights into the structural characterization of each of the oat predicted enzymes were analyzed using *in silico* approaches.

The results provide a detailed view of the main components involved in catalysis in these two enzymes.

Keywords: *Avena sativa*; Glycoside hydrolases; Bioinformatic analysis.

1. Introduction

The use of additives in industrial food processing is of high interest as they could improve or preserve food products. The promising additives are enzymes especially glycoside hydrolases (GHs), for instance, amylases

and chitinases which could have beneficial impacts on glycoside substrates enriched foods such as bread flour and its derivatives, i.e., starch, maltose, *β*-dextrin, etc. These enzymes are also potential candidates for anti-pathogen agents, e.g., anti-insects and antifungal agents. Fungi are a prominent source of contamination of all groups of food including cereals, fruits, vegetables, meat, milk, and their products. They are involved in food spoilage by secreting highly poisonous mycotoxins (Filtenborg et al., 1996).

The classification of GHs is commonly based on substrate specificity, mode of action or amino acid sequence similarities (Henrissat and Davies, 1997). *O*-glycoside hydrolases are classified, for instance, on the basis of substrate specificities as recommended by The International Union of Biochemistry and Molecular Biology (IUBMB) and are expressed in the EC number with the given code EC 3.2.1.x, where x generally represents the substrate specificity (Henrissat and Davies, 1997).

Chitin, an insoluble polysaccharide of *β*-(1,4)-linked N-acetyl-D-glucosamine residues (Muzzarelli, 1999), is the main constituent of the cell walls of many fungal plant phytopathogens. It can be decomposed by chitinases (EC 3.2.1.14) and herein plant chitinases are expressed during plant growth as well as plant and phytopathogen interactions. Therefore, plant chitinases have a major role as pathogenesis-related (PR) proteins that are involved in defense responses of a plant against its pathogens (Kasprzewska, 2003). Most characterized chitinases are clustered into families 18 and 19 of the GHs based on primary structures similarities of their catalytic domains including class III and V and class I, II, IV, VI and VII chitinases, respectively (Henrissat, 1991; Henrissat and Bairoch, 1993). However, some chitinases have also been identified as belonging to families' GH23 and GH48 (Arimori et al., 2013; Fujita et al., 2006).

The glycan metabolism involved many kinds of carbohydrate-active enzymes (CAZymes), which are grouped into sequence-based families on the CAZY database (Lombard et al., 2014), and the structural fold, as well as the catalytic mechanism, are highly conserved within these families. The most important CAZymes that depolymerize carbohydrate polymers are GHs (Munoz-Munoz et al., 2017). GH18 and GH19 chitinases are extensively characterized. Those from GH18 were exemplified to adopt the retaining mechanism, producing *β*-anomers after hydrolysis, while, GH19 commonly adopt the inverting mechanism, producing *α*-anomers after hydrolysis (Ohno et al., 1996; Udaya Prakash et al., 2010; Junges et al., 2014).

Extracts from different higher plants, especially from cereals, were proven to have class I chitinase isoforms and those from oat seed extracts were demonstrated to be more effective towards *Penicillium roqueforti*, a major contaminating fungal species in food, as opposed to extracts of other cereal seeds (Sørensen et al., 2010).

Starch, a polymer of glucose consists of two primary components, amylose, and amylopectin. Amylose is a linear polymer of glucose with α-1,4 linkages, while amylopectin is a branched glucose polymer in which linear chains of α-1,4 are interlinked with α-1,6 linkages (Muralikrishna and Nirmala, 2005).

Current thinking is that starch degradation in plants would certainly require the concerted activity of GHs, especially amylases that break down cereal starch into glucose, which is then taken up as the substrate for the plant germination and growth.

β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2; CAZY family GH14) is an inverting GH (Bourne and Henrissat, 2001; Cantarel et al., 2009), which is distributed throughout many organisms such as in prokaryotes or in eukaryotes, i.e., higher plants including cereals. β-amylase is a crucial enzyme that contributes to the complete degradation of starch into metabolizable or fermentable sugars during the germination or malting of cereal grains. However, different isoforms of β-amylase may exist in the same plant extract; thus, distinct structures and functions are ascribed to β-amylases (Fulton et al., 2008). To this, Daussant et al. (1994) have highlighted the diversity of cereal β-amylase isozymes when determined their activity in the kernels of 29 cultivars and inbred lines of seven kinds of cereal belonging to four tribes of the *Gramineae* such as *Hordeum vulgare* L. and *Avena sativa* L.

The common oat (*Avena sativa* L.) is a member of the *Poaceae* family (*Gramineae*) and is a promising plant for the future. It is edible and beneficial thanks to its nutritional, medicinal and pharmaceutical uses and, hence, recognized to be useful for a healthier world (Ben Halima et al., 2015a). The cultivated oat (*A. sativa* L.) is an allohexaploid ($2n = 6\times = 42$) species with a large genome which is still to be fully assessed.

Complex polyploidy genomes such as oats need substantial contributions to the bioinformatics pipeline and drafting of the genome. The oat genome has not yet been fully sequenced and the majority of GHs, especially

sequences of amylases and chitinases from oat and other cereals, has not been well characterized.

One important oat (*Avena sativa* L.) *β*-amylase sequence has already been characterized by functional proteomics in our previous study (Ben Halima et al., 2016).

Though Sørensen et al. (2010) have tried to characterize oat class I chitinase, this chitinolytic enzyme has not been subjected to further biochemical characterization. In this study, extracts of oat seedling were identified as a potential food additive through the catalytic activity of highly abundant proteins from GH14 and GH19 family. The proteins in the oat seedling extracts are isolated and characterized. Aiming to understand the mechanisms of hydrolytic action of oat *β*-amylases (AsBAMYs) and chitinases (AsChiIs), the sequencing of peptides resulting from tryptic digestion allowed identifying EST and TSA sequences from that the AsBAMYs and AsChiIs genes were compared by computational bioinformatics analysis.

Since an amino acid sequence of one oat chitinase is available in the protein data bank, an attempt has been made to more characterization and identification of the catalytic amino acid residues as well as the 3-D structure 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 GH14 and GH19 family based on mass spectrometry and bioinformatic tools and so far, to get insights about the oat genome.

2. Materials and methods

2.1. Plant material and chemicals

Oat (*Avena sativa* L.) was used as plant material in which seeds extract containing GH family, especially GH14 and GH19 families, were the subject of this study. *A. sativa* seeds were purchased from a local market in Sfax, a coastal town in South-East Tunisia.

Chemicals used in this study were of reagent grade and were supplied by Invitrogen and Sigma Chemical Co. (St Louis, France).

2.2. Extraction of GHs from oat seedling

Oat seeds (*A. sativa*) germinated on wet tissue paper in a plastic box. They were grown in the dark for 5 days at room temperature. On day 10 after planting, oat seedlings were ground using mortar and pestle with 0.02 M sodium acetate buffer (pH 5.6), filtered through two layers of cheesecloth to remove large particles and the supernatant obtained was centrifuged at 15,000 × g for 20 min. The supernatant was used as an oat crude extract of GHs as well as start material for purification procedure.

As a crude enzyme, acetone was added to the oat crude extract (2:1; v:v), sample centrifuged at 14,000 × g for 15 min and the 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 GH activity.

For the purification procedure, the oat proteins were extracted as described above following a purification procedure of some steps of a novel oat β-amylase of 25 kDa according to the report of Uno-Okamura et al. (2004).

2.3. 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 et al., 2009, 2012; Fendri et al., 2009) or manually treated as follows. Gel bands were manually excised in a sterile laminar flow hood, transferred individually to 1.5 mL microtubes and then cut into cubes of roughly 1 mm^3. 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 et al., 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 flow rate 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.

Moreover, a total of 17,776 ESTs were downloaded from GenBank for the oat cultivar (cv) CDC Dancer and a description of such EST generation can be accessed at the GrainGenes database site (Beattie et al., 2008). In fact, all CDC Dancer ESTs are from the seed and there are also 7097 oat ESTs from cv Ogle in GenBank, but are from a range of tissues and do not include enough seed ESTs to attempt seed protein sequence assemblies.

ESTs were assembled with the Seqman module of the Lasergene suite (DNAstar, Inc.) and all resulting contigs composed of three or more ESTs were annotated for the closest matching sequence of monocots (Anderson, 2014). 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 the 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.

2.4. *In silico* analysis

2.4.1. Retrieval of protein sequences

The amino acid sequences from the GHs that permitted comparison with the *de novo* sequencing of oat GH14 and GH19 proteins families were retrieved from the protein database of National Center for Biotechnology

Information (NCBI, http://www.ncbi.nlm.nih.gov/protein/). The sequences were saved in FASTA format. The *in silico* approach steps followed in this study consisted essentially on analysis of proteins from oat seedling by LC/MS/MS, MASCOT Search and Swiss-Prot Database as well as EST and TSA_*Avena sativa* Databases. Then, *de novo* sequencing of GH14 and GH19 proteins families from oat (*Avena sativa*) seedling were realized with structural characterization. This involved the prediction of primary and secondary structures through comparison with the retrieved protein sequences of GH14 and GH19 families and homology modeling analysis with some selected oat enzymes.

2.4.2 Sequence analysis

Bioinformatic analysis of the *A. sativa* peptide sequences, ESTs, genomic sequences and deduced protein sequences were performed using the following tools; Multiple sequence alignment was performed using the ClustalW algorithm (Thompson et al., 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 contains 25,400 entries (AM071411-CN180783) using BLAST (Altschul et al., 2005).

Primary structure analysis 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 et al., 2005; Gasteiger et al., 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 signal P 4.1 application (Petersen et al., 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 et al., 2013) were used.

Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) package version 7 (Kumar et al., 2016). The program MUSCLE (Edgar, 2004), implemented in MEGA7 package, was used to perform multiple alignments of amino acid sequences of AsBAMY

and AsChi and their homologous for phylogenetic analysis. The evolutionary history was inferred using either the Neighbor-Joining method (Saitou and Nei, 1987) or the unweighted pair group method with arithmetic mean (UPGMA) method (Sneath and Sokal, 1973). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 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).

2.4.3. Conserved protein motifs analysis and subcellular location prediction

Conserved protein motifs of the protein sequences from oat were analyzed using Multiple Expectation Maximization for Motif Elicitation (MEME) v.4.11.4 (Bailey and Elkan, 1994; Bailey and Gribskov, 1998; Bailey et al., 2015) (http://meme-suite.org) with the number of different motifs as 10, 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 et al., 2014). The mapping between Pfam (http://pfam.xfam.org) analysis and Gene Ontology (GO) is provided by InterPro (Sangrador-Vegas et al., 2016).

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

2.4.4 Secondary structure prediction

The prediction of the protein secondary structure was performed using The PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs. ucl.ac.uk/psipred/).

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

2.4.5. Molecular and homology modeling

PDB file of oat protein was generated by Swiss-Model server (http://swissmodel.expasy.org/). In order to build a model of a protein domain, multiple sequence alignments were performed between full-length chitinase or *β*-amylase protein sequence and another protein domain sequence in these databases. To build the model of the oat protein with more homology, high-resolution structure of protein model in Swiss model server was selected as a template.

3. Results and discussion

3.1. Results

A previous study has demonstrated the presence of both activities of chitinases and glucanases in the apoplastic compartment of oat (*Avena sativa* L.) primary leaves of 10-day old plants (Fink et al., 1988). Furthermore, Uno-Okamura et al. (2004) have shown the presence of a novel type of *β*-amylase from oat (*Avena sativa*) seedlings. Their results, especially on the catalytic properties of the purified enzyme (*β*-amylase) and the molecular size (approximately 25 kDa) are certainly different from those of known amylases obtained from *Gramineae* caryopses.

Taken together, these findings as well as the fact that oat seed extracts have previously been denoted for their catalytic activity of highly abundant class I chitinases (Sørensen et al., 2010), have proven the presence of many sequences of chitinases (GH19) and *β*-amylases (GH14) in 10-day old oat seedlings extract. Through LC/MS/MS technique and bioinformatics tools, novel amino acid sequences of both activities of oat *β*-amylases and chitinases could be reconstructed, in spite of the only one previously deposited sequence of oat chitinase in GenPept (P86181.1).

3.1.1. Extraction and identification of *A. sativa* seedling proteins from GH14 and GH19 families

Oat (*Avena sativa* L.) seedlings from 10-day old plants were used as starting material for extracting proteins from GH14 and GH19 families, i.e., *β*-amylases and chitinases. In fact, this extract was also enriched in amylolytic activity such as *β*-amylases as described by previous reports (Ben Halima et al., 2016; Ben Halima et al., 2015b). 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 1**).

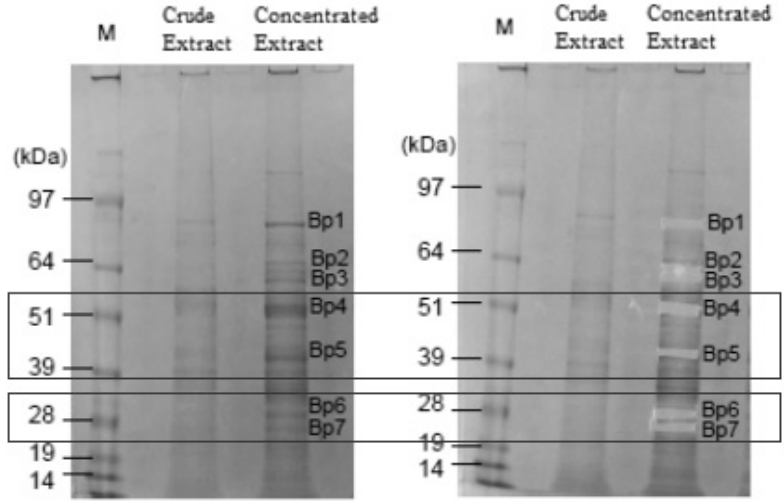

Figure 1. *The SDS-PAGE analysis of the 10-day old oat (Avena sativa) seedling extracts. Bp, Band of protein and M, molecular mass markers. The gel was stained with Coomassie blue. Bp 4 and 5 were matched to β-amylase according to Swiss-Prot database after in situ trypsin digestion and LC/MS/MS analysis. Bp 6 and 7 were matched to chitinase according to Swiss-Prot database after in situ trypsin digestion and LC/MS/MS analysis.*

An aliquot of the same oat extract was subjected to purification procedures of a glycoside hydrolase. The glycoside hydrolase activity recovered from oat seedlings was purified by precipitation with ammonium sulfate, and by chromatography on a gel filtration column (Superdex-75pg) in the FPLC system. To detect starch-degrading activity, the iodine method (Jones and Varner, 1967) was used and the activity was determined by monitoring the decrease in absorbance at 700nm of the starch-iodine complex and expressed as a relative starch-degrading activity. On the Superdex-75 column, a single peak of amylase activity was detected (data not shown). After this final purification step, SDS-PAGE with Coomassie blue staining revealed that the protein preparation migrated as a single band. Two aliquots of the pooled peak from Superdex-75 elution was analyzed by SDS-PAGE followed by a Coomassie blue staining step and the two resulting bands of proteins were excised from the preparative gel (data not