Journal of Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Label-free quantitative proteomic analysis of drought stress-responsive late embryogenesis abundant proteins in the seedling leaves of two wheat (*Triticum aestivum* L.) genotypes

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ARTICLE INFO

Keywords:
Drought
Heat- and acid-stable protein
LEA protein
Proteomics
Wheat

ABSTRACT

Late embryogenesis abundant (LEA) proteins are highly hydrophilic proteins with key roles in environmental stress responses. In this study, we performed the first survey of the LEA proteome in seedling leaves from two wheat genotypes subjected to drought stress, i.e., Shaanhe 6 (SH, drought-tolerant) and Zhengyin 1 (ZY, drought-sensitive). After isolating the LEA subpopulation by treating total soluble proteins with heating combined with 1% trichloroacetic acid treatment that was assessed by Western blotting of dehydrins, label-free proteomic analysis identified 38 LEA proteins or homologues belonging to seven LEA subfamilies in the two genotypes. The abundances of over half of the LEA proteins changed significantly after drought stress and they were involved in protection against drought, with at least 20 in SH and 14 in ZY. We found that the common differentially expressed LEA proteins increased in abundance more in the SH genotype compared with the ZY genotype, and six LEA proteins were significantly upregulated exclusively in the SH genotype, which may contribute to higher drought tolerance in SH. We also identified 221 non-LEA proteins from 12 functional categories. Our results provide a deeper understanding of the LEA expression patterns in response to drought stress in two wheat genotypes.

Significance: We identified 38 LEA proteins or homologues from different LEA families in two wheat genotypes, thereby indicating the complex and versatile protective roles of LEA proteins in drought stress resistance. Moreover, the abundance of differentially expressed LEA proteins increased more in the SH genotype compared with the ZY genotype, and several LEA proteins with significant upregulation only in the SH genotype may contribute to its higher tolerance of drought stress. 221 non-LEA proteins were differentially accumulated in at least one of the SH and ZY genotypes. They are involved mainly with 12 biological functions and they might explain different drought responses of the two genotypes. The differentially expressed LEA and non-LEA proteins may be potential markers of drought tolerance to facilitate wheat breeding, particularly those that were specifically upregulated in the SH genotype, or with opposing expression patterns in the two genotypes.

1. Introduction

Drought, one of the most adverse environmental factors, significantly impair the growth and consequently restrain productivity of plants such as the most important cereal crops, including wheat. Plants undergo various physiological, biochemical, and molecular changes in order to cope with the possible damage caused by unfavorable conditions [1–3]. Many specialized proteins are differentially expressed in plants during adaptation to stress, where they generally have roles as osmolytes, signaling molecules, ion homeostasis molecules, reactive oxygen scavengers, proteins with responses to abscisic acid (ABA), pathogen-related proteins, heat shock proteins, late embryogenesis abundant (LEA) proteins, and chaperones [4–7]. In particular, LEA proteins have been studied for many years because they accumulate in abundance in plant desiccation-tolerant structures such as seeds, as well as being induced in vegetative tissues in response to drought, extreme temperatures, and salinity, or after the exogenous application of ABA, even in bacteria and anhydrobiotic invertebrates [8–11]. However, the

Abbreviations: TES, *N*-[Tris (hydroxymethyl) methyl]-2-aminopropanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, DL-Dithiothreitol; GRAVY, grand average of hydropathicity; ASH, ascorbic acid; GSH, glutathione

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http://dx.doi.org/10.1016/j.jprot.2017.09.016
Received 21 February 2017; Received in revised form 27 September 2017; Accepted 29 September 2017

LEA proteome patterns have not been investigated in the seeds or vegetative tissues of wheat during drought stress.

LEA proteins are divided into at least six different groups based on their amino acid sequence similarities and the presence of repeated sequence motifs found in a publically available database (LEAPdb) [12]. Much of the evidence indicates that the expression of LEA proteins is linked to the acquisition of dehydration tolerance [8,13,14], although their precise cellular action remains obscure. Nevertheless, *in vitro* experiments have shown that different LEA proteins potentially have protective functions in water retention during dehydration, radical and ion scavenging, the stabilization of enzymes, proteins, and membranes, interactions with RNA and DNA, or their combinations [14,15]. LEA proteins may exhibit structural transitions when dried or associated with phospholipids [16–18].

About 170 LEA proteins are found in the *Triticum* plants listed in the latest UniProt release. Proteome-wide approaches are necessary to determine the physiological roles of LEA proteins in wheat by identifying the entire LEA profile during the response of wheat to drought stress, thereby understanding how LEA proteins might confer different levels of drought stress tolerance in various wheat genotypes. However, diverse LEA proteins profile have not been reported in the proteomics data obtained for wheat seeds or vegetative tissues in response to dehydration stress and only individual LEA proteins have been studied instead [19–22], although many studies have shown that the abundance of LEA proteins is low in the complex wheat proteome. Moreover, the low capacity of mass spectrometry (MS) for resolving and fragmenting large numbers of peptides can hinder the identification of proteins in complex samples by MS. Proteins with low abundances that are located in very specific subcellular compartments, or that are only present at certain stages of plant development, may only be detected if subpopulations of specific enriched proteins are analyzed [23]. Thus, reducing the complexity of samples before MS analysis is important for the successful identification of the wheat LEA proteome.

Most LEA proteins are highly hydrophilic and low complexity proteins, which contain a high proportion of charged and polar amino acids, and they have a largely unstructured conformation in the hydrated state. These features contribute to their tolerance of heat and acidity [10,24,25]. Disorder predictions for whole genomes have shown that nearly one-third of all eukaryotic genes encode proteins that are entirely or partially disordered [26]. These proteins are defined as intrinsically disordered proteins (IDPs). Most LEA proteins are intrinsically disordered proteins, which may exhibit mainly α-helical structures during drying or after binding to target molecules such as membranes [15]. Hydrophilins are defined as a group of proteins with a Gly content > 6% and a hydrophilicity index > 1 [25]. Moreover, it has been estimated that hydrophilins account for approximately 0.2% of the total proteins in a given genome. Most LEA proteins are also members of the hydrophilins group. These evidences suggest that heat and acid stability is not an exclusive feature of the LEA proteins found in plants. However, LEA proteins have been isolated from the seeds or radicles of some plant species, as well as invertebrates, according to proteomic identification based on their heat tolerance [11,23,27,28]. Moreover, a more effective strategy for detecting LEA proteins in the heat-soluble proteome has been investigated, which involves treating total protein extracts from *Arabidopsis thaliana* seeds by heating, followed by 3% trichloroacetic acid (TCA) treatment in order to obtain a soluble fraction that is highly enriched in LEA proteins, and this system is suitable for the large-scale identification of LEA proteins in seeds [29].

In the present study, we identified the LEA proteins present in the seedling leaves of two wheat genotypes in response to drought stress by using a label-free proteomics approach based on the MaxQuant algorithm. We identified 38 and 31 LEA proteins in the Shaanhe 6 (SH, drought-tolerant) and Zhengyin 1 (ZY, drought-sensitive) genotypes, respectively, which corresponded to 38 unique LEA proteins from seven LEA groups, and we also determined the changes in their abundances in the two wheat genotypes. We identified some differentially expressed proteins that did not belong to any known LEA family and we predicted their biological functions. Their physicochemical properties were also analyzed. Furthermore, the expression levels and functions of the LEA and non-LEA proteins with significant changes in abundance were investigated in detail. This study provides insights into the mechanisms employed by LEA proteins when responding to drought stress in the seedling leaves of two wheat genotypes.

2. Materials and methods

2.1. Plant material

Two genotypes of winter wheat (*Triticum aestivum* L.), drought-tolerant Shaanhe 6 (SH) and drought-sensitive Zhengyin 1 (ZH), were selected for this study. The seeds were grown in plastic pots filled with a mixture of peat substrate (Pindstrup, Denmark) and vermiculite (1:1, v/v). The experiment was performed in a rain shelter under natural temperature, light, and moisture conditions. Seedlings were watered well until the three-leaf stage. The plants were then subjected to drought stress by water withdrawal. Leaf samples were collected from the two genotypes using three biological replicates at 70%, 50%, 40%, 30%, and 20% of the maximum field capacity (FC), respectively. Some of the fresh leaves were used to measure physiological indicators, and the remainder were frozen immediately in liquid nitrogen and stored at − 80 °C until further analyses.

2.2. Physiological measurements

Physiological traits were investigated including the leaf relative water content (RWC) and relative electrolyte leakage in leaves from the two wheat genotypes subjected to water deficit. Fresh seedling leaves were weighed (fresh mass, FM) from three replicates treated with different water stress levels. The seedling leaves were then immersed in distilled water for 4 h. At fully turgid weight, the leaves were reweighed (TM) and the dry mass (DM) was then determined after incubating at 70 °C for 24 h. RWC was calculated according to the formula described by Turner [30]: $RWC = (FM - DM) / (TM - DM) \times 100$. Relative electrolyte leakage was measured in fresh leaves, as described by Dionisio-Sese and Tobita [31].

2.3. Protein extraction and western blot analysis

Soluble proteins were extracted from the leaves of control (70% FC) and stress-treated (20% FC) plants in triplicate, as described by Oliveira et al. [29] with some slight changes. Briefly, 1 g of leaf sample was ground into powder in liquid nitrogen and solubilized in salt buffer (20 mM TES-KOH, pH 8.0, 0.5 M NaCl) in the presence of standard protease inhibitors (1 mM PMSF, 50 μM leupeptin, 10 mM E-64). After two consecutive rounds of centrifugation at 10,000 × *g* for 10 min at 4 °C, the resulting supernatant was boiled for 10 min at 100 °C on a heating block, cooled for 30 min on ice, and centrifuged at 10,000 × *g* for 10 min at 4 °C. Subsequently, the heat-stable fraction was subjected to TCA treatment by slowly adding 40% TCA to a specific final concentration of TCA (0.5, 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, or 6.0%). Next, after 15 min on ice, the TCA-insoluble fraction was separated by centrifugation at 10,000 × *g* for 15 min. The acid-soluble proteins corresponding to the resulting supernatant were recovered by 15% TCA precipitation overnight. The pellets were washed twice with cold acetone and air-dried. For the MS experiments, the pellets were resuspended in 300 μL of SDT lysis buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl pH 8.0) [32], before ultrasonication ten times for 10 s with an interval of 15 s, and boiling for 10 min. The supernatants were obtained by centrifugation at 14,000 × *g* for 40 min at 25 °C, and then stored at − 80 °C until further use. The protein contents were determined with a BCA Protein Assay Kit (Bio-Rad, USA). All samples were preliminarily