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Original article

Changes in the proteome of grapevine leaves (*Vitis vinifera* L.) during long-term drought stress

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ABSTRACT

The essence of exploring and understanding mechanisms of plant adaptation to environmental stresses lies in the determination of patterns of the expression of proteins, identification of stress proteins and their association with the specific functions in metabolic pathways. To date, little information has been provided about the proteomic response of grapevine to the persistent influence of adverse environmental conditions. This article describes changes in the profile of protein accumulation in leaves of common grapevine (*Vitis vinifera* L.) seedlings in response to prolonged drought. Isolated proteins were separated by two-dimensional electrophoresis (2 DE), and the proteins whose level of accumulation changed significantly due to the applied stress factors were identified with tandem mass spectrometry MALDI TOF/TOF type. Analysis of the proteome of grapevine leaves led to the detection of many proteins whose synthesis changed in response to the applied stressor. Drought caused the most numerous changes in the accumulation of proteins associated with carbohydrate and energy metabolism, mostly connected with the pathways of glycolysis and photosystem II protein components. The biological function of the identified proteins is discussed with reference to the stress of drought. Some of the identified proteins, especially the ones whose accumulation increased during drought stress, may be responsible for the adaptation of grapevine to drought.

1. Introduction

Grapevine is one of the oldest cultivated crops in the world. The economy of many countries relies on the production of grapevines and wines. In 2011, the total acreage of grapevine plantations was nearly 8 million hectares (www.oiv.int). Abiotic stresses are the main factor that adversely affects the growth and yields of crops (Athar and Ashraf, 2009). It is estimated that around 30% of the land surface is exposed to a shortage of water, and the sum of annual precipitations over 10% of the world's land cover is just ¼ of the water needed for the proper growth of plants. This is the reason that drought stress is considered to be among the major factors limiting the geographical presence of plants. Drought also has a negative effect on the quantity and quality of yields, and causes large losses in agriculture (Bhatt and SrinivasaRao, 2005). The grapevine is particularly sensitive to drought in the early stages of growth. The consequences of drought include the inhibited growth and development of leaves and the root system, a lower number of set buds in internodes, and a lower average diameter of xylem vessels (Lovisolo et al., 2010). One of the earliest effects of drought observed in plants is the closure of stomata and the rolling of leaves. This response prevents excessive water transpiration, but simultaneously reduces gas exchange. It also causes considerable disturbances in the energy and carbohydrate metabolism (Jaleel et al., 2007). In addition to this, drought induces damage to cell membranes. The loss of turgor by cells leads to the collapse and rupture of the integrity of cell membranes. Moreover, dehydration of the protoplast results in an increased concentration of electrolytes. Drought also causes a change in the chemical potential of water inside cells, which often induces secondary osmotic stress (Dihazi et al., 2005). Reduction of the gas exchange and photosynthetic surface of leaves has a negative influence on the functioning of photosystems, impairs energy generation and interferes with the activity of the respiratory chain

Abbreviations: ABA, abscisic acid; AMT, aminomethyltransferase; APX, ascorbate peroxidase; CA, carbonic anhydrase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GADP, glyceraldehyde3-phosphate dehydrogenase; HSP, heat shock proteins; IFR, isoflavone reductase; MALDI TOF/TOF, matrix assisted laser desorption ionization time-of-flight/time-of-flight; MDH, malate dehydrogenase; NDK, nucleosidediphosphate kinase; PEPC, phosphoenolpyruate carboxylase; ROS, reactive oxygen species; RRF, ribosome recycling factor; RWC, relative water content; SAR, systemic acquired resistance; TLP, thaumatin-like protein.

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enzymes. Disturbances in the proper flow of electrons between the cytoplasm, chloroplasts and mitochondria that occur during drought affect the accumulation of reactive oxygen species (ROS). This process is also observed during other abiotic stresses (Tuteja, 2007; Jaleel et al., 2008). The signals heralding the shortage of water in soil are first received by roots. In response to this stimulus, plants intensify the synthesis of abscisic acid (ABA) in roots (Stoll et al., 2000), which is transported through the xylem and phloem to the aerial organs of the plant (Davies and Zhang 1991). When ABA reaches leaves, it induces a change in the expression of genes on to the so-called ABA-dependent pathway. Also, a higher concentration of ROS and calcium ions induces a change in the pursued genetic program (Shao et al., 2008). Products of the expression of genes activated during drought participate in the acclimatization of plants to the stress. In response to drought, the activity of the translation apparatus is also directed towards synthesis of stress proteins (Zhu et al., 2007). The role of stress proteins is to protect nucleic acids, cell membranes and other proteins from degradation. Moreover, stress proteins constitute transcription factors, control translation and stabilize cell membranes (Kosova et al., 2013). Longitudinal proteomic research will allow us to identify proteins whose expression changes under stress conditions. However, the functions of many of these proteins have not been recognized (Canovas et al., 2004; Rossignol et al., 2006).

Numerous proteins that accumulate differently in response to stressors enhance the adaptability of plants to new environmental conditions and cause changes in the activity of enzymes involved in key cellular processes (Budak et al., 2013). Analysis of the transcriptome does not completely explain the molecular response of plants to stress. The level of the expression of mRNA is often not correlated with the amount of the protein it encodes. Owing to the development of proteomic methods over the recent years, it has become possible to analyze the actual products of the transcription process while taking into account all post-translatory protein modifications (Siu et al., 2015).

Most of the ongoing proteomic studies focus on changes in the profile of protein synthesis in tissues of *Arabidopsis thaliana*, maize and rice (Bea et al., 2003; Amme et al., 2006; Lee et al., 2009). Recently, there have been some papers dealing with the effect of abiotic stresses on metabolism in grapevine (Król et al., 2014; Griesser et al., 2015). Despite the growing body of data, long-term drought tolerance mechanisms are not yet fully explained, especially for an early stage of grapevine development. Therefore, research in this area is of high importance.

This is the first experiment in which proteomic changes occurring in grapevine seedling leaves during persistent drought stress have been analyzed. All of the proteins isolated from grapevine leaves were separated by two-dimensional electrophoresis, after which the proteins whose accumulation level changed significantly during stress were identified on a mass spectrometer. The database contains information about plant stress proteins PlantPReS (www.proteome.ir/PlantStress.aspx), there is no data on the grapevine drought-response proteins. Therefore, this work provides a valuable complement to the largest database of plant stress protein.

2. Material and methods

2.1. Plant material

The material for the study consisted leaves of grapevine *Vitis vinifera* L. cultivar Kiszmisz Łuczistyj. The seedlings of grapevine for testing were purchased from the company "Professional grapevine seedlings in Józefosław (Poland).

2.2. Experimental conditions

The seedlings were transferred into large pots and grown for the next 8 weeks in a greenhouse under optimal conditions (soil moisture ca 70%, 25/18 °C day/night cycle and 14 h photoperiod at 180–200 lm m^{-2} s^{-1} irradiance). During the whole period, the plants were regularly watered to maintain the optimum soil moisture. The soil moisture was monitored using a VP-PRL-Nr 279462 moisture meter (Mera). When the plants adapted to greenhouse conditions had reached proper size, the experiment began. The seedlings were divided into two groups: control (LC) and drought stress (LS). The experimental conditions were as follows:

1. Control sample (LC) – plants were grown for 2 weeks in optimum conditions established in greenhouses (soil moisture content at the level of about 70%). Optimum soil moisture content was guaranteed by systematic watering.
2. Drought stress sample (LS) – plants were grown for 2 weeks under drought stress. In the first week, the moisture level was reduced from 70 to 30% and was maintained at 30% for the next week. After 2 weeks, leaf cuttings (only fully developed leaves) were cut and cleaned from soil by rinsing with room temperature water. Next, all samples were frozen in liquid nitrogen and stored at −20 °C until the time of analysis.

2.3. Relative water content (RWC) of leaves

The fresh weights (FW) of leaves were measured immediately after harvesting. The leaves were floated on deionized water for 3 h at 20 °C under low irradiance. The turgid leaves were then quickly weighed (TW) and dry weights (DW) were determined after oven-drying at 80 °C for 48 h. The relative water content (RWC) was calculated as:

$$\text{RWC}\ (\%) = (\text{FW} - \text{DW})/(\text{TW} - \text{DW}) \times 100$$

(Brass and Weatherley, 1962).

Data were analyzed with Statistica 10.0 (StatSoft, Poland), using a Student's *t*-test and reported as the means ± SD. Differences between the controls and stress samples were considered statistically significant at $p < 0.05$.

2.4. Proteome extraction

The leaves' proteome was extracted with the methods described by Hurkman and Tanaka (1986) with some modifications. In order to disrupt the leaf cells and extract proteins, 0.5 g of frozen material was ground in liquid nitrogen and suspended in 2.5 ml the extraction buffer containing: 0.7 M sucrose, 0.5 M Tris-HCl (pH = 8.6), 0.5 M ethylenediaminetetraacetic acid (EDTA), 2% β-mercaptoethanol, 1 Table/10 ml protease inhibitors Cocktail Complete Mini (Roche). The incubation of samples carried out on laboratory shaker for 10 min at 4 °C. After this, 2.5 ml saturated phenol solution (pH = 8.2) was added to samples and mixtures were incubated for 15 min on laboratory shaker at 20 °C. After the incubation time samples were centrifuged (12,000 × *g*, 10 min, 4 °C). The upper phenol phase was transferred to new falcons, and to lower phase was added 2.5 ml extraction buffer and 2.5 ml saturated phenol solution (pH = 8.2). Re-extraction of samples carried out 30 min on laboratory shaker at 20 °C. After incubation, the samples were centrifuged (14,000 × *g*, 10 min, 4 °C), and the upper phases were combined with phenol phase from first extraction. The proteins were precipitated from mixture using 0.1 M ammonium acetate (dissolved in methanol) for 24 h at −20 °C. After that the samples were centrifuged (15 min, 14,000 × *g*, 4 °C) and dry pellet of