Journal of Plant Physiology 231 (2018) 210–218

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Proteome of olive non-glandular trichomes reveals protective protein network against (a)biotic challenge

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ARTICLE INFO

Keywords:
Olea europaea
Olive
Trichomes
Non-glandular trichomes
Proteome
Two-Dimensional electrophoresis
Mass spectrometry

ABSTRACT

Olive is one of the most important fruit crop trees in the history of Mediterranean because of the high quality oil. Olive oil has a well-balanced fatty acid composition along with biophenols, which make it exceptional in human diet and provide an exceptional value to the olive oil. Leaf non-glandular peltate trichomes are specialized cell types representing a protective barrier against acute environmental conditions. To characterize the proteome of this highly differentiated cell type, we performed a comparative proteomic analysis among isolated trichomes and trichome-less leaves. Proteins were separated and identified using the 2-DE MALDI-TOF/MS method. A number of enzymes involved in abiotic and biotic stress responses are present and may be responsible for the adaptation to prolonged adverse environmental conditions. The results show that this highly differentiated cell type is physiologically active fulfilling the demands of the trichomes in furnishing the leaf with a highly protective mechanism.

1. Introduction

Trichomes are outgrowths of epidermal cells and appear on the surfaces of leaves and other vegetative and reproductive aerial organs of many plants. Their size, shape, composition and morphology vary among tissues and species. Trichomes represent physical and chemical barriers against harsh environmental conditions and they are divided into two categories, the glandular trichomes and the non-glandular trichomes (also known as simple trichomes). Glandular trichomes vary in cell number and constitute a plant secondary metabolite "factory" as they synthesize, store and secrete a variety of substances including terpenes, fatty acid derivatives, alkaloids and other defense proteins (Huchelmann et al., 2017). Most of these compounds serve as trichome-specific protection mechanisms against abiotic and biotic stresses (Iijima et al., 2004; Schilmiller et al., 2008; Glas et al., 2012). These phytochemicals, especially those derived from medicinal and aromatic plants, have gained much attention as they have high commercial value in pharmaceutical, food and cosmetic industry (Huchelmann et al., 2017). Like glandular trichomes, non-glandular hairs are unicellular or multicellular and exhibit a large variation in terms of structure and morphology, being branched or unbranched. Functionally, non-glandular trichomes protect the plants from insects, control water loss and leaf temperature and provide a protective mechanism against solar radiation (Glover, 2000; Wagner et al., 2004; Li et al., 2018). Additionally, trichomes have been extensively studied in model and non-model plant species to unravel the mechanisms of plant cell differentiation and the production of phytochemicals important for both plant defense and human pharmacopeia (Wang and Wagner, 2003; Hülskamp, 2004; Iijima et al., 2004; Ishida et al., 2008; Graham et al., 2010; Ioannidi et al., 2016).

The olive tree (*Olea europaea* L.) is one of the most common agricultural tree crops of the Mediterranean basin, being the main source of edible oil. Olive tree is an evergreen xerophytic plant carrying hypostomatic leaves with trichomes on both sides. During the growth and maturation of the olive leaf, the trichome density decreases at the adaxial (upper) surface. On the contrary, trichomes are dense on the epidermis of the abaxial (lower) surface covering the entire leaf. Olive leaf trichomes are proposed to associate with adaptation to arid conditions, control the respiration and regulate the leaf temperature (Fahn, 1986). Olive leaf hairs play an important role in the protection of guard

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https://doi.org/10.1016/j.jplph.2018.09.016
Received 26 July 2018; Received in revised form 19 September 2018; Accepted 20 September 2018
Available online 27 September 2018
0176-1617/

cell as they prevent stomatal closure by UV-B radiation (Grammatikopoulos et al., 1994). Furthermore, olive trichomes absorb UV-B radiation through UV-screening pigments, which have been considered as phenolics with considerable flavonoid contribution (Karabourniotis et al., 1992). Considerable effort has been made to elucidate the transcriptome of the olive leaf non-glandular trichomes resulting in the description of genes coding for enzymes that are involved in the synthesis of phenolic compounds with important roles in biotic and abiotic stress responses (Koudounas et al., 2015a). Despite the growing body of data, little is known about the exact proteins of olive trichomes which contribute to abiotic and biotic stress tolerance of leaves.

In this work, we present the proteome profile of non-glandular trichomes from the olive tree. A comparative proteomic analysis between trichome-less leaves and trichomes through Two-Dimensional Electrophoresis (2-DE) and Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) mass spectrometry for protein identification was performed to unravel the physiology and biosynthetic activity of olive trichomes.

2. Materials and methods

2.1. Isolation of non-glandular trichomes

Young, fully expanded leaves from *Olea europaea* L. cv. 'Koroneiki' were collected from the arboretum of the Agricultural University of Athens. Trichomes located on the abaxial and adaxial leaf surface were carefully dissected out by scraping the lower epidermis with razor blades and immediately frozen in liquid nitrogen.

2.2. Light and fluorescence microscopy

Small pieces of the leaf tissue and isolated trichomes were examined under a light microscope to verify no contamination from adjacent tissues using the BX-50 epi-fluorescence microscope (Olympus) equipped with the HBO 100 W/2 mercury short-arc lamp (Osram) and the following filter sets: (Endow GFP Bandpass filter set cat. no.41017 (excitation filter of 450–490 nm, beam splitter of 495-nm long-pass, emission filter of 500–550 nm; Chroma Technology), Chlorophyll autofluorescence, U-MSWG filter set (excitation filter of 480–550 nm, dichroic mirror of 570 nm, emission filter of > 590 nm; Olympus) and Ultraviolet filter using the U-MWU filter set (excitation filter of 330–385 nm, dichroic mirror of 400 nm, emission filter of.420 nm; Olympus). Images were captured with a model no. DP71 camera (Olympus) using CellˆA (Olympus Soft Imaging Solutions).

2.3. Protein extraction

Sample preparation of the isolated trichomes and trichome-less leaf tissues was carried out according to the protocol described previously (Wang et al., 2003) with modifications. Both tissues were grounded with liquid nitrogen in a mortar to fine powder and were washed several times with ice-cold 80% acetone until it became colorless. The powder was resuspended in SDS extraction buffer (30% sucrose, 2% SDS, 0.1 M Tris − HCl pH 7,4, 5% 2-mercaptoethanol (Sigma) 2,5 mM EDTA) and an equal volume of phenol (Tris-buffered, pH 8.0). The mixture was vortexed and centrifuged at 10.000 rpm for 30 min at 4 °C. The phenol phase was transferred to another tube, and proteins were precipitated by adding 5 volumes of cold (− 20 °C) 0.1 M ammonium acetate in 100% methanol and incubated at − 20 °C overnight. The precipitated proteins were collected by centrifugation (10.000 rpm, 30 min, 4 °C), and the pellet was washed twice with 0.1 M ammonium acetate in methanol and with ice-cold 80% acetone. The pellet was solubilized in 0.5 ml of lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X-100, 50 mM DTT, 0.2% [w/v] ampholytes [pH 3–10]) at 25 °C. To confirm reproducibility of profiles three independent biological replicates were analyzed for each tissue. Protein concentration was determined by the Bradford method.

2.4. Isoelectric focusing (first dimension)

One mg of protein of each tissue was separated in the first dimension by using immobilized pH gradient (IPG) strips with linear pH gradient 3–10 of 18 cm (Readystrip, Bio-Rad). Isoelectric focusing was performed on Protean IEF Cell (Bio-Rad). IPG strips rehydrated using constant current of 50 V for 16 h. The following settings were used for trichome protein extracts: 250 V for 4 h, linear increase from 250 V to 5000 V for 24 h, 5000 V for 24 h and 500 V for 24 h, and for trichome-less leaf protein extracts: 250 V for 2 h, linear increase from 250 V to 5000 V for 24 h, 5000 V for 24 h and 500 V for 24 h with a maximum setting of 99 mA/strip.

2.5. Transition from first to the second dimension

After isoelectric focusing electrophoresis, two steps of IPG strips equilibration were performed: for reduction, the strip was incubated for 10 min in buffer (50 mM Tris, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulfate) containing 0.5% w/v dithioerythritol, and for alkylation, for another 10 min with the same buffer containing 4.32% w/v iodoacetamide. Then the equilibrated IPG strips were placed on top of 12% polyacrylamide gel sealed with 1% agarose and subjected to electrophoresis in a vertical PROTEAN II xi Cell electrophoresis system (Bio-Rad, USA) at 4 mA per gel overnight.

2.6. Coomassie blue staining

Gels were incubated for 1 h in fixation buffer (40% methanol, 10% acetic acid), and then stained overnight with "Coomassie Colloidal Blue" staining solution (10% phosphoric acid, 10% ammonium sulfate, 20% methanol, 0.12% Brilliant Blue G-250). At the end, the gels were destained using Milli-Q water for 40 min and this step was repeated 3 times.

2.7. Image acquisition and analysis

The gels were scanned with a GS800 Calibrated Densitometer (BioRad) using the PDQuest 2D-Gel Software v. 7.2.0 (BioRad). A number of spots were detected in gel images from trichome-less leaves and trichomes. Similar results were obtained in three independent experiments.

2.8. Automated spot cutting and in-Gel Protein Digestion

Spots were detected automatically and selected spots were exported as a peak list to the SPControl3 software (Bruker Daltonics). Spots were excised using the automated spot picker Proteineer SpII (Bruker Daltonics) and gel plugs were transferred into 96-Well microtiter plates. Individual spots were destained with 40% acetonitrile in 50 mM ammonium bicarbonate, washed several times with ultrapure water until there was no evidence of remaining Coomassie blue stain and dried in a speed vacuum concentrator (MaxiDry Plus).

Collected spots were digested with 3 µL Trypsin solution (Roche Diagnostics) per spot (10 ng/µl in 10 mM NH_4HCO_3 pH 8.5) and incubated overnight at room temperature in a sealed 96 well plate. Peptide fragments were recovered by adding 10 µl 50% acetonitrile containing 0,1% trifluoroacetic acid to each spot and incubated for 15 min with constant shaking at room temperature. The peptide mixture (1 µl) was applied on the sample target with 1 µl of matrix solution. The extracted peptide masses were analyzed by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS, Bruker Ultraflex).