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Proteomic analysis of Holm oak (*Quercus ilex* subsp. *ballota* [Desf.] Samp.) pollen

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ABSTRACT

This paper presents an analysis of Holm oak pollen proteome, together with an evaluation of the potentiality that a proteomic approach may have in the provenance variability assessment. Proteins were extracted from pollen of four Holm oak provenances, and they were analyzed by gel-based (1- and 2-DE in combination with MALDI-TOF/TOF) and gel-free (nLC-LTQ Orbitrap MS) approaches. A comparison of 1- and 2-DE protein profiles of the four provenances revealed significant differences, both qualitative and quantitative, in abundance (18 bands and 16 spots, respectively). Multivariate statistical analysis carried out on bands and spots clearly showed distinct associations between provenances, which highlight their geographical origins. A total of 100 spots selected from the 402 spots observed on 2-DE gels were identified by MALDI-TOF/TOF. Moreover, a complementary gel-free shotgun approach was performed by nLC-LTQ Orbitrap MS. The identified proteins were classified according to biological processes, and most proteins in both approaches were related to metabolism and defense/stress processes. The nLC-LTQ Orbitrap MS analysis allowed us the identification of proteins belonging to the cell wall and division, transport and translation categories. Besides providing the first reference map of Holm oak pollen, our results confirm previous studies based on morphological observations and acorn proteomic analysis. Moreover, our data support the valuable use of proteomic techniques as phylogenetic tool in plant studies.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 1-DE, one dimensional electrophoresis; 2-DE, two dimensional electrophoresis; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem mass spectrometry; nLC-LTQ Orbitrap MS, nano liquid chromatography electrospray ionization tandem mass spectrometry coupled to a Fourier Trap (Orbitrap); CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; IEF, isoelectrofocusing; BSA, bovine serum albumin; TFA, trifluoroacetic acid; ACN, acetonitrile; CID, collision-induced dissociation; DW, dry weight; RG, Granada; GSE, Sevilla; BCA, Cádiz; SAA, Almería.

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1. Introduction

Holm oak (*Quercus ilex* subsp. *ballota* [Desf.] Samp.) is the dominant tree species in natural forest ecosystems over large areas of the Western Mediterranean Basin [1]. In Spain, it is widely used for forest systems and forest practice conservation, covering a surface of 2489000 ha approximately, which present estimated values of over 120 million Euro in fruit acorn production per year [2]. Holm oak acorns are a major component in the feeding systems of many Mediterranean wild and livestock species and, at the same time, they are the basic feed ingredient for domestically-bred pigs to obtain high quality meat [2].

Q. ilex subsp. *ballota*, like other oak species, has some features that favor a high intra-provenance genetic diversity: long life span, allogamy, wind pollination, monoecy, and a continuous geographical distribution [3]. Spanish Holm oak provenances have showed a prolonged isolation, in spite of being native and distributed throughout diverse forest areas with complex geographic patterns of genetic variation [4,5]. Some provenances have survived under the extreme dry climate conditions that prevail in southern Spain. However, and despite these differences, the classification of individual trees according to a given provenance based on phenotypes is quite challenging. In this context, proteomic studies have already proved their value to assess provenance variability in Andalusia Holm oaks. Proteomic approaches have been carried out to study in this species the following elements: i) the leaf proteome [6]; ii) the leaf proteome at several plant developmental stages with different provenances and under drought conditions [7,8]; and iii) the variability through acorn protein profile analysis [9]. Our results generally showed a remarkable difference between the different Andalusia provenances. According to these findings, we started a study focusing on the Holm oak pollen proteome, expected to be less variable than the leaf or acorn proteomes.

Quercus is considered a moderate cause of pollinosis in many areas of Europe, especially in those with high pollen levels [10]. Not only is the study of *Quercus* pollen useful because of their allergenic potential, but it is also essential for evaluating the ecological conservation of the Spanish woodland. Several pollen proteomic studies have been generally carried out in herbaceous plants [11–14], and to a lesser extent in forest trees and woody crops, focusing on allergen proteins [15–17]. An analysis of *Quercus ilex* subsp. *ballota* pollen proteome would help to gain insight into the molecular nature of allergens, as well as into other proteins involved in the functional specialization of oak pollen.

A comprehensive analysis of Holm oak pollen proteome has been performed by using complementary gel-based (1-DE and 2-DE with MALDI-TOF-TOF) and gel-free (nLC-LTQ Orbitrap MS) approaches. Moreover, we used the available protein, DNA and EST sequences accessible in public databases for protein identification. The potentiality of these approaches in assessing provenance variability has also been evaluated. Multivariate statistical analyses were performed to compare the 1- and 2-DE protein profiles of four provenances, and to establish phylogenetical distances among them. Holm oak pollen proteome has been partially described, with 355 protein species identified from peptide spectra techniques, allowing a functional protein characterization, as well as with the identification of provenance-specific protein markers.

2. Materials and methods

2.1. Plant material

Holm oak flowers were collected from two major Andalusia regions: south (SSA, RG and BCA) and northwest (GSE) (see reference [18] for details). Pollen grains were collected from freshly open flowers by shaking the anthers on a glass slide. Flower debris was removed using a microsieve, and pollen was visualized under a light microscope (Supplementary Fig. 1). Pollen was either used immediately, or stored at −70 °C after freezing in liquid nitrogen. Three different trees (biological replicates) per provenance were used for the protein extraction to observe their natural variability.

2.2. Protein extraction

Proteins were extracted from 500 mg of pollen using the TCA-phenol protocol [19,20]. The final pellet was solubilised in 100 μL of a solution of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.5% (w/v) Triton X100, and 100 mM DTT. Insoluble material was removed by centrifugation, and the protein content in the supernatant was quantified according to the Bradford method [21] using BSA as standard. Samples were stored at −80 °C.

2.3. Gel electrophoresis

Protein extracts (70 μg BSA equivalent) were subjected to SDS-PAGE [22] on 13% polyacrylamide gels by using PROTEAN II cells (Bio-Rad, Hercules, USA). Gels were stained employing a Colloidal Coomassie procedure as reported in [23]. Images were digitized using a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, USA), and then they were analyzed with Quantity One software (Bio-Rad, Hercules, USA).

Preliminary 2-DE experiments were made with the Mini-Protean 3 system (Bio-Rad, Hercules, USA), using 7-cm pH 3–10 linear gradient strips (Bio-Rad, Hercules, USA) and 13% polyacrylamide gels. The 2-DE analysis was carried out using the large system (17 cm). IEF was performed using 17-cm, 5–8 pH linear range, IPG strips (Bio-Rad, Hercules, USA). These strips were actively rehydrated applying 50 V for 12 h with 250 μL of a solution of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.2% (v/v) IPG ampholite buffer pH 5–8, 100 mM DTT, and 0.01% (w/v) bromphenol blue, containing 300 μg BSA equivalent of proteins [24]. The strips were loaded onto a Protean IEF Cell system (Bio-Rad, Hercules, USA) and electrofocused at 20 °C using the following parameters: 250 V for 20 min, followed by 150 min linear gradient from 250 V to 10000 V, and finally focused on up to 40000 V at 10000 Vh. After IEF, the strips were immediately reduced and alkylated according to [24]. The second dimension was performed on 13% polyacrylamide gels using the Protean Dodeca Cell (Bio-Rad, Hercules, USA). The gels were run at 150 constant volts until the dye reached the bottom of the gel. Gels were stained employing