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Differential Expression and Sequence Polymorphism of the Olive Pollen Allergen Ole e 1 in Two Iranian Cultivars

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ABSTRACT

Molecular evidence on the heterogeneity present in the Ole e 1 allergen of the olive pollen is emerging. Such polymorphism is dependent on the cultivar origin of pollen, which also determines wide differences in the expression of this protein. Determination of biochemical and molecular characteristics of Ole e 1 pollen allergen in two Iranian olive cultivars, namely 'Rowghani' and 'Zard' is necessary to assess their allergenicity potential.

SDS-PAGE and immunoblotting analysis of pollen extracts showed that both cultivars present high and low expression of Ole e 1, respectively. These protein levels correlated with similarly different levels of transcripts, as determined by RT-PCR. Two-dimensional protein profiles also showed conspicuous differences in the distribution and the level of expression of those spots reacting to an anti-Ole e 1 antibody.

Bioinformatic analysis of four Ole e 1 sequences corresponding to 'Rowghani' and two sequences for 'Zard', showed numerous heterogeneities when compared with those Ole e 1 and Ole e 1-like sequences present in databases. Nucleotide substitutions resulted in many cases in changes over the predicted amino acid sequences. A cladistic analysis of the sequences showed Iranian entries in a central position between West-European sequences, and Ole e 1-like sequences from other *Oleaceae* species.

Moreover, amino acid changes affected key epitopes of the protein involved in the recognition of the protein by the human immune system. Putative implications of polymorphism in both the biological role and the allergic reactivity of Ole e 1 are discussed.

Keywords: Allergen, Cultivar; Gene Expression; Ole e 1; Olive; Pollen; Polymorphism

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INTRODUCTION

The olive tree (*Olea europaea* L.) is one of the most important oil-producing plant species all over the world.[1] Olive industry was first introduced on a large scale in Iran almost a decade ago. Iranian olive production is expected to notably increase over the next few years. Among the wide olive germplasm, the cultivars denominated 'Rowghani' and 'Zard' are considered the two most important and extensively cultivated olive cultivars in Iran. They have been characterized on the basis of morphological characters, in conjunction with a molecular genetics program for molecular characterization of Iranian olive cultivars.[2]

Characterization of pollen proteins and their patterns of expression are a widely used approach to improve basic knowledge, prior to the design of breeding and biotechnological approaches.[3-5] As a result of clinical studies, many of these proteins have been characterized like allergens as well.[6,7]

Ole e 1 names the "pollen proteins of the Ole e 1 family" within the Pfam protein families database (Accession PF01190). This family is formed by 115 structurally related proteins, including proteins present in the pollen of other Oleaceae (*Fraxinus excelsior* and *Ligustrum vulgare*), several species of *Nicotiana*, *Oryza* and *Gossypium*, as well as in *Brassica rapa*, *Arabidopsis thaliana*, *Zea mays*, *Capsicum annuum*, *Solanum lycopersicum*, *Phleum pratense*, *Plantago lancelolata* and *Lolium perenne* among others. They have been suggested to play important events during pollen formation, probably involved in pollen hydration, pollen germination and pollen tube growth.[8] In olive pollen, Ole e 1 is expressed from the late microspore stage onwards, and is newly synthesized and released to the culture medium during in vitro pollen germination. The protein localizes in the endoplasmic reticulum and the walls of the mature pollen, and in the anther tapetum before it degenerates.[8,9]

Seasonal allergy to olive pollen is a major health problem for humans throughout the Mediterranean area. Much attention has been paid to the study of allergenic proteins in the pollen of this species, because of both their biological and allergenic interest. Numerous details are known particularly in regard to the biochemical characteristics and the allergenicity of Ole e 1, which is considered the major allergen from olive tree pollen.[10,11] Ole e 1 produces IgE-reactivity in >70% of patients allergic to olive pollen. It exhibits different variants, from which the glycosylated (20 kDa) and the non-glycosylated (18.5 kDa) forms are the most common.[12] Ole e 1 is highly abundant in olive pollen extracts, representing up to 25% of the total protein extracted in saline media.[10] The protein has been expressed *in vitro* and B-cell, T-cell epitopes and IgE and IgG B-cell immunodominant regions of Ole e 1 have been experimentally determined.[13-15]

Several studies have recently revealed the existence of significant differences in the level of expression of this protein throughout the wide germplasm of the olive tree.[16] Like many other allergens, Ole e 1 is highly polymorphic, showing numerous microheterogeneities all through its amino acid sequence. However, this polymorphism is much lower within a given variety than between different varieties, thus revealing the genetic origin of these changes.[17]

These observations have a number of implications for both the reproductive biology of the species and for the pollen allergy issue, which have to be dealt with in detail.[18] A first step to elucidate these implications is to precisely determine the nature of the differences in both the expression and the molecular characteristics of Ole e 1 for cultivars of different origins and characteristics.

To our knowledge, no research has been carried out up to date regarding neither the expression of pollen proteins, nor their allergenicity in olive cultivars from Iran. The present work describes these different aspects in the two major varieties cultivated in Iran.

MATERIALS AND METHODS

Pollen Samples

Olive pollen was obtained during May 2006 from selected trees of the two main Iranian cultivars: 'Rowghani' and 'Zard', growing at the olive research centre of Tarom- Zanjan (Iran) (36º 52' 60" N; 48° 55' 0" E). The collected pollen was sieved through a 150 µm mesh, immediately frozen in liquid nitrogen and stored at -80ºC.

Preparation of Crude Protein Extracts

Crude protein extracts were obtained by stirring 1gr of each pollen sample in 10 ml of extraction buffer [40mM Tris-HCl (pH 8.8), 2% (v/v) Triton X-100, 1mg/ml ascorbic acid, 100µl protease inhibitor cocktail (Sigma), 5mM 1,4-dithiothreitol (DTT), 5% (w/v) polyvinylpolypyrrolidone (PVPP)], for 4h at 4ºC.

Samples were centrifuged at 12,000 g for 15 min, and the protein concentrations of supernatants were measured according to Bradford[19] using the Bio-Rad (CA, USA) reagent, and bovine serum albumin as the standard.

Separation of Olive Pollen Proteins

Analytical gel electrophoresis was performed according to Laemmli[20] in 4% stacking and 15% resolving acrylamide gels, using a Mini-Protein II system (Bio-Rad, CA, USA).

For two-dimensional gel electrophoresis, crude protein extracts were dissolved in chloroform/methanol (2:1) and precipitated in trichloroacetic acid/acetone. After 3X washing with cold acetone, the final pellets were dried and dissolved in 2-D rehydration solution [40mM Tris-HCl (pH 8.8), 7M urea, 2M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylamonium]-1-propanesulphonate (CHAPS), 0.2% (v/v) IPG buffer pH 3-10, tributyl phosphine and 0.002% (w/v) bromophenol blue]. The protein samples were alkylated by adding iodoacetamide (IAA) to a final concentration of 200 mM. Samples (100 µg protein) were applied to 18 cm Immobiline Dry Strip (pH 3-10, Bio-Rad, CA, USA) for 12 h using in-gel rehydration. Isoelectric focusing (IEF) was performed in a IPGphor device (Pharmacia, Uppsala, Sweden) at 20ºC, applying 300 and 1,000 V for 1h each followed by a linear increase from 1,000 to 8,000 V during 3h, and finally 8,000 V for 12h. Focused strips were equilibrated using a DTT and IAA solution. For SDS-PAGE, the Laemmli buffer system[20] was used to cast 4% stacking and 13% resolving gels.

Immunoblotting

Gels were transferred onto (polyvinylidene difluoride) PVDF membranes at 15V for 25 min (25V/30 min for 2-D gels) using a Semi-Dry Transfer Cell (Bio-Rad, CA, USA). The membranes were blocked in Tris-buffered saline buffer (TBS) containing 0.3% (v/v) Tween 20 and 10% (w/v) skimmed dried milk (blocking buffer), and then probed with a monoclonal antibody to Ole e 1[21] diluted 1/1,000 in the same buffer. An Alexa Fluor 488 anti-mouse IgG antibody made in goat (Molecular Probes, CA, USA) was used as the secondary antibody, diluted 1/2,000 in TBS. The reaction was detected using a Molecular Imager PharosFX System (Bio-Rad, CA, USA). Gel and blot quantitation were performed by using the Quantity One v.4.6.2 software (Bio-Rad, CA, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen Iberia, Madrid, Spain). Pollen samples were ground in a mortar with liquid nitrogen and processed according to manufacturer's instructions. Quantity and quality of the extracted RNA were assessed by spectrophotometric measurements at A260/280 nm in a NanoDrop microspecto-photometer (Thermo, MA, USA) and by agarose gel electrophoresis. First strand olive pollen cDNA was synthesized from total RNA (1µg) using an oligo$(dT)_{17}$ and SuperScript II reverse transcriptase (Invitrogen, CA, USA) following the manufacturer's instructions. Relative quantisation of cDNA in the samples, as the result of the RT reaction was tested by amplification of ubiquitin transcripts as previously described.[8] As the template, 100 ng of cDNA were used for further PCR amplifications. An Ole e 1 sense primer (5' ACCTCCAGTTTCTCAATTTCAC 3') and oligo$(dT)_{17}$ antisense primer (both at a concentration of 0.1 µM) were used for amplification, which was carried out as follows: prior denaturation at 95 ºC for 5 min, 40 cycles of 94 ºC/1 min denaturation, 57 ºC /1 min annealing and 72 ºC/1 min extension, final extension at 72 ºC/15 min. The PCR-amplified fragments were analysed by agarose gel electrophoresis.

cDNA Cloning and Sequencing

Selected bands were purified from the agarose gel using a Prep-A-Gene DNA Purification System (Bio-Rad, CA, USA) and cloned into pGEM-T Easy vector (Promega, WI, USA). The ligation mixture was used to transform *E. coli* DH5α competent cells. Recombinant clones were identified by colour screening on indicator plates and further tested by restriction analysis of the purified plasmids. The nucleotide sequence of cDNA inserts was determined after sequencing reactions using the vector specific primers SP6 and T7 which flanked the insert.

Bioinformatic Tools

The sequences obtained were analyzed using the BLAST program at NCBI. Sequence alignments and nucleotide sequences were analyzed by the nucleotide-nucleotide BLAST (blastn) program[22]. BLAST

pairwise alignments and tree view were generated by using the fast minimum evolution method and maximum sequence difference of 0.75. Nucleotide and protein sequence alignments were performed by the ClustalW software,[23] and viewed using the Jalview viewer 2.2.[24] Conservation index was calculated as described.[25] Ole e 1 protein sequences were searched for different motifs with the ScanProsite on-line facilities at the ExPASy Proteomics Server.[26]

RESULTS

Analysis of Olive Pollen Protein Extracts

SDS-PAGE analysis of crude pollen protein extracts from the two major Iranian olive cultivars showed the band patterns displayed at Figure 1 (a and b) after Coomassie and silver staining, respectively. A conspicuous band of c.a. 20 kDa was clearly visible in the extracts corresponding to the 'Rowghani' cultivar, whereas the band showed reduced intensity in the 'Zard' cultivar. Quantitation of these bands indicated that they corresponded, respectively to 8.2 % and 3.2 % of the total amount of protein present in the samples. After probing Western blots with a monoclonal antibody to Ole e 1, three immunoreactive bands of c.a. 18.0, 20.0 and 22.5 kDa were observed in the 'Rowghani' cultivar. Only the 20.0 kDa immunoreactive band was observed in the 'Zard' cultivar, also displaying lower intensity than the equivalent band in the 'Rowghani' extract (Figs. 1 c and d). The sizes of these bands are in good agreement with the three major glycosylation variants of Ole e 1 already described in the literature (non-glycosylated, -18.0 kDa- and glycosylated -20.00 and 22.5 kDa-). [12,21,27,28]

Figure 2 illustrates the two-dimensional gel electrophoresis patterns obtained from olive pollen extracts of the cultivars 'Rowghani' (Figure 2a, b) and 'Zard' (Figure 2a' and b'). Approximately 750 spots could be detected by silver staining in the 2-D map of each sample. The observed 1-D conspicuous bands of 18-22.5 kDa shown in Fig. 1 appeared now separated into different spots focusing at different pHs ranging between 5.0 and 7.0.

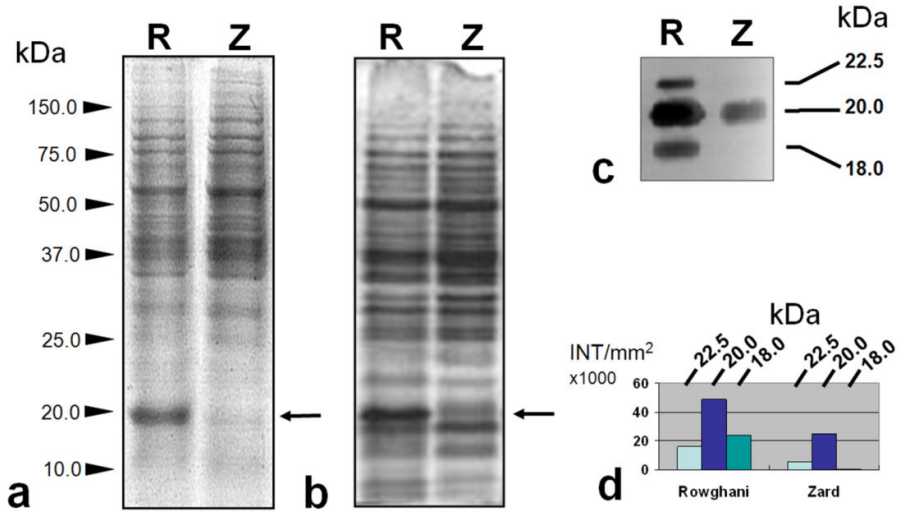

Figure 1. Comparative SDS-PAGE pattern of crude protein extracts from mature pollen of two Iranian olive cultivars (R: 'Rowghani' and Z: 'Zard'). a: Coomassie blue staining pattern. b: Silver staining pattern. The arrows point to a 18 kDa conspicuous band corresponding to the monoglycosylated form of Ole e 1 c: Western blotting using a monoclonal anti-Ole e 1 antibody. d: Quantitative analysis of the intensity of the three cross-reactive bands observed in the blot, corresponding respectively to the non-glycosylated form (18.0 kDa), and two glycosylated forms (20.0 and 22.5 kDa) of Ole e 1.

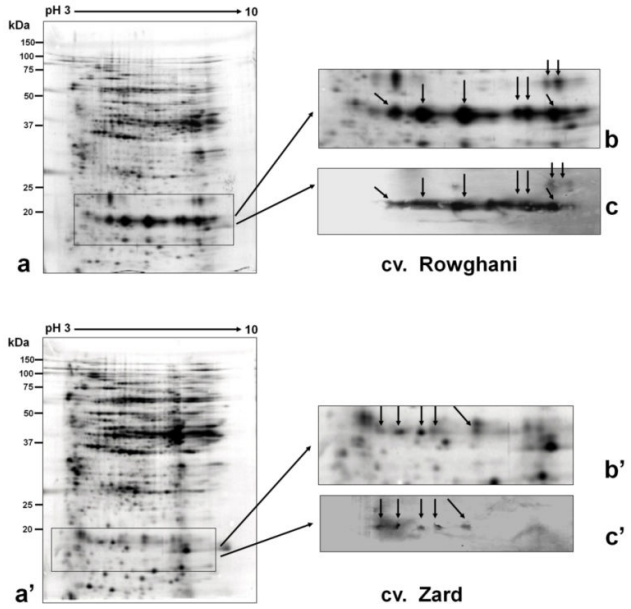

Figure 2. Two-dimensional gel analysis of the olive pollen proteome from cultivars 'Rowghani' (upper panel) and 'Zard' (lower panel). a and a': overall view of the silver-stained gels. b and b': Details of the silver-stained gels corresponding to the 18-20 kDa region. c and c': immunoblots corresponding to equivalent gels, probed with the Ole e 1 antibody. Arrows show the reactive spots.

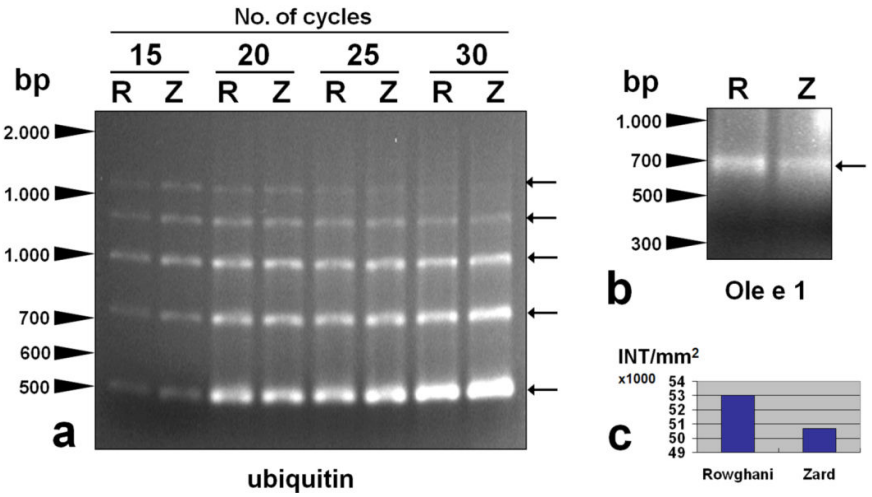

Figure 3. RT-PCR analysis of Ole e 1 transcripts in the pollen of the two olive cultivars (R: 'Rowghani' and Z: 'Zard'). a: assessment of cDNA loading by ubiquitin RT-PCR amplification (monomers to pentamers). b: RT-PCR amplification of Ole e 1 transcripts. c: Quantitative analysis of the bands observed in B.

These spots were more abundant and of higher intensity in the cultivar 'Rowghani' compared to the cultivar 'Zard'. Up to 9 of these spots were immunoreactive to the Ole e 1 antibody after Western blotting experiments in the cultivar 'Rowghani' (Figure 2c) whereas up to five immunoreactive spots only were detected in the cultivar 'Zard' (Fig. 2c'). This pattern was highly reproducible for independent extraction from the same batch of pollen.

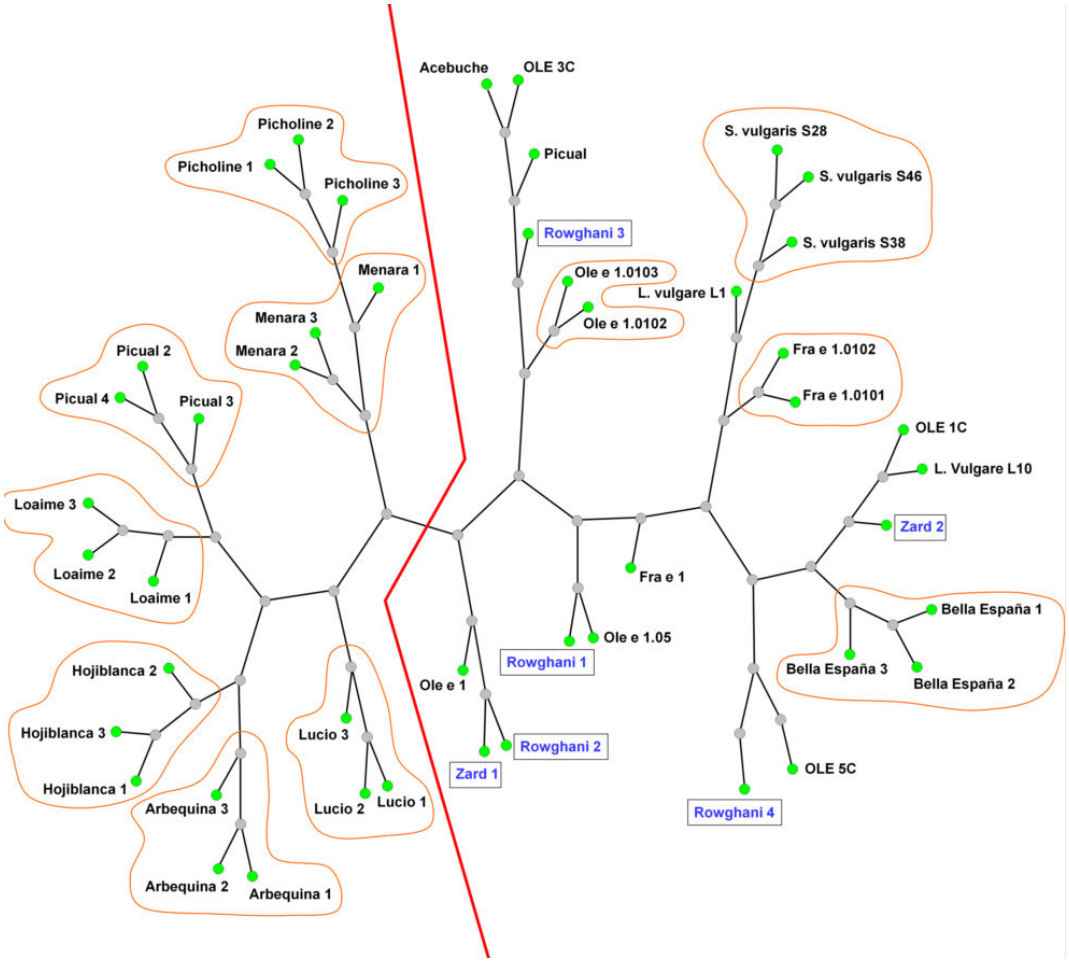

Figure 4. Tree view corresponding to the higher scores of a BLAST pairwise alignment of the 'Rowgani' and 'Zard' sequences of Ole e 1 sequences within the nr databases. With minor exceptions, the red line separates Ole e 1 sequences from olive cultivars widely grown in Europe (particularly in Spain) and the group formed by the Ole e 1 sequences from olive cultivars widely cultivated outside Europe (Spain) and the Ole e 1-like sequences from different members of the *Oleaceae* family. Sequences from the same cultivar are sometimes grouped in individual branches.

RT-PCR Quantitation of Ole e 1 Transcripts

The amount of Ole e 1 transcripts present in the pollen of both 'Rowghani' and 'Zard' cultivars was analyzed by PCR amplification after carefully calibrating the relative amounts of cDNA obtained from the RT reaction (Figure 3a). After RT-PCR, the pollen from the 'Rowghani' cultivar showed significantly higher levels of Ole e 1 transcripts (c.a. 700 bp band) compared to the pollen of the 'Zard' cultivar (Figure 3b and c).

Analysis of Ole e 1 Sequences

Cloning and sequencing of the products of the RT-PCR amplification resulted in four raw sequences from the cultivar 'Rowghani' (Rowghani 1 to 4, corresponding to GenBank accession numbers EF541386 to EF541389) and two sequences from the cultivar 'Zard' (Zard 1 and 2, corresponding to GenBank accessions EF541390 and EF541391). These sequences were individually analyzed by the nucleotide-nucleotide BLAST (blastn) program. Higher identity scores included those sequences from Ole e 1 and Ole e 1-like proteins present in the databases. The identity analysis involved the whole sequence of these variants, with the exception of a short 5' fragment of 38–44 nucleotides including the ATG initiation codon which was absent in the RT-PCR amplified fragments as the result of the amplification strategy used.

Different tree views were computed based on BLAST pairwise alignment of the query sequences to the sequences searched in the databases. Figure 4 shows an example of the tree views generated, in this case after applying the neighbour joining algorithm. Although 'Rowghani' and 'Zard' sequences did not grouped within the same cluster, most of them were located in close proximity.

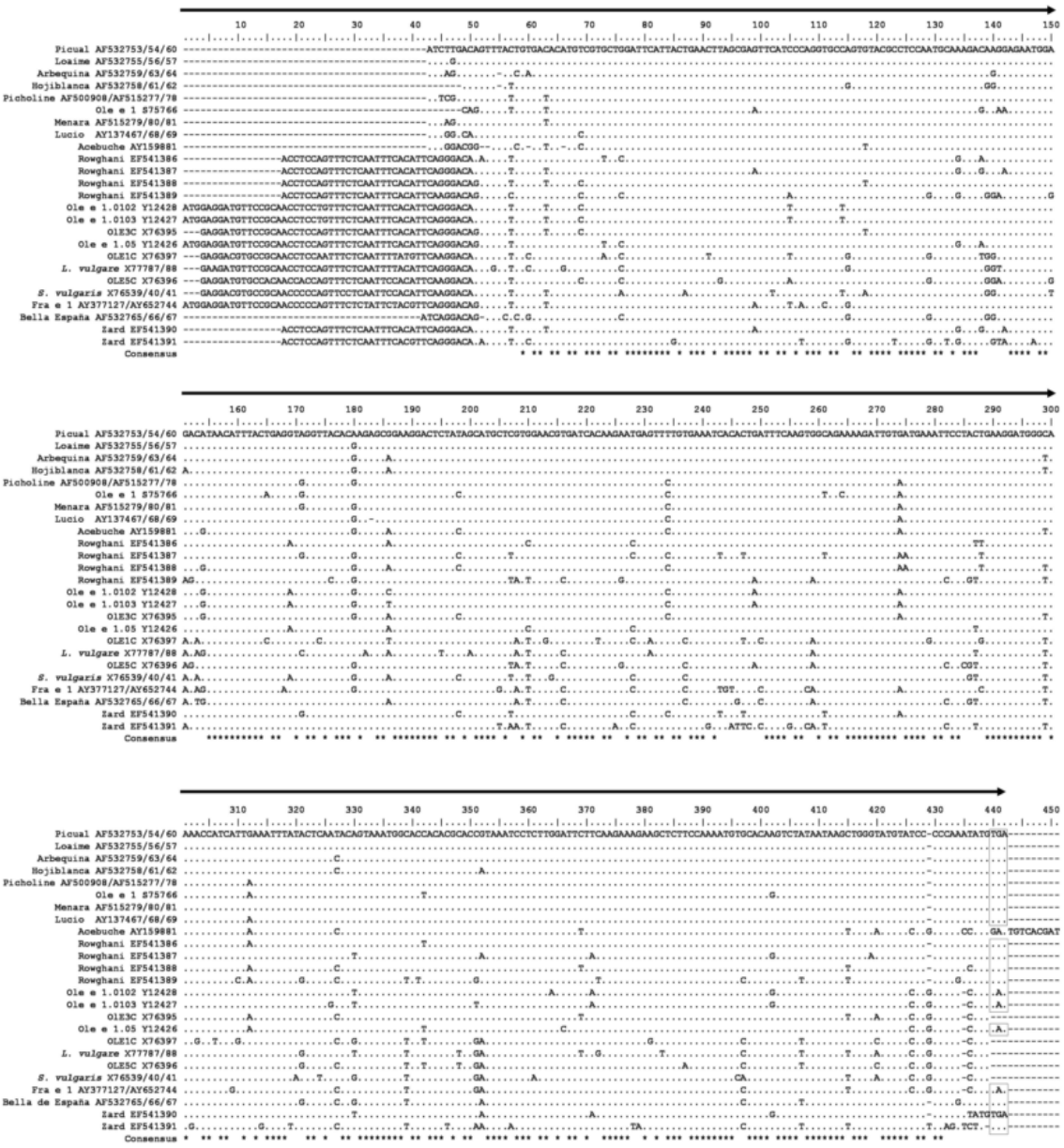

Figure 5. Multiple alignment of the nucleotide sequences of Ole e 1 displayed in Fig. 4. Stop codon is showed in boxes. Redundant sequences from the same variety are only showed once.

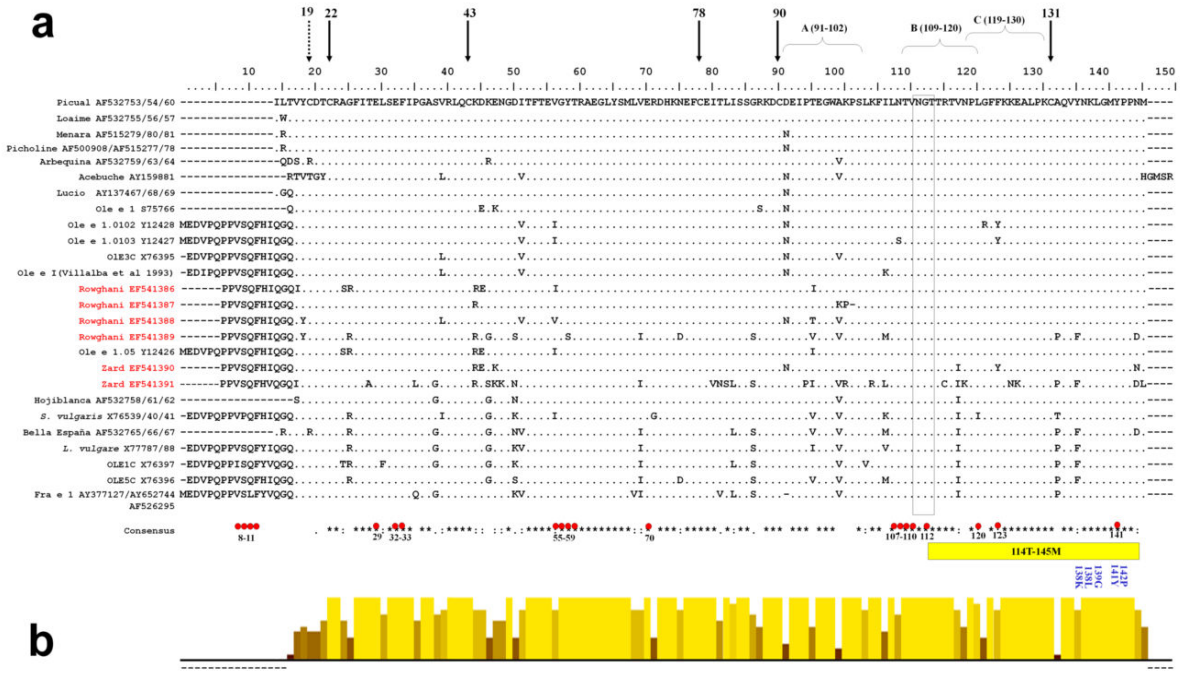

Figure 6. a: Multiple alignment of the deduced amino acid sequences corresponding to the Ole e 1 sequences displayed in Fig. 4, and the experimentally determined sequence of Ole e 1 determined by Edman degradation.[34] Arrows at the top of the alignment indicate the positions of the conserved Cys at the positions 22, 43, 78, 90 and 131 (semi-conserved at position 19). The boxes show predicted N-glycosylation motifs. Brackets A, B and C at the top of the alignment show the position of experimentally determined immunodominant T-cell epitopes.[13] Red circles and bars at the bottom of the alignment mark the positions of IgE and IgG B-cell immunodominant regions of Ole e 1, as determined.[15] b: Sequence conservation index (Jalview viewer).

The alignment of the Ole e 1 sequences from 'Rowgani' and 'Zard' cultivars and those consensus sequences obtained from other olive cultivars and members of the *Oleaceae* family allowed us to identify a large number of nucleotide substitutions in these sequences (Figure 5). The percentage of nucleotide identity between the sequences ranged from 99.25 ('Loaime') to 83.96% ('Zard') when compared to the sequence of Ole e 1 corresponding to 'Picual', used as a reference.

Many of these nucleotide substitutions also resulted in amino acid changes throughout the deduced amino acid sequences. In this case, the percentage of identical amino acids ranged from 98.47% ('Menara' and 'Picholine') to 74.81% ('Zard') (Figure 6a). No substitutions were detected in the Iranian Ole e 1 sequences as regard to the 6 Cys conserved residues at positions 19, 22, 43, 78, 90 and 131, nor to the putative N-glycosylation site at position 111. However, an additional Cys was detected at position 115 of the deduced sequence of 'Zard' EF541391.

Detailed analysis of the immunodominant T-cell epitopes of the Ole e 1 allergen, as previously characterized,[13] and the amino acid residues implicated in IgG and IgE binding[15] (Figure 6a) showed that several of these epitopes were fully conserved in the Iranian Ole e 1 sequences (i.e. positions 8-11, 29, 32-33, 70, 107-110, 112). On the contrary, some other relevant positions for the allergen interaction with the immune system were widely polymorphic in the Ole e 1 from Iranian varieties (positions 55-59, epitopes A: 91-102, B: 109-120 and C: 119-130, and particularly the region 114-145). The graph displaying the conservation indexes for each amino acid position

(Figure 6b) also reflected a relatively high level of conservation, combined with the presence of micro-heterogeneities in certain positions.

DISCUSSION

Clear differences in the expression of the Ole e 1 allergen have been detected in the pollen of the two major Iranian cultivars 'Rowghani' and 'Zard'. They correspond to cultivars with high and low levels of Ole e 1 expression, respectively, as revealed by the SDS-PAGE and RT-PCR experiments showed here. Such strong differences in the allergen content have also been described for several Spanish and Italian cultivars.[16,29] A number of functions have been attributed to this protein, which is clearly involved in pollen hydration and germination.[8,9] These putative functions include the participation of Ole e 1 as a signalling molecule, involved in pollen-pistil interactions and pollen tube guidance.[18] The high degree of polymorphism displayed by Ole e 1 in a cultivar-specific manner, as shown by the present and previous papers[17] may represent part of a unique recognition system.

The Ole e 1 variants of the Iranian cultivars 'Rowghani' and 'Zard' share most key signatures with Ole e 1 variants and Ole e 1-like proteins already described in the literature,[17] namely the presence of 6 cysteines conserved in the sequence, the presence of several glycosylation variants, and similar molecular sizes. In spite of these conserved characteristics, micro-heterogeneity also seems to be a common feature of the Iranian Ole e 1 sequences, which permits performing sequence comparisons leading to genetic classifications. Although 'Rowgani' and 'Zard' sequences do not separate into individual, clearly-detached branches into the computed tree-view, the cluster analysis interestingly allowed us to make a clear separation between the Ole e 1 sequences from olive cultivars widely grown in Europe (particularly in Spain) and the group formed by the Ole e 1 sequences from olive cultivars widely cultivated outside Europe (Spain) and the Ole e 1-like sequences from different members of the *Oleaceae* family. This later group includes both the Iranian cultivars and the Spanish cultivar 'Bella de España' whose sequences are closer to those of the Ole e 1-like proteins from members of the *Oleaceae* family compared to Ole e 1 from olive species.[17]

It is only recently when the use of molecular approaches has been extended to the identification of Iranian cultivars.[30] In this study, several accessions of the 'Rowghani' and 'Zard' cultivars have been characterized, showing different degrees of genetic relationship. This early characterization has also shown the existence of homonyms, synonyms and mislabeling within Iranian accessions after using microsatellite markers. The use of Ole e 1 as an additional marker would help to elucidate these intricate relationships.

Information regarding pollinosis in Iran is still scarce and fragmentary. The available studies date from the 70s, and point to willow, cedar, sycamore, pine, alder, walnuts, elm, oak and several other species as the most relevant tree pollens present in the atmosphere of Tehran, the capital city of the country.[31,32] Although olive pollen has not been significantly reported in the atmosphere of Iran, the presence of ash pollen (genus *Fraxinus*, Fam. *Oleaceae*), highly allergenic pollen presenting broad cross-reactivity with olive pollen is frequent during the months of March to May.[31,32] The current and foreseeable increase in olive production taking place in Iran over the last years raises the question of whether a parallel increase in the number of allergic patients to olive pollen will also occur. Several population studies comparing the prevalence of olive pollen allergy between different communities (some of them settled in a geographical area since relatively short time), seem to indicate that new sensitizations to this allergenic sources are likely to occur.[33] Therefore, olive pollen allergy is liable to constitute an important public health issue in Iran in the near future.

Olive cultivars have been shown to contain quite variable allergen profiles.[16,17] It is therefore of particular importance to determine the allergenic characteristics of the pollen of the major Iranian olive cultivars, which are beginning to be extensively cultivated.

As demonstrated in this paper, 'Rowghani' and 'Zard' cultivars are themselves essentially different in terms of the level of expression and the predicted allergenicity of Ole e 1, the olive pollen major allergen. They are also different from other forms of Ole e 1, as a result of their genetic dissimilarity. These differences may represent important factors that should be taken into account in order to define the methods of diagnosis and immunotherapy to be used in this country.

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