



**HAL**  
open science

## Proteomic analysis of pollen and allergen profiling by an immunoproteomic approach

Marilisa Barranca, Simona Fontana, Simona Taverna, Giovanni Duro, Isabelle Zanella-Cleon, Michel Becchi, Giacomo Leo, Riccardo Alessandro

► **To cite this version:**

Marilisa Barranca, Simona Fontana, Simona Taverna, Giovanni Duro, Isabelle Zanella-Cleon, et al.. Proteomic analysis of pollen and allergen profiling by an immunoproteomic approach. *Biotechnology Letters*, 2009, 32 (4), pp.565-570. 10.1007/s10529-009-0180-5 . hal-00568368

**HAL Id: hal-00568368**

**<https://hal.science/hal-00568368>**

Submitted on 23 Feb 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Section of the journal: Plant Cell Technology**

**Proteomic analysis of *Parietaria judaica* pollen and allergen profiling by an immunoproteomic approach**

Marilisa Barranca<sup>1\*</sup>, Simona Fontana<sup>1\*</sup>, Simona Taverna<sup>1</sup>, Giovanni Duro<sup>2</sup>, Isabelle Zanella-Cleon<sup>3</sup>, Michel Becchi<sup>3</sup>, Giacomo De Leo<sup>1</sup> and Riccardo Alessandro<sup>1°</sup>

<sup>1</sup> *Sezione di Biologia e Genetica, Dipartimento di Biopatologia e Metodologie Biomediche, Palermo, Italia;* <sup>2</sup> *Istituto di Biomedicina ed Immunologia Molecolare “Alberto Monroy”, Consiglio Nazionale delle Ricerche, Palermo, Italia;* <sup>3</sup> *UMR 5086 CNRS, Institut de Biologie et Chimie des Proteines, IFR128, Lyon, France*

\* These authors contributed equally to this work

° Corresponding author:

Prof. Riccardo Alessandro

Dipartimento di Biopatologia e Metodologie Biomediche

Università di Palermo, Via Divisi 83

90133 Palermo

Tel: 091 655 4600

Fax: 091 655 4624

e-mail: [ricale@unipa.it](mailto:ricale@unipa.it)

## **Abstract**

*Parietaria judaica* pollen is one of the most common causes of airway allergic disease in the Mediterranean area. Proteome analysis of mature *Parietaria judaica* pollen by two-dimensional gel electrophoresis (2-DE) and mass spectrometry allowed to establish the first reference proteome map of this weed. Proteins involved in a variety of cellular functions as well as the occurrence of allergens were detected. By using 2-DE and immunoblotting with sera from *Parietaria judaica* allergic patients we obtained a more detailed characterization of *Parietaria judaica* allergen profile so to improve our comprehension of the pathogenesis of pollen-induced allergic reaction.

**Keywords:** *allergens, Parietaria judaica, immunoproteome, pollen*

## Introduction

*Parietaria judaica* belongs to the Urticaceae family, is commonly found in urban and rural areas and its pollen is one of the main causes of allergy in the Mediterranean area (Masullo et al. 1996). *Parietaria judaica* pollen allergens have been identified and characterized by immunochemical and molecular-biological techniques (Amoresano et al. 2003; Stumvoll et al. 2003). *Par j 1* and *Par j 2* represent two major allergens belonging to a family of glycoproteins known as non-specific lipid transfer proteins (Salcedo et al. 2004). Recently, the presence of minor allergens like profilin (*Par j 3*) and 2-EF-hand (*Par j 4*) proteins have been also described (Asturias et al. 2004; Bonura et al. 2008). However, other proteins, specific isoforms or posttranslational modifications (PTM) of known allergens can contribute to hypersensitivity reactions in allergic individuals. The accurate diagnosis of hypersensitive patients as well as the in-deep knowledge of the mixtures for a effective immunotherapy depend on the molecular characterization of all allergenic components from a biological source.

Proteomic profiling of pollen extracts coupled with classical immunological approaches have been applied to examine the presence of novel allergenic components in maize, grass, and other species (González-Buitrago et al. 2007; Petersen et al. 2006). An understanding of *P. judaica* pollen proteome would provide insight in the molecular nature of allergens as well as of other proteins involved in the functional specialization of pollen. Using 2-DE followed by LC-MS/MS analyses, we generated the first reference map of the *P. judaica* mature pollen proteome. In total, we identified 70 protein spots including isoforms, and by using an immunoproteomic approach with serum from *P. judaica* allergic patients, we have obtained a more detailed characterization of *Parietaria judaica* allergen profile so to improve our comprehension of the molecular determinants of *P. judaica*-induced allergic reaction. In particular, we have evidenced that the IgE from patients recognise *Par j 1* and the *Par j 2* P2 allergens while they don't recognise the polypeptide *Par j 2* P8. These results could provide important indications to develop new and more efficient vaccines for allergic patients.

## **Materials and methods**

### **Pollen collection and protein extraction**

Pollen from *Parietaria judaica* was purchased from Allergon (Ängelholm, Sweden) and extracted as previously described (Alessandro et al. 2009). Briefly, the pollen grains were defatted with diethyl ether, extracted in PBS at 4°C, dialysed against water and finally lyophilised and stored at -80°C until use.

### **2-DE, image acquisition and analysis**

Aliquots of the lyophilised proteins, 60 µg for analytical gels and 1mg for preparative gels, were solubilized in a buffer containing 8 M Urea, 4% w/v CHAPS, 40 mM TRIS and 65 mM DTE and a trace amount of bromophenol blue.

The first dimension IEF was carried out using immobilized pH gradient (IPG) strips, pH 3-10 non linear and pH 4-9 linear, 18 cm long. The first dimension, equilibration steps and the second dimension were performed as previously described (Fontana et al. 2007).

Gels were stained with ammoniacal silver nitrate for analytical gels (Hochstrasser et al. 1988) or comassie staining for preparative gels. The gels were scanned by densitometer ImageScanner II (GE Healthcare Life Science, NJ, USA ) and the ImageMaster 2D Platinum 6.0 software (GE Healthcare Life Science, NJ, USA) was used to perform the qualitative and quantitative analysis of the 2-DE maps. Human serum was used as internal standard for isoelectric point (pI) and Molecular Weight (MW) calibration of the 2-DE gel with pH 3-10 non linear gradient (Bjellqvist et al. 1993).

### ***In-gel* protein digestion**

Coomassie blue or silver-stained protein spots were excised from the preparative gels and cut into 1-mm pieces. In-gel digestion was performed as described by Shevchenko et al (Shevchenko, Wilm, Vorm and Mann 1996) with minor modifications. De-staining was obtained by successive washes with 20 mM NH<sub>4</sub>HCO<sub>3</sub> buffer and H<sub>2</sub>O/CH<sub>3</sub>CN (50/50) mixture for Coomassie blue. For proteolytic digestion, the gel was treated by 5-15 µl of a trypsin solution (20ng/ul in 50 mM NH<sub>4</sub>HCO<sub>3</sub>) for 5 h at 37°C. The resulting tryptic peptides were extracted from the gel by centrifugation and the supernatant fraction was recovered. A second extraction step was performed using 10-15 µl H<sub>2</sub>O/CH<sub>3</sub>CN/HCOOH (60/36/4; v/v/v) mixture for 30 min and, finally, all extracts were pooled. For LC-ESI/MS/MS experiments, extracts were dried in a vacuum concentrator and re-suspended in 0.1 % TFA solution.

### **NanoLC/nanospray/tandem mass spectrometry (LC-ESI/MS/MS)**

Experiments were performed on a Q-STAR XL instrument (Applied Biosystems, Courtaboeuf, France) equipped with a nanospray source using a distal coated silica-tip emitter (FS 150-20-10-D-20, New Objective, USA) set at 2300V. Information Dependent Acquisition (IDA) mode allowed +2 to +4 charged peptide ions within a m/z 400-2000 survey scan mass range to be analyzed for subsequent fragmentation. MS/MS spectra were acquired in the m/z 65-2000 range. The collision energy was automatically set by the software (Analyst 1.0) and was related to the charge of the precursor ion. The MS and MS/MS data were recalibrated using internal reference ions from a trypsin autolysis peptide at m/z 842.510 [M+H]<sup>+</sup> and m/z 421.759 [M+2H]<sup>2+</sup>. Deconvolution of the TOF MS spectra were done with Bayesian Peptide Reconstruct tool of Analyst. Multicharged ions in 700 to 1800 m/z range with 0.2 Da mass tolerance were used to calculate glycopeptide masses from 2000 to 4000 Da.

Tryptic peptides were separated using an Ultimate-nanoLC (Dionex, Voisins Le Bretonneux, France) with a C<sub>18</sub> PepMap micro-precolum (5 μm; 100 Å; 300 μm x 5 mm ; Dionex) and a C<sub>18</sub> PepMap nano-column (3 μm; 100 Å; 75 μm x 150 mm ; Dionex). After a 3 min. wash with 0.05% TFA in H<sub>2</sub>O/CH<sub>3</sub>CN (98/2) of the injected sample solution on the micro-precolum, the chromatographic separation was developed using a linear 60 min gradient from 0 to 50 % B, where solvent A was 0.1 % HCOOH in H<sub>2</sub>O/CH<sub>3</sub>CN (95/5) and solvent B was 0.08 % HCOOH in H<sub>2</sub>O/CH<sub>3</sub>CN (20/80) at approximately 200 nl/min flow rate.

Protein identifications were performed using the Paragon<sup>TM</sup> Algorithm thorough search mode in ProteinPilot<sup>TM</sup> software (Applied Biosystems, Courtaboeuf, France).

### **Patient sera**

For the identification of IgE-reactive proteins, sera from patients (n = 10) sensitized to *Parietaria judaica* whose radioallergosorbent test (RAST) exhibited scores over 3, were used. Control sera were from healthy, non-allergic subjects and from subjects sensitized to other allergic sources (dust mite, Olea and Cupressus). All sera were stored at -80 °C until use.

### **Immunoblotting**

For immunoblot analysis, 2-DE gel was transferred to nitrocellulose membrane (Hybond-ECL, Amersham Bioscience, Little Chalfont, UK) and the membrane was incubated in blocking solution (5% w/v non-fat dry milk, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) (TBST) at room temperature. After several washings in TBST buffer, the filters were incubated overnight at room

temperature with sera from allergic patients diluted 1:10 followed by horseradish peroxidase (HRP)-conjugated rabbit anti human IgE (1:2000 dilution). After washings in TBST buffer, immunocomplexes were detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL, USA).

## Results and Discussion

### Proteome map of *Parietaria judaica* pollen

*Parietaria judaica* represents the main cause of allergy in the Mediterranean area where up to 80% of pollinosis patients are sensitized against the proteins composing the *P. judaica* pollen. With the aim to obtain a comprehensive and detailed analysis of *Parietaria judaica* pollen content, we extracted total protein from 1.5 gr of mature pollen. Aliquots of the protein samples were subjected to 2-DE analysis and representative silver-stained 2D gel is shown in Fig. 1. Good reproducibility and resolution were achieved throughout the experimental procedures. On average 975 protein spots were clearly resolved using IPG strips pH 3-10 non-linear.

### Protein identification

To obtain a first reference proteomic map of *P.j* pollen, 100 protein spots were excised from Coomassie blue stained gels and among these, 70 protein spots (corresponding to 21 different proteins) were identified by LC-MS/MS technology (Table 1; Supplementary Material). Identified proteins ranged in calculated molecular mass from 92 to 7.5 kDa and in calculated pI from 4.30 to 7.10. All proteins were designated with arbitrary spot numbers as shown in Fig. 1. While the identification of proteins by proteomics methodology has found extensive application with organisms which have a complete sequence of their genomes already available, cross species identification has been used to identify proteins from several plant species (Faulkner et al. 2005; Fernando 2005; Wang, et al 2005). In fact, in our work, due to the poor protein and DNA sequence database coverage in *Parietaria judaica* (only 7 proteins are present in Uni-Prot database for this plant), MS analyses allowed the recognition of 25 protein spots from *P.j* and 45 from other species (Table 1; Supplementary Material). Multiple spots corresponding to the same protein were also identified, as described in other proteomic studies, and reasonably corresponding to post-translational modifications, protein degradation, different isoforms derived from different genes of a multigene family or products of alternatively spliced mRNAs. Fig. 2 shows the distribution of *P.j* pollen proteins according to their functional classification. Briefly, the proteins were grouped in four distinct functional classes: proteins involved in carbon metabolism and energy generation, cytoskeletal and calcium-binding proteins, stress-related proteins, pollen allergens and proteins involved in the methionine metabolism. As concerns specifically *P.j* allergens, we identified 11 spots (37-47) correspond to *Par j 2* P2 protein, 13 spots (49-61) correspond to *Par j 2* P8 protein,

while 1 spot (36) corresponds to *Par j 1* P1 protein. A search of *Par j 1* and *2* protein sequence, using specific softwares supported by ExPaSy Proteomics Server evidenced both O-glycosylation sites and phosphorylation sites (<http://ogpet.utep.edu/OGPET> and <http://www.cbs.dtu.dk/services/NetPhos> ).

In particular, *Par j 1* P1 protein has 1 glycosylation sites and 5 phosphorylation sites, *Par j 2* P8 protein has 5 phosphorylation sites and *Par j 2* P2 protein has 3 phosphorylation sites. These post-translational modifications do not change significantly the molecular weight of a protein but they may be responsible of the shifts in isoelectric points. At the same time, post-translational modifications might have an influence on the Ig binding so that the knowledge of the structure of allergens is important to improve diagnosis and therapy of allergy (Ferreira et al. 2006).

### **Identification of IgE reactive spots by 2-DE and pollen allergens**

Although molecular biology and immunological methodologies have allowed the identification of *Par j 1* and *Par j 2* as the major pollen components responsible of allergic reaction and profilin as a minor allergen (Asturias et al.), the study of P.j pollen allergens have never been completed by using an immunoproteomic approach. Since *Par j 1* and *Par j 2* are basic proteins, to improve the separation of their several isoforms, the pollen protein samples were run on IPG strips 6-9 linear, which allows a better separation of polypeptides with a pI higher than 7. Figure 3A shows a representative Coomassie-stained 2 D gels and corresponding immunoblot obtained by using pooled sera of allergic patients (Fig. 3B). In order to better characterize the biochemical identities of immunoreactive spots (Fig. 3A, # 1-7) they were excised from Coomassie stained 2-DE gel and subjected to LC-ESI MS/MS. Table II in Supplementary Material shows the results of protein spot identifications. Protein spots n° 1 and 2 are isoforms of *Par j 1* allergen, protein spots n° 3-5 are isoforms of *Par j 2* P8 while protein spots n° 6 and 7 are isoforms of *Par j 2* P2 allergen. Fig. 3B, shows a representative western blot using the pooled sera from patients. In particular, the spot indicated with arrows corresponds to *Par j 1* and spot included in the square corresponds to *Par j 2* P2. Control experiments, carried out with pooled sera from 10 nonsensitized subject, indicated the specificity of antibody detection (Figure 3 C). Interestingly, no reaction was ever observed against *Par j 2* P8 suggesting that *Par j 2* P2, together with *Par j 1*, are the main allergenic components present in *Parietaria judaica* pollen.

The data presented in our study show for the first time the identification of *Parietaria judaica* pollen allergens through bidimensional western blot thus representing satisfactory tools for immunological characterization and classification of the proteins from a raw plant extract.

### **Acknowledgments**

M.B. was a fellow of Regional Agency for Environmental Prevention, ARPA Sicilia. This work was supported by ARPA Sicilia; Università di Palermo (International Cooperation) to R.A.; ex 60 % MURST to R.A. and to G.D.L.

## References

- Alessandro R, Gallo A, Barranca M, Principe S, Taverna S, Duro G, Cassata G, Becchi M, Fontana S, De Leo G (2009) Production of an egg yolk antibody against *Parietaria judaica* 2 allergen. *Poultry Sci* 88 (8): 1773-1778
- Amoresano A, Pucci P, Duro G, Colombo P, Costa M, Izzo V, Lamba D, Geraci D (2003) Assignment of disulphide bridges in Par j 2.0101, a major allergen of *Parietaria judaica* pollen. *Biol Chem* 384 (8): 1165-1172
- Asturias J, Ferrer A, Arilla M, Andreu C, Madariaga B, Martinez A (2007) Tolerance and immunological changes of chemically modified allergen vaccine of *Parietaria judaica* in accelerated schedules. *Clin Exp Immunol* 147 (3): 491-496
- Asturias J, Ibarrola I, Eseverri J, Arilla M, González-Rioja R, Martínez A (2004) PCR-based cloning and immunological characterization of *Parietaria judaica* pollen profilin. *J Investig Allergol Clin Immunol* 14 (1): 43-48
- Bjellqvist B, Hughes G, Pasquali C, Paquet N, Ravier F, Sanchez J, Frutiger S, Hochstrasser D (1993) The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14 1023-1031
- Bonura A, Gulino L, Trapani A, Di Felice G, Tinghino R, Amoroso S, Geraci D, Valenta R, Westritschnig K, Scala E, Mari A, Colombo P (2008) Isolation, expression and immunological characterization of a calcium-binding protein from *Parietaria* pollen. *Mol Immunol* 45 (9): 2465-2473
- Faulkner C, Blackman L, Cordwell S, Overall R (2005) Proteomic identification of putative plasmodesmatal proteins from *Chara corallina*. *Proteomics* 5 (11): 2866-2875
- Fernando D (2005) Characterization of pollen tube development in *Pinus strobus* (Eastern white pine) through proteomic analysis of differentially expressed proteins. *Proteomics* 5 (18): 4917-4926
- Ferreira F, Briza P, Inführ D, Schmidt G, Wallner M, Wopfner N, Thalhamer J, Achatz G (2006) Modified recombinant allergens for safer immunotherapy. *Inflamm Allergy Drug Targets* 5 (1): 5-14

Fontana S, Alessandro R, Barranca M, Giordano M, Corrado C, Zanella-Cleon I, Becchi M, Kohn E, De Leo G (2007) Comparative proteome profiling and functional analysis of chronic myelogenous leukemia cell lines. *Journal of Proteome Research* 6 (11): 4330-4342

González-Buitrago J, Ferreira L, Isidoro-García M, Sanz C, Lorente F, Dávila I (2007) Proteomic approaches for identifying new allergens and diagnosing allergic diseases. *Clinica Chimica Acta* 385 21-27

Hochstrasser D, Harrington M, Hochstrasser A, Miller M, Merrill C (1988) Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal Biochem* 173 (2): 424-435

Masullo M, Mariotta S, Torrelli L, Graziani E, Anticoli S, Mannino F (1996) Respiratory allergy to parietaria pollen in 348 subjects. *Allergol Immunopathol* 24 (1): 3-6

Petersen A, Dresselhaus T, Grobe K, Becker WM (2006) Proteome analysis of maize pollen for allergy-relevant components. *Proteomics* 6 (23): 6317-6325

Salcedo G, Sanchez-Monge R, Diaz-Perales A, Garcia-Casado G, Barber D (2004) Plant non-specific lipid transfer proteins as food and pollen allergens. *Clin Exp Allergy* 34 (9): 1336-1341

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 68 (5): 850-858

Stumvoll S, Westritschnig K, Lidholm J, Spitzauer S, Colombo P, Duro G, Kraft D, Geraci D, Valenta R (2003) Identification of cross-reactive and genuine *Parietaria judaica* pollen allergens. *J Allergy Clin Immunol* 111 (5): 974-979

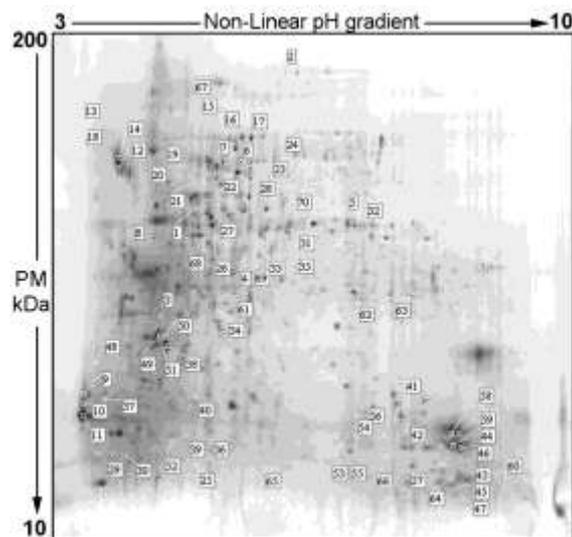
Wang S, Chen F, Sommerfeld M, Hu Q (2005) Isolation and proteomic analysis [corrected] of cell wall-deficient *Haematococcus pluvialis* mutants. *Proteomics* 5 (18): 4839-4851

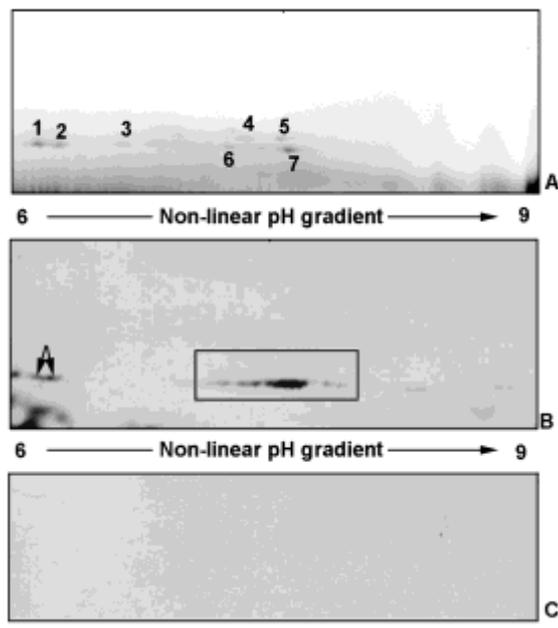
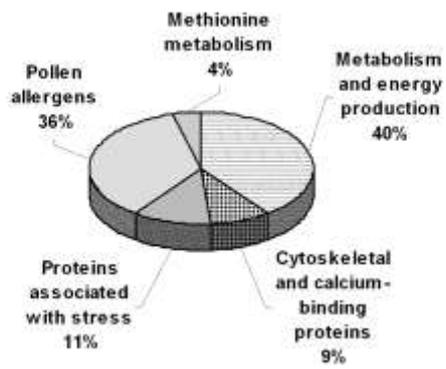
## Figure Legends

**Figure 1** Representative proteomic map of *Parietaria judaica* pollen, proteins (60 µg) were loaded on IPG strips (18 cm, 3.5–10 nonlinear pH). The second dimension was performed on a vertical linear-gradient slab gel (9–16%). Gel was silver stained.

**Figure 2** Pie chart representing the percentage distribution of identified pollen proteins within functional classes

**Figure 3** 2-DE analysis of *Parietaria judaica* pollen extract using a non linear pH 6-9 gradient in the first dimension followed by a 9-16% SDS-PAGE gradient in the second dimension. The separated proteins were stained by Coomassie (A) or transferred in to a membrane for the detection with patients sera (B) or control subjects (C). In B arrows indicate *Par j 1* polypeptides detected by patients sera while spots corresponding to *Par j 2* P2 are bordered by a square





Supplementary Table 1. Proteins identified by LC-MS/MS analysis

Nr Spot (a)	Entry name	AC number (b)	Protein Name	Theoretical <i>pI MW (Da)</i> (c)		Experimental <i>pI MW (Da)</i> (d)		Score (e)	No. of matched peptides (f)
1	ACOC_CUCMA	P49608	Aconitate hydratase, cytoplasmic	5,74	98005	5,28	41029	8,08	5
2	ACOC_CUCMC	Q42669	Aconitate hydratase [Fragment]	6,34	83273	5,74	91193	4,26	2
3	ALF_SPIOL	P29356	Fructose-bisphosphate aldolase, cytoplasmic isozyme	5,96	38471	4,98	19630	5,76	4
4	ALF_SPIOL	P29356	Fructose-bisphosphate aldolase, cytoplasmic isozyme	5,96	38471	5,55	30161	4,17	3
5	ALF_SPIOL	P29356	Fructose-bisphosphate aldolase, cytoplasmic isozyme	5,96	38471	6,08	34151	4,01	2
6	ATPBM_MAIZE/ORYZA	P19023	ATP synthase subunit beta, mitochondrial [Precursor]	5,19	54062	5,42	48662	10,07	5
7	ATPBM_ORYZA/MALIZE	Q01859	ATP synthase subunit beta, mitochondrial [Precursor]	5,18	53990	5,37	49663	4,53	3
8	BIP2_MAIZE	P24067	Luminal-binding protein 2 [Precursor]	5,07	70937	4,68	30076	3,09	3
9	CALM1_ORYSA	Q7F8I8	Calmodulin-1	4,1	16715	4,34	13828	4,07	3
10	CALM1_ORYSA	Q7F8I8	Calmodulin-1	4,11	16700	4,34	13001	2,74	1
11	CALM1_ORYSA	Q7F8I8	Calmodulin-1	4,11	16716	4,34	12401	4	2
12	CALR_ORYSA	Q9SLY8	Calreticulin	4,43	45623	4,63	46341	6,55	4
13	CALR_PRUAR	Q9XF98	Calreticulin	4,38	46091	4,23	48071	5,26	4
14	CALR_PRUAR	Q9XF98	Calreticulin	4,38	46091	4,63	49663	9,3	4
15	CH60B_ARATH	Q8L7B5	Chaperonin CPN60-like 1, mitochondrial	5,33	58351	5,17	58943	4,02	2
16	CH60B_ARATH	Q8L7B5	Chaperonin CPN60-like 1, mitochondrial	5,33	58351	5,36	58943	4,03	2
17	CH60B_ARATH	Q8L7B5	Chaperonin CPN60-like 1, mitochondrial	5,33	58351	5,47	59097	5,4	3
18	ENO_ALNGL	Q43321	Enolase	5,41	47597	4,3	45208	8	4
19	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,1	48267	9,72	6
20	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,15	48267	7,22	3
21	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,37	45880	10,12	6
22	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,41	45923	13,76	6
23	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,52	46720	14,53	7
24	ENO_ALNGL	Q43321	Enolase	5,41	47597	5,54	48464	4	2
25	ENO1_HEVBR	Q9LEJ0	Enolase 1	5,57	47830	5,24	9050	4	2
26	ENO1_HEVBR	Q9LEJ0	Enolase 1	5,57	47830	5,35	33107	6,7	4
27	ENO1_HEVBR	Q9LEJ0	Enolase 1	5,57	47830	5,39	41287	6,14	5

**Supplementary table 2. Proteins identified by LC-MS/MS analysis**

Nr Spot (a)	Entry name	AC number (b)	Protein Name	Theoretical <i>pI</i> <i>MW (Da)</i>	ProtScore (c)	%
1	NLT13_PARJU	Q40905	Probable non-specific lipid-transfer protein 1 (Protein P1)	7,03 10922,66	19,7	
2	NLT13_PARJU	Q40905	Probable non-specific lipid-transfer protein 1 (Protein P1)	7,03 10922,66	13,52	
3	NLT22_PARJU	O04403	Probable non-specific lipid-transfer protein 2 (Protein P8)	8,63 11109,99	9,15	
4	NLT22_PARJU	O04403	Probable non-specific lipid-transfer protein 2 (Protein P8)	8,63 11109,99	15,2	
5	NLT22_PARJU	O04403	Probable non-specific lipid-transfer protein 2 (Protein P8)	8,63 11109,99	25,52	
6	NLT21_PARJU	P55958	Probable non-specific lipid-transfer protein 2 (Protein P2)	8,16 11345,19	10	
7	NLT21_PARJU	P55958	Probable non-specific lipid-transfer protein 2 (Protein P2)	8,16 11345,19	23,68	

(a) Spot number reported in the maps in Figure 3; (b) Accession number used in the SWISS-PROT database; (c) and (d) data from (peptide sequences).