Research Article

# Proteomic analysis of the major soluble components in Annurca apple flesh

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Apple is one of the most worldwide-consumed fruits and a number of cultivars, differing in organoleptic and nutritional characteristics, are available for the market. Annurca apple is a regional variety from Southern Italy, which is known for crispness, excellent taste and long shelf life of fruits. These features have renewed the interest in the investigation of their genetic potential and different studies have lead to their partial genetic and metabolic characterisation. In this study, we present the analysis of the protein repertoire of the pseudocarp tissues of three accessions of *Malus* x *domestica* Borkh. cv. Annurca, as first example of the systematic annotation of the apple proteome. Proteins were extracted from fruit tissues and resolved on 2-DE gels; commonly expressed proteins were *in-gel* digested and analysed by MALDI-TOF-MS and μLC-ESI-IT-MS/MS approaches. Peptide MS and MS/MS data were searched against publicly available protein and EST databases, and 44 spots were identified and associated to 28 different species. They were related to important physiological processes such as energy production, ripening and stress response. The occurrence of allergens causative of widespread food allergy syndromes was also detected. Integration of genomic, metabolomic and proteomic data will be indispensable for future molecular characterisation and hence full exploitation of the peculiar organoleptic, nutritional and agronomic traits of local cultivars of fruits.

**Keywords:** 2-DE / Apple germplasm / *Malus* x *domestica* cv. Annurca / Mass spectrometry / Proteome

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## 1 Introduction

Among fleshy fruits, apple is one of the most consumed worldwide and hence a very relevant dietary source of vitamins and other beneficial compounds (*i. e.* antioxidants) for human health [1]. Different apple cultivars are commonly available for consumers, although over the years only a limited number of elite varieties adapted to modern intensive cultivation, relegating many other cultivars to marginal production, despite their favourable nutritional and taste features. Selection of new high quality varieties with peculiar composition of beneficial compounds is therefore relevant for human nutrition and disease prevention. It relies on a better comprehension of the underlying genetics of individual cultivars as well as of the molecular changes brought about by treatments or environmental factors. This renewed attention to apple germplasm resulted in a number of molecular studies on metabolic [2 – 5] and genetic [6 – 8] profiling.

In this context, 'Annurca' apple represents a worth noting example; it is a regional variety of apple, cultivated in the South of Italy, which produces crisp and white flesh fruits with peculiar flavour and aromas characteristics. This variety is present on about 430 ha of the national surface, producing 120 000 quintals (1 quintal = 220.5 pounds) of the product *per* year. It represents 60% of the Campania region apple production and is considered Indicazione Geografica Protetta (IGP) by the European Union in the framework of the authentication and protection of characteristic agroalimentary products. Fruits of Annurca are usually harvested before the complete maturity and subjected to a peculiar postharvest treatment during which they acquire their redness. Apple is a major source of dietary polyphenols with antioxidant activity and it has been reported that the content and composition of antioxidants varies greatly among cultivars, owing to both genetic features and environmental fac-

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**Abbreviation: PR,** pathogenesis-related

tors [9]. It has been demonstrated that flesh of Annurca fruits have a very high content of polyphenols and that the particular harvest conditions and postharvest treatments increase their levels [5].

In a recent study, a genetic characterisation of the genetic diversity of different accessions of Annurca cultivar, in relation to apple elite varieties has been accomplished by the Simple Sequences Repeats (SSR) technique [8]. From this investigation, the genetic diversity of Annurca germplasm clearly emerged, as well as its homogeneity among all the 15 different tested accessions available from the apple germplasm repository of the Campania Region.

In the last decades, high-throughput techniques opened up new scenarios on genetic information on plants used for vegetables/fruits production, such as nucleotide sequences, genetic maps, and DNA markers. At the same time, it has been realised that this information does not necessarily always match quantitatively or qualitatively the translated protein repertoire. In fact, different processes affect gene products, including the stability, post-transcriptional, cotranslational and degradative modifications of proteins along with the environmental factors. Hence, integration of genetic data with the actual protein complement of a cell or tissue is highly desirable and potentially informative for crop breeding and amelioration. In this study, we report the first systematic proteomic analysis of the pseudocarp tissues of *Malus* x *domestica* Borkh. cv. Annurca, using 2-DE for protein separation and different MS procedures for protein identification. Mature fruits from three different accessions were selected among the ones previously characterised at the gene level and compared as far as their protein repertoire.

## 2 Materials and methods

### 2.1 Plant material

Apple fruits of individuals of three accessions of *Malus* x *domestica* Borkh. cv. Annurca, namely 'S. Agata dei Goti', 'Bella del Sud' and 'Sangue di Bue', were obtained from the apple germplasm repository of the Campania Region, Italy, near the forest training ground of Bucciano, Benevento, (Italy) after their reddening. Each accession consisted of nine pools of fruits at the same reddening state. The maturation of fruits has been measured by starch degradation analysis, using a hyperspectral imaging system [10]. All fruits were quickly washed in sterile distilled water, and the receptacle or pseudocarp was separated from the sepal making up the calyx and the ovary with seeds. Total proteins of the apple fruit pseudocarp were extracted by a phenol-based method described by Saravanan [11].

### 2.2 Protein extraction

Fruit parts were ground to a fine powder in liquid nitrogen, suspended in extraction buffer, containing 700 mM sucrose, 500 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM KCl, 2% v/v β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride (PMSF), vortexed and incubated for 15 min at 4°C. After addition of an equal volume of Tris-HCl pH 7.5-saturated phenol, the mixture was vortexed extensively for 10 min and then centrifuged at $10\,000 \times g$ for 15 min at 4°C. The upper phenol phase was removed and was incubated at −20°C in cold saturated ammonium acetate in methanol, overnight. Precipitated proteins were pelletted at $10\,000 \times g$ for 30 min. Then, the protein pellet was washed twice in cold methanol followed by a cold acetone washing. The resulting pellet was vacuum dried, solved in freshly prepared lysis buffer containing 9 M urea, 4% w/v CHAPS, 20 mM DTT and 1% w/v ampholyte pH 3–10 (BioRad, Hercules, CA, USA), and 0.5% v/v Triton X-100, extensively vortexed at room temperature, overnight, and then centrifuged at $10\,000 \times g$ for 10 min at 20°C. Protein concentration was determined by the Bradford assay [12] using a SmartSpec Plus Spectophotometer (BioRad).

### 2.3 2-DE

IEF was performed on a Protean IEF Cell (BioRad), using 18-cm ReadyStrip IPG strips with a linear pH gradient of 4–7 (BioRad). Protein samples (400 µg for preparative gels), were loaded onto strips and soaked in rehydration solution (final volume 315 µL), containing 8 M urea, 2% w/v CHAPS, 0.3% w/v DTT, 2% IPG buffer pH 3–10, and 0.002% w/v bromophenol blue for 16 h, at 22°C. IEF was then performed by applying a voltage of 250 V for 1 h, ramping to 1000 V over 5 h, and holding at 8000 V until a total of 52 kVh was reached. Prior to the second dimension, the gel strips were equilibrated in 6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.8, 0.01% w/v bromophenol blue, and 2% w/v DTT for 20 min, followed by 20 min in the same buffer containing 2.5% w/v iodoacetamide. Electrophoresis in the second dimension was carried out using a Protean apparatus (BioRad) and 12% polyacrylamide gels (18 cm × 24 cm × 1 mm) in 25 mM Tris pH 8.3, 1.92 M glycine and 1% w/v SDS, with 70 V (~35 mA) applied for 16 h. Two samples for each Annurca accession were independently phenol-extracted and run in triplicate. Protein spots were annotated only if detectable in all gels.

### 2.4 Image acquisition and analysis

2-DE gels were stained with colloidal Coomassie G250 and scanned using a GS-800 calibrated densitometer (BioRad). Image analysis was performed using the PDQuest 2D image analysis software (BioRad). Spot detection and matching between gels were performed automatically, followed by manual verification. For quantitative analysis, after normalisation of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for three

 

different gels and Student's *t*-test analysis ($P < 0.01$) was performed.

### 2.5 Protein digestion and MS analysis

Spots from 2-DE were excised from the gel and digested with trypsin, as previously reported [5]. Samples were desalted using μZipTipC18 tips (Millipore) before MALDI-TOF-MS analysis and/or directly analysed by μLC-ESI-IT-MS/MS. Peptide mixtures were loaded on the MALDI target together with CHCA as matrix, using the dried droplet technique. Samples were analysed with a Voyager-DE PRO spectrometer (Applera, USA). Peptide mass spectra for peptide mass fingerprint (PMF) experiments were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applera). Peptide mixtures were also analysed by using a LCQ Deca Xp Plus mass spectrometer (ThermoFinnigan, USA) equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan) [10]. Peptide mixtures were separated on a capillary Hypersil-Keystone Aquasil C18 Kappa column (100 × 0.32 mm, 5 μm) using a linear gradient from 10 to 60% of ACN in 0.1% formic acid, over 60 min, at flow rate of 5 μL/min. Spectra were acquired in the range 200–2000 *m/z*. Data were elaborated using the BioWorks 3.1 software provided by the manufacturer.

### 2.6 Protein identification

ProFound software was used to identify spots from NCBI nonredundant database by PMF experiments. Candidates with ProFound's Est'd *Z* scores > 2 were further evaluated by comparison with $M_r$ and p*I* experimental values obtained from 2-DE. Sequest software was used to identify proteins with data deriving from μLC-ESI-IT-MS/MS experiments. Candidates from NCBI apple EST database with identified CID spectra of peptides and Sequest Xcorr values > 2.5 were further evaluated by the comparison with experimental $M_r$ values obtained from 2-DE. Identified nucleotide sequences in EST databases obtained by MS/MS analysis were translated using the Translate program (http://www.expasy.org/tools/dna.html). Protein functional classification was done according to literature data and the Swiss-Prot/TrEMBL database.

## 3 Results and discussion

Extraction of proteins suitable for 2-DE analysis from Annurca pseudocarp proved challenging. In fact, apple fruits are notoriously recalcitrant tissues for proteomic analysis, due to a low protein content and high concentration of interfering substances such as pigments, carbohydrates, polyphenols, polysaccharides and starch. Phenol extraction followed by ammonium acetate/methanol precipitation gave best results, providing protein samples sufficiently concentrated for IEF and devoid of interfering sub-

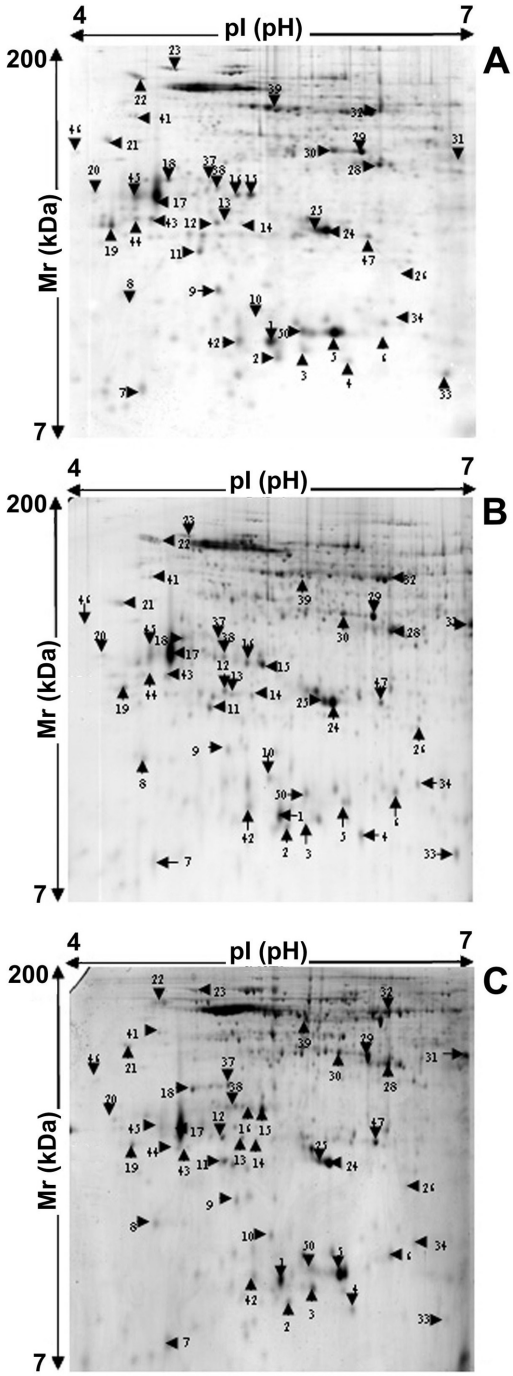


**Figure 1.** Representative 2-DE gels of total protein extracts from pseudocarp of three accessions of *Malus* x *domestica* cv. Annurca. (A) Accession Bella del Sud; (B) accession S. Agata dei Goti; (C) accession Sangue di Bue. Gels were stained with colloidal CBB G-250. Spot numbering refers to Table 1 showing protein identification as obtained by MS analysis.

stances (data not shown). Proteins were solved from freshly prepared pseudocarp tissues from selected Annurca accessions, namely S. Agata dei Goti, Bella del Sud and Sangue di Bue, sampled at the same red stage. An effective separation was obtained by 2-DE, applying a linear 4 – 7 pH gradient in the first and 12% polyacrylamide in the second dimension. Representative gels for Bella del Sud, S. Agata dei Goti and Sangue di Bue accessions are shown in Fig. 1. Average proteomic maps showed 470, 425 and 303 spots for S. Agata dei Goti, Bella del Sud and Sangue di Bue, respectively. Accordingly, the degree of similarity between the accession considered as reference (Bella del Sud) and the others was 64 and 55% with S. Agata dei Goti and Sangue di Bue, respectively. Common spots, matched among the three different accessions, were 203.

### 3.1 Identification of the major commonly expressed proteins

To obtain a first reference proteomic map of the pseudocarp of apple fruit, 50 among the most abundant and or best resolved spots matched in all the three Annurca accessions, were manually picked, digested with trypsin and subjected to MS analysis, to determine protein identity. Owing to the poorly solved apple genome, identification by MALDI-TOF mass fingerprint experiments proved successful only for a limited number of protein species. In fact, this analysis allowed the identification of nine spots from *Malus* x *domestica* and six from other plant species. Difficulties in protein identification of samples showing clear MALDI-TOF mass spectra were solved by μLC-ESI-IT-MS/MS experiments, which allowed the identification of further 29 spots. In conclusion, this combined mass fingerprint/MS/MS approach allowed the identification of 44 protein spots, of which 39 were from *Malus* x *domestica* and five from other species. This analysis also allowed a definitive determination of the direction and the frame of reading for the identified nucleotide sequences deposited in EST databases, as shown in Supporting Table 1. The resulting list of the identified proteins, grouped according to their functional classification, is reported in Table 1.

### 3.2 Proteins associated with carbon and nitrogen metabolism or energy production

Different key enzymes of glycolysis were identified in the pseudocarp of Annurca accessions, namely fructose-1,6-bisphosphate aldolase (spot 31), glyceraldehyde-3-phosphate dehydrogenase (spot 31), triose-phosphate isomerase (spot 24) and enolase (spot 32). Their abundance very likely reflected the pivotal role of this pathway that fuels substrates for respiration and organic acid and pigments synthesis from imported sugars during ripening [13]. It is worth noting that, since all of these identifications were on the basis of the homology with proteins of other plant species, determined peptide sequences (Supporting Table 1) may provide useful information for future cloning experiments and marker selection in *Malus* x *domestica*. The NAD-dependent malate dehydrogenase (spot 28) has been detected as a homologue of the cytosolic isoform from *Prunus persica.* Malic acid is one of the predominant organic acid molecules in ripe fruits and greatly contributes to overall organoleptic quality. Its accumulation during maturation is mainly dependent on the activity of cytosolic NAD-dependent malate dehydrogenase. Whereas no information is available in apple, in peach it has been demonstrated that the expression of this enzyme is regulated during ripening [14]. On the other hand, two spots with different p*I* values, namely spot 29 and 30, were associated to glutamine synthetase, an enzyme which plays a pivotal role in the $NH_3$ mobilisation between metabolites that occurs during the ripening transition [15]. Eukaryotic glutamine synthetase is an octameric enzyme occurring as a cytosolic and a plastidial isoform, hence the detection of two distinct spots may be due to different subunits as well as to post-translational modifications. Some enzymes related to energy production were also detected, namely ATP-synthase D chain (spot 9), adenine-phosphoribosyltransferase I (spot 11), nucleoside-diphosphate kinase I (spot 33), cytochrome C oxidase 6B subunit (spot 20) and mitochondrial processing peptidase (spot 39). The occurrence of these proteins as abundant ones in the apple flesh is not surprising considering the respiratory increase that accompanies the ripening process in climacteric fruits.

### 3.3 Proteins associated to stress and ripening

Fruit ripening has been described as a controlled oxidative process whereby $H_2O_2$ and ROS accumulation are balanced by the activity of cellular antioxidant systems [16]. In this respect, the identification of [Mn]-SOD (spot 26) as an abundant protein is not surprising. In fact, SOD is a major component of the antioxidant circuitry in the cell and it has been reported that its activity increases during fruit maturation [16]. The identification of type 2 peroxiredoxin (spot 10) was also related to protection against oxidative stress. In fact, this class of enzymes can reduce $H_2O_2$, alkyl hydroperoxides and hydroxyl radicals thereby protecting lipids, enzymes and DNA against ROS insult. Whereas in *Arabidopsis* seeds it has been shown that the AtPER1 gene is expressed in the embryo during maturation [17] and is involved in the inhibition of germination during stress [18], no information is available about expression and role of peroxiredoxins in the maturation of fruits. Other identified proteins generally related to the ripening process and/or response to stress were Hsp70 protein (spot 25), putative glucanase (spot 21), abscisic stress ripening protein homolog (spot 25), acidic endochitinase (spot 19). Heat shock proteins of different classes occur in plants and their expression is modulated by a wide range of stresses, including oxi-

 

**Table 1.** Spots/proteins identified in *Malus* x *domestica* Borkh. cv. Annurca classified according to functional categories. Spots identified by peptide mass MALDI-TOF fingerprint (MF) or μLC-ESI-IT-MS-MS (MS/MS) are reported. Spot number, protein name, accession/EST number, organism, method of identification, PROWL Est'd *Z* score, number of peptides identified, experimental and theoretical p*I* and $M_r$ values are listed

| Spot | Protein name | Gene | Swiss–Prot Acc. | Gen Bank gi | Peptides/ Sequence coverage (%) | Organism | Identification method (Est'd *Z* score) | Theoretical MW (Da) | Theoretical p*I* (pH) | Experimental MW (Da) | Experimental p*I* (pH) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon/nitrogen metabolism or energy | | | | | | | | | | | |
| 9 | ATP Synthase D Chain | Atp5h | | *51912938* | 22/60 | *M. domestica* | MS/MS | 25.736 | 8.48 | 24.112 | 5.26 |
| 11 | Adenine phosphoribosyltransferase 1 | APT1 | | *51563580* | 8/27 | *M. domestica* | MS/MS | 18.624 | 5.49 | 27.137 | 5.15 |
| | | | | *51865226* | 5/37 | | | 18.490 | 9.03 | | |
| 20 | Subunit 6B of cytochrome C oxidase | COX6b | | *51560933* | 3/10 | *M. domestica* | MS/MS | 25.734 | 4.82 | 31.654 | 4.25 |
| 24 | Triosephosphate isomerase | TPIP1 | | *52025086* | 3/31 | *M. domestica* | MS/MS | 17.033 | 5.66 | 28.321 | 5.97 |
| 28 | NAD-dependent malate dehydrogenase | mdh1 | Q946Y0 | | 12/33 | *P. persica* | MF (2.32) | 35.497 | 6.60 | 35.615 | 6.0 |
| 29 | Glutamine synthetase | Gln1-5 | Q42951 | | 14/17 | *N. tabacum* | MF (2.33) | 39.176 | 5.38 | 39.260 | 5.50 |
| 30 | Glutamine synthetase | Gln1-5 | Q42951 | | 10/17 | *N. tabacum* | MF (2.00) | 39.176 | 5.38 | 39.260 | 5.50 |
| 31 | Glyceraldehyde 3-phosphate dehydrogenase + fructose-biphosphate aldolase | GAPC | | *51293399* | 7/48 | *M. domestica* | MS/MS | 23.591 | 6.49 | 34.450 | 7.0 |
| | | | | *51713317* | 7/46 | | | 17.006 | 6.66 | | |
| | | | | *51241377* | 5/27 | | | 23.894 | 8.31 | | |
| | | | | *51098631* | 1/10 | | | 24.958 | 6.39 | | |
| | | | | *49632440* | 8/54 | | | 21.537 | 7.02 | | |
| | | | | *50889010* | 2/24 | | | 17.072 | 9.80 | | |
| | | | | *48486994* | 1/4 | | | 21.806 | 5.62 | | |
| 32 | Enolase | | P42896 | | 12/32 | *R. communis* | MF (2.18) | 47.912 | 5.56 | 46.556 | 5.23 |
| 33 | Nucleoside diphosphate kinase I | NDPK1 | | *52024523* | 14/46 | *M. domestica* | MS/MS | 23.793 | 7.12 | 19.213 | 6.72 |
| 39 | Mitochondrial processing peptidase | MPP | | *48485396* | 5/35 | *M. domestica* | MS/MS | 18.381 | 5.07 | 54.213 | 5.83 |
| | | | | *48389152* | 5/50 | | | 21.714 | 4.84 | | |
| | | | | *48418211* | 2/22 | | | 24.737 | 6.43 | | |
| 47 | Triosephosphate isomerase | TPIP1 | | *51913259* | 11/44 | *M. domestica* | MS/MS | 25.929 | 8.31 | 28.123 | 6.31 |
| | | | | *51912257* | 4/26 | | | 16.554 | 9.49 | | |
| Stress and ripening | | | | | | | | | | | |
| 6 | Putative actin depolymerizing factor | ADF | | *51865630* | 6/43 | *M. domestica* | MS/MS | 20.132 | 7.92 | 20.316 | 6.41 |
| 7 | Profilin | | | *51565608* | 1/5 | *M. domestica* | MS/MS | 26.747 | 7.08 | 18.507 | 4.76 |
| 10 | Type 2 peroxiredoxin | PrxII | | *51865528* | 16/52 | *M. domestica* | MS/MS | 22.773 | 5.58 | 23.345 | 5.54 |
| 19 | F3H7.1 protein + Acidic Endochitinase SE2 | SE2 | | *51912579* | 5/24 | *M. domestica* | MS/MS | 24.478 | 4.37 | 26.206 | 5.0 |
| | | | | *51097167* | 1/18 | | | 24.314 | 4.93 | | |
| | | | | *51560882* | 5/31 | | | 26.206 | 4.51 | | |
| 21 | Putative glucanase | | | *48939066* | 3/22 | *M. domestica* | MS/MS | 19.651 | 4.53 | 39.512 | 4.46 |
| 23 | Hsp70 protein | T22-A6.110 | Q9STW6 | | 14/19 | *A. thaliana* | MF (2.35) | 76.508 | 5.07 | 76.200 | 4.48 |
| 24 | Abscisic stress ripening protein homolog | | | *52024974* | 1/15 | *M. domestica* | MS/MS | 15.237 | 5.86 | 28.321 | 5.97 |
| | | | | *52024714* | 6/50 | | | 25.197 | 5.72 | | |
| 25 | Abscisic stress ripening protein homolog | | | *52024974* | 1/15 | *M. domestica* | MS/MS | 15.237 | 5.86 | 28.321 | 5.97 |
| | | | | *52024714* | 6/40 | | | 25.197 | 5.72 | | |
| 26 | Superoxide dismutase [Mn] | SODA | | *50890378* | 6/40 | *M. domestica* | MS/MS | 18.617 | 6.12 | 26.456 | 6.59 |
| | | | | *51292622* | 3/24 | | | 14.997 | 9.30 | | |
| 43 | Acidic Endochitinase SE2 | SE2 | | *51865118* | 3/23 | *M. domestica* | MS/MS | 30.625 | 5.51 | 29.321 | 4.82 |
| | | | | *52025053* | 1/8 | | | 18.924 | 8.86 | | |
| 44 | Acidic endochitinase SE2 | SE2 | | *51865118* | 2/7 | *M. domestica* | MS/MS | 30.625 | 5.51 | 29.409 | 4.71 |
| | | | | *52025053* | 4/49 | | | 18.924 | 8.86 | | |
| Allergenes | | | | | | | | | | | |
| 1 | Major latex-like protein | RAS1 | | *46612948* | 16/68 | *M. domestica* | MS/MS | 15.231 | 5.65 | 21.302 | 5.61 |
| 2 | Ribonuclease-like PR-10b | YPR10*b | Q941P8 | | 7/48 | *M. domestica* | MF (2.24) | 17.563 | 5.50 | 17.234 | 5.0 |
| 3 | Ribonuclease-like PR-10b | YPR10*b | Q941P8 | | 5/47 | *M. domestica* | MF (2.01) | 17.563 | 5.50 | 17.234 | 5.0 |
| 5 | Major allergen Mal d 1 | | Q40280 | | 13/72 | *M. domestica* | MF (2.41) | 17.408 | 5.63 | 17.123 | 5.86 |
| 17 | Thaumatin-like protein 1a – Mal d 2 | TL1 | Q9FSG7 | | 7/38 | *M. domestica* | MF (2.36) | 23.211 | 4.72 | 23.500 | 4.80 |

**Table 1.** Continued

| Spot | Protein name | Gene | Swiss–Prot Acc. | Gen Bank gi | Peptides/ Sequence coverage (%) | Organism | Identification method (Est'd Z score) | Theoretical MW (Da) | Theoretical p/ (pH) | Experimental MW (Da) | Experimental p/(pH) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 42 | Major latex-like protein | RAS1 | | *46612948* | 4/26 | *M. domestica* | MS/MS | 15.231 | 5.65 | 21.467 | 5.41 |
| 45 | Thaumatin-like protein 1a – Mal d 2 | TL1 | Q9FSG7 | | 4/17 | *M. domestica* | MF (2.08) | 23.211 | 4.72 | 23.400 | 4.35 |
| 50 | Major allergen Mal d 1 | | Q40280 | | 7/38 | *M. domestica* | MF (2.17) | 17.408 | 5.63 | 17.580 | 5.0 |
| Other proteins | | | | | | | | | | | |
| 4 | Glycine-Rich RNA-Binding Protein | GRP | | *51241112* | 4/22 | *M. domestica* | MS/MS | 19.231 | 6.59 | 20.310 | 6.15 |
| | | | | *51863876* | 2/29 | | | 6.080 | 10.61 | | |
| 8 | Translationally controlled tumour protein homolog | | | *51865641* | 10/46 | *M. domestica* | MS/MS | 23.872 | 4.68 | 24.106 | 4.66 |
| 12 | F13M22.16 Protein | | | *51912587* | 5/25 | *M. domestica* | MS/MS | 26.418 | 5.78 | 28.991 | 5.25 |
| | | | | *51561177* | 2/6 | | | 17.491 | 9.15 | | |
| 13 | Arg10 | | | *51560124* | 3/15 | *M. domestica* | MS/MS | 23.599 | 5.61 | 29.101 | 5.32 |
| | | | | *51292997* | 2/54 | | | 7.978 | 4.60 | | |
| | | | | *51560831* | 2/30 | | | 9.754 | 5.02 | | |
| 14 | F13M22.16 Protein | | | *51912587* | 5/12 | *M. domestica* | MS/MS | 26.418 | 5.78 | 28.901 | 5.43 |
| | | | | *51561177* | 3/46 | | | 17.491 | 9.15 | | |
| 15 | Plasma membrane intrinsic polypeptide | | | *51864816* | 12/66 | *M. domestica* | MS/MS | 23.379 | 5.04 | 31.202 | 5.50 |
| | | | | *51560648* | 4/22 | | | 18.127 | 5.90 | | |
| 16 | Plasma membrane intrinsic polypeptide | | | *51864816* | 9/45 | *M. domestica* | MS/MS | 23.379 | 5.04 | 31.123 | 5.40 |
| | | | | *51560648* | 4/22 | | | 18.127 | 5.90 | | |
| 18 | Atranbp1b protein | RANBP1B | | *51913615* | 8/36 | *M. domestica* | MS/MS | 23.281 | 5.41 | 32.345 | 4.93 |
| 22 | clone AAFA001430 | | | *47966193* | 5/35 | *M. domestica* | MS/MS | 88.440 | 5.23 | 80.876 | 4.76 |
| 34 | F26k24.22 protein | F26-K24.22 | | *52024867* | 12/45 | *M. domestica* | MS/MS | 25.309 | 8.47 | 23.098 | 6.57 |
| 37 | 20S Proteasome alpha 6 subunit | PAF1 | Q8H1Y2 | | 8/30 | *N. benthamiana* | MF (2.04) | 29.876 | 5.07 | 30.336 | 4.96 |
| 38 | Plasma membrane intrinsic polypeptide | | | *51563760* | 7/42 | *M. domestica* | MS/MS | 19.785 | 5.91 | 31.786 | 5.27 |
| | | | | *51238172* | 4/50 | | | 11.732 | 4.46 | | |
| | | | | *51864816* | 3/18 | | | 23.379 | 5.04 | | |
| 41 | RAD23 PROTEIN | RAD23 | | *54684737* | 5/33 | *M. domestica* | MS/MS | 24.127 | 4.55 | 52.432 | 4.70 |
| 46 | clone AVBC007680 | | | *48413158* | 5/51 | *M. domestica* | MS/MS | 18.694 | 4.02 | 34.876 | 4.01 |

dative stress. Recently, in tomato it has been shown that the cytosolic isoform of the Hsp70 increases during ripening [19]. Profilin (spot 7) and actin-depolimerizing factor (spot 6) were also detected; they are involved in the regulation of actin cytoskeleton whose organisation is influenced upon different kind of stresses [20]. They also classify as general fruit allergens (see next paragraph). Finally, proteins involved in the response to biotic stresses were identified, such as acidic endochitinase (spot 19, 43 and 44), thaumatin-like protein 1a (spot 17) and glucanase (spot 21). Also these polypeptides, which function as a defense against chitin containing fungal pathogens and accumulate in leaves upon fungal infection [21] are described as important food allergens (see next paragraph).

### 3.4 Allergens

Different classes of allergens associated with the birch pollen-related food allergy or with the latex-fruit syndrome were detected in the pseudocarp of Annurca apples. The birch pollen-related food allergy is due mainly to the Mal d 1 allergen, the major apple allergen, which is cross reactive to the homologous Bet v 1 allergen from birch [22]. Identifications of proteins related to this syndrome were major allergen Mal d 1 (spots 5 and 50) and ribonuclease-like PR-10 b (major allergen Mal d 1.03D) (spots 2 and 3). In fact, Mal d 1 belongs to a group of pathogenesis-related (PR)-10 proteins which are induced by pathogens and also increase during ripening [23]. The Mal d 1 class appears to be represented at least by 18 members and apple cultivars differ consistently in allergenicity. Densitometric analysis of normalised spot densities for Coomassie-stained spots revealed that among the Annurca accessions, levels of Mal d 1 varied, with the S. Agata dei Goti cultivar containing the smallest antigen amount, nearly three-fold less than that of other accessions. The latex-fruit syndrome concerns people allergic to natural rubber latex that show an associated hypersensitivity to plant food, especially fruits; it is caused by different classes of allergens and it is similarly due to allergen crossreactivity [24]. Latex-fruit syndrome allergens identified in the Annurca flesh were major latex-like protein (spot 1 and 42), thaumatin-like protein 1 a (allergen

 

Mal d 2) (spots 17 and 45), glucanase (spot 21), acidic endochitinase (spot 19) and profilin (spot 7). Mal d 2 protein was by far the most prominent spot and its quantity differed among the three accessions; in fact, S. Agata dei Goti cultivar contained a two-fold higher content than the other accessions. Our investigation showed that Mal d 1 and Mal d 2 proteins are the predominant allergens occurring in Annurca flesh and suggests that a detailed characterisation of their quantitative and isoform profile among accessions could be very helpful in the breeding of hypoallergenic cultivars.

### 3.5 Other proteins

Diverse proteins that did not group in the above classes were also identified. For the majority of them information was very poor; in particular, functions were unknown or very hypothetical and based on sequence homology criteria. In few cases, a relation to important physiological processes emerged. In this respect, it is worth noting the identification of two proteins that appear involved in the control of programmed cell death (PCD), namely the 20S Proteasome alpha 6 subunit (spot 37) and the RAD23 protein (spot 41), which contains a ubiquitin-like domain and interacts with catalytically active proteasomes [25]. While increasing evidence indicates that PCD plays a pivotal role in diverse plant processes, such as embryogenesis, senescence or pathogen defence [26, 27], data concerning its involvement in the ripening of fruits are lacking. A homologue of the Ran-binding protein1 b from *Arabidopsis* (AtRanBP1b) was also identified (spot 18). Ran-binding proteins are activators of Ras-related nuclear small GTP-binding proteins (Ran) and in *Arabidopsis* have been shown to be involved in the regulation of auxin-induced mitotic progression [28]. A protein showing sequence relation to the translationally controlled tumour protein homologue from rice was also identified (spot 8). Mammalian counterparts of these proteins have calcium binding ability and microtubule stabilisation activity, from which a function of the plant homologues in the maturation of fruits might be envisaged. Finally, other proteins for which information is very scarce or lacking were identified: (i) the glycine-rich RNA-binding protein (spot 4), (ii) the Arg10 protein (spot 13), which in *Arabidopsis* has been shown to be down-regulated by auxin [29], (iii) the F26k24.22 protein (spot 24), which shows 90% identity to the putative ethylene-responsive protein from *Arabidopsis* (accession Q8L9C2), (iv) the F13M22.16 protein (spot 12, 14), which is putatively a chloroplastic sugar epimerase, (v) the plasma membrane intrinsic polypeptide (spot 15, 16, 38), and (vi) the AVBC007680 clone (spot 46) and the AAFA001430 clone (spot 22), with unknown function.

## 4 Concluding remarks

In conclusion, this first proteomic study on the pseudocarp of Annurca apple is very informative about the composition of proteins abundantly expressed in ripe fruit and its conservation among different accessions. The molecular characterisation of germplasm collections is essential to the identification of markers which can help plant breeders to a better exploitation of the genetic resources of crop species. In this respect, future investigation will be aimed to a more detailed description of the protein pattern of the Annurca pseudocarp and to its quantitative comparison to that of elite varieties, in order to identify genetic-based differences and possibly define a repertoire of molecular markers which could account for at least some of the peculiar nutritional and taste characteristics of this variety. Finally, since this study allowed identifying different allergens, causative of widespread food allergy syndromes, a detailed characterisation of the Annurca allergen profile will be carried out, in view of the possibility to breed less antigenic cultivars and to provide information about crossreactivity risk to allergic consumers.

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