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The identification of allergen proteins in two different varieties of strawberry by two different approaches: Proteomic and western blotting method

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ABSTRACT

Strawberry (*Fragaria ananassa* Duch.) is one of most important fruit crops cultivated widely in world. In the present study, we have investigated the proteome variation to different commercially available red strawberry varieties, to gain better insight into the variation in allergen content between the different varieties, as well as their proteome variation. In fact, a combination of proteomic tools have been used to build a complete allergen map of strawberry. The water-soluble fraction of a strawberry extract was precipitated using a phenol-based procedure and separated by 2-DE. Further, all known strawberry allergens were localized on a 2-DE map and they were matched with spots recognized by sera of patients with different allergic patterns. A number of constitutive or differentially accumulated proteins were found. The role of the identified proteins, in particular of the allergen proteins, is discussed in relation to the different variety of strawberry.

1. Introduction

In human diets, the main source for vitamins and minerals are fresh vegetables and fruits though contain significant level of allergens (Nakamura and Teshima, 2013; Ahsan et al., 2016) which commonly presents as oral allergy syndrome (OAS).

Recently (January 2016) a database online AllergenOnline (http://www.allergenonline.org/) reports 778 allergen protein groups and 1956 sequences (Goodman et al., 2014) of which 388 (46%) allergen proteins from plants, 338 (40%) from animals, and 110 (13%) from fungi. Therefore, this database contains over 3066 allergenic molecules, considered as the largest allergen database (Alm et al., 2007).

Proteomics approaches have been used extensively to detect and quantify food allergens in the last decades. In fact, only 20% of the studies on allergy focused on food allergens wherein only 5% of proteomic studies on allergy used a targeted proteomic approach (Guarino et al., 2007; D'Amato et al., 2010; Song et al., 2015).

The proteomic method is based on the properties of allergens, in that, many allergens of plant consist mainly in proteins that have low molecular weight and include disulfide bonds. Therefore, using these properties, including peptide ligand libraries to detect low abundant allergens (Scala et al., 2011), triple-one-DE and MS to analyze carboxyl modifications of allergens (Kitta et al., 2006) is possible to improve the separation of low molecular weight allergens (Yano and Kuroda, 2008), and a proteomic method targeting disulfide bonds (Bianco et al., 2009).

One of the most common methods for detection and quantification of allergens in food was the 2D-immunoblot method, proteins are separated by IEF, electrically transferred onto polyvinylidene fluoride (PVDF) membranes, and then proteins binding to IgE in patients' serum are detected using an antibody to human IgE. This method separates proteins quickly, it does not require isolation of proteins, and it allows comprehensive detection of several IgE-binding proteins simultaneously. In future, a combination of proteomics and these biological assays can yield much information on IgE-binding proteins.

Strawberry is one of the most popular fruit and it is worldwide appreciated for its unique flavor (Pastorello et al., 2001). The total worldwide production is estimated at 4.59 million tonnes in 2011 (FAOSTAT, Production, 2015).

Plant allergens have various roles; some such as are storage proteins, some are enzymatic, and others are structural proteins (Breiteneder and Radauer, 2004; Breiteneder and Mills, 2005; Radauer and Breiteneder, 2007; Matthes and Schmitz-Eiberger, 2009). Plant allergens are classified into families according to their functions and structures. The expression levels of allergens can vary due to various factors, such as plant cultivars (Brenna et al., 2004; Sancho et al., 2006; Ciardiello et al., 2009; Pedreschi et al., 2009), growth and storage

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conditions (Erler et al., 2011) and ripening stages. Proteomics can also yield much information about the expression levels of plant allergens in various environmental conditions.

One of the factors that affect allergen expression levels is the genetic background of plant cultivars. In fact, in a study (Song et al., 2015) proteomics data by DIGE and proteins identified with MALDI-MS/MS have showed that the amount of the strawberry allergen Fra a1 varied between different strawberry varieties and in particular between red and white strawberries and that all the white strawberry cultivars were low in allergen. Thus, the allergen variation due to growth conditions must be taken into consideration in attempts to obtain a low-allergen strawberry. Moreover, allergenicity varies among cultivars, since each cultivar expresses different allergen isoforms or allelic variants (Karlsson, et al., 2004; Fang et al., 2013).

In recent years, several cross-reactive allergens have been identified and sequenced in fruits of the Rosaceae family. The major allergens in apple (Mal d1), pear (Pyr c1), apricot (Pru ar1), and sweet cherry (Pru av1) are structural homologs to the birch pollen major allergen Bet v1, which belongs to class 10 of pathogenesis-related proteins. Other major allergens from apple (Mal d2) and cherry (Pru av 2) have been identified as thaumatin-like proteins. More recently, a lipid transfer protein has been reported to be an important allergen in peach (Pru p3), apricot (Pru ar 3), and apple (Mal d3). The strawberry allergen Fra a1 is such a homologue to the major birch pollen allergen Bet v1 (Okie, 2004; Muñoz et al., 2010).

In the present study, we have investigated the proteome variation to different red strawberry varieties, to gain better insight into the variation in allergen content between the different varieties, as well as their proteome variation. In fact, a combination of proteomic tools have been used to build a complete allergen map of strawberry. The water-soluble fraction of a strawberry extract was precipitated using a phenol-based procedure and separated by 2-DE. Further, all known strawberry allergens were localized on a 2-DE map and they were matched with spots recognized by sera of patients with different allergic patterns.

Therefore, we have identified the allergen proteins in two different varieties of strawberry (*Fragaria ananassa* Duch.) by proteomic approach compared to conventional methods involving protein isolation processes for detecting the binding between a patient's IgE and separated plant allergenic protein on a membrane.

2. Materials and methods

2.1. Strawberry material for proteome analysis

Two different varieties of strawberry, *F. ananassa*, were cultivated outdoors in June - July 2010, harvested at the same site, within a program directed by prof. Carmine Guarino at the experimental station of University of Sannio (Italy). This set, selected for analysis, was composed of biological duplicates of two commercially available varieties of strawberry ("*Queen Elisa*" and "*Camarosa*"). The first, "*Queen Elisa*" is derived from a cross made in 1994 between the Italian variety "*Miss*" and selection American USB 35 (*Lateglow* × *Seneca*). It is early maturing, suited for use in the northern plains and is valid for that protected crops, open field. It differs from other varieties for good fruit quality, admirable consistency, combined with high aroma and sweetness due to the high sucrose content (Pastorello et al., 2001; Carbone et al., 2006). The comparison of the cultivar *Queen Elisa* with its parental genotypes Miss and USB35 indicated up- and down-regulation of several genes, some of which were involved in the expression of quality traits such as aroma (Pastorello et al., 2001; Faedi et al., 2006).

The other "*Camarosa*" is a cultivar of American origin, obtained from the University of California, widespread commercially since 1992. The fruits are beautiful conical-oblong, very large, of good taste. The color although rather intense is well accepted by the market, because it always maintains bright. This cultivar has been shown to adapt well to the environment Campanian and this is highlighted by an excellent reproductive behavior.

Ten fruit samples for each variety and ripening stage (ca. 30 days after anthesis), were harvested, cut into quarters, pooled and immediately frozen in liquid nitrogen and kept at -80 °C until use for protein extraction. Three biological replicates (1–3) were performed for each variety.

2.2. Protein concentration

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 b-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 × *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 × *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 × *g* for 10 min at 20 °C. Protein concentration was determined by the Bradford assay using a SmartSpec Plus Spectophotometer (BioRad).

2.3. Two-dimensional gel electrophoresis

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 6 × 1 mm) in 25 mM Tris pH 8.3, 1.92 M glycine and 1% w/v SDS, with 70 V (about 35 mA) applied for 16 h. Two samples for each Fragaria accession were independently phenol-extracted and run in triplicate. Protein spots were annotated only if detectable in all gels. For mass spectrometric analysis gels were stained with CBB, while other gels were transferred onto suitable membranes for western blotting analysis using IgE.

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 normalization 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 by Guarino et al. (2007). 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). 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 non-redundant database by PMF experiments. Candidates with ProFound's Est'd Z scores > 2 were further evaluated by comparison with Mr and pI 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 Mr 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.

2.7. IgE western blotting

Blotting transfer was performed on to a PVDF membrane by a semidry blotter (BioRad, Hercules, USA). PVDF membranes were rinsed briefly in distilled water and then blocked by submersion in 5% non-fat dry milk (NFDM) in PBS buffer for 2 h at room temperature. Blocked membranes were incubated overnight at room temperature in sealed plastic pouches with individual strawberry fruit allergic sera or control sera, which had been diluted 1:50 (v/v) in 2% NFDM in PBST (PBS with 0.05% Tween 20) 60 min prior to adding to the membrane. Following incubation membranes were washed four times in PBST, then incubated with goat anti-human IgE conjugated with horseradish peroxidase (ICL, Newberg, USA) at a ratio of 1:1000 v/v for 4 h at room temperature. Reacting spots were visualized by ECL (Amersham Biosciences) reaction and chemiluminescence using the Molecular Imager ChemiDoc XRS (BioRad Hercules, USA).

2.8. Serum donor

Sera of 20 strawberry- allergic subjects (male and females) were used to identify distinct allergens and to confirm the strawberry allergen map, including five subjects for each pooled sera. Immediately after collection, fasting blood samples were allowed to clot at room temperature for four hours, and the serum were collected and centrifugated at 3000 rpm/min for 15 min. Before pooling the samples, the donor were clinically diagnosed as allergic to strawberry based on clinical histories and specific IgE tests (ImmunoCAPTM, Pharmacia Diagnostica). Five serum samples were the Pooled sera 1 (STR01-5-mean IgE 1347 KU/L), while other five serum samples were the Pooled sera 2 (STR06-10- mean IgE 1715 KU/L), and five serum samples were the Pooled sera 3 (STR011-15-mean IgE 588 KU/L). Five serum samples of Pooled sera 4 (CON01-5-mean IgE 0 KU/L) were mixed as control-pool. The four pooled sera were diluted respectively to ~20 mg/mL with 100 mM phosphate buffer (pH 2.0, containing 5% ACN). Then, the pooled sera were filtered through 0.22 μm filters (Agilent technologies) by spinning at 10 000*g* at 4 °C for 30 min and dialyzed to 100 mM phosphate buffer (pH 2.0, containing 5% ACN). Historical information and diagnostic results for all strawberry fruit allergic donors are presented in Table 1 along with data for donor of non-strawberry fruit allergic control donors collected by the University of Sannio.

Table 1
Characteristics of pooled sera of 20 donors. Pooled sera from strawberry fruit- allergic groups 1, 2 and 3 (STR01-5, STR06-10, andSTR011-15), diagnosed based on clinical histories, and specific IgE (ImmunoCap from Pharmacia Diagnostics) and pooled sera from five individual (CON 01-5) who is not allergic to strawberry fruit. AE: AngioEdema; AP: Abdominal Pain; FE: Facial edema; OE: Oropharyngeal Edema; U: Urticaria.

Pooled sera	Serum donors	Age (years)	Age onset	Symptoms strawberry	IgE (kU/L)	Other known allergies
1	STR01	13	6	AE, OE, U	1344	Birch pollen, cat, tree nuts
	STR02	13	7	AE, OE, U	1350	Birch pollen, cat
	STR03	11	5	AE, OE, U	1345	Birch pollen, tree nuts
	STR04	11	6	AE, OE, U	1375	Cat, tree nuts
	STR05	12	6	AE, OE, U	1321	Birch pollen, cat, tree nuts
	Mean	12	6	AE, OE, U	1347	Birch pollen, cat, tree nuts
2	STR06	36	26	AP, FE	1745	Peanuts, house dust mite
	STR07	32	27	AP, FE	1713	House dust mite
	STR08	36	25	AP, FE	1742	Peanuts, house dust mite
	STR09	36	25	AP, FE	1703	Peanuts, house dust mite
	STR010	35	27	AP, FE	1672	Peanuts, house dust mite
	Mean	35	26	AP, FE	1715	Peanuts, house dust mite
3	STR011	14	8	U	588	None
	STR012	13	8	U	598	None
	STR013	15	7	U	578	None
	STR014	12	8	U	597	None
	STR015	16	9	U	579	None
	Mean	14	8	U	588	None
4	CON01	47	–	–	< 0.35	None
	CON02	48	–	–		None
	CON03	45	–	–		None
	CON04	49	–	–		None
	CON05	47	–	–		None
	Mean	47	–	–	< 0.35	None

3. Results and discussion

Proteins extracted from "*Camarosa*" and in "*Queen Elisa*" strawberry variety were separated by 2D-PAGE and spots were visualized by CBB staining. Analysis of spots on the 2-DE gel using the PDQuest 2D image analysis software (Bio- Rad) indicated approximately 200 proteins having a *Mr* less than 100 kDa and a *pI* in the range 3–10 (Fig. 1).

Taking into account the spot areas and their intensity, 69 protein spots were selected along each 2-DE gel for "*Camarosa*" and in "*Queen Elisa*". 15 out of 69 proteins were differentially expressed in two varieties of strawberry ($P < 0.01$) while 54 protein spots were found to be common to two variety of *F. ananassa*.

Owing to the poorly solved strawberry genome, 27 protein spots were from *F. ananassa* and 42 from other species.

The relative quantitative variations of the identified proteins, grouped according to their functional classification, are reported in supplementary online material (Table S2).

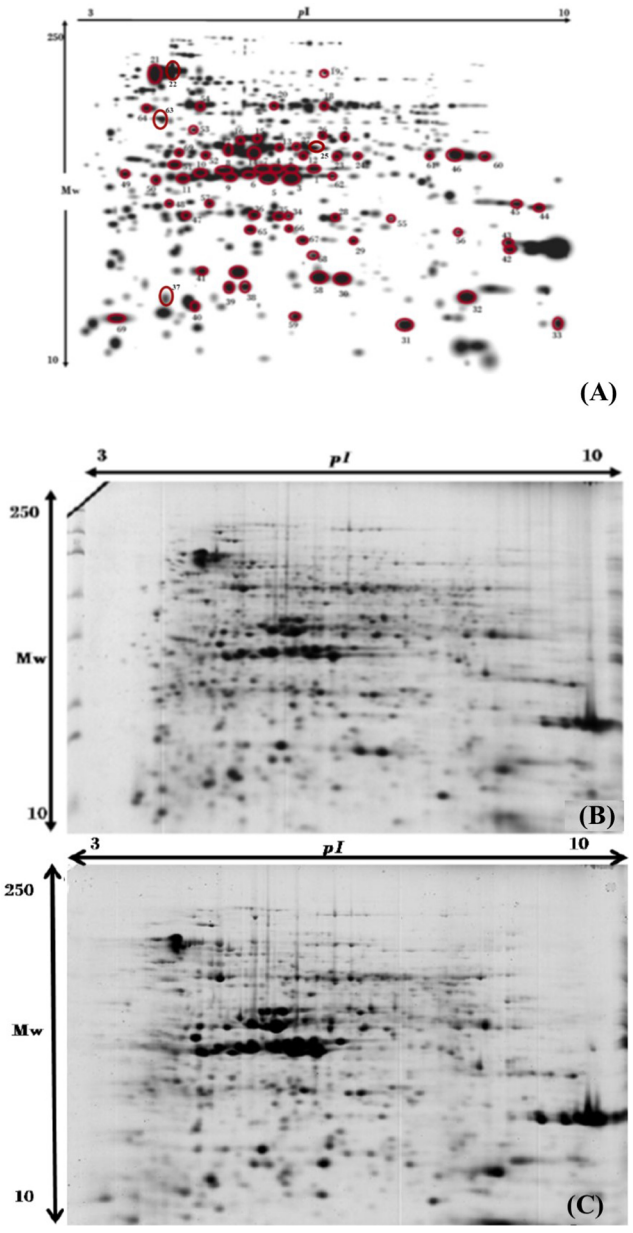

Fig. 1. Representative 2-DE gels of total protein extracts from two varieties of *Fragaria* × *ananassa*. (A) Master gel; (B) "*Camarosa*" variety; (C) "*Queen Elisa*" variety. Gels were stained with colloidal CBB G-250. Spot numbering refers to Table 1 showing protein identification as obtained by MS analysis.

The resulting list of the identified proteins were subdivided in eight categories, as follows: 1) Allergens; 2) Cellular proteins involved in the organization; 3) Proteins involved in energy metabolism and carbon; 4) Heterogeneous proteins; 5) Proteins involved in the metabolism of nucleotides, in the synthesis and metabolism of proteins; 6) Response to stress proteins; 7) Proteins involved in secondary metabolism and in the biosynthesis of cellular components; 8) Proteins involved in the facilitated transport (Fig. 2).

3.1. Allergens

Precisely seven allergens are identified (spots 1, 2, 3, 4, 5, 6, 7) into two groups: that of PR-10 (pathogenesis-related proteins 10) and that of storage proteins of seeds (Table 2). The first group includes the two proteins identified as "Fra a1", the second group the Prunine and its precursors. Fra a1 (spots 1 and 2), is the first specific allergen of the strawberry to have been identified and characterized (Nessler et al., 1985). It is a homologue of Bet v1 (the major allergen of birch), and therefore, as the latter, is also a PR-10 (Okie, 2004). The PR-10 proteins are ubiquitous and present in angiosperms (mono-and dicotyledons), both in gymnosperms. The members of the family PR-10 proteins are associated with plant defense and tissue differentiation. Their expression is in fact triggered by pathogenic infections by plant hormones, to injury, stress or cold saline. Phylogenetic analysis of PR-10 isoforms, show that the multiple sequences of each family of plants form a monophyletic group and that the sequences of each family are much more similar than that from other families. This suggests that genes are regulated by multigene families concerted evolution. They present a well-defined domain, characterized by an extended and antiparallel β-sheet wrapped around a residue C-terminal α-helix, which includes a large cavity inside the protein. Within which different ligands can be bound hydrophobic. It has recently been discovered that the allergen Fra a1 is present only in the red fruit stage of mature *F. ananassa* (Hjernø et al., 2006). In fact, in the white fruit, characterized by a mutant genotype and known to be tolerated by people with allergies, the allergen is missing. The proteomic analysis has shown that the Fra a1 as well as the enzymes of the biosynthetic pathway of flavonoids, which determine the color red fruits, are down-regulated in strawberries white in color. While, the down-regulation of this allergen provides an explanation of why individuals with allergy to strawberries can tolerate white strawberries instead, the other is to assume that the homologues of Bet v1, are involved in the transport of flavonoids. These include a wide range of compounds with different functions, among which is precisely the pigmentation of the fruits and flowers. Further confirmation that the Bet v1 homologues are involved in the biosynthetic pathway of flavonoids is given by 45% sequence identity between Bet v1 and Hyp-1, the enzyme responsible for the formation of hypericin, a color naftodiantrone red, present in *Hypericum perforatum* (Bais et al., 2003). The naftodiantrone is a compound of the family of anthraquinones, which in turn belong to the family of quinones, colored pigments present in many plants. Hypericin is a natural product synthesized in the medicinal plant *Hypericum perforatum*, (known as St. John's Wort), which is arousing the interest of the pharmaceutical sector because of its potential application in various therapies, including antidepressant treatment. The 'Hyp-1' is a small protein of 17.8 kDa, which because of its similarity to the allergen Bet v1 (45%) has been tentatively classified as a PR-10 plant. As mentioned previously, the PR-10 are the main responsible of the clinical manifestations of 'OAS (Oral Allergy Syndrome)'.

The prunine represent the second group of differentially expressed allergens identified in the work. They are the "seed storage protein" and belong to the family of globulins. Globulins along with albumins are the major storage proteins found in seeds of angiosperms. As already mentioned, the globulins are divided into two groups according to the sedimentation coefficient: viciline the 7S and 11S legumine. Globulins

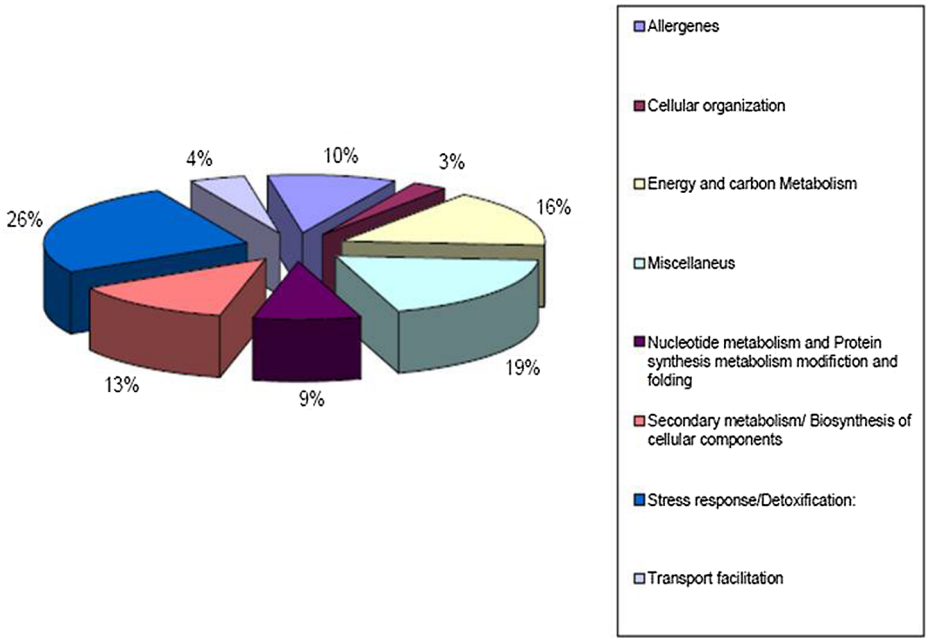

Fig. 2. Distribution of the proteins identified in *Fragaria* × *ananassa* classified according to functional categories.

(or legumine) 11S are present in the endosperm of many seeds. They are synthesized at the level of the rough endoplasmic reticulum and transferred through the Golgi apparatus, up to the vacuole. The latter then form by fragmentation of the protein bodies. This information allows us to deduce that the assembly of storage proteins in protein bodies is a coordinated event, (as it involves several organelles RER, Golgi, etc.), and orderly. The synthesis of storage proteins occurs in a precise period during the process of formation of the seed. In this period, the mRNA transcripts that encode these proteins are present exclusively in the reserve tissues. Understanding of the mechanisms of regulation of genes for storage proteins are derived, mainly, from studying their expression in seeds of transgenic plants. In general, the storage protein genes are expressed in the appropriate tissue in transgenic seeds also. For example, it was found that by transferring genes from legumes, which coded for proteins, in tobacco, their expression occurred mainly in the cotyledons. This has to deduce that the regulatory regions of these genes are similar and bear the same information, (being so recognized), even in very different species. It was found also that in developing seeds in the abscisic acid (ABA), that a growth regulator positively influences the synthesis of many proteins, including those of the reserve. It is seen that the mRNAs of 11S globulin of rapeseed and to a vicilina 7S soy, increase when the seeds are treated with ABA. The synthesis of storage proteins of seeds is not only regulated at the transcriptional level, but also by post-transcriptional mechanisms. An example is the shortage of sulfur. We must premise that the 11S globulins are unlike viciline 7S, sulfur-rich proteins. The seeds being formed to meet a condition of nutritional deficiency of sulfur by altering the relationship that exists between the sulfur-rich proteins (11S globulins) and poor of this element (global-7S globulins), in favor of the latter. Naturally, the increase in synthesis of vicilina 7S, in conditions of deficiency of sulfur, reflects the increased transcription of the corresponding gene. The allergenicity of the 11S globulin has been confirmed in several studies. For example, research on the identity of the allergenic soybeans (a dicotyledonous herbaceous), point to it as the major allergen of this plant (Shibasaki et al., 1980). Another study of mustard seeds (Palomares et al., 2005) refutes the idea to be valid until some time ago that the head of the allergenic profile was only mustard 2S albumin. In fact, it was found that there are two families of storage protein to be involved in allergic responses to this plant: the 2S albumin and 11S globulin given as Sin a2. The 11S globulins are of oloproteine of 350–400 kDa whose six subunits are frequently encoded by a multigene family from a single precursor. The fact that they were found prunine and precursors of prunine with a high heterogeneity of both molecular weight, pI of what is probably justified by the fact that the globulins are of the 11S protein complex consists of 6 different subunits. Each subunit, characterized by a molecular weight of 60 kDa, consists in turn of two polypeptides linked by disulfide bridges. An acid of 40 kDa and a basic of 20 kDa. The use in the extraction buffer of a high salt concentration and the addition of ß-ME has disassembled 11S globulin. In fact, the high concentration of salts induces the oloproteine dissociation in its subunits because of interference with the non-covalent bonds of electrostatic nature, which keep them together. The reducing agent ß-ME then breaks the disulfide bonds that hold together each pair within a polypeptide and an acid base balance.

This analysis showed that a down-regulation of allergen Fra a1 corresponded to a down-regulation of enzymes of the phenylpropanoid pathway, responsible for its red pigmentation of the fruit. Our results, however, varies from what is reported in the literature. First, because the variety "*Camarosa*" is that the "*Queen Elisa*" enzymes in the biosynthetic pathway of flavonoids are not co-regulated with PR-10. "*Camarosa*" in fact a down-regulation of allergen Fra a1 is associated with an up-regulation of enzymes of the biosynthetic pathway of flavonoids, while the "*Queen Elisa*" to up-regulation of allergen Fra a1 is associated with a down-regulation of enzymes of phenylpropanoid pathway. Secondly, why in "*Camarosa*", where the up-regulation of enzymes responsible for the pigmentation of the fruit is also expressed at the phenotypic level (the color of this fruit is much more intense and brilliant than that of *Queen Elisa*), we expected an increased allergenicity, but the level of PR-10 is less than of that of the *Queen Elisa*.

As regards the second group of allergens found, the prunine, we can say that they are of the 11S globulin. As already mentioned, the globulins are distinguished according to their sedimentation coefficient in viciline 7S and 11S legumine. A further difference between the two is that the globulin proteins are poor viciline sulfur while the legumine are proteins rich in sulfur. Our results indicate a down regulation of prunine the *Camarosa* variety. The literature attributed to a deficiency of sulfur (of which they are rich) decreased levels of 11S legumine seeds. In fact, the seeds in formation respond to a condition of nutritional deficiency of sulfur by altering the ratio of the sulfur-rich proteins and poor of this element. This means that the absence of sulfur

Table 2
Allergenes identified in *Fragaria* × *ananassa*. 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 pI and Mr values are listed. Increasing/decreasing index (fold change) was calculated as the ratio of spot intensities (relative volumes) for "*Camarosa*" variety and "*Queen Elisa*" variety. Proteins were considered as differentially expressed when a relative fold change > 2.0 or < 0.5 was measured.

Spot	Protein name	Swiss – Prot Acc.	Gene Bank gi	Sequence coverage (peptide)	Organism	Identification method (Est'd Zscore)	Experimental *pI*/ (pH)	Theoretical *pI*/ (pH)	Experimental MW (Da)	Theoretical MW (Da)	Fold change (Cam) student's *t*-test $p < 0.01$
	Allergenes										
1	Major strawberry allergen Fra a 1-C	\	90185686	41(7)	Fragaria × ananassa	MS/MS	5.97	5.97	17.500	17.740	0.4
2	Major strawberry allergen Fra a 1-B	\	90185680	9(1)	Fragaria × ananassa	MS/MS	6.32	5.97	17.500	17.820	0.02
3	Prunin	Q43607		17(2)	Prunus dulcis	MF	8.50	6.59	24.100	63.020	0.4
4	Prunin	Q43607		17(2)	Prunus dulcis	MF	8.50	6.59	22.100	63.020	0.1
5	Prunin1 precursor	\	14272197	13(2)	Fragaria × ananassa	MS/MS	4.52	6.59	34.600	63.020	0.4
6	Prunin 1 precursor	\	14272197	13(2)	Fragaria × ananassa	MS/MS	4.35	5.21	36.600	63.020	0.3
7	Prunin 1 precursor	\	14272197	11(1)	Fragaria × ananassa	MS/MS	4.45	6.59	34.800	63.020	0.4

induces an increase in the synthesis of viciline 7S (poor of this element), which in turn reflects an increase in the transcription of the corresponding gene. In our study comparing the two varieties have shown a decrease in the expression of the variety *Camarosa* prunine than the *Queen Elisa*. Based on what has been said, to a shortage of legumine would bind due to an increase in viciline. This increase was not recorded, or rather to emphasize that the protein is also expressed in two varieties, in fact, was not identified any vicilina. Therefore, as a down-regulation of 11S globulins in *Camarosa*, without an associated up-regulation of viciline 7S, is not possible to attribute the result to a deficiency of sulfur, as suggested in the literature. Given the limited information available to us, even in this case we can assume that the different regulation of the globulins is to be attributed to a "phenotypic plasticity". This may motivate the different gene expression that exists between two varieties whose biological diversity is not so high.

3.2. Other proteins identified

Different key enzymes of glycolysis were identified in the *Fragaria* variety, namely cytosolic aldolase (spot 61), glyceraldehyde-3-phosphate dehydrogenase (spots 46, 60), triose-phosphate isomerase (spot 36), pyruvate dehydrogenase (spot 63), phosphoglyceromutase (apgm) (spot 21). 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.

The malate dehydrogenase (spot 24) has been detected from *F. ananassa*. 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 strawberry, in peach it has been demonstrate that the expression of this enzyme is regulated during ripening (Etienne et al., 2002). On the other hand, some enzymes related to energy production were also detected, namely ATP-synthase (spot 28), ribulose bisphoshate carboxylase (spot 18).

The presence of a large number of proteins related to stress and defense supports the idea that significant oxidative processes (Ramakrishna et al., 2003) accompany the ripening of the fruit. Oxidative stress arises from conditions that promote the formation of reactive oxygen species (ROS), which damage or kill cells (such as pollution, drought, heat stress or cold sores, infections and UV light, and senescence). Plants detoxify and eliminate these reactive molecules through antioxidant defense systems. Many proteins identified, such as ascorbate peroxidase (spots 34, 35), glutathione S-transferase (spot 67), are involved in the cycle of ascorbate-glutathione, which is an important route of antioxidant. Also have been identified of proteins known as heat shock proteins (HSP). The expression of these pro-protein is observed, when the plants are exposed to temperatures of at least 5 °C higher than their optimal growth temperature. Some of the major HSPs are conserved in all eukaryotic organisms is that prokaryotes. Many of these proteins function as chaperones and are involved in the folding of proteins denatured by heat. Some HSP are expressed during development. So some proteins HSP defined, are not induced by heat, but rather classified as such for sequence homology or similarity to a possible function. Interesting is also the identification of the LMW heat shock protein (spots 32 and 38). It is believed that this protein is involved in the change of color of the fruit during ripening of tomatoes, as it induces the transition of the chloroplast in chromoplast (Neta-Sharir et al., 2005).

The phenolic components are a major class of secondary metabolites, which includes a broad spectrum of substances that are very heterogeneous, but all characterized by the presence of an aromatic ring with one or more hydroxyl substituents. Their importance in fruit and vegetables is due to the prominent role that they play in determining the color and flavor. In particular is associated to phenolic acids the acid taste, astringency to tannins, while the bitter flavor is

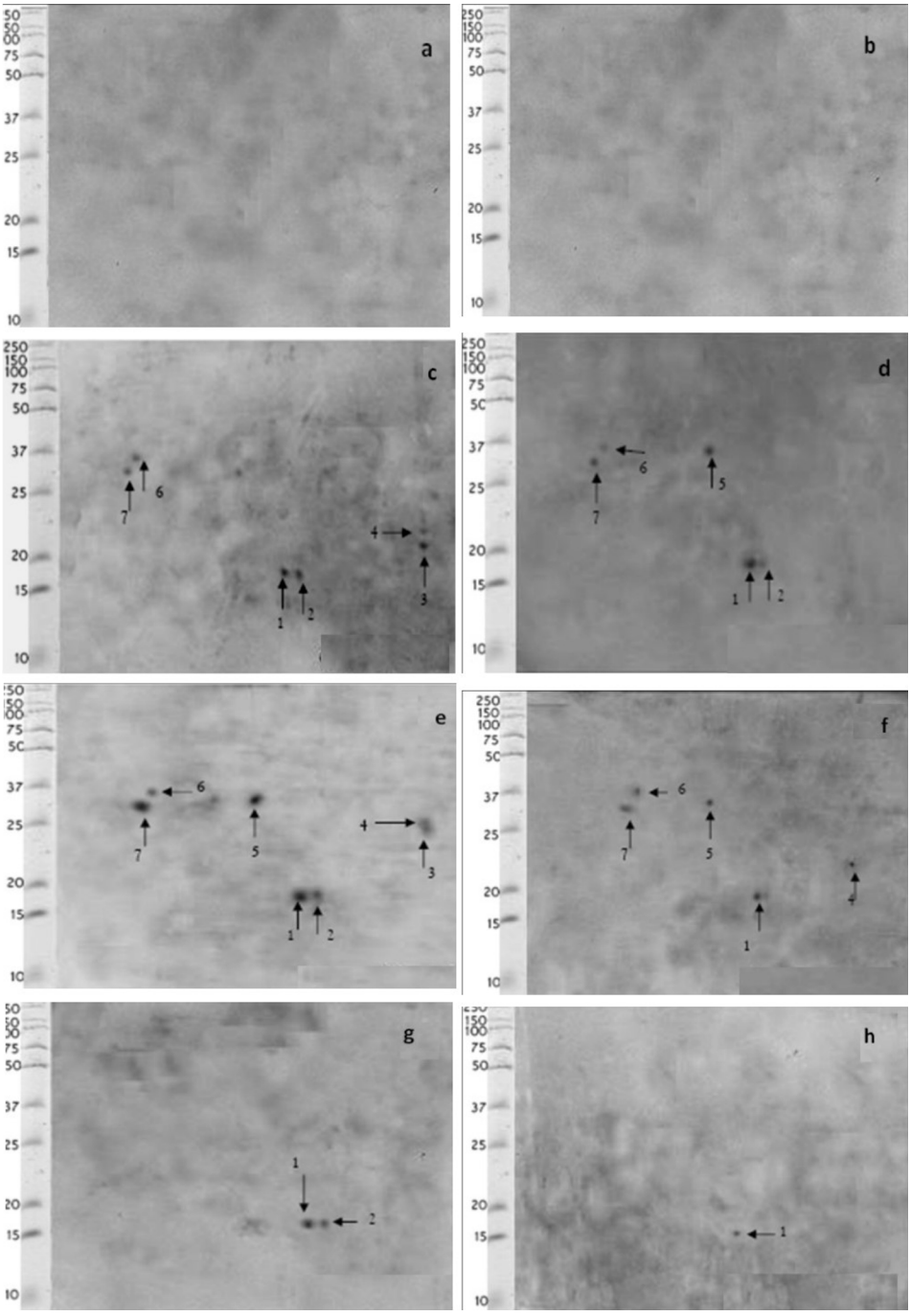

Fig. 3. Identification of allergenic proteins using four pooled sera in "Camarosa"variety (3a, 3c, 3e, 3g) and in "Queen Elisa" variety (3b, 3d, 3f, 3h). a–b: Pooled sera 4 (CON01-5-mean IgE 0 KU/L); c–d: Pooled sera 1 (STR01-5- mean IgE 1347 KU/L); e–f: Pooled sera 2 (STR06-10- mean IgE 1715 KU/L); g–h: Pooled sera 3 (STR011-15-mean IgE 588 KU/L).

associated with some flavonoids such as naringenin and neohesperidin. The color, finally, is determined by the presence of anthocyanins and their characteristics co-pigmentation reactions. The content of phenolic compounds in plant tissues varies depending on the species, variety, the organ in question, and physiological condition of the soil and climatic conditions. Currently have been identified several thousand phenolic structures, among these, the most consistent class is that of flavonoids (C6C3C6). The chemical structure of these compounds, present in all parts of the plant, is based on a skeleton with 15 carbon atoms with an in-color in the ring attached to a second color. In the present study identified a number of key enzymes in the biosynthesis of flavonoids, such as chalcone isomerase (spot 68) and the flavonone 3-hydroxylase (spots 12 and 13).

Under this name are grouped different proteins. The most numerous and interesting are undoubtedly the Ripening-induced protein, or proteins associated with the maturation process (14, 15, 17, 26, 51 spots). The maturation is a complex process that determines important changes within the fruit, both from an organoleptic point of view both from a nutritional point of view. A process that involves the biosynthesis of pigments, the production of volatile components and aromatic and the accumulation of important nutrients. The ripening process involves both the cytoskeleton that the biological membrane, as the cell wall. The Ripening-induced protein, the proteins are rich in proline. The development of the fruit and its maturation are associated with

progressive changes in the tissue of the fruit. It is generally accepted that they are determined by changes in the composition of the cell wall and its structure (Brady, 1987; Nunan et al., 1998). It is thought that these proteins are involved in the composition and structure of the cell wall (the changes faced by the cell wall are among the most important among those who register in the early stages of maturation) (Showalter and Rumeau, 1990; Ye and Varner, 1991). This group of proteins represent major subgroups of idrossiproline rich glycoproteins (HRGPs) (Sommer-Knudsen et al., 1998).

3.3. Serological analysis of strawberry allergen 2-DE maps

To identify the allergens in two varieties of strawberry, western blotting, which is an immunochemical method for detecting the binding between a patient's IgE and separated plant proteins on a membrane, has been employed.

Fig. 3 show the sensitization profiles of four fruit allergenic patients. Immunoblotting revealed differences in reactivity of pooled sera from two varieties. The pooled serum of Group 1 [STR01-5 (IgE_{tot} = 1347)] in the "*Queen Elisa*" occurs much reactive about the intensity of the present spots that in the number of the same. In fact, "*Camarosa*" lacking the spots indicated by number 3 and number 4 (Fig. 3d). The pooled serum of Group 2 (STR06-10), which is the one with the highest (IgE_{tot} = 1715) differs between two varieties only for the intensity of the spots. The reactivity is more evident and intense in "*Queen Elisa*" (Fig. 3e). The pooled serum of Group 3 (STR011-15), with the low value of IgE_{tot} (588), means little reactivity both in "*Queen Elisa* "that "*Camarosa*" (Fig. 3g–h). However, it remains a slight difference in the intensity of reaction that again sees the most reactive the "Queen Elisa" respect the "*Camarosa*".

Mass spectrometry has identified the spots reactive and numbered from 1 to 7 as belonging two classes of allergens, Fra a1 and Prunina. Fra a1 is a homologue of the major allergen of birch (Bet v1); the prunina is an 11S globulin and it is a seed storage protein.

4. Conclusion

The results obtained in this work allow us to show the "*Queen Elisa*" as the variety between the two analyzed, with greater allergenic potential. Motivating these data was not yet simple. In the literature, for example, the power allergenicity of PR-10 is associated with the pigment content in the fruit. A study carried out precisely on *Fragaria* × *ananassa*, has compared a variety with fruits normally colored, with a mutant strain, the fruits of which are devoid of pigmentation. The results, therefore, differ from the only work in the literature, unfortunately for these subjects is still very sparse. Thus, the change in protein between the two varieties could only be attributed to a phenomenon of "phenotypic plasticity", which is an expression of a plant adaptation to the environment that surrounds them. To note is that the different regulation of allergen Fra a1 between the two varieties, may offer a plausible explanation of why some individuals with allergies can tolerate well some varieties of strawberries.

In conclusion, our results demonstrate that the proteomics analyses has accelerated identification of multiple allergens in plants, compared to conventional methods involving protein isolation processes. Therefore, in addition to conventional methods for detecting allergens, the use of proteomic method has broad applicability in research on allergens as well as practical applications, such as allergen detection and characterization.

Therefore, in future, a combination of proteomics and these biological assays can yield much information on IgE-binding proteins.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aoas.2018.11.003.

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