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Applicability of 2-DE to assess differences in the protein profile between cold storage and not cold storage in nectarine fruits

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ABSTRACT

Cold storage is being used to increase nectarine fruits' postharvest life. However, low temperatures lead to chilling injury and limit their commercial quality and value. In this study a proteomic approach was used to compare the protein profile between control and cold storage nectarine fruits. Protein extracted from tissue was resolved by two-dimensional gel electrophoresis (2-DE) in the 4–7 pH and 10–200 kDa Mr range. Around 350 spots were well determined, and 11 from 17 spots that showed significant differences were identified by peptide mass fingerprinting (PMF) using matrix-assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)-mass spectrometry (MS). Four differentially expressed proteins were characterized as allergens and were further assessed at the transcription level using quantitative real time-RT-PCR (qRT-PCR).

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1. Introduction

Nectarine [*Prunus persica* (L.) Batsch, var. *nectarina*] belongs to the Rosaceae family; it originated from China [1] and, together with peach, represents one of the most important species within the stone fruit market, with a total world production of 1,257,700 tons per year [2]. Stone fruit production plays a key role in the agricultural sector.

Nectarine is highly perishable and deteriorates quickly when stored at room temperature [3]. The rapid ripening of this fruit results in a short shelf-life, which represents a serious constraint for efficient handling and transportation. Cold storage is one of the most frequently employed methods for extending its shelf-life. However, prolonged cold storage can negatively affect fruit quality and result in chilling injury (CI) [3]. This disorder, which is also known as internal breakdown, is the major reason for low consumption of this fruit in comparison with other fresh fruits such as apples and bananas [4]. Typical CI symptoms include lack of juiciness (mealiness or woolliness), flesh browning, black pit cavity, flesh translucency (gel

breakdown), red pigment accumulation (bleeding), loss of flavor, development of off flavors, and failure to ripen [3]. Most of these symptoms are developed during fruit ripening after cold storage, and CI problem is usually not noticed until fruit reaches consumers, thereby reducing consumer satisfaction [5,4]. CI results in a loss of commercial value [6]. The physiology of CI has received considerable attention. However to date, no really effective solutions have been found to prevent CI [7] and the molecular mechanisms leading to this physiological disorder are not completely understood. Nevertheless recent studies have shown that low temperatures themselves lead to the synthesis of new proteins and a decrease in the levels of others [8]. Proteomics is a powerful tool for studying and identifying global changes in structure and abundance of plant proteins in response to developmental and environmental signals [9,10]. For this reason, there has been an increasing use of proteomic methods in fruit and vegetable postharvest physiology over the last few years [11–14]. So the main objective of the present paper was to screen for differentially expressed protein between non storage and cold storage in nectarine fruit using a proteomic

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analysis by 2DE coupled with mass spectrometry and to confirm the 2DE-PAGE results with qRT-PCR analyses in order to increase our understanding of this physiological process of CI in stone fruit.

2. Materials and methods

2.1. Plant materials

The nectarine [*P. persica* (L.) Batsch, var. *nectarina*, cultivar “Venus”] was harvested at commercial maturity from trees of the same age, grown under the same edaphoclimatic conditions in commercial orchards in Extremadura (Spain). Nectarines were transported to the proteomic laboratory of the Food Technological Institute of Extremadura (INTAEX, Spain). Fifty fruits were manually selected for uniformity of size, good appearance and the absence of physical damage. Then they were divided into two groups. Half of the fruits were exposed to 20 °C for 24 h (control) and the other half (cold) were refrigerated, after having been precooled in a forced air flow refrigerate tunnel at 2 to 3 m s⁻¹ at 0° for 1 h (precooled), in chambers at 5 °C and 85–90% relative humidity for 5 weeks. After removing from cold storage, the fruits were exposed to 20 °C for 24 h for ripening. In order to reduce the inherent variation from the biological samples used in this study, four different biological samples of five fruit tissues (mesocarp) were tested for each treatment, “control” and “cold”. Consequently, four extractions were performed, so the fruits were ground powdered under liquid nitrogen using a pestle and mortar to obtain 5 g of fine powder which was then stored at –80 °C prior to protein and RNA extraction.

2.2. Physiological parameters

Flesh firmness and internal CI symptoms were evaluated for 2 years. Flesh firmness was measured according to [15] on two opposite sides of each fruit, after removal of 1 mm thick disk of peel, using a Stable Micro Systems Texture Analyzer TA-XT2i (Aname, Pozuelo, Madrid, Spain). An 8 mm cylindrical probe penetrated 8 mm into the sample with a test speed of 1 mm s⁻¹ and the maximum force (in N) was recorded. Mean values represent the average of 20 fruits. After firmness internal CI symptoms were visually assessed on flesh browning according to the method of Wang [16], and the percentage of fruits with dry mealy texture and “wooliness”. The score of CI was assessed by measuring the browning area in each fruit of 20 fruits based on the following scale: 0 = no browning; 1 = less than 1/4 browning; 2 = 1/4–1/2 browning; 3 = 1/2–3/4 browning area; 4 = more than 3/4 browning. The CI index was calculated from the following formula: $(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4) \times 100 / (4 \times N)$, where N = total number of fruits measured and N1, N2, N3 and N4 were the numbers of fruit showing the different degrees of browning.

2.3. Protein extraction

Protein extraction was performed according to [17], based on the initial protocol of [18]. Fruit tissue from the mesocarp

(approximately 5 g of finely ground powdered flesh) was homogenized with 10 ml of extraction buffer (100 mM Tris pH 8.8, 5 mM EDTA, 20 mM DTT, 2 mM PMSF and 30% saccharose). Samples were shaken vigorously and then an equal volume of phenol saturated with Tris–HCl, pH 7.5 was added; the mixture was shaken for 3 min at 4 °C and then left for 10 min on ice. The mixture was centrifuged at 10,000 g for 10 min at 4 °C to separate the phenolic and the aqueous phases. The upper phenolic part was then collected and mixed with 5 volumes of ice cold 0.1 M ammonium acetate in methanol before being incubated overnight at –20 °C to precipitate the proteins. The protein pellet was collected by centrifuging at 10,000 g for 10 min at 4 °C and then washing with ice-cold 0.1 M ammonium acetate and 80% ice-cold acetone. Each washing step was followed by 10 min of centrifugation as described above. The pellet was air-dried at room temperature and solubilized in a 300 µl solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 2% pharmalyte and 40 mM DTT. Extractions were performed four times. Samples were then cleaned using the PlusOne 2-D Clean-Up kit (GE Healthcare) in accordance with the manufacturer's instructions. Finally, the protein extracted was solubilized in a sample preparation solution containing 8 M urea, 2% (p/v) CHAPS, 1% (v/v) pH 4–7 immobilization buffer (GE Healthcare), 0.018 M DTT and 0.002% (p/v) bromophenol blue, with one tablet of Mini Protease Inhibitor Cocktail (Roche) per 10 ml buffer. Protein concentration was determined by the Plus One Quant Kit (GE Healthcare) based on the BSA standard.

2.4. 2-DE

150 µg of proteins in a final volume of 250 µl, was used overnight to passively rehydrated IPG strips of 13 cm and 4–7 pH range (GE Healthcare). After rehydration, the strip was electrophoresed with a Multiphor apparatus (GE Healthcare, Milan, Italy) at 20 °C using the following program: at 100 for 3 h, 500 and 1000 V for 1 h each; at 1000 V up to 8000 V for 1 and 3 h respectively; and finally at 8000 V for 1 h, giving a total of 24.575 V h. IEF gels were run four times for each treatment. IEF strips were equilibrated before the second dimension separation for 15 min with gentle shaking in an SDS equilibration buffer [75 mM Tris, 6 M urea, 29.3% (v/v) glycerol, 2% (w/v) SDS, and traces of 0.002% (w/v) bromophenol blue] and then 0.065 M DTT was added, followed by alkylation in a fresh aliquot of equilibration buffer containing 2.5% (w/v) iodoacetamide for 15 min. The IPG strips were then transferred onto 12.5% polyacrylamide gels and the second dimension separation was done with an SDS-electrophoresis buffer (Tris base 250 mM, glycine 1.92 M, SDS 1% (w/v)). Gels were run in a SE 600 Ruby™ vertical gel electrophoresis unit (GE Healthcare) at a constant voltage of 60 V until the dye reached the bottom. Finally, gels were stained using the silver staining plus kit (GE Healthcare), according to the manufacturer's recommendations with some modifications, glutaraldehyde was replaced by formaldehyde to be suitable for MALDI-TOF analysis.

2.5. Scanning and comparison of gels

Scanned gel images were obtained using an ImageScanner III (GE Healthcare) coupled with ImageMaster Labscan version

6.0 software (GE Healthcare). Image analyses and gel comparisons were subsequently performed using ImageMaster™ 2D Platinum 7.0 software (GE Healthcare) which allowed gel matching and spot quantification. All statistical analyses were performed with the software SPSS 17.0 (SPSS Inc., Chicago, IL). Differential variations in spot abundance between the “control” condition and the “cold” fruit were tested with one-way ANOVA ($p < 0.05$). A total of four 2DE gels for each treatment, resulting from different biological replicates, were analyzed. The gel with the highest spot-count and the best resolved spots was assigned as master gel. Gel images were automatically matched to the master gel by ImageMaster. Gels were compared within a match set created and in addition were visually verified. Each spot included on the standard gel met several criteria: it was present in at least three of the four gels and was qualitatively consistent in size and shape in the replicate gels. Low quality spots were eliminated from further analysis. The remaining high quality spot quantities were used in the analysis. The quantity of a protein spot was calculated based on the volume. Spot volumes were corrected for background and normalized as a percentage of the total volume in all the spots in the gel. The protein spots with significant changes in intensity in a consistent direction (increase or decrease) were considered to be different. The identified protein spots were visually rechecked. Quantitative analyses were carried out after normalizing the spot volumes in all gels, in order to compensate for abundance related variations. Quantitative comparisons between the two sample sets were carried out taking into account statistically significant spots only ($p < 0.05$). Qualitative comparisons were also done, in order to detect the presence or absence of proteins. Estimates of M_r and pI were calculated from digitized two-dimensional electrophoresis images using standard molecular mass and pI markers.

2.6. MS analysis and protein identification

Spots which showed qualitative or quantitative statistically significant differences between treatments were subjected to MALDI-TOF analysis after trypsin digestion. Spots of interest were manually excised. Digestion was performed as previously described [19], but with minor variations. Silver-stained gel

plugs were destained with a 1:1 working solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate, to remove the silver and were then rinsed with water and dehydrated with 100% acetonitrile. Next, plugs were treated with 10 mM DTT in 25 mM ammonium bicarbonate at 56 °C and subsequently with 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature. Gel pieces were then rinsed with 100% acetonitrile and 20 mM ammonium bicarbonate. Modified porcine trypsin, at a final concentration of 3 ng/ μ l in 20 mM ammonium bicarbonate (Promega, Madison, Wis.), was then added to the dry gel pieces and incubated at 37 °C for 16 h. Tryptic peptides were recovered and dried in a speed vacuum system and resuspended with 0.1% trifluoroacetic acid and 50% acetonitrile in a final extraction volume of 5 μ l. For MALDI TOF peptide mass fingerprinting, a 0.5 μ l aliquot of matrix solution (5 g/l 2,5-dihydroxybenzoic acid in 33% aqueous acetonitrile plus 0.1% trifluoroacetic acid) was manually loaded onto a 400 μ m diameter AnchorChip™ Target plate (Bruker Analytic GmbH, Bremen, Germany) probe and 1 μ l of the above peptide extraction solution was added and allowed to dry at room temperature. Samples were analyzed on a Bruker Ultraflex MALDI-TOF mass spectrometer (Bruker-Franzen Analytic GmbH, Bremen, Germany). The acquisition mass range was set to 600–4000 Da. The equipment was first externally calibrated employing protonated mass signals from a standard peptide calibration mixture that contained 7 peptides (Bruker Peptide Calibration Standard #206196, Bruker Daltonics) covering the 1000–3200 m/z range. Thereafter, each spectrum was internally calibrated using selected signals arising from trypsin autolysis (842.510 and 2211.105-Da peptides) to reach a typical mass measurement accuracy of ± 30 ppm. Each raw spectrum was opened in FlexAnalysis 3.0 (Bruker Daltonics) software and processed and analyzed using the following parameters: a signal-to-noise threshold of 1; a Savitzky–Golay algorithm for smoothing; a tangential algorithm for baseline subtraction; and centroid algorithm for monoisotopic peak assignment. In all cases, the resolution was greater than 9000. All known contaminants (trypsin autolysis and known keratin peaks) were excluded during the process. The peaks generated were checked with Mascot Server (version 2.2) using Bio Tools 3.1 (Bruker Daltonics) software. They were then searched against

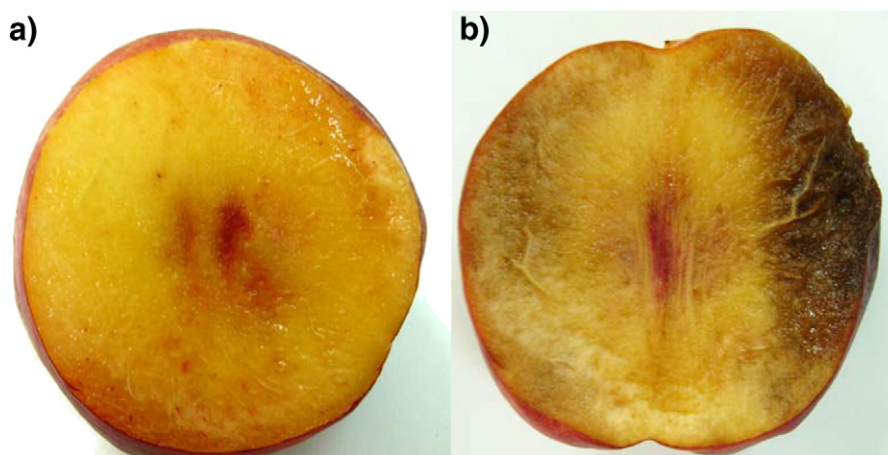


Fig. 1 – Representation of the “Venus” nectarine fruit: a) “control” fruit without cold storage. b) Chilling injury in “cold” nectarines after 5 weeks of storage at 5 °C and 90% relative humidity plus ripening period at 20 °C.

NCBI nr 20100507 (10,930,288 sequences; 3,721,510,947 residues) and the local database *Rosaceae* 20100528 (11,376 sequences; 2,615,317 residues). The search parameters against the NCBI nr database were set as follows: searches were restricted to Viridiplantae (green plants) (742,951 sequences); up to one missed tryptic cleavage; mass accuracy of 100 ppm; MH+ monoisotopic masses; carbamidomethyl cysteine as a fixed modification; and methionine oxidation as a variable modification. In both searches (NCBI nr and local database) Mowse scores with values greater than 69 were considered significant ($p < 0.05$). Value of the score is $-10 \cdot \log(P)$, where P is the probability for a random result.

2.7. Total RNA extraction

Total RNA was extracted from the same finely ground powdered flesh (~1.5 g) used for protein extraction. RNA was extracted using the Purelink RNA mini kit for plants (Invitrogen) followed by DNase treatment with Purelink DNase set (Invitrogen), used according to the manufacturer's instructions. Extractions were performed in triplicate. The concentration of total isolated RNA was calculated from the absorbance at a 260 nm Synergy™ HT Multi-Mode Microplate Reader using Gen 5™ 182 V1.09 data analysis software (BioTek). Purity was verified by optical density (OD) absorption ratios (OD 260 nm/OD 280 nm) of between 1.80 and 2.05. OD 260 nm/OD 230 nm readings ranging from 2.00 to 2.60 revealed polysaccharide/polyphenolic contaminants and protein contaminants [20].

2.8. Primer design

Translation elongation factor 2 (TEF2) was selected and used as a housekeeping gene, as previously described by [21]. Pru p 1.06A, Pru p 1.05, Pru p 2.01B and Pru p 2.01A were chosen as the genes of interest based on previous results from proteomic assays. Four primer pairs were designed (one primer pair for each of the 4 genes of interest) based on the nucleotide sequence of *P. persica* deposited in the GenBank database of the National Centre for Biotechnology Information (Supplementary material). The primers were designed using UniProt software (<http://www.uniprot.org/uniprot/Q2I6V8>) and according to the following criteria: 60 °C melting temperature and 120 pb average size of amplified fragments. The primer pairs were tested by 2.5% agarose gel electrophoresis and ethidium bromide staining. Finally qRT-PCR analysis was performed.

2.9. Preparation of cDNA

One microgram of each RNA sample was reverse transcribed to cDNA with Hc RNA–cDNA Mm W/No- kit (ABI Applied Biosystems), following the manufacturer's recommendations. RT-PCR was performed in triplicate from each one of three biological samples using Absolute Blue Qpcr SYBR Green (Thermo Scientific Abgene). For this, the cDNA sample was diluted in a total volume of 20 µl. Thermal cycling was performed under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Data for the RT-PCR assay were collected with an ABI 7500 fast real time PCR system (Applied Biosystems). The expression levels of the target genes were quantified in the “control” and “cold” treatments. The reference gene in the reverse

transcription reactions was the housekeeping gene: translation elongation factor 2 (TEF2).

2.10. Statistical analysis

Expression levels of the tested reference genes were determined by the number of amplification cycles (CT) needed to reach a specific threshold level of detection. The PCR efficiency was calculated for each gene using the SAS software system (version 9.1) program, as previously described [22]. The ΔCT value for each sample was determined by subtracting the CT value of the reference gene from the CT value of the target gene. The relative

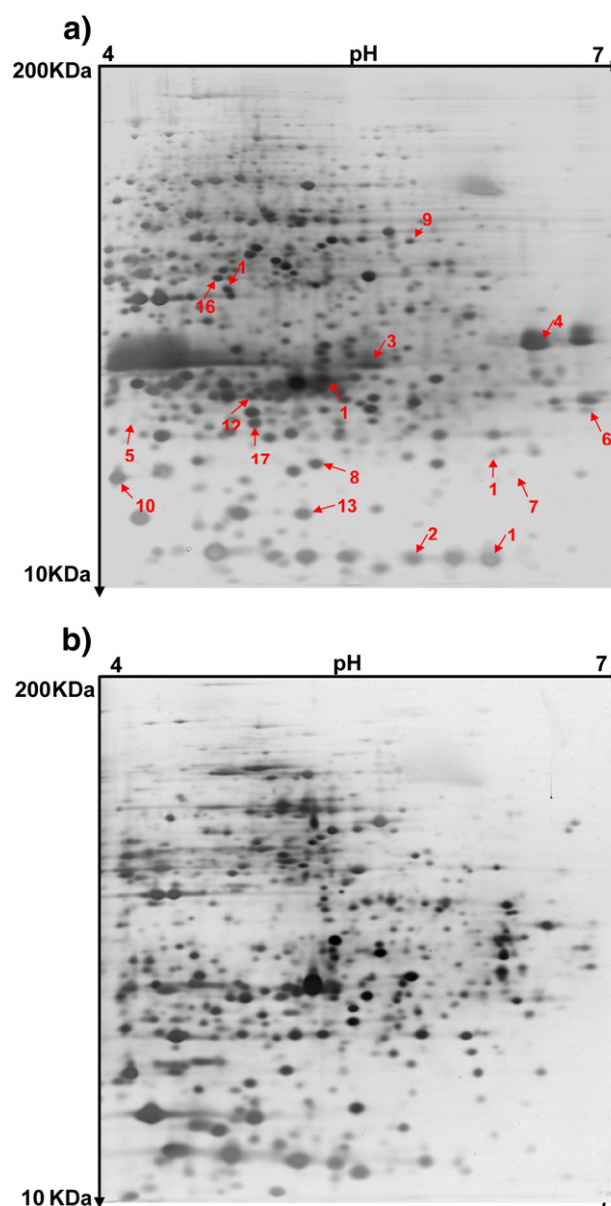


Fig. 2 – Silver stained 2D gel of proteins extracted from the “Venus” nectarine fruit tissue. a) Proteome from the “cold” stored fruit. b) Proteome from the “control” fruit. About 150 µg of protein from the nectarine fruit phenol extracted is resolved by 2-DE with pH 4–7 Immobiline DryStrip. The gels were visualized with 0.25% (v/w) silver.

quantification of target gene expression was evaluated using the $\Delta\Delta CT$ method. The $\Delta\Delta CT$ was calculated by subtracting the ΔCT value of the cold storage fruit from the ΔCT value of the healthy fruit. The gene expression levels are shown as means $\pm$ standard deviations (SD). Statistical differences were analyzed by SAS procedure PROC GLM where p values of less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Development of chilling injury in nectarine fruit

Symptoms that appeared in the “cold” fruit (stored at 5 °C for 5 weeks plus 1 d of shelf-life) were visually assessed by cutting 20 nectarine fruits/year (Fig. 1). The fruit was observed visually and determined organoleptically. 95% of these fruits developed CI. Mainly in the first year the fruit presented flesh browning (62.5%), a dry mealy texture (15.78%) or “wooliness” (61%). In the second year all the “cold” fruits developed CI, 100% of the fruits presented wooliness and 41.25% presented browning. There were no visible symptoms of CI in the control fruit (Fig. 1). These results show that temperature and storage are involucre in CI symptom expression [23,24]. Fruits harvested at commercial time were exposed to 20 °C for 24 h with an average texture of 36.8 N and 26.15 N after storage.

3.2. Proteome map of nectarine flesh

2-D gel electrophoresis analysis was performed in order to analyze changes in protein abundance patterns of the nectarine fruit as a result of cold storage (5 °C) (Fig. 2). Four replicate gels for each of the different extracts per sample were processed concurrently with the ImageMaster Platinum 7 software. Proteins were phenol extracted [17], and subjected to 2-DE. Preliminary results (Supplementary material) for 2-DE in the 3–11 pH range revealed that the spots were concentrated in the 4 to 7 pH range, and in order to increase the resolution the pH range was reduced from 3–11 to 4–7. The total number of spots detected was 569, from which 324 were used for further analysis because they presented high quality and match set and they were well-focused and redundant in the 4 replicated gels (Table 2). Most of the proteins (52.9%) presented a M_r of between 25 and 50 kDa and pH between 4 and 6.5.

3.3. MALDI-TOF analysis and protein identification

Results revealed a total of 17 differential spots ($p \leq 0.05$) which were MALDI-TOF analyzed, resulting in 12 proteins with a significant match (MASCOT score > 69 ; Table 1) from which one protein was eliminated because it was identified in another organism, probably because of the small number of sequences for *Prunus* available in the databases. The other

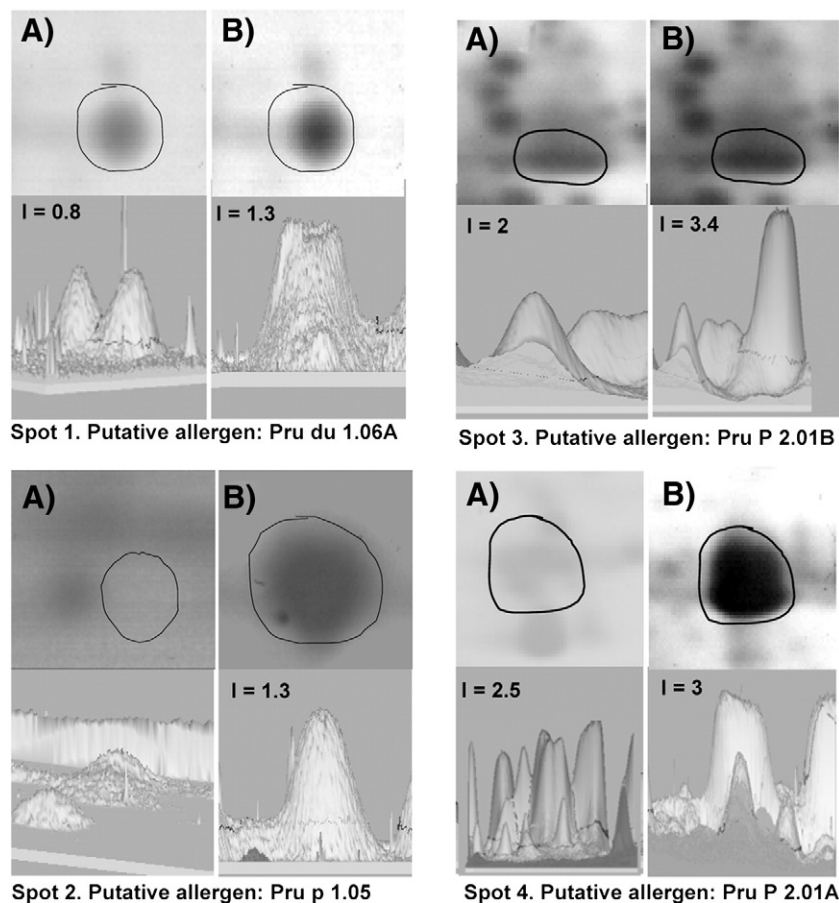


Fig. 3 – Accumulation pattern of proteins differentially expressed in control fruit (A) and cold stored (B) condition. Images from sections of two-dimensional gels and 3D spot images are shown with intensity peaks.

Table 1 – Identification of differentially variant proteins after cold storage of nectarine fruits.

Spot ID ^a	Protein name ^b	Accession UniProtKB ^c	Gene name ^d	Sequence similarities ^e	Function ^f	Score ^g	MP ^h	Organism	Theor.		Expe.		Treat ⁱ .	
									Mr ^j	pI ^k	Mr ^j	pI ^k	C ^l	CS ^m
1	Putative allergen Pru du 1.06A	B6CQS9	Pru du 1.06A	BetVI family	Plant defense/pathogenesis-related protein	202	10	<i>Prunus dulcis</i> × <i>Prunus persica</i>	17.40	4.98	15.40	6.00	+	++
2	Putative allergen Pru p 1.05	B6CQS6	Pru p 1.05	BetVI family	Plant defense/pathogenesis-related protein	198	10	<i>P. dulcis</i> × <i>P. persica</i>	17.40	5.22	15.80	5.70	–	++
3	Putative allergen Pru p 2.01B	B6CQT7	Pru p 2.01B		Signal peptide	157 _N	10	<i>P. dulcis</i> × <i>P. persica</i>	25.80	8.43	29.98	5.69	–	++
4	Putative allergen Pru p 2.01A	B6CQT5	Pru p 2.01A		Signal peptide	98 _N	10	<i>P. dulcis</i> × <i>P. persica</i>	25.80	5.13	31.50	6.10	+	+++
5	Catalase	Q7XTK9	cat1	Catalase family	Hydrogen peroxide catabolic process	108	10	<i>P. persica</i> (peach)	56.90	6.77	27.00	4.31	+	–
6	Putative class IV chitinase	A7Y7L3			Chitin catabolic process	70 _R	10	<i>P. dulcis</i> (almond)	11.20	4.99	25.99	6.52	–	+
7	NBS-containing resistance-like protein	Q000D4			ADP binding	69 _R	10	<i>Prunus avium</i> (cherry)	28.80	7.76	20.01	6.12	+	–
8	Glutathione peroxidase	A1Z1Z7		Glutathione peroxidase family	Response to oxidative stress/ oxidoreductase/peroxidase	138 _N	10	<i>P. avium</i> (cherry)	19.10	8.97	21.98	5.15	–	++
9	Actin	D2DJ29	ACT1	Actin family	ATP binding/cytoplasm/ cytoskeleton	221	10	<i>P. avium</i> (cherry)	41.30	5.46	46.95	5.62	++	+
10	Cl small heat shock protein 1	C9EIM3		Small heat shock protein (HSP20) family	Stress response	127	10	<i>Prunus salicina</i>	18.10	5.57	22.98	4.21	+	–
11	Enoyl-ACP reductase	B8K204			Nucleotide binding	154	10	<i>Malus domestica</i> (apple)	41.40	8.72	37.51	4.90	+	++
								(<i>Pyrus malus</i>)						

List of protein identities by MALDI-TOF analyses. Relative expression protein intensity in different treatments: – representing no expression, + low expression and +++ the highest level of expression.

^a Spot number represented the number on the master gel (Fig. 1).

^b Name of the protein obtained via the MASCOT software.

^c Accession number from the UniProtKB database.

^d Name of the gene.

^e Sequence similarities to identified protein.

^f Function of the identified protein.

^g Search was done by consulting the NCBI and *Rosaceae* databases by using the MASCOT algorithm – $10 \log(P)$, where P is the probability that the observed match is a random event. Score >69 indicates identity or extensive homology ($p < 0.05$).

^h MP: matched peptides.

ⁱ In this table we can see that 11 spots from 17 spots corresponded to those showing qualitative or statistically significant quantitative ANOVA differences ($p < 0.05$).

^j Molecular weight (kDa).

^k Isoelectric point.

^l Control fruit.

^m Fruit after 5 weeks of cold storage at 5 °C plus 1 d of shelf-life.

Table 2 – Values are mean±SD of four biologically replicated fruits. All the differences were statistically significant according to the ANOVA test ($\alpha=0.05$).

Protocol	Tratamiento	Protein yield (mg/g fresh weight)	Resolved spots (consistent spots)	Differential spots	
				Qualitative difference	Quantitative difference
				Spot detected only in	Value more intense in
Phenol	Control	0.25±0.044	452±137.58 (113)	3	1
	Cold	0.24±0.024	569±48.35 (324)	6	7

proteins were matched with proteins from the *Prunus* genus (spots 1, 2, 3, 4 and 5 correspond to *P. persica*; spots 7, 8 and 9 to *Prunus avium*, spot 6 to *Prunus dulcis* and spot 10 to *Prunus salicina*). The exception was spot 11, which matched an enoyl-ACP-reductase from *Malus domestica* (Table 1). This was not surprising, because linkage maps between *M. domestica* and *Prunus* suggest a high level of synteny between the linkage groups of these two genera [25].

Most of the identified proteins (81.82%), experimental *Mr* and *pI* showed a good correlation with theoretical values for matched proteins, and this conferred confidence to the identification. For spots 5 (catalase) and 6 (putative chitinase) less experimental than theoretical *Mr* was found; this could be explained by breakdown of products associated with protein degradation. This phenomenon has been previously reported [26].

Most of the identified proteins were represented by allergen proteins (Pru du 1.06A, Pru p 1.05, Pru p 2.01B and Pru p 2.01A), which are included on the international allergen list (<http://www.allergen.org>). These proteins induce allergic responses mediated by immunoglobulin E in susceptible individuals and they are defined as allergens. The highest number of allergens present in the (*Rosaceae*) fruit belongs to four families: pathogenesis-related protein 10 (PR-10 protein, birch allergen Bet v 1 homologous); thaumatin-like proteins (TLP, PR-5 proteins); nonspecific lipid transfer proteins (nsLTPs, PR-14 proteins); and profilins (PRF) [27].

Pru p 2.01B and Pru p 2.01A proteins with less than 5% dissimilarity between them belong to a different isoallergen and regarding nomenclature they are distinguished by adding a capital letter after their isoallergen name. In the same form, additional numbers can be added to distinguish different isoallergen names. Pru p 1.05 and Pru p 1.06 are isoallergens that are differentiated by adding 5 aa. Allergen proteins were more abundant in “cold” than in “control” fruits (Fig. 3); this would seem to make sense as these are typical stress-responsive proteins. Pru p 1 belongs to the PR-10 family and Pru p 2 to the TLP family. *Mr* and *pI* values of the allergens detected (Pru p 1.05/Pru du 1.06 and Pru p 2.01 B/Pru p 2.01 B) matched quite well with the values respectively described for PR-10 and the TLP protein family by [28]. PR-10 proteins are neither directly involved in pathogen perception nor in defense, but have a more common function as biochemical components in the signal transduction pathways of pathogen interactions and stress. On the other hand, TLP families are high-molecular-weight proteins that are classified as class 5 pathogenesis-related proteins (PR-5) [29]. TLP are reported to have various functions, including: fungal activity [30]; antifreeze activity [31]; and involvement in fruit ripening [32]. Allergen proteins can provide some form of protection to fruits during periods of stress [11]. Changes in the level of expression of specific defense proteins in association with biotic or abiotic stress may cause

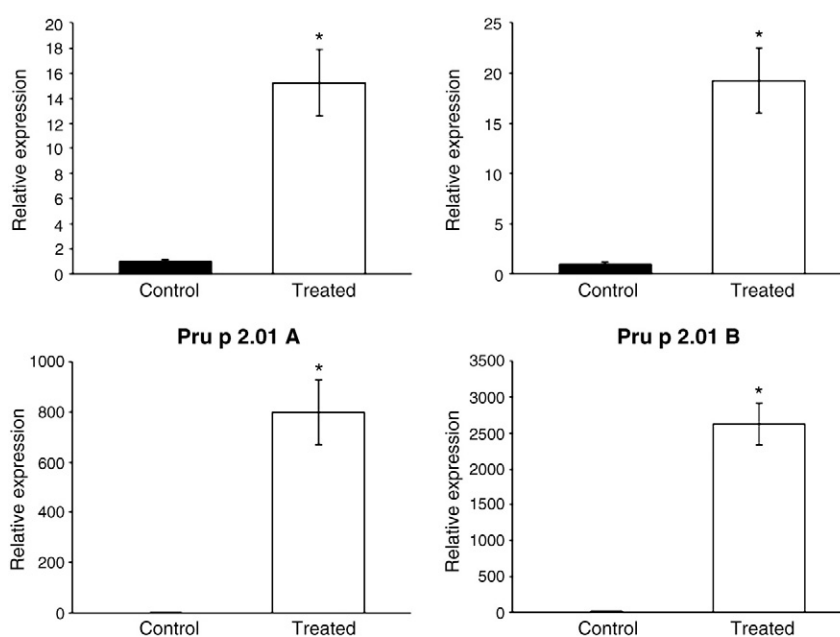


Fig. 4 – Changes in mRNA expression levels of Pru p 1.05, Pru du 1.06A, Prup 2.01A and Pru p 2.01B associated with cold storage and control fruit samples. Data are represented as relative expression, mean±SD. The asterisk means $p < 0.05$.

significant variations in the amount of allergen present. Similarly, storage conditions and postharvest treatments can affect the quality of the final product. The post-storage up-regulation of this set of genes may be related with the cause of CI in the fruit. Similar results with regard to transcript abundance of TLP were previously reported [14,33]. Nilo defended that this protein (TLP) was very relevant in differentiating chilling injured fruit from a healthy fruit. The early accumulation of the TLP1 transcript in CI-resistant peaches as opposed to sensitive cultivars suggests that TLP1 may be involved in protection against CI [34]. However, a greater number of cultivars and spots need to be studied in order to confirm this. The other protein identified was peroxidase (spot 8), which was more abundant after cold storage (Table 1). This result confirms previously published reports in which peroxidase levels increased during softening. This was because peroxide plays an important role in cellular redox regulation [35], and because it has been associated with fruit ripening [36]. Peroxidase has also been found to respond to abiotic stress stimuli, such as CI and drought [37].

Some of the proteins that were identified either disappeared or suffered significant decreases in the “cold” fruit. These included; catalase (spot 5), NBS-containing resistance-like protein (spot 7), actin (spot 9) and small HSP 1 (spot 10) (Table 1). A decrease in catalase has previously been reported in peach fruit upon cold storage and this has been proposed as a marker for CI [14]. In pitaya amarilla fruits, catalase activity also markedly decreased 6 days after harvesting [38]. Catalase, peroxidase and polyphenoloxidase from pitaya amarilla fruits could be related to ripening and senescence. This reduction in catalase could be related to a reduced capacity to cope with oxidative stress [39]. A similar expression trend was found in apricot [39] and in tomato [40]. Small HSP 1 (spot 10) has been linked to the stabilization of cell components under such stress conditions as heat and oxidative stress [41]. The lowest level of this protein in chill injured fruit would reduce its capacity to cope with stress-related damage [14]. Finally, the level of actin (spot 9) diminished in “cold” fruit; this decrease has been related to tissue softness as a consequence of CI [14].

3.4. Gene expression analysis by real-time qPCR

To validate the proteomic data and the hypothesis of allergen induction in response to cold storage, we analyzed the level of relative mRNA accumulation in target genes (Pru p 1.05, Pru du 1.06 A, Pru p 2.01 A and Pru p 2.01 B) in nectarine “cold” fruit.

The qRT-PCR analysis revealed that the relative expression of all target genes tested was significantly increased in “cold” fruit (Fig. 4). The qRT-PCR analysis corroborated the results of the 2-DE analysis.

4. Conclusion

Using a comparative proteomic approach with 2-DE and MALDI-TOF allowed us to identify proteins that showed stage specific changes in their accumulation pattern. Four allergen proteins were up-regulated in cold storage nectarine fruit and they were identified. Results found with qRT-PCR

analyses suggest that the studied set of genes encoding proteins Pru p 1.05, Pru du 1.06 A, Pru p 2.01 A and Pru p 2.01 B (allergen proteins) was up-regulated in cold-treated mesocarp tissues. These results confirm the result previously obtained by 2-DE analysis.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2012.08.005>.

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Supplementary material A

Name	Forward Primer sequence [5'-3']	Reverse Primer sequence [5'-3']	Amplicon Size (bp)	Tm (°C)
TEF2	GGTGTGACGATGAAGAGTGATG	TGAAGGAGAGGGAAGGTGAAAG	129	60
Pru du 1.06A	CCTCATCCCCAAGATTGCTCCCCA	GGTTCCAACACCTCCATCTCCTCA	73	59
Pru p 1.05	CAGCAGCTACCACACCAAAGGTGA	GCATCGGGGTTGGCCACAAGAT	120	59.5
Pru p 2.01B	CCGGTCGCTTCTTTGGCCGAA	CTGCGCCGTTACCGTTGCATG	111	59.8
Pru p 2.01A	GCCAGGAACCCTAACCGGTGAC	CGGCCAAAGAAGCGACCGGA	120	59.4

Table A. Primer sequences of housekeeping TEF2 and four genes of interest designed, the amplification length and temperature of the amplified product

Supplementary material B

SPOT ID	ACCESSION UNIPROTKB	HOMOLOGOUS PROTEIN BY MASCOT	ORGANISM	SCORE	TOP HIT BY MS/MS-MS BLAST	ORGANISM	SCORE BLAST
1	B6CQS9	Putative allergen Pru du 1,06A	<i>Prunus dulcis x Prunus persica</i>	813	Putative allergen Pru du 1.06B	<i>Prunus dulcis x Prunus persica</i>	800
2	B6CQS6	Putative allergen Pru p 1,05	<i>Prunus dulcis x Prunus persica</i>	810	Putative allergen Pru du 1.05	<i>Prunus dulcis x Prunus persica</i>	794
3	B6CQT7	Putative allergen Pru p 2,01B	<i>Prunus dulcis x Prunus persica</i>	1340	Thaumatococcus-like protein 1	<i>Prunus persica</i> (Peach) (<i>Amygdalus persica</i>)	1322
4	B6CQT5	Putative allergen Pru p 2,01A	<i>Prunus dulcis x Prunus persica</i>	1338	Thaumatococcus-like protein 2	<i>Prunus dulcis x Prunus persica</i>	1306
5	Q7XTK9	Catalase	<i>Prunus persica</i> (Peach) (<i>Amygdalus persica</i>)	2695	Catalase	<i>Ziziphus jujuba</i> (Chinese jujube) (<i>Ziziphus sativa</i>)	2593
6	A7Y7L3	Putative class IV chitinase	<i>Prunus dulcis</i> (Almond) (<i>Prunus amygdalus</i>)	564	Class IV chitinase	<i>Corylus heterophylla</i>	473
7	Q000D4	NBS-containing resistance-like protein	<i>Prunus avium</i> (Cherry)	844	NBS-containing resistance-like protein	<i>Prunus cerasus</i> var. <i>caproniana</i>	838
8	A1Z1Z7	Glutathione Peroxidase	<i>Prunus avium</i> (Cherry)	895	Glutathione peroxidase	<i>Malus domestica</i> (Apple) (<i>Pyrus malus</i>)	691
9	D2DJ29	Actin	<i>Prunus avium</i> (Cherry)	1942	Actin1	<i>Morella rubra</i>	1927
10	C9EIM3	Cl small heat shock protein 1	<i>Prunus salicina</i>	813	Low molecular weight heat shock protein	<i>Prunus salicina</i>	714
11	B8K204	Enoyl-ACP reductase	<i>Malus domestica</i> (Apple) (<i>Pyrus malus</i>)	1969	Predicted protein	<i>Populus trichocarpa</i> (Western balsam poplar) (<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>)	1661

Table B. Identification of Differentially Expressed Proteins from Peach Fruit with MASCOT and MS-BLASTa

The Search string was submitted to MS BLAST sequence similarity searching via the Web interface <http://www.uniprot.org/blast/uniprot/>
MS BLAST match was defined by the score was higher



Fifty fruits were manually selected for uniformity of size, good appearance and the absence of physical damage.



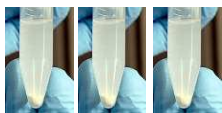
A) "Control": The fruits were exposed to 20 °C for 24hrs before testing



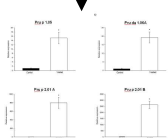
B) "Cold": fruits were stored at optimal conditions of 5°C and 85–90% relative humidity for 5 weeks plus ripening period at 20°C for 24 hrs



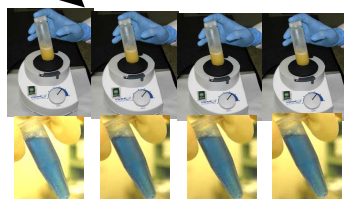
B) "Control": Four different biological samples of five fruits tissue (mesocarp) were tested



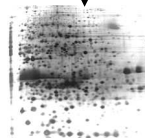
Three extractions of ARN were performed (1.5 g x 3 was used)



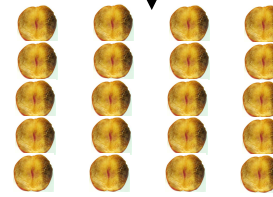
RT-PCR was performed in triplicate from each one of three biological samples



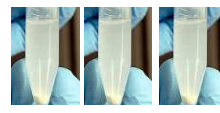
Four extractions of protein were performed (5 g x 4 was used)



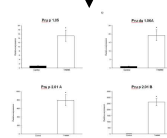
A total of four 2DE gels for each treatment



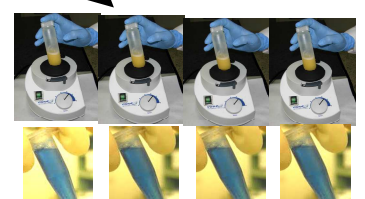
A) "Cold": Four different biological samples of five fruits tissue (mesocarp) were tested



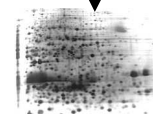
Three extractions of ARN were performed (1.5 g x 3 was used)



RT-PCR was performed in triplicate from each one of three biological samples



Four extractions of protein were performed (5 g x 4 was used)



A total of four 2DE gels for each treatment

supplementary material D

SEQUENCES OF IDENTIFIED PROTEIN

>tr|B6CQS9|B6CQS9_9ROSA Putative allergen Pru du 1.06A OS=Prunus dulcis x Prunus persica GN=Pru du 1.06A PE=3 SV=1

MGIFTYTDESTSVIPPPRLFKALVLEADTLIPKIAQSVKSAEIVEGDDGGVGTIKKISFEGEGSHYSYVKHQIDGLDKDNFVYN
YSLVEGDALSDKVEKISYEIKLVASADGGSVIKSTSNYHTKGDVEIKEEDVKAGKEKATGLFKLIENYLVANPDAYN

>tr|B6CQS6|B6CQS6_9ROSA Putative allergen Pru p 1.05 OS=Prunus dulcis x Prunus persica GN=Pru p 1.05 PE=3 SV=1

MGVFTYATEFTSVVPPARLFKALVLDADNLVPKIAQSAEIVEGDDGGVGTIKKTSFGEGSEYSYVKHQVDALDKDNFV
YNYSLIEGDALSDKIEKISYEIKLVASADGGSVIKNTSSYHTKGDVEIKEEHVKAGKEKAHALFKIETYLVANPDAYN

>tr|B6CQT7|B6CQT7_9ROSA Putative allergen Pru p 2.01B OS=Prunus dulcis x Prunus persica GN=Pru p 2.01B PE=4 SV=1

MMKSQAALLGLTTLAILFFSGAHAAKITFTNKCSYTWPGTLTGDQKPQLSLTGFKLATGISRSVDAPSPWSGRFFGRTR
CSTDASGKFTCATADCGSGQVSCNGNGAAPPATLVEITIASNNGGQDFYDVSLVDGFNLPMSVAPQGGTGKCKASTCPA
DINKVCPAPLQVKGSDGSVIACKSACLAFNQPKYCCTPPNDKPETCPPTDYSKLFKTQCPQAYSAYDDKSSTFTCSGR
PDYLITFCP

>tr|B6CQT5|B6CQT5_9ROSA Putative allergen Pru p 2.01A OS=Prunus dulcis x Prunus persica GN=Pru p 2.01A PE=4 SV=1

MMKSQAALLGLTTLAILFFSGAHAAKITFTNKCSYTWPGTLTGDQKPQLSLTGFEATGISNSVDAPSPWSGRFFGRTR
CSTDASGKFTCATADCGSGQVSCNGNGAVPPATLVEITIAENGGQDFYDVSLVDGFNLPMSVAPQGGTGCKASTCPA
DINKVCPAPLQVKGSDGSVIACKSACLALNQPKYCCTPPNDKPETCPPTDYSKLFKTQCPQAYSAYDDKSSTFTCSGR
PDYLITFCP

>tr|Q7XTK9|Q7XTK9_PRUPE Catalase OS=Prunus persica GN=cat1 PE=2 SV=1

MDPYKYRPSSAFDSPFWTTNSGAPVWNNSSSLTVGSRGPILLEDYHLVEKLANFDRERIPERVVHARGASAKGFFEVT
DISQLTCADFLRAPGVQTPVIVRFSTVIHERGSPETLRDPRGFAVKFYTRETNFDLVGNFVPVFFVRDGMKFPDMVHALK
PNPKSHIQEPWRILDFFSHHPESLHMFTFLFDDLGVQDYPYRHMEGSGVNTYTLINKAGKAQYVFKHWKPTCGVKCLLED
EAIKVGGANHSHATKDLYSIAAGNYPEWKLYIQITIDPDHEDRFDFDPLDVTKTWPEDILPLQPVGRLVLNKNIDNFFAEN
EQLAFCPAIVVPGVYYSDDKLLQTRIFSYSDTQRHRLGPNYLQLPVNAPKCSHHNNHHEGFMNFMHRDEEVNYPFSRY
DPVRHAERFPPIPNILSGKREKCIIEKENNFKQPGERYRSWAPDRQERFIHRVWDALSDPRVTHEIRSVWISYWSQADKS
LGQKLASRLNVRPSI

>tr|A7Y7L3|A7Y7L3_PRUDU Putative class IV chitinase (Fragment) OS=Prunus dulcis PE=2 SV=1

YGRGPLQLTWNYNNGAAGNSIGFDGLNSPESVASDPVLAFKTALWFWITNVRPVISQFGGATIRAINGAVECDGKQPAL
VQARINYTYDYCTQLSVDPGTNLSC

>tr|Q000D4|Q000D4_PRUAV NBS-containing resistance-like protein (Fragment) OS=Prunus avium PE=4 SV=1

TCAKEVYNHGDVQTFDFDCRAWVYVSQQFRTRDILQSIKQVATRTDMDTKLLEEELEEMLYKLLQGRRYLVLDDIWSST
DFDSLAKAFPSDCSGNKLLLTTRNNVALHADAQSLPHELFRNQEDSWKLLCRKAFTESIDRVCPQQLEETGQEIVAK
CS

>tr|A1Z1Z7|A1Z1Z7_PRUAV Glutathione peroxidase OS=Prunus avium PE=2 SV=1

MASQSGSGSKSIHDFTVKDAKGDVDLSIYKGVLLIVNVASQCGLTNSNYTELSQLYEKYKNQGLEILAFPCNQFGAQE
PGSNDEIVEFACTRFKAEPYIFDKVDVNGDNAPIYKFLKSSKGGLFGDSIKWNFSKFLVDKKGMLLTVCPTLLSYIRRV
TVGLQAPSVIRN

>tr|D2DJ29|D2DJ29_PRUAV Actin (Fragment) OS=Prunus avium GN=CACT1 PE=2 SV=1

GEDIQPLVCDNGTGMVKAGFAGDDAPRAVFPISVGRPRHTGVMVGMGQKDAYVGDQAQSKRGILTLYPIEHGIVSNW
DDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNVPAMYVAIQAVLSLYASGRRTTGIVLDSGDGV
SHTVPIYEGYALPHAILRLDLAGRDLTDALMKILTERGYMFTTTAEREIVRDMKEKLAYVALDYEQELETAKSSSSVEKNY
ELPDGQVITIGAERFRCPEVLFQPSLIGMEAAGIHETTYNSIMKCDVDIRKDLYGNIIVLSGGSTMFPPIADRMSKEITALAP
SSMKIKVVAPPERKYSVWIGGSILASLSTFQMQMWISKGEYDESGPSIVHRKNH

>tr|C9EIM3|C9EIM3_9ROSA CI small heat shock protein 1 OS=Prunus salicina PE=2 SV=1

MSIIPSFRRGTIFDPFSLNVWEPFKDFPFPSSSSSLSTFPEFSRENSAFLDTRIDWKETPEAHLFKADLPGLKKEEVKVEVE
DDRVLQISGERKIEKEDKNDQWHRVERSSGKFLRRFQLPENAKVDEIKAAMENGVLSTVPKAEVKKADVKAIEISG

>tr|B8K204|B8K204_MALDO Enoyl-ACP reductase OS=Malus domestica PE=2 SV=1

MATTAASSLQMATARPCISSRRAFSGSSTAMLNGNFKVASWTKLSSACHISSVQSFQRCFTSSSMKLDKFVTKAMAGA
SENKPVSGPLPINLKGKRAFIAGVADDNGYGWAIKSLAAGAEILVGTWVPALNIFESSLRGRKFDESRLPDGSLMEITK
VYPLDAVFDNPDVPEEIKTNKRYAGSSNWTVQEAECVKNDFGSIDVLVHSLANGPEVVKPLLETSRKGYLAAISASSY
SYVSLKHLFLPIINPGGSSISLTYSERIPGYGGGMSSAKAALESSTRVLAFEAGRKKGIRVNTISAGPLRSRAAKAIGFID
MMIDYSSANAPLEKELSAEEVGNTAAFLASPLASAITGGVIYVDNGLNAMGVGVDSPIFENLNIPKAQH