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Proteomic analysis of a segregant population reveals candidate proteins linked to mealiness in peach

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ABSTRACT

Peaches are stored at low temperatures to delay ripening and increase postharvest life. However some varieties are susceptible to chilling injury, which leads to fruit mealiness, browning and flesh bleeding. In order to identify potential markers associated with chilling injury, we performed proteomic analyses on a segregating population with contrasting susceptibility to chilling-induced mealiness. Chilling-induced mealiness was assessed by measuring juiciness in fruits that have been stored in cold and then allowed to ripen. Fruit mesocarp and leaf proteome from contrasting segregants were analyzed using 2-DE gels. Comparison of protein abundance between segregants revealed 133 spots from fruit mesocarp and 36 from leaf. Thirty four fruit mesocarp proteins were identified from these spots. Most of these proteins were related to ethylene synthesis, ABA response and stress response. Leaf protein analyses identified 22 proteins, most of which related to energy metabolism. Some of the genes that code for these proteins have been previously correlated with chilling injury through transcript analyses and co-segregation with mealiness QTLs. The results from this study, further deciphers the molecular mechanisms associated with chilling response in peach fruit, and identifies candidate proteins linked to mealiness in peach which may be used as putative markers for this trait.

1. Introduction

World production of peaches and nectarines exceeds 21 million tones [1]. Chile is the principle peach/nectarine exporter from the Southern hemisphere [2]. However, the time it takes for Chilean exports to reach their distant consumer markets is a problem, because the fruits quickly ripen and deteriorate at room temperature. In order to delay ripening during shipping, fruits are harvested unripe and may be stored at low temperatures (0 °C) for more than one month [3]. Nevertheless, some varieties are susceptible to physiological disorders associated with long-term cold storage, such as chilling injury. The late season varieties are more susceptible to chilling injury than those that are harvested early [4]. Chilling injury symptoms include mealiness (manifested as a lack of juice), flesh browning and flesh bleeding (visualized as red pigmentation in the fruit flesh near the stone). These symptoms are evident during ripening and when the fruits reach the consumer [3]. Mealiness is the most common chilling injury symptom that negatively affects the quality of Chilean exports and represents a major problem, due to consumer rejection of fruits that lack juice [5]. Stone fruits that present mealiness appear to have good quality externally, but have a mealy or wooly texture and less juice. This mealy texture has been associated to an abnormal cell wall modeling during cold exposure following by ripening at room temperatures [6]. Flesh browning and bleeding are often associated to the chilling-induced mealy phenotype and are related to lack of membrane integrity which lead to interactions between polyphenol oxidase and its substrates resulting in the formation of brown pigments [7]. Some authors have associated chilling injury to a change in the redox status of the cells and triggering of an alternative cold response regulated by abscisic acid (ABA), auxins and ethylene [8,9]. It is also suggested that a differential cold response program in susceptible and tolerant chilling injury genotypes is activated before the fruits are stored in the cold, which indicates that there is a genetic component controlling the chilling-induced mealiness trait [10].

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Looking for strategies to extend postharvest life and avoid chilling injury, some treatments such as controlled atmosphere [11], ethylene [12,13], intermittent warming and conditioning [14,15] among others have been developed. However, these treatments are not effective in all cultivars. For this reason, the development of new cultivars less susceptible to chilling injury is another option to find a solution. The use of molecular markers and other genomic tools to assist breeding programs could allow a much more efficient selection of outstanding genotypes [16]. In the last decade, several studies have been performed to associate molecular markers to fruit quality traits, some of which have focused on chilling injury symptoms. Significant quantitative trait loci (QTLs) for mealiness, graininess, leatheriness and flesh bleeding were identified in linkage group 4 (LG4) through the use of a intraspecific cross between 'Venus' and 'Big Top' nectarines, which correlated with QTLs previously reported for chilling injury symptoms in LG4 from an unrelated progeny population [17]. In another recent work, SNPs associated with chilling injury susceptibility traits like mealiness, flesh browning and flesh bleeding were identified through the evaluation of a peach intraspecific cross between 'Dr. Davis' and 'Georgia Belle', two cultivars displaying contrasting behavior for different quality traits including chilling injury [18]. Another association mapping study in peach populations, allowed the construction of a SNP linkage map with full coverage from two mapping populations. Using the linkage map and phenotypic data collected from three seasons, QTLs associated with mealiness and flesh bleeding were identified on LG4, and browning on LG5 [19].

In the present work, the segregation of chilling-induced mealiness was analyzed in 67 siblings from a F2 population of 'Starkred Gold' × 'Flamekist' (obtained by self-pollinating 'Venus' nectarine). Proteome profiles of fruits and leaves from segregants showing contrasting phenotypes were analyzed by two-dimensional electrophoresis (2-DE). Proteins with differential accumulation among the segregants were identified. Genes codifying for some of the identified proteins co-localize with QTLs for chilling injury previously mapped. The results of this study further deciphers the molecular mechanisms associated with tolerance to chilling injury in peach and the proteins identified that correlate with phenotypically contrasting segregants may be putative molecular markers for the selection of new cultivars less susceptible to chilling injury symptoms including mealiness. The main objective of this work was to identify candidate proteins associated with the predisposition of fruit juiciness or mealiness, upon cold storage.

2. Material and methods

2.1. Plant material and phenotyping

Sixty seven siblings of a F2 population, derived from self-pollinating 'Venus' nectarine, were used in this study. 'Venus' was originally obtained from a cross between 'Starkred Gold' and 'Flamekist'. Fruits were harvested at "commercial maturity" based on flesh firmness and ground color. The firmness of the fruit was evaluated using a penetrometer with a 8 mm plunger in two opposite sides of the fruit that had previously been peeled to remove the epidermis, and firmness range data among segregants varies between 50 to 70 N. Ground color was evaluated by an hedonic commercial harvest index color chart (from DN1 = green to DN7 = orange), currently used by the peach industry, and all the segregants were harvested at DN = 2 and 3 values. Fruits were stored at 4 °C during 21 days and then ripened at 20 °C to reach 1 kgf (9.8 N) flesh firmness. Chilling-induced mealiness of ripe fruits was assessed as a percentage of fruit juice measured in 5–10 fruits from each segregant as described previously [20]. Fruit phenotyping was performed during three consecutive harvest seasons (2008–2010). Four leaves per fruit close to the fruit insertion were collected within each segregant at harvest. The mesocarp of the fruits and leaves were frozen in liquid nitrogen and stored at −80 °C until its use for protein extraction.

Statistical analyses of phenotypic data were performed using a mixed model for repeated measures. Variance components were estimated by restricted maximum likelihood (REML) and the phenotypic and values of family were predicted by best linear unbiased prediction (BLUP). The mixed model 63 on the software Selegen-REML/BLUP [21] was used. The mixed model is expressed as:

$$y = Xm + Wp + e,$$

where y, m, p and e are vectors of the data, fixed measurements (harvest seasons) effects added to the general mean, random permanent individual plant effects (genotypic effects + environmental effects) and random errors, respectively. Capital letters are matrices of incidence of m and p.

2.2. Protein extraction

Three representative segregants of each category (mealy vs juicy) were selected for proteome analysis. One gram of fruit mesocarp was taken from three fruits per segregant and pooled together for protein extraction. For the leaves, 1 g of a pool of leaves from each segregant was used for protein extraction. Each biological replicate was composed by an independent pool of fruits or leaves. The protein extraction protocol used was phenol extraction followed by ammonium/acetate precipitation [22]. The obtained pellet was air dried at room temperature and resuspended in isoelectrofocusing (IEF) buffer (5 M urea, 2 M thiourea, 2% SB3-10 (*w*/*v*), 2% CHAPS (*w*/*v*), 0.5% ampholites pH 5–7 and 0.25% ampholites pH 3–10 (*v*/*v*)) [23]. Protein quantification was carried out using Bradford protein assay [24].

2.3. 2D-SDS running and staining

Proteins were separated by two-dimensional electrophoresis (2-DE). Isoelectrofocusing was carried out in PROTEIN IEF (Bio-Rad) and SDS-PAGE was performed in Ettan Dalt Twelve system (General Electric). Before IEF, 100 μg of protein samples were supplemented with DTT and TCEP (final concentrations of 20 mM and 2 mM, respectively) and IEF buffer to reach a final volume of 450 μl.

Strips (24 cm) were actively rehydrated at 20 °C under 50 μA per strip for about 9 h. The next step was the IEF which consisted of 30 min at 150 V, then successive voltage increments: 105 min at 2000 V, 210 min at 6000 V and 420 min at 9500 V. After focusing, strips were incubated with 2% (*w*/*v*) DTT for 10 min to reduce the proteins and alkylated with 2.5% (*w*/*v*) iodoacetamide for 10 min, both solutions also contained 50 mM Tris–HCl (pH 8.8), 6 M urea, 30% (*w*/*v*) glycerol and 2% (*w*/*v*) SDS. Each strip was then embedded using 0.5% (*w*/*v*) agarose in running buffer containing traces of bromophenol blue and positioned on the top of an 11.5% to 14.5% 24 cm polyacrylamide gradient gel. SDS-PAGE was performed at 20 mA for each gel. Gels were stained with Deep Purple Protein Stain (GE Healthcare) following the manufacturer's instructions.

2.4. Image acquisition

Stained gels were digitalized at 100 μm per pixel using the Quantity One software in a Phosphoimager FX from Bio-Rad and analyzed using Delta 2D software v4.0 (Decodon). This program allows the images to be adjusted to generate a fused image, creating a proteome map representative from the whole experiment. The spot detection step was performed on the proteome map. All detected spots on the fusion image were transferred to all other images. This procedure led to a 100% spot matching, strengthening the statistical analysis [25]. To improve the analysis, spots were manually checked.

Subsequently, spots were quantified and normalized dividing the raw quantity of each spot in a gel by the total quantity of the

valid spots present in the same gel and expressed as percentage of the volume (%V) [26].

2.5. Statistical analyses

Two statistical analyses were performed, Wilcoxon (non-parametric) and t-test (parametric). Wilcoxon method was implemented with a cut off P-value <0.05. The analysis was executed in MeV v4.8 software [28]. Parametric analyses require a normal distribution of data. Since 2-DE data tends not to distribute normally, spots %V raw data were transformed with Box–Cox power transformation [29] (Fig. S1). Following this transformation of the data, a t-test was performed with a cut off P-value <0.05. This analysis was executed in MeV v4.8. Differentially accumulated spots detected by both analyses (Wilcoxon and t-test) were selected for further analyses.

2.6. Multivariate principal component analysis

Principal component analysis (PCA) was performed using Box-Cox transformed data of spots volume percentage. From 837 total spots detected in fruit 2D gels, 533 spots with a sufficient volume percentage to be visualized in 2D gel images were selected. For leaf spot data the same treatment was applied, from 276 spots detected in leaf 2D gels 190 were selected. Those spots were analyzed with PCA using InfoStat 2014 software.

2.7. Experimental LC/MS/MS

Total protein (200 μg) from Venus fruit mesocarp or leaf extract from the Venus segregants were run on 2D gels. These gels were stained with colloidal Coomassie where differentially accumulated spots and spots correlated to PCA were manually excised and sent to the Proteomics Core Facility in Michigan State University. Gel bands were subjected to in-gel tryptic digestion [27]. The extracted peptides were then automatically injected by a Waters nanoAcquity Sample Manager (www.waters.com) and loaded for 5 min onto a Waters Symmetry C18 peptide trap (5 μm, 180 μm × 20 mm) at 4 μL/min in 5% ACN/0.1% formic acid. The bound peptides were then eluted onto a MICHROM Bioresources (www.michrom.com) 0.1 × 150 mm column packed with 3 μ, 200A Magic C18AQ material over 16 min with a gradient of 2% B to 30% B in 9 min using a Waters nanoAcquity UPLC (buffer A = 99.9% water/0.1% formic acid, buffer B = 99.9% acetonitrile/0.1% formic acid) with a flow rate of 1 μL/min.

Eluted peptides were sprayed into a ThermoFisher LTQ linear ion trap mass spectrometer outfitted with a MICHROM Bioresources ADVANCE nano-spray source. Spectra were acquired in a data

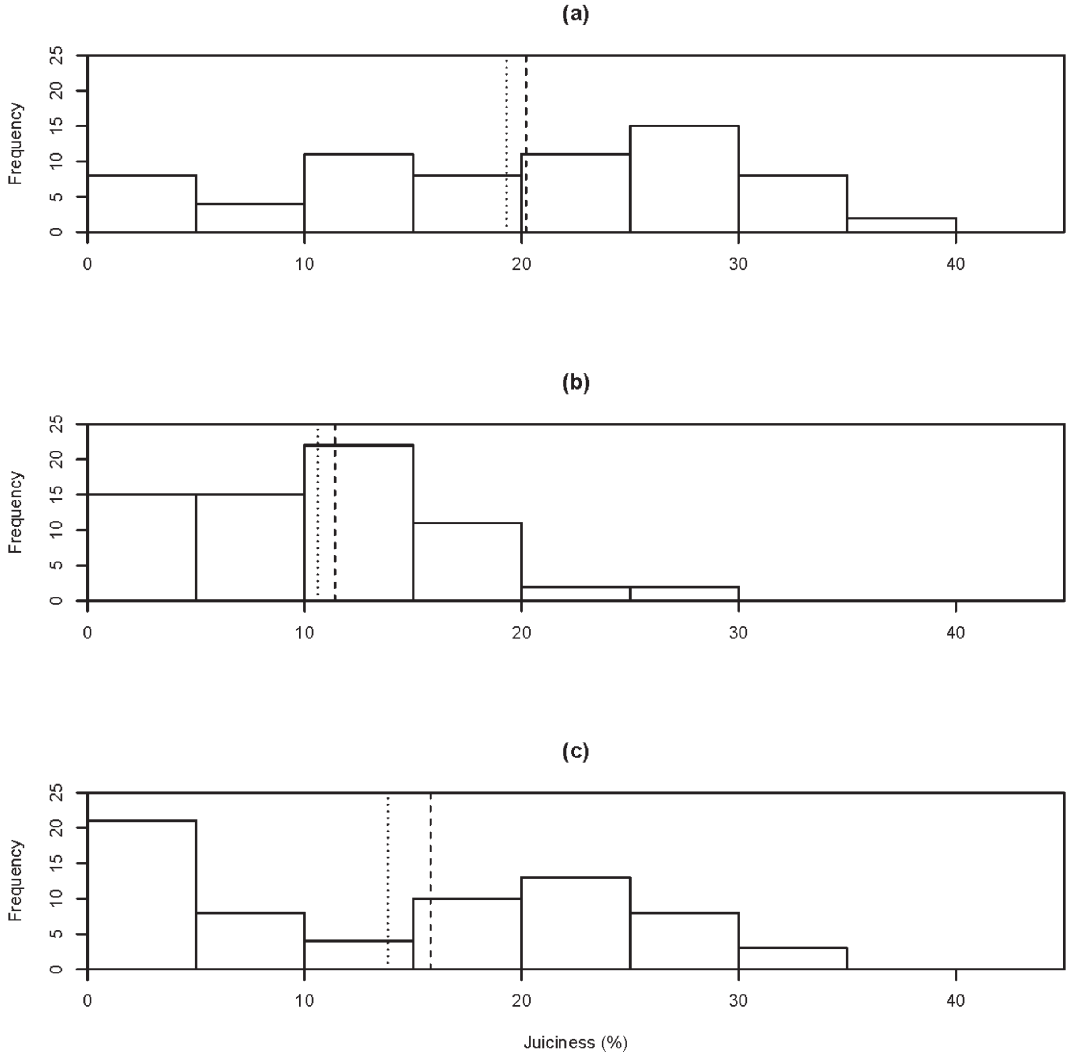

Fig. 1. Distribution of juiciness character in three harvest seasons in the F2 Venus population. Histograms show the frequency of individuals showing different fruit juice content expressed as % juiciness in (a) 2007–2008, (b) 2008–2009 and (c) 2009–2010 harvest seasons. Dotted lines indicate the median and dashed lines indicate the average % juiciness.

Table 1
Estimates of phenotypic and genetic parameters of juice content in fruits of Venus segregant family in three harvest seasons.

Parameter	Value
Vfp	33.1469
Vet	101.3578
Vf	134.5047
r	0.8826
Acm	0.9395
m	15.4174

The results are based in three harvest seasons.
Vfp: phenotypic variance; Vet environmental variance; Vf individual phenotypic variance; r repeatability of the average of m harvests or m repeated measurements; Acm selection accuracy; *m* general average.

dependent fashion where the top ten ions in each survey scan are subjected to low energy collision induced dissociation (CID) in the LTQ. Minimum intensity threshold of $1e^4$ was used and collision energy was set to 30. Dynamic exclusion was enabled and peaks were excluded from re-analysis for 45 s. The resulting MS/MS spectra were converted to peak lists using Mascot Distiller, v2.5.1.0 (www.matrixscience.com) using the default LTQ tripleplay instrument parameters. Peak lists were searched against the translated *Prunus persica* Whole Genome Assembly v2.0 and annotation v2.1 (v2.0.a1) downloaded from Phytozome (URL: phytozome.jgi. doe.gov) [28] using the Mascot searching algorithm v2.4 (www.matrixscience.com). The following parameters were used: missed cleavages 2, peptide tolerance 200 ppm, MS/MS tolerance 0.6 Da, peptide charge state limited to +1, +2 and +3, fixed modification of carbamidomethyl cysteine and variable modification of oxidation of methionine.

3. Results

3.1. Population phenotyping and selection of segregants for proteomic analysis

Chilling-induced mealiness phenotyping of the fruits of segregants from self-pollinated Venus was performed in three consecutive harvest seasons. Fig. 1 shows the distribution of juiciness in the segregating population. Fruits that presented more than 10% juice were considered juicy segregants, while fruits that presented 10% or less juice were considered mealy. It can be observed that the juiciness in the population varies between 0% and 40% juice which shows that there is segregation of the character in the population. The maximum juice %, median (dotted line) and average (dashed line) juice values vary in the population in the different harvest seasons reflecting an environmental influence on chilling-induced mealiness (Fig. 1).

Estimates of the phenotypic and genetic parameters for juice content are presented in Table 1. The estimate of repeatability of the average of the three consecutive seasons or repeated measures (r) was 0.88. The square root of this value indicates the accuracy in the selection of the genotypes. The accuracy (Acm) was 93.94% which indicates that the data obtained from the three seasons allowed a good precision and the regularity of the superior juicy individuals in the different harvest seasons.

BLUP analysis was performed in order to select contrasting segregants on juice content. Table S1 shows the ranking of the segregants according to the permanent phenotypic effect (fp) that considers the genotypical and the permanent environmental variance among the three harvest seasons evaluated. Individuals VS 121 17B (6th in the ranking), VS 177 15B (9th in the ranking) and VS 174 17 B (13th in the ranking) were selected as juicy segregants while VS 165 15B (63rd

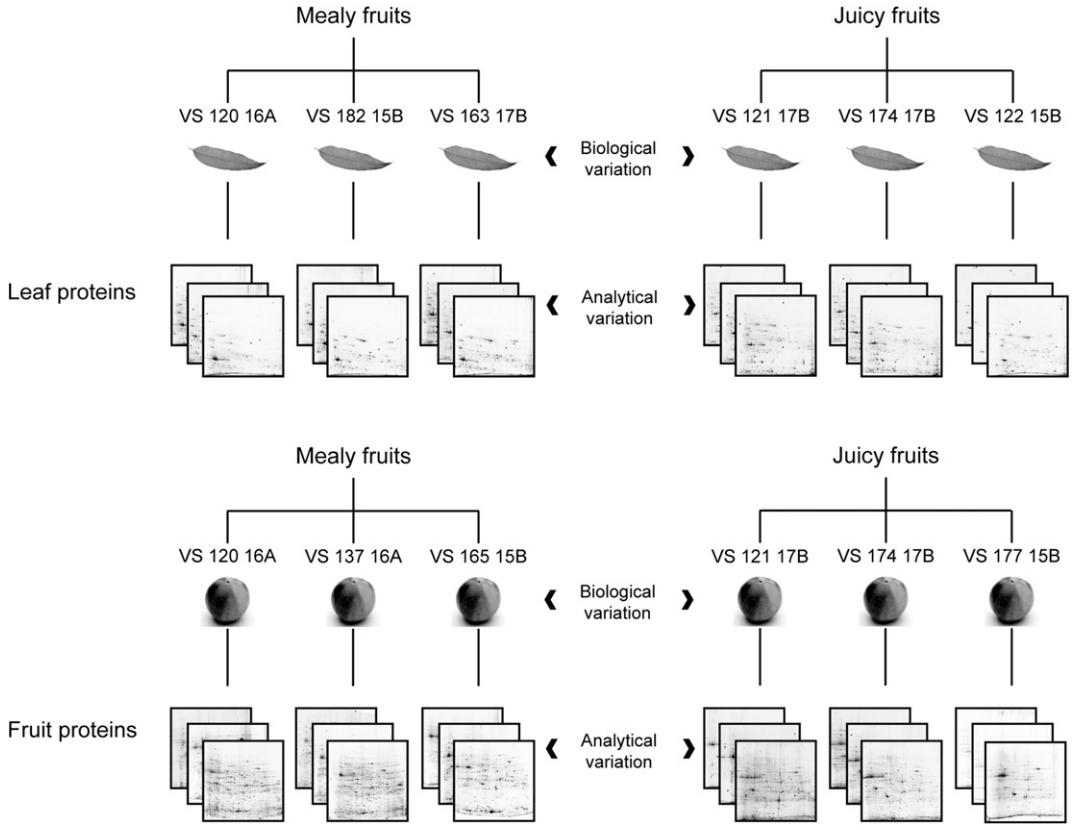

Fig. 2. Experimental design used for proteomic analysis of *Prunus persica* 'Venus' segregants. Leaf and mesocarp proteins of three segregants that produce mealy fruits and three segregants that produce juicy fruits were evaluated using 2D gel electrophoresis. Three independent pools of 3–4 leaves or three independent pools of mesocarp from 3 fruits from each segregant were used for protein extraction. These proteins were used to generate the 2D gels. Three gels from each segregant were generated and considered as technical replicates. The ensemble of gels from each segregant was considered as a biological replicate.

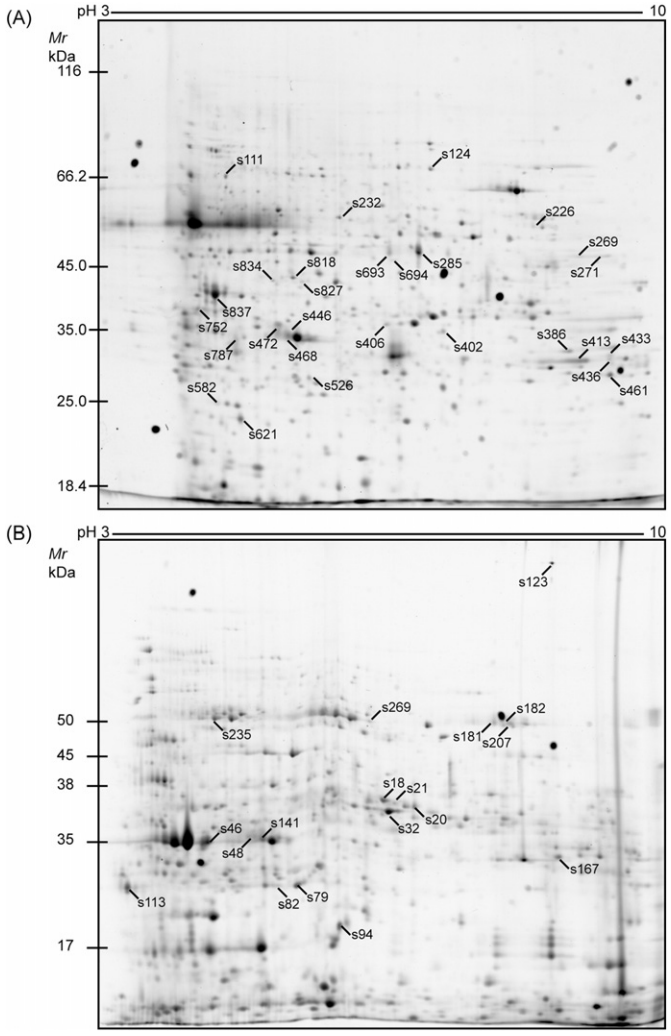

Fig. 3. Representative deep purple stained 2D gels from *P. persica* variety 'Venus' proteins. (A) Fruit mesocarp and (B) leaf proteins sampled at commercial harvest. Proteins extracts focused from pH 3 to 10 followed by separation by SDS-PAGE. Proteins spots that showed differential abundance between juicy and mealy phenotypes and were identified by mass spectrometry are indicated with numbers attributed by Delta 2D software.

in the ranking), VS 137 16A, (64th in the ranking) and VS 120 16A (67th in the ranking) were selected as mealy segregants and used for proteomic analysis.

The experimental design used for proteomic analysis is shown in Fig. 2. Three independent pools of 3–4 leaves and three independent pools of mesocarp of 3 fruits from each selected segregant were used for total protein extraction.

3.2. Spot detection and quantification

Proteins from fruit mesocarp and mature leaves were separated by 2D-PAGE using a pH gradient from 3 to 10. Gel analyses allowed the detection of 837 spots in fruit mesocarp samples that were consistently detected in samples from all segregants (Fig. 3A). On the other hand, 276 spots were detected on 2D gels from leaf protein extract (Fig. 3B).

3.3. Identification of differentially accumulated proteins

Two statistical analyses were carried out to define the fruit mesocarp spots to be sequenced. Using the raw data, the non-parametrical Wilcoxon test [29] allowed the detection of 183 spots differentially accumulated between all segregants. A parametrical t-test, using Box–Cox transformed raw data with a normal distribution, resulted in the detection of 165 differentially accumulated spots (Supplementary Fig. S1). A total of 133 spots were detected as differentially accumulated by both analyses (Fig. 4).

The same analyses were performed for leaf 2D gels raw data of 276 spots detected, Wilcoxon test allowed us to detect 53 spots differentially accumulated between leaves from mealy and juicy segregants. On the other hand, t-test was used with transformed data of 276 spots detected in leaf 2D gels, of which 44 spots were detected as differentially accumulated. As Fig. 4 shows, 36 spots were detected as differentially accumulated by both analyses in leaf data.

3.4. Multivariate principal component analysis

In order to identify the projection and distribution of the data obtained, principal components analysis (PCA) was performed. In the case of mesocarp fruit protein spot abundance principal component 1 (PC1), which explains 29.5% of the variability could separate mealy fruit segregants (regular letters) from juicy fruit segregants (bold letters) (Fig. 5). These results showed that the ensemble of the fruit proteomes can segregate the two phenotypic classes of segregants. Spots correlated with PC1, which were not detected as differentially accumulated by statistical analyses, were selected for identification by mass spectrometry. The complete list of spots and their respective correlation values is in Supplementary Table 2. On the other hand, in principal component analysis of leaf spots abundance, PC1 explaining 33.6% of variability and could partially separate between juicy and mealy segregants (Supplementary Fig. 2). In this analysis the juicy segregant VS 122 15B clustered with mealy segregants, and the mealy segregant 163 17B clustered with juicy segregants. In this case, the ensemble of leaf proteome could not segregate the two phenotypic classes. Therefore only the spots that were detected as differentially accumulated by statistical analyses between the two classes of segregants were identified by mass spectrometry.

3.5. Protein identification by MS

Spots detected as differentially accumulated between mealy and juicy fruit segregants were identified by mass spectrometry (MS). Thirty

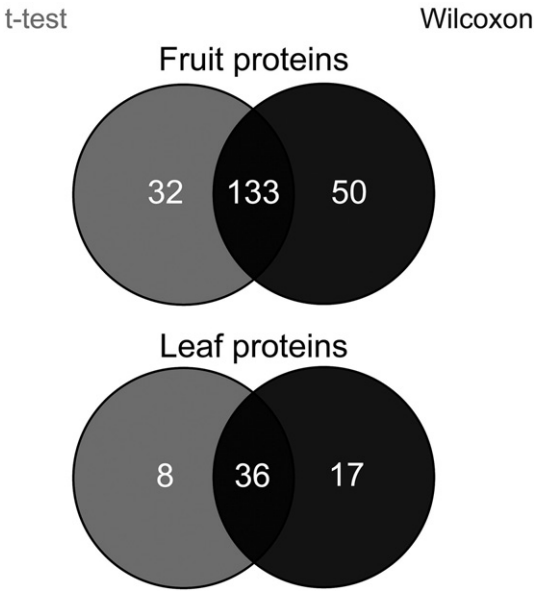

Fig. 4. Comparison of statistical tests used to detect differentially accumulated proteins from fruit mesocarp and leaves of 'Venus' segregants with differential juice content. Of the 837 spots detected in fruit mesocarp protein extracts, 133 were commonly identified by t-test and Wilcoxon statistical tests. In leaves, from the 276 spots detected, 36 were commonly identified by both statistical tests. Spots detected by both analyses were selected for protein identification.

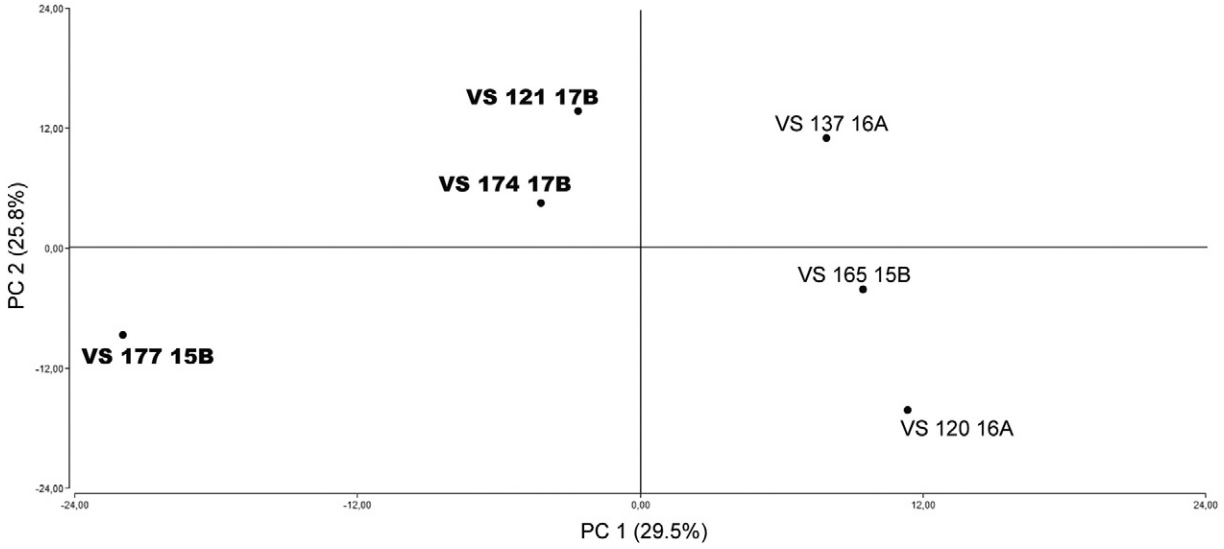

Fig. 5. Principal component analysis of abundance of all spots detected in 2D gels of mesocarp fruit protein extracts. Loading data was the quantitative values of the spots and score plot the protein samples from the different 'Venus' segregants. Bold type segregants correspond to juicy phenotype and regular type segregants correspond to mealy phenotype.

four spots were identified from fruit 2D gels and 36 spots from leaf 2D gels. These proteins were classified in processes such as response to stress, cell wall metabolism, transmembrane transport, oxidation–reduction reactions, energy metabolism, and other functions (Table 2). All proteins identified presented more than two unique peptides and high coverage percentage (Table 2, Supplementary Fig. 3). The experimental isoelectric points (pI) and relative molecular weight (MW) of the proteins showed a good relationship with their theoretical pI and MW. In some cases, it was obtained the same protein identification in spots with different experimental pI and/or MW. These could be related to post-translational modifications or abnormal electrophoretic mobility. In terms of their accumulation in juicy and mealy segregants, some displayed the same patterns and others showed opposite. In some cases, more than one protein was identified within the same spot (s827, s752, s402, s621, s18, s32 and s46). As in all cases more than 5 unique peptides were identified for the proteins, the phenomenon indicates co-migration of the proteins in the gel. The co-migration of proteins in a single spot is a limitation of gel-based proteomics and may render comparative quantification inaccurate [30].

Proteins preferentially accumulated in juicy fruits in comparison to mealy fruits include: response to stress proteins and proteins associated with hormone synthesis (e.g. S-adenosylmethionine synthetase [SAMS, s694], 1-aminocyclopropane carboxylate oxidase [ACO, s837], or hormone inducible proteins (e.g. abscisic acid [ABA/WDS, s446, s468, s472, s837] induced protein) (Table 2).

Other proteins related to response to stress, cell wall metabolism and transmembrane transport were also accumulated preferentially in juicy fruits in comparison to mealy fruits. In contrast, proteins that accumulated preferentially in mealy fruits include proteins associated with oxidation–reduction processes as well as some proteins related to stress. Some proteins identified in different spots, exhibited opposite variations in their accumulation patterns. For example, three spots classified as SAMS (s693, s694 and s827) showed opposite patterns of accumulation. The difference between these spots was the experimental pI obtained, suggesting that this protein may undergo post-translational modifications. Similar findings were seen for ABA/WDS (s468, s472 and s446).

Fig. 6A shows a detail of spots with differential abundance between juicy and mealy segregants. Some are highly abundant in one condition (e.g. s837, s86 and s142), while barely accumulated in the other condition.

Most of the proteins identified from leaf 2D gels were involved in energy metabolism (Table 2). The data indicate that identified proteins in spots s18, s32, s46, s107 are involved in photochemical process, while the proteins identified in the spots s32 and s235 are involved in photosynthetic carbon fixation step. Oxygen-evolving enhancer protein 2, a protein involved in the photochemical step of photosynthesis, was found in two spots, both of them showed increased accumulation levels in leaves of juicy segregants compared to mealy ones. On the other hand, proteins such as malate dehydrogenase (s20) and serine hydroxymethyltransferase (s181 and s182) were accumulated preferentially in leaves of mealy segregants (Fig. 6B). The accumulation pattern of spot s113, identified as cysteine synthase is shown in Fig. 6B.

4. Discussion

Peaches stored for long periods at cold temperatures develop a physiological disorder known as chilling injury. The main symptoms associated with chilling injury are mealiness (perceived as a dry texture of mesocarp), flesh browning and internal reddening [3]. However, some varieties are more susceptible to develop chilling injury, being the late season varieties more prone than early harvested cultivars [4]. Several studies have been done with the objective of understanding the molecular processes involved in chilling injury [31–34], however, this problem is still not well understood. In this work, we analyzed the differential proteome in fruits and leaves of individuals from a F2 population which segregated for chilling injury resistance or susceptibility. Most of the proteins identified were related to abiotic stress and oxidation–reduction processes. During cold storage of both chilling injury resistant and susceptible segregants, cold stress is perceived and a response is triggered, however, this appears at different intensities and/or time points. In previous publications based on transcriptomic analyses, some authors had suggested that an alternative cold response, regulated by the hormones ethylene, ABA and auxin, is acting in chilling injury tolerant varieties [9] and that these differential responses are active prior to cold exposure [10]. Our results support these hypotheses, since we observed an increase in the abundance of proteins related to the hormones such as ABA and ethylene in the mesocarp of segregants resistant to chilling injury. On the other hand, susceptible segregants that develop mealy fruits, proteins related to late response to cold stress were more abundant. Particularly, proteins related to oxidative stress such as glutathione S-transferase, heat shock protein and leucoanthocyanidin dioxygenase. It appears as though the tolerant segregants are prepared

Table 2
Identification of differentially accumulated proteins from fruit mesocarp and leafs. Comparison between juicy and mealy segregants.

spot	Identity	Accession N°	Coverage %	pI theo/exp	MW theo/exp	Unique peptides	Mascot Score	Volume % Juicy fruit	Volume % Mealy fruit	Ratio J/M
	Fruit mesocarp proteins									
	Accumulated in juicy segregant									
	Response to stress									
s226	Catalase	Prupe.5G011300.1	51	6.9/8	56.9/57	21	672	0.13	0.05	2.60
s468	ABA/WDS induced protein	Prupe.8G034100.1	36	5.7/5.1	20.7/35	6	433	0.26	0.08	3.25
s472	ABA/WDS induced protein	Prupe.8G034100.1	48	5.7/5.2	20.7/36	8	549	0.24	0.04	6.00
s446	ABA/WDS induced protein	Prupe.8G034100.1	56	5.7/5.3	20.7/36	9	991	0.45	0.27	1.67
s693	S-adenosylmethionine synthetase	Prupe.1G107000.1	47	5.7/5.9	42.8/49	12	663	0.11	0.03	3.67
s694	S-adenosylmethionine synthetase	Prupe.1G107000.1	33	5.7/6.0	42.8/48	9	628	0.14	0.05	2.80
s752	ABA/WDS induced protein	Prupe.8G034100.1	40	5.7/3.8	20.7/35	9	480	0.43	0.26	1.65
s837	aminocyclopropanecarboxylate oxidase	Prupe.3G209900.1	54	5.2/4.3	36.1/41	12	987	2.37	1.09	2.17
	Cell wall metabolism									
s285	Polygalacturonase	Prupe.4G262200.1	51	6.2/6.2	41.2/49	9	1539	0.32	0.06	5.33
	Transmembrane transport									
s433	Porin/voltage-dependent anion-selective channel protein	Prupe.3G271800.1	54	9.3/9.2	35.3/37	14	1237	0.17	0.05	3.40
s436	Porin/voltage-dependent anion-selective channel protein	Prupe.6G221700.1	60	8.8/9.1	29.4/36	15	1083	0.17	0.04	4.25
s461	Porin/voltage-dependent anion-selective channel protein	Prupe.1G086700.1	15	9.1/8.9	29.5/35	3	163	0.12	0.02	6.00
	Other functions									
s582	Nuclear transport factor 2	Prupe.1G224200.1	26	5.7/4.2	13.6/28	2	236	0.1	0.07	1.43
s752	Syntaxin of plants SYP7	Prupe.6G238700.1	43	5.3/3.8	29.9/35	9	348	0.43	0.26	1.65
	Accumulated in mealy segregant									
	Response to stress									
s111	Dehydrin	Prupe.7G161100.1	61	6.4/4.3	48.1/69	20	1290	0.04	0.11	0.36
s124	Heat shock protein STI	Prupe.5G076500.1	54	6.3/6.4	64.1/67	31	1560	0.07	0.17	0.41
s406	Annexin	Prupe.6G186600.1	46	6.2/5.9	36/38	12	592	0.04	0.1	0.40
s269	3-ketoacyl-CoA thiolase B, peroxisomal	Prupe.1G003300.1	43	8.3/8.5	48.5/52	17	1468	0.16	0.24	0.67
s271	3-ketoacyl-CoA thiolase B, peroxisomal	Prupe.1G003300.1	59	8.3/8.7	48.5/52	21	1853	0.04	0.09	0.44
s526	Glutathione S-transferase	Prupe.2G227100.1	73	6/5.4	23.6/31	14	743	0.15	0.25	0.60
s818	Zinc-binding oxidoreductase	Prupe.3G284300.1	40	5.9/5.1	34.4/44	7	311	0.38	0.56	0.68
s827	S-adenosylmethionine synthetase	Prupe.1G107000.1	23	5.7/5.2	42.8/47	4	270	0.07	0.1	0.70
s827	Leucoanthocyanidin dioxygenase	Prupe.5G086700.1	43	5.4/5.2	40.5/47	10	420	0.07	0.1	0.70
s834	Zinc-binding oxidoreductase	Prupe.3G284300.1	63	5.8/4.5	34.4/44	20	883	0.37	0.46	0.80
	Oxidation–reduction process									
s402	Malate dehydrogenase	Prupe.5G194200.1	41	8.6/6.4	43.5/38	13	915	0.05	0.17	0.29
s402	Thioredoxin reductase (NADPH)	Prupe.7G187600.1	51	8.7/6.4	40.7/38	14	977	0.05	0.17	0.29
s406	Aldo/keto reductase family	Prupe.1G175800.1	48	5.7/5.9	37.5/38	17	884	0.04	0.1	0.40
	Other functions									
s232	UTP–glucose-1-phosphate uridylyltransferase	Prupe.3G015000.1	63	5.9/5.7	51.6/56	24	1648	0.21	0.33	0.64
s386	20S proteasome subunit alpha 4	Prupe.7G170800.1	49	7.7/8.5	27.1/38	11	828	0.15	0.26	0.58
s413	Remorin	Prupe.2G164400.1	54	7.7/8.6	21.8/38	13	359	0.03	0.08	0.38
s621	CYSTEINE PROTEINASE INHIBITOR 12	Prupe.2G047300.1	74	5.5/4.6	10.6/24	8	306	0.19	0.30	0.63
s621	Profilin	Prupe.1G504400.1	47	5/4.6	14.1/24	4	290	0.19	0.30	0.63
s787	Ubiquinone biosynthesis methyltransferase COQ5	Prupe.1G079700.1	15	6.1/4.8	32.4/32	3	261	0.04	0.15	0.27
s818	Glutamine synthetase	Prupe.3G166500.1	43	5.9/5.1	38.9/44	14	809	0.38	0.56	0.68
	Leaf proteins									
	Accumulated in juicy segregant									
	Energy metabolism									
s18	Malate dehydrogenase, mitochondrial	Prupe.4G170500.1	60	9.0/6.2	35.7/39	11	1066	1.42	1.11	1.28
s18	Ferredoxin–NADP reductase, leaf isozyme, chloroplastic	Prupe.7G169200.1	43	8.8/6.2	40.8/39	12	513	1.42	1.11	1.28
s32	Quinone oxidoreductase-like protein At1g23740, chloroplastic	Prupe.3G284800.1	22	8.9/6.3	41.8/37	6	285	2.14	1.6	1.34
s32	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	Prupe.5G155800.1	35	6.4/6.3	36.8/37	7	522	2.14	1.6	1.34
s46	Oxygen-evolving enhancer protein 1, chloroplastic	Prupe.7G145900.1	46	6.1/4.2	35.4/35	10	434	3.13	2.49	1.26
s46	Putative L-ascorbate peroxidase, chloroplastic	Prupe.1G522500.1	32	6.1/4.2	38.5/35	10	585	3.13	2.49	1.26
s48	Oxygen-evolving enhancer protein 1, chloroplastic	Prupe.7G145900.1	41	6.1/5.0	35.4/35	8	277	1.36	0.57	2.39
s48	Putative L-ascorbate peroxidase, chloroplastic	Prupe.1G522500.1	31	6.1/5.0	38.5/35	8	470	1.36	0.57	2.39
s82	Glutathione S-transferase DHAR1, mitochondrial	Prupe.2G227100.1	41	6.0/5.2	23.7/25	7	326	0.61	0.3	2.03
s94	Ribulose bisphosphate carboxylase small chain, chloroplastic	Prupe.1G084500.1	30	9.1/5.7	20.5/21	5	301	0.51	0.24	2.13
s141	Putative L-ascorbate peroxidase, chloroplastic	Prupe.1G522500.1	38	6.1/5.1	38.5/32	13	731	1.41	0.43	3.28
s235	Phosphoglycerate kinase, chloroplastic	Prupe.4G133800.1	31	8.4/4.4	50.3/50	11	393	0.96	0.89	1.08
	Other functions									
s21	Cysteine synthase, chloroplastic/chromoplastic	Prupe.2G172800.1	50	8.8/6.3	40.8/38	34	1348	0.65	0.33	1.97
	Accumulated in mealy segregant									
	Energy metabolism									
s20	Malate dehydrogenase, mitochondrial	Prupe.4G170500.1	39	9.0/6.6	35.7/38	19	811	0.16	0.94	0.17
s20	Ferredoxin–NADP reductase, leaf isozyme, chloroplastic	Prupe.7G169200.1	39	8.8/6.6	40.8/38	15	523	0.16	0.94	0.17
s113	Cysteine synthase, chloroplastic/chromoplastic	Prupe.2G172800.1	18	8.8/3.0	40.8/26	5	168	0.11	0.59	0.19
s167	Malate dehydrogenase, glyoxysomal	Prupe.8G157300.1	29	8.2/9.2	37.8/31	8	458	0.16	0.46	0.35
s167	Ribulose bisphosphate carboxylase small chain, chloroplastic	Prupe.1G084500.1	38	9.1/9.2	20.5/31	7	282	0.16	0.46	0.35
s181	Serine hydroxymethyltransferase, mitochondrial	Prupe.6G167300.1	46	8.4/7.8	57.2/50	16	672	0.12	0.46	0.26
s182	Serine hydroxymethyltransferase, mitochondrial	Prupe.6G167300.1	39	8.4/8.0	57.2/50	12	566	0.7	1.14	0.61
s207	Serine hydroxymethyltransferase, mitochondrial	Prupe.6G167300.1	47	8.4/8.3	57.2/50	17	721	1.2	0.71	1.69
	Other functions									
s79	Whole genome shotgun sequence of line PN40024, scaffold_5.assembly12x	Prupe.5G237100.1	42	7.0/5.3	18/26	11	234	0.64	1.17	0.55

in advance (acclimated) to cope with cold stress, thereby preventing the appearance of mealiness and other chilling injury symptoms. In contrast, the susceptible segregants are not acclimated and, therefore, need to combat cold stress once they are exposed to it. To understand what proteins may be involved in this acclimation process, a detailed description about the different proteins identified are discussed below.

4.1. Changes related to response to stress

Abscisic acid (ABA) is a phytohormone that participates in various processes, such as stomatal closure, growth inhibition and adaptation to abiotic stress [6,35]. An ABA stress ripening (ASR) protein has been identified that accumulates under abiotic stress conditions such as osmotic stress, aluminum tolerance and extreme temperatures [36]. We found an ABA/WDS (ABA/water-deficit-stress) protein in spots that accumulate preferentially in fruit mesocarp of juicy segregants (spots s446, s468, s472 and s752) (Table 2). This accumulation pattern suggests that segregants with juicy fruits are more prepared to cope with temperature stress than mealy segregants. In previous studies, the ASR protein was showed to increase in the mesocarp of juicy 'O'Henry' fruits that were stored for a prolonged period of time [33]. Therefore, there is a reproducible correlation between the accumulation of members of the ASR protein family and juicy ripened fruits that had undergo prolonged cold-storage post-harvest.

Ethylene is a volatile hormone that regulates growth, development, leaf and flower senescence as well as fruit ripening [37]. In plants, ethylene is synthesized from S-adenosylmethionine (SAM), which is an active form of methionine produced by S-adenosylmethionine synthetase (SAMS). Subsequently, two steps are necessary to produce ethylene. The first is the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. The second step is mediated by ACC oxidase which converts ACC to ethylene [38]. It has been reported that ethylene treatment of nectarine fruits during cold storage prevented chilling injury in 85% of the fruits [12]. We have identified two enzymes related to ethylene biosynthesis that accumulate preferentially in the mesocarp of juicy fruit segregants, SAMS (spots s693 and s694) and ACC oxidase (spot s837) (Table 2). This increased level of S-adenosylmethionine synthetase and ACC oxidase may lead to an increased fruit ethylene production in juicy fruits when compared with mealy fruits. A S-adenosylmethionine synthetase protein was identified in a spot that was more abundant in mealy fruits (s827). Therefore it may be an alternative transcript or post translation modification as these isoforms presented different pI and/or MW. The increase in the abundance of ethylene biosynthesis related proteins in juicy fruits was previously observed in 'O'Henry' mesocarp submitted to cold postharvest treatment [33]. Proteomic analyses have shown that these proteins correlated with normal fruit ripening in peach and other climacteric fruits [39,40]. Additionally, we found that the gene that encodes the S-adenosylmethionine synthetase isoform (Prupe.1G107000.1) identified in this work, is located within a previously reported QTL for flesh bleeding (qBL1) [19]. One of the most significant SNP markers associated with qBL1 is located within close proximity of the SAMS locus. The UCD_SNP_821 marker is located at 8,409,875 bp on Pp01 (corresponding to scaffold 1), whereas the SAMS locus is located between 8,141,239 and 8,143,517 bp on the same scaffold.

Oxidative stress is a secondary response to chilling injury and also contributes to the loss of cell membrane integrity. Oxidative stress occurs when cellular homeostasis is perturbed [6]. Catalase, one of the principle plant antioxidant enzymes, is activated early in response to oxidative stress [41]. In this work, we identified a catalase (s226) that is highly accumulated in juicy fruits. Increased levels of this catalase, may lead to a greater ability of these fruit to resist oxidative stress, in comparison to those that develop mealiness. This finding supports previously observations of an increased accumulation of catalase proteins in chilling injury tolerant fruits [33,34].

Some proteins related to stress are accumulated preferentially in the mesocarp of mealy fruit segregants. One of these proteins, annexin (s406), is a membrane-binding protein [42] capable of interacting with membrane lipids and proteins. Annexins play an important role in stress adaptation by regulating different signaling pathways [43]. Another enzyme identified was glutathione S-transferase (s526), a protein that plays a role in cellular detoxification by conjugating xenobiotic and endobiotic compounds to glutathione [44]. These proteins, annexin and glutathione S-transferase, presented similar accumulation pattern in a previously reported proteomic analysis of chilling injured peach fruit [33,34].

Dehydrins are proteins that are induced under abiotic stress conditions, including low temperatures, water stress and ABA application [45]. Dehydrins can be found in the cytoplasm and the nucleus. Cytoplasmic dehydrins may bind to membranes, improving membrane stability [46]. These proteins can also act as chaperones for other proteins, helping proper protein folding, thereby preventing aggregation under heat or freezing stress conditions [47]. We identified one dehydrin in fruit protein 2D gels (s111) that presented higher accumulation levels in the mesocarp of mealy fruit segregants. Another spot which did not show statistically significant differential abundance between juicy and mealy segregants was also identified as dehydrin (not shown). These two spots were located within the same region according to pI but have distinct MW. This difference is probably due to post-translational modifications or alternative splicing. It has been reported that dehydrins, under stress conditions are subjected to post-translational modifications, mainly phosphorylation [48,49]. This phosphorylation of dehydrin in some cases, is controlled by casein kinase II and could be associated to the translocation of dehydrins from the cytoplasm to nucleus [50].

Leucoanthocyanidin dioxygenase (LDOX) is an enzyme that participates in anthocyanin biosynthesis by converting leucoanthocyanidins to anthocyanidins, which are substrates for the synthesis of anthocyanins [51]. Anthocyanins are phenolic compounds that are responsible for flower and fruit pigments, acting as insects and animals attractants [52]. Anthocyanidins have red color [53] and after some enzymatic reactions, they can be converted to compounds that can be oxidized by polyphenol oxidase (PPO) and peroxidase (POD) generating brown coloration [54,55]. It has been reported that *LDOX* gene co-localizes with a QTL (qP-Brn5.1^{m}) affecting browning in peach, making the LDOX gene potentially responsible for this QTL [56]. In a more recent work, it was found that a QTL for flesh browning (qBrL5) in peach [19] appears to be similar to the previously identified qP-Brn5.1^{m} locus [56]. This QTL was located on Pp05 (corresponding to scaffold 5), specifically between 5,812,837–16,758,189 bp. One of the most significant SNP markers (UCD_SNP_872) in this QTL is located at 9,812,134 bp, very close to the position of the LDOX gene that codifies for the protein identified in this work (s827), which is located on Pp05 between 9,817,673–9,819,380 bp. It is probable that this SNP could affect the LDOX regulation. In our work, as well as previous studies [33], this protein presented higher accumulation levels in fruits that presented chilling injury symptoms such as mealiness.

4.2. Changes related to cell wall metabolism

Softening and texture changes in fruits during ripening are associated with cell wall modifications. These modifications include depolymerization of the glycan matrix, pectin solubilization and loss of neutral sugars, among others. However, they are not common for all species [57].

The firmness changes in peach fruit have been related to depolymerization and solubilization of pectins, which is carried out by pectolytic enzymes [58,59]. An enzyme associated with pectin depolymerization is polygalacturonase, which catalyzes the hydrolytic cleavage of $\alpha\ (1 \rightarrow 4)$ galacturonan linkages from demethyl esterified homogalacturonan chains [60,61].

Several studies in *P. persica* have reported a lower activity and protein accumulation of endo-polygalacturonase in mealy fruits compared to juicy ones [62,15,33]. In this work, the results corroborate previous studies, suggesting that this protein is of great importance for correct cell wall dismantling.

Another very interesting point is that the polygalacturonase protein (s285) identified is encoded by a gene (Prupe.4G262200.1) that is located inside a mealiness QTL (qML4.1) previously mapped in *P. persica* [19]. In that study, it was found a QTL in linkage group 4 associated to mealiness. The most significant SNP markers near to qML4.1 are located between 27,275,348 and 27,336,281 bp on scaffold Pp04. The polygalacturonase gene identified in this work is located very close to these SNPs between 22,684,500 and 22,687,159 bp on Pp04 (corresponding to LG4).

4.3. Transmembrane transport

The regulation of mitochondrial physiology needs an efficient metabolite exchange system between mitochondria and cytosol through the mitochondrial outer membrane. In this function, the voltage dependent anion channel (VDAC) is quite important in the regulation of energetic and metabolic functions [63]. It has been reported that VDAC is involved in Ca^{2+} transport across the mitochondrial outer membrane and that Ca^{2+} controls its permeability and VDAC [64]. We found this protein in three spots (s433, s436 and s461, Table 2) showing increased accumulation in the mesocarp of juicy fruits when compared to mealy fruits. This differential accumulation could affect, the metabolic and energetic balance associated with mitochondria in mealy fruits.

4.4. Changes related to oxidation-reduction processes

Malate dehydrogenase (s402), an enzyme that catalyzes the conversion of malate to pyruvate and NADPH [65] accumulated more abundantly in the fruit mesocarp of mealy fruit segregants when compared to juicy fruit segregants. This enzyme is involved in cellular oxidation–reduction processes.

Most of the leaf proteins differentially accumulated between juicy fruit segregants and mealy fruit segregants are related to energy metabolism. A protein related to photochemical reactions of photosynthesis as oxygen-evolving enhancer (OEE) protein 1 was identified in two different spots (s32, s48). In higher plants OEEs are involved in the splitting of water in photosystem II and are present in different isoforms [66]. It is believed that these isoforms help plants to adjust photosynthesis in response to different stimulus [66].The differential accumulation of these and other proteins in leaves from contrasting segregants indicates that the photosynthetic rate, oxidative respiration and consequently carbon exportation and partitioning in leaves are different between the segregants that produce juicy fruits and those that produce mealy fruits [67]. This variation in the leaves may lead to differential metabolite composition in the fruits and increased/decreased susceptibility to chilling injury [68].

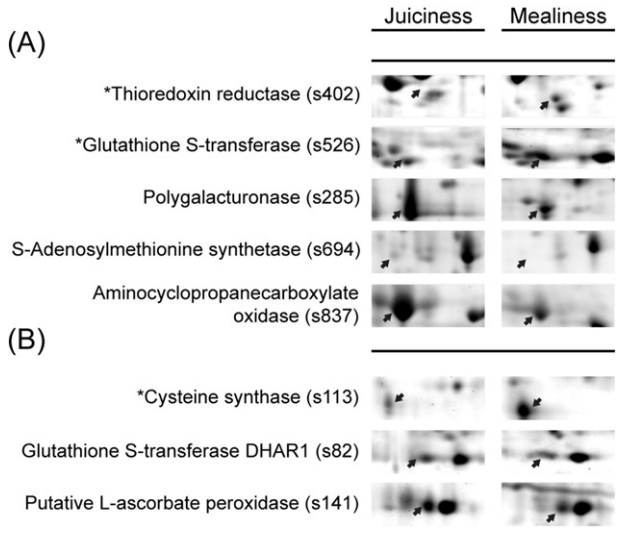

Fig. 6. Accumulation pattern of differentially abundant proteins in juicy and mealy fruit segregants. Total protein extracts of fruit mesocarp (A) and leaf (B). Proteins marked with asterisk are more accumulated in mealy segregants. Images are sections of 2D gels.

4.5. Changes in proteins related to other functions

Other enzyme that presented higher accumulation levels in mealy fruits was UTP-glucose 1-phosphate uridylyltransferase (s232). This protein also known as UDP-glucose pyrophosphorylase (UGlcPP) is a key enzyme in carbohydrate metabolism. UGlcPP catalyzes the reversible conversion of glucose 1-phosphate and UTP- to UDP-glucose and pyrophosphate [69]. UDP-glucose is the precursor of several nucleotide sugars, which are associated to glycan synthesis [70]. That is why a disorder in precursors for cell wall synthesis could affect the correct cell wall composition and structure, which directly impact the fruit texture and could be associated with mealiness.

β-oxidation of fatty acids is catalyzed by three proteins, acyl-CoA oxidase, a multifunctional protein and the 3-ketoacyl-CoA thiolase (KAT) [71]. This enzyme catalyzes the formation of propionyl-CoA by removal of acetyl-CoA from 2-methylacetoacetyl-CoA [72]. Propionyl CoA is converted via methylmalonyl-CoA to succinyl-CoA by the actions of propionyl-CoA carboxylase methylmalonyl-CoA racemase and methylmalonyl-CoA mutase [73], the resulting succinyl-CoA is part of the tricarboxylic acids. We found two KAT proteins in two distinct spots (s269 and s271, Table 2), both accumulated preferentially in mealy fruit segregants.

5. Conclusions

In this work we have shown that there is differential protein accumulation in the fruits and leaves of a F2 population that is segregating for chilling injury susceptibility. Our results indicate that this differential susceptibility involves the crosstalk of at least two hormones, abscisic acid and ethylene, that trigger a cold response within the fruit. Moreover, the susceptibility or resistance to fruit chilling injury may be established during the preharvest conditions due to the genetic contribution to the trait. Some of the proteins with differential abundance identified in this work, have previously been correlated with susceptibility or tolerance to chilling injury and co-localize with QTL which confirms their potential as putative markers for the chilling injury susceptibility/tolerance.

Abbreviations

ABA/WDS	abscisic acid/water deficit stress
ACC	1-aminocyclopropane 1-caboxylic acid
Acm	Selection accuracy
ACO	1-aminocyclopropane 1-caboxylate oxidase
ASR	abscisic acid stress ripening
BLUP	best linear unbiased prediction
fp	permanent phenotypic effect
KAT	3-ketoacyl-CoA thiolase
kDa	kilo Dalton
K-W	Kruskal–Wallis
LDOX	Leucoanthocyanidin dioxygenase
m	General average
OEE	Oxygen evolving enhancer
PC	Principal component
PCA	Principal component analysis
POD	Peroxidase
PPO	Polyphenol oxidase

PTM	Post-translational modification
QTL	Quantitative trait loci
r	Repeatability of the average of m harvests or m repeated measurements
REML	Restricted maximum likelihood
SAM	S-adenosylmethionine
SAMS	S-adenosylmethionine synthetase
SNP	Single nucleotide polymorphism
TCEP	Tris(2-carboxyethyl)phosphine
u + fp	Permanent phenotypic value
UDP	Uridine diphosphate
UGlcPP	UDP-glucose pyrophosphorylase
UPLC	Ultra performance liquid chromatography
UTP	Uridine triphosphate
VDAC	Voltage dependent anion channel
Vet	Environmental variance
Vf	Individual phenotypic variance
Vfp	Phenotypic variance
VS	Venus segregant

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.10.011.

Conflict of interest

The authors declare no conflict of interest.

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