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Alterations in protein expression associated with the development of mealiness in peaches

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SUMMARY
Two-dimensional electrophoresis (2-DE) combined with mass spectrometry was used to identify proteins related to the development of mealiness in peaches. Five proteins were identified that differed significantly in amount between peaches that had mealy flesh and those that remained juicy following 3 weeks of storage at 5°C and subsequent ripening for 3 – 4 d at 23°C. The accumulation of ACC oxidase (ACO), a key enzyme of ethylene biosynthesis, decreased in mealy peaches. Immunoblots probed with antibodies against ACO confirmed the 2-DE results, and showed that the relationship between ACO levels and mealiness also existed within individual fruit. Phosphoglycerate kinase, an enzyme in the glycolytic pathway, was reduced in amount in mealy fruit. In contrast, two heat shock proteins and hydroxymethylbutenyl-4-diphosphate synthase increased in amounts in mealy peaches. The results regarding ACO levels are of interest due to the confirmed association of mealiness and ethylene and, given the regulatory effect of ethylene on cell wall degrading enzymes, suggest that the reduction in ACO expression during cold storage and subsequent ripening may be pivotal in the development of mealiness.

Mealiness is a disorder of stone fruit that occurs during cold storage, or following subsequent ripening, that causes the flesh to lose juiciness and take on a mealy or woolly texture. The development of mealiness is often associated with flesh browning and reddening, gel formation, and poor flavour. This disorder is one of the most important that limit the storability of stone fruit, due to its relatively rapid onset and common occurrence. Since the fruit can appear, externally, to be of good quality, and the onset of symptoms occurs during ripening, consumers often encounter mealy fruit in the marketplace. Consumer research with fresh peaches has shown that mealiness is one of the primary factors causing consumer dissatisfaction (Bruhn *et al*., 1991). Although stone fruit cultivars vary in susceptibility (Crisosto *et al*., 1999), mealiness will generally develop in most cultivars following 3 or more weeks at 0ºC, or as early as 1 – 2 weeks if fruit are stored at 2º – 5ºC (Crisosto *et al*., 1999; Lill *et al*., 1989). Various methods to inhibit the development of mealiness in stone fruit have been explored, including the use of controlled atmospheres (Zhou *et al*., 2000b), the addition of ethylene (Palou *et al*., 2003), intermittent warming (Buescher and Furmanski, 1978), and delayed storage (Crisosto *et al*., 2004). Commercially, delayed storage has been the most successful and is used extensively to extend the market-life of Californian- and Chilean-grown peaches, based upon a protocol developed by Crisosto *et al*. (2004).

Although treatments have been developed to slow down the onset and progression of mealiness in stone fruit, the ultimate goal is to understand this disorder at the biochemical level and, using this information, to develop cultivars that will remain juicy even after long periods of cold storage. The current state of knowledge regarding the biochemical basis of mealiness in peaches and nectarines has been reviewed recently (Lurie and Crisosto, 2005). In brief, mealiness in stone fruit develops due to an accumulation of soluble, high molecular weight pectic compounds, with a low degree of methyl esterification, that are capable of sequestering free water in the fruit by forming gels (Ben-Arie and Lavee, 1971). Numerous studies (e.g., Brummell *et al*., 2004; Buescher and Furmanski, 1978) have compared the extractable cell wall pectins from juicy and mealy fruit and have presented data supporting this hypothesis.

Research has focussed on cold-induced alterations in the activities of polygalacturonase (PG) and pectin methylesterase (PME), two enzymes believed to be key in the depolymerisation of pectin in stone fruit (Lill *et al*., 1989), as the primary cause for the differing pectins found in mealy fruit. An imbalance in the activities of these two enzymes, where pectin depolymerisation but not de-esterification is inhibited, could lead to the formation of gel-forming pectins. Accordingly, reductions in PG activity have been found to occur as a result of cold storage (Buescher and Furmanski, 1978), while results concerning cold storage and PME have been inconsistent (Ben-Arie and Sonego, 1980). Obenland *et al*. (2000) observed that the development of mealiness can also be enhanced by heat treatment, but that it was possible to induce mealiness without a corresponding decrease in PG. Other enzymes have also been investigated for their potential links to the development of mealiness. Endo-1,4-β-glucanase, endo-1,4-β-mannanase, β-galactosidase, and α-arabinosidase were all lower in mealy compared to

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juicy fruit (Brummell *et al*., 2004). Expansin, a cell wall protein that does not have enzymatic activity, decreased in amount as fruit became mealy, and may have a role in controlling the interaction between PG and pectin (Obenland *et al*., 2003).

While much has been learned regarding the potential mechanism by which mealiness develops, the large number of metabolic changes that have been found to occur when fruit become mealy make it difficult to understand the entire picture by extracting and assaying a small number of enzymes, in separate experiments, as has been done previously. Since it is known that a decline in ethylene production accompanies the development of mealiness (Zhou *et al*., 2000a), it is clear that many proteins related to ethylene synthesis and the ripening process are being altered in amount and activity compared to fruit that ripen normally. In addition to the large number of proteins whose synthesis is influenced by ethylene, it is also possible that there may be other proteins involved which have not yet been investigated.

The goal of the present work was to gain a broader picture of the changes in protein expression using two-dimensional electrophoresis (2-DE) and mass spectrometry, to perform a proteomic analysis of the development of mealiness in peach tissues. A previous report (Uthairatanakij *et al*., 2005) used 2-DE to investigate chilling injury in peaches, but studied the response of different cultivars to ethylene and carbon dioxide during cold storage, rather than directly studying chilling injury. Also, these authors did not make any attempt to determine the identity of any of the proteins that responded to the treatments they imposed. In this study, we have identified five proteins that differ in amount between mealy and juicy peaches that had been in cold storage for 3 weeks, and compared their levels to those in peaches sampled at the start of storage.

MATERIALS AND METHODS

Fruit samples

Peaches (*Prunus persica* cv. O'Henry) of commercial-harvest maturity were acquired from a local packinghouse, and 80 fruit were placed into storage at 5ºC, a temperature conducive to the development of mealiness (Crisosto *et al*., 1999). Twenty fruit were removed from storage each week, beginning with a pre-storage (week-0) sample, and placed at 23ºC. The fruit were checked daily for firmness using a penetrometer (University of California Firmness Tester; Western Industrial Supply Co., San Francisco, CA, USA) and allowed to ripen until they reached a firmness value of 13.3 N, or less. Percent free juice, a quantitative determination of mealiness, was measured for ten individual fruit each week using the procedure first described by Crisosto and Labavich (2002), and in detail by Obenland *et al*. (2003). This was continued until week-3 when a large proportion of the fruit were mealy. The portion of each fruit not used for free juice measurement was peeled and the mesocarp tissue frozen immediately in liquid nitrogen and stored at –80ºC.

In a separate storage experiment, peaches ('O'Henry') of commercially-harvested maturity were acquired from a local packinghouse and 200 fruit were placed into storage at 5ºC. After 2 and 3 weeks of storage, 100 fruit were removed from cold storage, placed at 23ºC, and ripened to a firmness of 13.4 N, or less. After ripening all 100 fruit per storage period were sliced transversely, split in half, and visually examined for sharply-defined areas of mealiness within individual fruit. A small number (*ca*. 5%) of fruit had easily separated regions within the fruit that were mealy, along with regions that were very juicy. These regions (both juicy and mealy) were excised, placed into separate vials labelled by fruit number, stored at –80ºC, and later used for western blot analysis.

Protein extraction and 2-DE analysis

Frozen tissue samples were finely ground in liquid nitrogen in a cold mortar and pestle and processed by a modification of the method of Wang *et al*. (2006b). Following addition of 13 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) in 100% (v/v) acetone per g of tissue, the mixture was mixed thoroughly, centrifuged at 4ºC for 3 min at 8,000 × *g*, and the supernatant discarded. The pellet was re-suspended in 13 ml ice-cold 100% acetone and centrifuged as above, again discarding the supernatant. The pellet was then washed in 13 ml ice-cold 0.1 M ammonium acetate in 80% (v/v) methanol, following the same procedure. The resultant pellet was incubated for 1 h at 50ºC to evaporate the methanol. Proteins were then extracted from the pellet by adding 2 ml Tris-saturated phenol (pH 8.0) plus 2 ml extraction buffer [0.5 M Tris-base, pH 8.8, 1% (v/v) 2-mercaptoethanol, 1% (w/v) SDS, 30% (w/v) sucrose]. This mixture was vortexed, incubated at room temperature for 5 min, and centrifuged at 8,000 × *g* for 3 min at room temperature. The phenol phase was transferred to a new tube, and five volumes of cold 0.1 M ammonium acetate in 100% (v/v) methanol was added. This was incubated at –20ºC for 10 min and centrifuged at 8,000 × *g* at 4ºC for 5 min to precipitate proteins. After discarding the supernatant, the pellet was washed once with 2 ml cold 100% (v/v) methanol by completely re-suspending the pellet and centrifuging at 10,000 × *g* at 4ºC for 3 min. The pellet was then washed once with ice-cold 80% (v/v) acetone and the final protein pellet air-dried for 30 min and stored dry, or under 80% (v/v) acetone at –20ºC. Extracted proteins were suspended in buffer containing 7 M urea, 2 M thiourea, 65 mM dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), and 0.4% (v/v) immobilised pH gradient (IPG) buffer (GE Heathcare, Piscataway, NJ, USA) and assayed using a modified Bradford protein assay from Bio-Rad (Hercules, CA, USA) with BSA as the standard (Bradford, 1976).

Immobilised pH gradient strips (18 cm, pH 4 – 7; GE Healthcare) were each loaded with 100 – 200 μg protein and rehydrated under oil overnight at room temperature. The strips were then run overnight using Bio-Rad's IEF Cell for a total of 125,000 Vh, with cooling set to 20ºC. Previous experimentation had established that this duration of iso-electric focussing provided optimum separation. Second dimension gels (20 cm × 20 cm) consisted of 10% (w/v) acrylamide [with 2.6% (w/v) 1,4-bis(acryloyl) piperazine (PDA) as crosslinker], 12.5% (w/v) Tris-base buffer, pH 8.8, 20% (w/v) sucrose, 0.05% (w/v) ammonium persulphate, and 0.05% (v/v) tetramethyl ethylene diamine (TEMED). The gel

solution was degassed for 15 min and the cast gels were allowed to polymerise overnight.

After focussing, strips were equilibrated (Barraclough *et al*., 2004) and sealed on top of the polyacrylamide gels with 1.0% (w/v) agarose. Gels received either 5 µl of 1:20-diluted broad-range SDS-PAGE markers (Bio-Rad) on filter paper sealed with agarose, or one-half of a Precision Plus Protein Standard Plug (Bio-Rad). Electrophoresis in the second dimension was performed using a Dodeca Plus unit (BioRad) under the following conditions: 200 V for 6 h, 100 V for 16 h, and 200 V for 5 h, with the circulating cooler set at 17º – 17.5ºC. Following electrophoresis, gels were fixed for 30 min in 50% (v/v) methanol, 10% (v/v) acetic acid and stained overnight in Sypro Ruby (Bio-Rad) diluted 1:5 in ultrapure water in the dark (Krieg *et al*., 2003). Three extractions from individual fruit were performed for each type of tissue (i.e., pre-storage, juicy, or mealy). Six replicate gels were run for each extraction.

Gel-imaging analysis

Prior to imaging, gels were rinsed for 1 h in deionised water to reduce the background stain. Gels were imaged with a Molecular Imager FX Pro Plus (Bio-Rad) using the high intensity setting for Sypro Ruby. Gels were analysed using PDQuest Version 8.0 software (Bio-Rad), which normalised gels according to a regression model. Spots that had quality factors of ≤ 30 were excluded; quality factors being based upon the degree of streaking, spot overlap, and whether or not the spot intensity was within the linear range of the scanner. Spot volume was calculated using the following formula:

$$\text{Spot height} \times \pi\delta_x\delta_y$$

Spot height was the peak of the Gaussian distribution of the spot in optical density units, and δ_x, δ_y were the standard deviations of the spot intensity in the *x*- and *y*-axis directions. Spots were selected for further analysis if there was at least a two-fold difference in mean spot volume between tissue types. Analysis of variance was conducted using SPSS software (Chicago, IL, USA) to determine if the mean spot volume values for each tissue type were statistically different ($P \leq 0.05$) from each other.

Protein identification

Gel spots of interest were placed into 96-well reaction plates for subsequent in-gel tryptic digestion with an automated protein digester (DigestPro; Intavis, Langenfeld, Germany). The DigestPro was programmed to destain the gel piece and carry out reduction with DTT, alkylation with iodoacetamide, enzymatic digestion with trypsin, and elution of the peptides thus generated into a 96-well plate that was subsequently inserted into the autosampler of a mass spectrometer.

Samples were analysed with a QSTAR PULSAR i quadrupole time-of-flight (TOF) mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) as previously described (Vensel *et al*., 2005). Briefly, 20 µl samples from the 96-well sample-containing plate were loaded into a loop and pumped at a flow rate of 20 µl min^{-1} onto a 5 µm, C-18, 300 Å, Nano-Precolumn (300 µm i.d. × 1 mm; LC Packings/Dionex, Sunnyvale, CA, USA). After 4 min, the loading valve was switched, in order to place the trap in-line with the LC pump (Ultimate; LC Packings/Dionex). Samples were eluted from the trap onto the C-18 monomeric, Vydac Everest™ column (W.R. Grace & Co.; Deerfield, IL, USA) at a rate of 200 – 250 nl min^{-1}. The spray tip was an 8 µm i.d. PicoTip emitter (New Objective; Woburn, MA, USA). Spray voltage was maintained at 1.8 kV. The mobile phase A was 0.5% (v/v) glacial acetic acid (Fisher Scientific; Pittsburg, PA, USA) in HPLC-grade water (Burdick and Jackson, Muskegon, MI, USA). Analytical column elution solvents were: A [0.5% (v/v) acetic acid] and B [80% (v/v) acetonitrile with 0.5% (v/v) acetic acid]. Samples were eluted with the following gradient profile: 8% (v/v) B at 0 min, to 80% (v/v) B by 12 – 13 min, to 8% (v/v) B by 14 min, continuing at 8% (v/v) B to 28 min.

Data were acquired using the Analyst QS Version 1.1 software (Applied Biosystems). From an initial survey scan of mass range *m*/*z* 400 – 2,000, the most abundant doubly or triply charged ion above a threshold of 20 counts was selected for fragmentation. The quadrupole mass filter (Q1) was adjusted to allow ions of the selected *m*/*z* to enter the collision cell. Collision-induced disassociation of the mass-selected ion in the collision cell was carried out using ultra-high purity nitrogen as the collision gas. Analysis of the resulting fragment ions by the TOF mass analyser was set to a range of *m*/*z* 70 – 2,000. The selected precursor ion was precluded from further MS/MS experiments. An AnalystQS script was used to determine the optimum collision energy for each precursor ion, automatically. Following the 3 s MS/MS fragmentation period, the MS survey scan was repeated until another MS/MS period was triggered. The wiff (proprietary binary format) files created by the QSTAR AnalystQS software for each sample were processed using a locally installed copy of Mascot Daemon (http://www.matrixscience.com/) and the resulting MS/MS data were analysed using Mascot Version 2.1.04 (Matrix Science, London, UK). Mascot was set to search a local version of a National Center for Biotechnology Information (NCBI) non-redundant green plant database (1 March 2006) that contained 188,169 protein sequence entries. Trypsin was selected as the cleavage enzyme. The database was created by parsing the NCBI non-redundant database (10 October 2006) into taxonomic groupings using the Sequence Database Management Wizard (Genomic Solutions, Ann Arbor, MI, USA). The local version of the green plant database contained non-redundant-*Arabidopsis-thaliana*.fasta, non-redundant-Other-*Viridiplantae*.fasta, and non-redundant-*Oryza-sativa*.fasta, as well as protein sequences contained in the common Repository of Adventitious Proteins (4 June 2006) available from the Global Proteome Machine Organization (ftp://ftp.thegpm.org/fasta/cRAP). The post-analysis software package Scaffold (Proteome Software Inc., Portland, OR, USA) was used to analyse both Mascot and X! Tandem search results (Craig and Beavis, 2004). The results were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.00 Da. The iodoacetamide derivative of cysteine was specified in Mascot and X! Tandem (www.thegpm.org; version 2006.04.01.2) as a fixed modification. The methionine oxidation was specified in Mascot as a

variable modification. In X! Tandem, oxidation of methionine and the iodoacetamide derivative of cysteine were specified as variable modifications.

Criteria for protein identification

Scaffold (Version 01.05) was used to validate the combined Mascot and X! Tandem MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al*., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al*., 2003). Proteins that contained similar peptides, and could not be differentiated based on MS/MS analysis alone, were grouped to satisfy the principles of parsimony.

ACO immunoblots

Frozen tissue samples were finely ground in liquid nitrogen in a cold mortar and pestle. Powdered peach tissue samples (0.5 g) were homogenised in 1 ml grinding buffer (40 mM sodium phosphate, pH 7.0, 3 mM EDTA, 0.5 mM DTT) and centrifuged at 12,000 × *g* for 10 min. The pellets were suspended in 0.75 ml SDS loading buffer [62.5 mM Tris-base pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.5 mM DTT], boiled for 10 min, mixed, then boiled again. Following centrifugation as above, the pellets were discarded and the supernatant was assayed for protein using the DC assay (Bio-Rad). Samples and biotinylated markers (Bio-Rad low-range) were prepared for SDS-PAGE by adding 1 µl β-mercaptoethanol and 1 – 2 µl running dye [0.1% (w/v) bromphenol blue, 50% (w/v) sucrose] to each. Samples (80 µg per lane) were run into 10% (w/v) acrylamide gels at 125 V and 98 mA for 95 min. Gels and nitrocellulose membranes were equilibrated in transfer buffer [25 mM Tris-base, 192 mM glycine, 20% (v/v) methanol] for 30 min and the proteins were transferred using a Semi-dry Transfer Cell (BioRad) for 35 min at 25 V and 100 mA per blot.

Blots were blocked for 1 h with 5% (w/v) non-fat dry milk in TBST buffer [50 mM Tris-base, 2 mM $MgCl_2$, 140 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5] or with 1% gelatin (w/v) in TBST buffer (for gels containing biotinylated markers). Membranes were then probed overnight with goat polyclonal ACO antibodies raised against an ACO peptide from *Arabidopsis* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 in 0.4% (w/v) non-fat dry milk in TBST buffer. Blots were washed six-times, for 5 min each, in 60 ml TBST buffer. Blots were incubated for 1 h with donkey anti-goat IgG-HRP (Santa Cruz Biotechnology,) at 1:100,000, or with avidin-HRP (Bio-Rad) at 1:50,000 in 1% (w/v) gelatin in TBST buffer for blots with markers. Blots were washed again, as above, and placed in Super Signal West Dura chemiluminescence substrate (Pierce, Rockford, IL, USA) for 5 min. Film was exposed for 1 – 30 s and processed with Kodak developer and fixer.

RESULTS

Stored peaches were analysed for free juice content in order to identify juicy and mealy peaches and to select a storage regimen for sample collection. All peaches remained juicy up to 2 weeks of storage at 5ºC. The average free juice contents were 43.9%, 48.3%, and 54.4% for weeks-0, -1 and -2, respectively. The increase in overall juiciness may have been due to softening of the fruit during storage and more complete pressing during determination of the free juice content. After 3 weeks, the average free juice decreased to 30.9%. The free juice content of individual fruit ranged from 49.3% (considered juicy) to 13.8% (considered mealy with a paste-like consistency). Fruit above 45%, and below 26% free juice content were selected from fruit stored for 3 weeks for 2-DE analysis. This enabled a comparison of fruit differing in juiciness, but stored for the same length of time. Three fruit from the pre-storage time point (week-0) were also analysed to provide an estimate of the effect of storage on protein expression.

The 2-DE imaging software detected at least 700 spots in gels of proteins extracted from peach fruit stored for 0 or 3 weeks. Matching gel patterns for proteins extracted from mealy and juicy tissue revealed that they were quantitatively similar for all samples. Figure 1A shows a representative 2-DE gel of proteins extracted from mealy mesocarp tissue. Proteins were well-resolved, and ranged in size from 17 – 150 kDa. When spot volumes were compared, five spots were found that were significantly different ($P \leq 0.05$) between mealy and juicy tissue (Figure 1A, B). The proteins in these spots were identified by LC-MS-MS. The key properties of these proteins are listed in Table I. They include protein name, theoretical and experimental MW, pI, peptide number, sequence coverage, and sequence information.

Spot 1 was identified as ACC oxidase (ACO), which catalyses the final step in the ethylene biosynthetic pathway. ACO was significantly less abundant in mealy tissue compared to both juicy and pre-storage tissues (Figure 2). Western blot analysis, using an *Arabidopsis* ACO antibody, identified a single immunoreactive protein band at 40 kDa (Figure 3), almost identical to the molecular mass estimated from 2-DE (Table I). The two mealy peach samples (14% and 26% juice) had markedly less immunoreactive protein than did the stored and pre-storage juicy samples (Figure 3A). In addition, regions within individual fruit that were mealy had less immunoreactive protein than regions of the same fruit which were juicy (Figure 3B). These results supported the 2-DE analysis, and indicated that fruit with a higher degree of mealiness had correspondingly less ACO protein.

Spot 2 was identified as a heat shock protein 70 (Hsp70), and spot 5 as a luminal binding protein (BiP), a member of the Hsp70 family (Table I). Both of these stress-related proteins behaved similarly in that they increased greatly in amount in response to 3 weeks of cold storage, and that the amount of protein present in mealy tissue was at least twice as much as in juicy tissue (Figure 2).

Spot 3, identified as hydroxymethybutenyl 4-diphosphate synthase, is a protein involved in carotenoid biosynthesis. This protein was nearly twice as abundant in mealy compared to juicy tissue, while the amounts in pre-storage and juicy (stored) tissues were the same (Figure 2). Spot 4, which was identified as phosphoglycerate kinase (PGK), a key enzyme in glycolysis, was reduced in

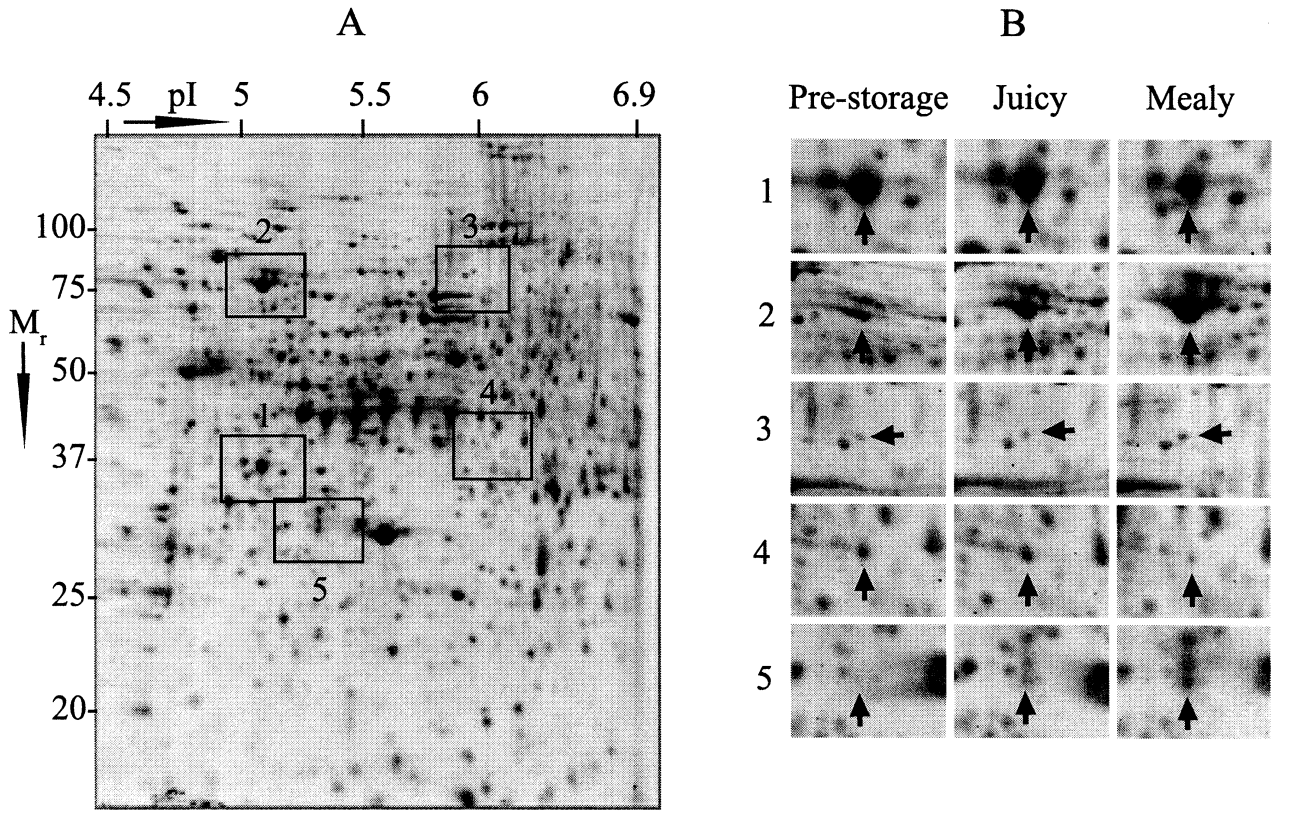

FIG. 1
Representative 2-DE gel of peach fruit proteins showing the locations of the five proteins identified (Table I) that showed differential expression in comparisons of juicy and mealy mesocarp tissues (Panel A). Enlargements of the corresponding areas in additional gels from pre-storage, juicy and mealy peach tissues (Panel B). Numbers to the left of the enlargements indicate the arrowed spots.

amount by ≥ 2.5-fold in mealy compared to juicy tissue (Figure 2). The amount of this protein was the same in pre-storage and juicy (stored) peaches.

DISCUSSION

Initial attempts at performing 2-DE on peach fruit using standard published extraction methods for plant tissues proved unsatisfactory due to excessive streaking and poor resolution in the first dimension. The methods tried included the technique most commonly used for difficult-to-extract plant tissues in which the proteins are first solubilised in phenol, followed by precipitation with methanol and ammonium acetate (Hurkman and Tanaka, 1986). Abdi *et al.* (2002) reported success in 2-DE separations of peach fruit tissue using a phenolic extraction technique, but this method also did not give good results in our hands. In this case, one reason may have been that the authors used tube gels for the first dimension step, while we were using IPG strips in order to improve the reproducibility of the separations. Especially problematic, using the standard extraction methods in our study, was achieving the same degree of focus in gels for both juicy and mealy tissue types. Successful 2-DE separations were finally achieved using a method for recalcitrant plant tissues published by Wang *et al.* (2006b) that extracts tissue sequentially with TCA and acetone, followed by phenol. When proteins were extracted from peach fruit expressing or not expressing the mealiness disorder, using this method, and separated by 2-DE, protein spots were well-resolved for both tissue types.

The finding that ACO accumulation was reduced in mealy *vs.* juicy tissues is significant, in that ethylene production has been closely linked to the development of mealiness (Zhou *et al.*, 2000a). Numerous studies have shown that either the addition of ethylene to the storage environment (Dong *et al.*, 2001; Zhou *et al.*, 2000a), or

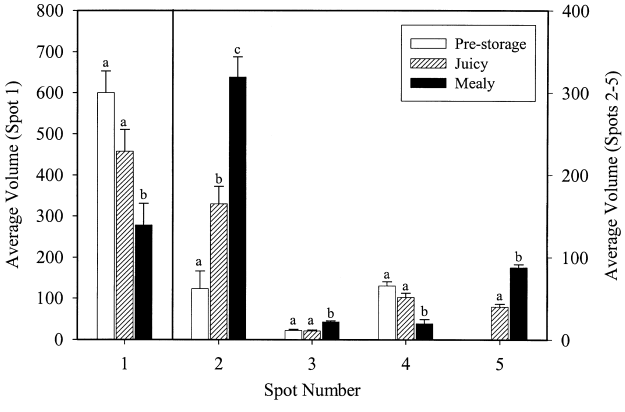

FIG. 2
Average spot volumes of the five proteins (1–5; Figure 1B) expressed differentially in juicy and mealy peach tissues from samples taken prior to cold storage (pre-storage), and following storage for 3 weeks at 1ºC (juicy and mealy). Spot volume is expressed in units of OD × $(IU)^2$, where OD is the peak spot height in optical density units, and IU (image unit) is the product of the standard deviations of the Gaussian distributions of the spot in the *y*- and *x*-axis directions and π (see text). All peaches were removed from cold storage and ripened at 23ºC to a firmness of 13.3 N, or less, prior to sampling. Bars indicate SE, and bars with a different lower-case letter are significantly different for each spot ($P \leq 0.05$).

TABLE I
Identification of proteins differing in expression between juicy and mealy peaches

Spot no.	NCBI Accession number/ Protein Name/ Organism	Theoretical MW (kDa)/pI	Experimental MW (kDa)/pI	No. unique peptides/ sequence coverage (%)	Calculated peptide mass (AMU)[X]	Spectrum charge	Deviation from expected (AMU)	Unique sequence[Y] with flanking amino acids
1	CAA54449/ ACC oxidase (ACO)/ *Prunus persica*	36/5.2	39/5.0	16/59	1360.65	2	0.06	(K)AFYGTNGPTFGTK(V)
					1713.88	3	0.06	(R)AHTDAGGLILLFQDDK(V)
					2552.41	3	0.13	(R)AHTDAGGLILLFQDDKVSGLQLLK(D)
					3068.41	3	0.18	(K)DACENWGFFELVSHGIPTEFLDTVER(L)
					921.54	2	0.03	(R)FKELVASK(G)
					1950.07	3	0.08	(R)HSIVINLGDQLEVITNGK(Y)
					3309.59	4	0.13	(K)IKDACENWGFFELVSHGIPTEFLDTVER(L)
					1488.75	3	0.07	(K)KAFYGTNGPTFGTK(V)
					2432.27	3	0.13	(K)LAEQLLDLLCENLGLEQGYLK(K)
					2560.37	3	0.14	(K)LAEQLLDLLCENLGLEQGYLKK(A)
					2570.29	3	0.13	(R)MSIASFYNPGSDAVIYPAPTLVEK(E)
					1664.78	2	0.04	(K)SNISEVPDLEDQYR(N)
					1905.83	2	0.08	(K)TEVNDMDWESTFYLR(H)
					960.51	2	0.04	(R)VIAQTDGTR(M)
					857.55	2	0.04	(K)VSGLQLLK(D)
					1627.82	2	0.05	(K)VSNYPPCPNPELIK(G)
2	AAB99745/ heat shock protein 70/ *Triticum aestivum*	71/5.1	77/5.0	16(37)	1540.76	3	0.00	(R)ARFEELNMDLFR(K)
					1675.73	3	0.01	(K)ATAGDTHLGGEDFDNR(M)
					2658.27	3	0.03	(K)EQVFSTYSDNQPGVLIQVYEGER(A)
					1313.62	2	0.00	(R)FEELNMDLFR(K)
					1240.55	2	–0.01	(R)FSDPSVQSDMK(L)
					1927.93	3	0.01	(R)FSDPSVQSDMKLWPFK(V)
					2588.22	3	0.04	(K)GEGPAIGIDLGTTYSCVGVWQHDR(V)
					1787.99	3	–0.01	(R)IINEPTAAAIAYGLDKK(A)
					1017.57	2	0.01	(K)ITITNDKGR(L)
					1358.62	2	–0.02	(K)NALENYAYNMR(N)
					1680.83	2	0.02	(K)NAVVTVPAYFNDSQR(Q)
					2925.55	3	0.03	(K)NVLIFDLGGGTFDVSLLTIEEGIFEVK(A)
					1565.82	2	–0.01	(K)QFAAEEISSMVLIK(M)
					3025.49	3	0.04	(R)TLSSTAQTTIEIDSLYEGVDFYTTITR(A)
					1487.70	2	–0.03	(R)TTPSYVAFTDTER(L)
					1436.75	2	0.01	(K)VQQLLQDFFNGK(E)
3	AAO15447/ GcpE/ *Lycopersicon esculentum*	82/5.8	81/5.9	7(13)	1811.96	3	0.05	(R)AIEMEHATDALIQLIK(D)
					1215.70	2	0.03	(K)DLATVDSILLR(E)
					1259.57	2	0.02	(R)GMVESAFEFAR(I)
					1204.53	2	0.02	(R)IMSYYGDSPR(G)
					1309.63	2	0.03	(R)NTSFNLLQGCR(M)
					2110.14	3	0.04	(K)NYNIPLVADIHFAPSVALR(V)
					1799.99	3	0.04	(K)SAIGIGTLLQDGLGDTIR(V)
4	CAA88840/ phosphoglycerate kinase/ *Nicotiana tabacum*	42/5.5	43/6.0	2(5)	970.50	2	0.05	(R)AHASTEGVAK(Y)
					978.53	2	0.03	(K)FAAGTEAIAK(K)
5	P49118/ luminal binding protein/ *Lycopersicon esculentum*	73/5.1	34/5.3	2(6)	2384.29	3	–0.04	(K)DILLLDVAPLTLGIETVGGVMTK(F)
					2116.11	3	0.02	(K)LGTVIGIDLGTTYSCVGVYK(N)

[X]Mono-isotopic mass of the pseudo-molecular ion (MH^+).
[Y]Sequences of the matched peptides.

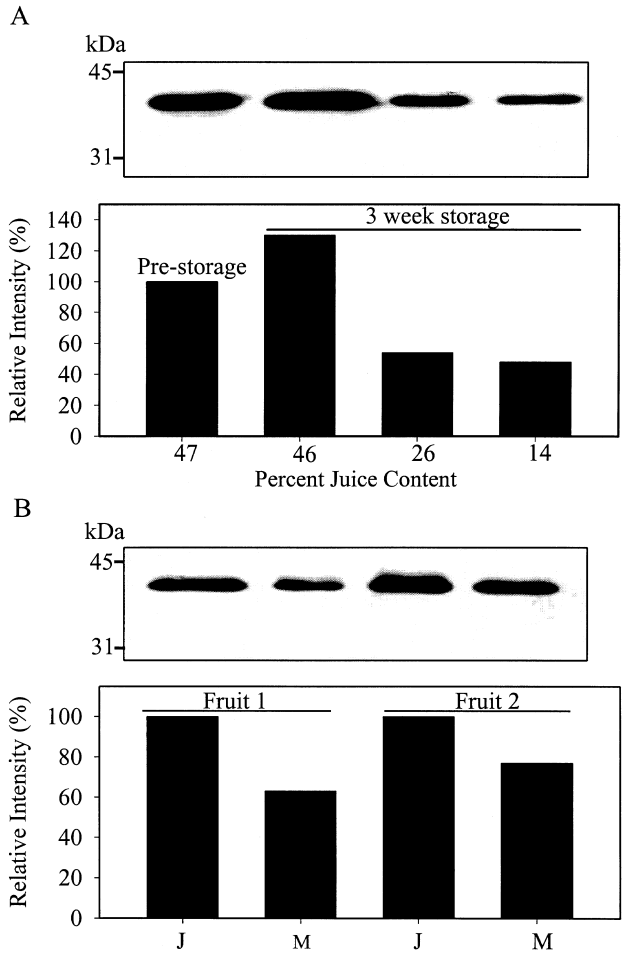

FIG. 3
Western blot analysis of ACO expression in peach mesocarp tissue with differing degrees of mealiness. Panel A, comparisons among fruit sampled prior to, and following storage at 1ºC for 3 weeks that had differing percentages of free juice content (amounts of mealiness). Each sample represents an individual fruit. Panel B, comparisons of regions within individual fruit that were visibly juicy or mealy. Immunodetection used a goat polyclonal anti-ACO antibody derived from an *Arabidopsis* ACO peptide. Molecular masses are listed to the left of the blots (in kDa).

using delayed storage, or intermittent warming to enhance the natural production of ethylene during and following storage (Crisosto *et al*., 2004; Zhou *et al*., 2000b), delay the onset of mealiness. Treatment of fruit with the ethylene inhibitor, 1-methylcyclopropene (1-MCP), on the other hand, caused mealiness to be enhanced (Dong *et al*., 2001). Changes in ethylene production, or inhibition of its action, precede the onset of mealiness and support the view that ethylene has a direct role in mediating the development of the disorder. This is not surprising, as cell wall hydrolytic activity, which has long been linked to mealiness (Lill *et al*., 1989), is induced by ethylene (Bonghi *et al*., 1998; Sitrit and Bennett, 1998).

Mealiness in peaches and nectarines is believed to be a chilling injury disorder (Lurie and Crisosto, 2005). It is known that the ethylene biosynthetic step catalysed by ACO is sensitive to low temperatures (Wang, 1982). Accordingly, ACO activity was shown to be lower in chilling-injured fruit that became mealy, compared to fruit that remained juicy (Zhou *et al*., 2000a). Zhou *et al*. (2001) found that, in chilling-injured peaches, ACO gene transcripts increased upon transfer of fruit to a warmer temperature, while ACO activity and ethylene production did not recover, indicating that the effect of chilling on this enzyme occurred after transcription. Our results suggest that the loss in ACO activity that occurs in mealy peaches is due to a decline in the amount of enzyme, although changes in ACO activation cannot be ruled-out. Two other spots identified as ACO were found on our 2-DE gels at a similar pI and MW as spot 1; but these were not reported in this study as the two spots were not present in some of the replicate gels. It is not known whether these spots represented other members of the multi-gene ACO family that is known to occur in peach (Ruperti *et al*., 2001), or whether they may be expressed differentially in mealy tissue.

In a number of different plant species it has been shown that the synthesis of Hsp70 proteins is induced by low temperatures (Neven *et al*., 1992; Wu *et al*., 1993) and is related to enhanced resistance to low temperature damage (Sabehat *et al*., 1996). Since the peaches in this study were stored for 3 weeks at low temperatures (5ºC), increased production of Hsp70 and BiP in both juicy and mealy peaches is probably not surprising.

Ramakrishna *et al*. (2003) reported that expression of the heat-shock protein gene *vis 1* was negatively associated with juice viscosity and pectin depolymerisation in tomatoes. Although it is not known if *vis 1* is present in stone fruit, this research provides a potential link between heat-shock protein expression and characteristic alterations in pectin metabolism that occur during the development of mealiness in stone fruit (Ben-Arie and Lavee, 1971). In a recent report, it was shown that treatment of peaches with salicylic acid was associated with a reduction in the incidence of chilling injury, alterations in the anti-oxidant system, and induction of expression of Hsp101 and Hsp73 (Wang *et al*., 2006a). The authors speculated that the induction of heat-shock proteins may play a role in the reduction of chilling injury. This idea is not supported by our research, which found higher levels of heat-shock proteins in fruit which developed chilling injury and became mealy, than in fruit which remained juicy. It is possible that those fruit that became mealy simply had a greater stress response as a consequence of the development of the disorder, leading to an enhanced build-up of heat-shock proteins that have no real consequence in lessening the disorder. It cannot be ruled out, however, that there are heat-shock proteins other than those we detected, which do have an impact on the prevention of the disorder.

The other two proteins that differed in amount between juicy and mealy tissues, GCPE and PGK, do not have an obvious linkage to the development of the disorder. Jung *et al*. (2003) found that *PGK* gene expression was strongly induced by cold in *Arabidopsis* leaves, and suggested that enhanced energy production *via* glycolysis played a role in surviving cold stress. PGK has also been shown to be induced by salt and nitrogen-starvation in plants (Umeda *et al*., 1994), again suggesting a role in adaptation to stress. In this study, the abundance of PGK was greatly reduced in mealy peach tissue; but it is unclear if this reduction had any impact on the development of the disorder.

In conclusion, this is the first study that we are aware of that has used 2-DE to obtain an overall perspective of

the changes that occur in protein expression during the development of mealiness in peaches, and to identify some of those proteins. It was extremely difficult to accomplish this in the past, due to the absence of a suitable method to extract proteins from peach tissue for 2-DE analysis. Although five proteins were identified that differed significantly between juicy and mealy peaches, ACO may be the most significant due to its role as a key regulatory enzyme in ethylene biosynthesis, and the known association of ethylene and mealiness.

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