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ORIGINAL ARTICLE

Crucial roles of membrane stability and its related proteins in the tolerance of peach fruit to chilling injury

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Abstract Proteome patterns in peach fruit (*Prunus persica* L.) stored at different low temperatures were examined in order to gain a better understanding why peach fruit is less prone to chilling injury when stored at 0°C than at 5°C. Some differently expressed proteins in peach fruit stored at 0 and 5°C were identified using electrospray ionization quadrupole time-of-flight tandem mass spectrometry. Among these proteins, four membrane stability related proteins, i.e., enolase, temperature-induced lipocalin, major allergen Pru p 1, and type II SK2 dehydrin were enhanced, but three proteins related to phenolic compounds metabolization, cinnamyl-alcohol dehydrogenase 5, cinnamyl-alcohol dehydrogenase 1, and chorismate mutase, were repressed in peach fruit at 0°C as compared to that at 5°C. The abundance of glucose-6-phosphate dehydrogenase, NADP-dependent isocitrate dehydrogenase, and NADP-denpendent malic enzyme, which catalyze the reactions during sugar metabolism and energy pathways, was found to decrease in peach fruit stored at 0°C. In addition, our data revealed that low temperature of 0°C might regulate the endogenous H_2O_2 level, resulting in activating the transcriptional level of genes encoding the proteins related to membrane stability. These results provide a comprehensive knowledge to understand the mechanisms by which peach fruit stored at 0°C showed a higher chilling tolerance than that at 5°C.

Keywords Chilling stress · Hydrogen peroxide · Membrane stability · Peach fruit · Proteomics

Abbreviations

CI	Chilling injury
CBB	Coomassie brilliant blue
2-DE	Two-dimensional gel electrophoresis
ESI-Q-TOF–MS/MS	Electrospray ionization quadrupole time-of-flight tandem mass spectrometry
IEF	Isoelectric focusing
OA	Oxalic acid
ROS	Reactive oxygen species
SA	Salicylic acid
UFAs	Unsaturated fatty acids

Introduction

Low temperature is one of the major environmental factors limiting plant growth and survival. Cold-sensitive plants or specific plant organs may become injured when exposure to temperature below optimum growth (Thomashow 1999). However, low-temperature storage, an important approach to prolong the postharvest life of fresh horticultural crops, has been widely used in commercial treatments. In general, many tropical and subtropical fruits are susceptible to

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chilling injury (CI) when exposed to low, non-freezing temperatures, leading to serious losses in quality and market value (Saltveit and Morris 1990). A better understanding of the mechanisms through which fruits respond to low temperature stress at the biochemical and molecular levels is necessary, as this may lead to important agricultural and economic benefits.

Maintenance of membrane stability at low temperature is important for plant resistance to cold stress (Wongsheree et al. 2009). The chemical composition of membranes, especially the unsaturated fatty acids (UFAs) in cell membrane might be one of the ways for plant to maintain membrane fluidity, and to acclimatize low-temperature stress (Hazel 1995). Ishizaki-Nishizawa et al. (1996) reported that UFAs level of membrane lipid was greatly increased in transgenic tobacco plants expressing a chloroplast omega-3 fatty acid desaturase (FAD) gene, resulting in a significant increase in chilling resistance. In a recent study, we provided evidence that higher linolenic acid (C18:3) and membrane lipid unsaturation are beneficial for maintaining membrane fluidity, leading to an enhanced tolerance of peach fruit to low temperature (Zhang and Tian 2009). In addition, some evidences showed that CI was accompanied by membrane lipid degradation caused by an imbalance between the production and elimination of reactive oxygen species (ROS) inside the fruit (Franck et al. 2007; Wise 1995). These authors considered that ROS accumulation may induce loss of cell membrane integrity which becomes macroscopically visible through the enzymatic oxidation of phenolic compounds to brown-coloured polymers. Protection of cell membrane from oxidative injury is thought to be a major mechanism of resistance to chilling stress, and this resistance is likely to depend on the competence of the antioxidant system (Knorzer et al. 1999). Our previous study showed that treatment with 5 mM oxalic acid (OA) or 2 mM salicylic acid (SA) could enhance the resistance of mango fruit to low temperature, which was attributed to inhibiting ROS accumulation, delaying H_2O_2 decrease and inducing higher reducing status of ascorbate and glutathione (Ding et al. 2007). These results partially explored the mechanism underlying the CI-induced changes in fruit physiology. However, very few data are available for the molecular mechanism of fruit in response to low temperature, especially at the proteomic level.

For most of the chilling-susceptible commodities, fruits tend to suffer severer CI when exposed to the lower temperature and longer periods (Saltveit and Morris 1990), but we found that CI is prevented in peach fruits stored at 0°C, but not at 5°C. To investigate the mechanism underlying this novel phenomenon at the proteomic level, we analyzed the temporal changes of total proteins in peach fruit stored at 0 and 5°C, and identified some differentially expressed proteins, including many novel proteins related to membrane stability. The physiological and biochemical implications of these proteins were discussed.

Materials and methods

Fruits and treatments

Peach (*Prunus persica* L. cv. Hongtao) fruits were harvested at 97 N firmness and 9.1 °Brix total soluble solids from an orchard in Beijing, China, and were immediately transported to our laboratory. Fruits were selected for uniformity without any damage and randomly divided into two groups. One group was stored at 5°C and served as control; the other was stored at 0°C. All fruits were placed in plastic boxes (40 × 30 × 25 cm) wrapped in polyethylene film (0.04 mm thickness, with five holes of 20 mm in diameter on upper and side surfaces) to maintain about 95% relative humidity (RH).

Determination of CI and the relative electrolyte leakage

The browning, as a CI symptom, was assessed by measuring the extent of the total browned area on each fruit flesh, on the following scale (Wang et al. 2005): 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 browning index was calculated using the formula: $\sum$ (browning scale × percentage of corresponding fruit within each class). Three replicates (30 fruit per replicate) of each treatment were carried out.

The relative electrolyte leakage was measured as described by Saltveit (2002). Nine discs, of 10-mm diameter and 4-mm thickness, from equatorial regions of nine fruits, were washed with 0.4 M mannitol solution and dried with filter paper. After incubating the discs in 0.4 mol L^{-1} mannitol solutions at 25°C for 180 min, the initial electrolyte leakages of the solutions were determined by conductivity meter. Then the solutions and discs were heated at 95°C for 30 min before the total electrolyte leakages were measured. The relative electrolyte leakage was calculated as the percentage of the initial to total electrolyte leakage.

Protein extraction from fruits

Total protein extracts were prepared from the sample on day 21 according to the method of Saravanan and Rose (2004) with some modifications. All procedures described below were carried out at 4°C. Briefly, 4 g of flesh from ten fruits was ground in liquid nitrogen and then homogenized in 4 mL of homogenization buffer (20 mM Tris–HCl,

pH 7.5, 250 mM sucrose, 10 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, and 1% Triton X-100). The mixture was extensively homogenized on ice, and subsequently extracted with an equal volume of Tris–HCl (pH 7.8) buffered phenol for 30 min. After centrifugation at 10,000*g* for 40 min, the phenol phase was re-extracted three times with extraction buffer as previously mentioned. Proteins were precipitated from the final phenol phase with five volumes of ice-cold saturated ammonium acetate in methanol overnight at −20°C. The proteins were collected by centrifugation at 10,000*g* for 30 min and washed twice with cold saturated ammonium acetate in methanol and acetone each. The precipitate was air-dried and kept at −80°C until use.

Two-dimensional gel electrophoresis (2-DE) and image analysis

Aliquots of proteins (600 μg) resolved in 250 μL thiourea/urea lysis buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT and 2% (v/v) carrier ampholytes mixture (pH 5–8: pH 3.5–10, 1:1) were applied to rehydrate gel strips with an immobilized nonlinear pH gradient from 4 to 7 (Immobiline DryStrip pH 4–7 NL, 13 cm; GE Healthcare) for 16 h. The IEF was performed on an Ettan IPGphor unit (GE Healthcare) following the manufacturer's instruction. After IEF, the immobiline strips were equilibrated for 15 min in each of the following solutions: (1) 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT; (2) 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide. The equilibrated strip was placed onto the stacking gel and sealed with 0.5% agarose prepared in equilibration buffer. SDS-PAGE in the second dimension was performed with 15% separation gels and 7% stacking gels at a constant current of 30 mA. After electrophoresis, the 2D gels were stained with Coomassie Brilliant Blue (CBB) R-250 solution containing 50% (v/v) methanol, 15% (v/v) acetic acid and 0.1% (w/v) CBB R-250.

Gel images were acquired using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden) with 600 dpi resolution and saved in TIF format. Image analysis was performed by Image Master 2D Elite software (Amersham Biosciences, Uppsala, Sweden). For each treatment analyzed, at least triplicate gels were prepared from three different protein extractions. Protein spots were selected for quantitative analysis if they were consistently visible in three replicates. To correct experimental variations in 2D gels, the spot volumes were normalized as a percentage of the total volume in all of the spots in the corresponding gel. The relative change between 5 and 0°C samples was subjected to statistical analysis with SPSS (SPSS Inc., Chicago, IL, USA), and only spots that changed significantly in averaged normalized spot volume were excised for protein identification.

In-gel digestion

In-gel digestion was performed as described by Chan et al. (2007). CBB stained protein spots were manually excised from the gels and destained with 50 mM NH_4HCO_3 in 50% (v/v) methanol, changing the solutions every 1 h until the blue color of CBB was removed. Then, gel particles were mixed with 10 mM DTT in 25 mM NH_4HCO_3 for 1 h at 60°C to reduce the proteins. The gels were dried in a vacuum centrifuge for 30 min, and then incubated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 for 45 min at room temperature in the dark. After being washed several times with water and after being completely dried in a vacuum centrifuge, gel pieces were rehydrated with a digestion buffer of 10 ng μL^{-1} trypsin (sequencing grade, modified; Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 and digested for 16 h at 37°C. After digestion, the gel slices were washed three times with 0.1% trifluoroacetic acid (TFA) in 50% (v/v) acetonitrile to extract the peptides. The collected solutions were concentrated to 10 μl, and then desalted with ZipTipC18 (Millipore, Bedford, MA, USA). Peptides were eluted from the column in 2 μL of 0.1% TFA in 50% acetonitrile.

Protein spot identification by ESI-Q-TOF–MS/MS

ESI–MS/MS analysis of the digests was performed using an ESI quadrupole time-of-flight hybrid mass spectrometer (ESI-Q-TOF; micro, Micromass, Altrincham, UK) with a z-spray source (Nouwens et al. 2002). Before loading the digested peptides, the instrument was externally calibrated with the fragmentation spectrum of the doubly charged 1,571.68 Da (785.84 m/z) ion of fibrinopeptide B. The peptides were introduced by nanoelectrospray needle (gold-coated borosilicate glass capillaries, Micromass). The applied spray voltage was 800 V, with a sample cone working on 30 V. The collision energy was varied from 14 to 40 V depending on the mass and charge state of the peptides. MS/MS data were processed using the MaxEnt3 (Micromass), and the data searched in NCBInr protein databases (version 20081010) with the Mascot MS/MS Ions Search program on the Matrix Science public web site. A peptide tolerance of ±2.0 Da for the precursor ions and a MS/MS tolerance of ±0.8 Da for the fragment ions were set. Peptide charges of +1, +2, and +3 monoisotopic mass were chosen, and the instrument type was set to ESI-Q-TOF. Viridiplantae was chosen for the taxonomic category, trypsin was specified as the proteolytic enzyme, and one missed cleavage site was allowed for each search. Carbamidomethylation of cysteine (+57.02 Da) and oxidation

of methionine (+16 Da) were designated as variable modifications for searching.

Measurements of phenolic compounds

Phenolic compounds were measured according to the method of Liu et al. (2005). Frozen tissue (10 g) was homogenized with 5 mL ice-cold 1% HCl-methanol solution and then centrifuged at 10,000*g* for 30 min. The supernatant was collected and absorbance measured at 280 nm. Phenolic compounds were expressed as $OD_{280}\ kg^{-1}$.

Extraction and analysis of membrane lipids

The total lipids were extracted as described in our previous study (Zhang and Tian 2009). About 10 g powder of frozen fruit was mixed with 9 mL of chloroform: methanol (2:1, v/v) and vortexed vigorously. To improve layer separation, a volume of 5.4 mL of 1 mM KCl and 3 mL of chloroform were added with stirring for 5 min. Then, the mixture was centrifuged at 10,000 *g* for 10 min at 4°C. The chloroform layer was collected and dried under N_2. Total lipids were dissolved in 3 mL petroleum ether (b.r. 90–120°C) pre-equilibrated with 95% methanol and extracted three times with equal volumes of 95% methanol pre-equilibrated with petroleum ether. The three methanol extracts were combined and back-extracted with 1 mL of petroleum ether. The polar lipids were obtained by evaporation of the methanol phase under N_2.

Fatty acids of the polar lipid were quantified by gas chromatography (GC) after conversion to the corresponding methyl esters by hot methanolic sulphuric acid. Methylated fatty acids were separated using a temperature program (the initial column temperature of 140°C was held for 5 min, increased by 4°C per min to 240°C and held for 0 min, and then increased by 50°C per min to a final temperature of 255°C for 5 min) on LECO Pegasus® IV GC-TOFMS fitted with a 30 m × 0.25 mm × 0.25 μm SUPELCO wax™10 capillary column. Authentic methylated fatty acid (Sigma 47801) was used as external standard to identify and quantify peaks; corrections were made at this stage for losses using the C17:0 internal standard. The double-bond index (DBI), a measure of the degree of fatty acid desaturation, was calculated as described by Zhang and Tian (2009).

Analysis of H_2O_2

To quantify H_2O_2 content, 10 g peach fruit was extracted in ice-cold acetone, and the supernatant was measured as described by Patterson et al. (1984). One unit of H_2O_2 content was defined as 1 mmol H_2O_2 per kg of pulp. To visualize cytochemical localization of H_2O_2 in pulp cell, pulp tissue pieces of approximately 1 mm^3, located 0.5 cm near the fruit surface and in equatorial regions of fruit, were cut and incubated in freshly prepared 5 mmol L^{-1} $CeCl_3$ in 50 mmol L^{-1} MOPS buffer (pH 7.2) for 1 h. As controls, samples were incubated with (1) catalase; and (2) without $CeCl_3$. Then the H_2O_2 was examined using a previous procedure (Romero-Puertas et al. 2004).

Cloning of genes encoding Enolase, Temperature-induced lipocalin, Major allergen Pru p 1, and Type II SK2 dehydrin as well as Northern blot analysis

Total RNA was extracted from the sample using the hot-phenol protocol as described by our previous study (Chan et al. 2007). Part gene sequence of *enolase*, *temperature-induced lipocalin*, *major allergen Pru p 1,* and *type II SK2 dehydrin* (accession nos. DY648587, DQ251187, DQ222997, and DQ111949, respectively) of peach fruit was used to design the following DNA primers: *Enolase*: 5′-GTG AGT GAG TAC CCC TAT TGT-3′ (forward primer) and 5′-TTC ATT TCA TCC CGT CCC GC-3′ (reverse primer); *Temperature-induced lipocalin*: 5′-GAT GTG GTG AAG GGT CTG GA-3′ (forward primer) and 5′-CGG TGT CTT GTG GAG TTT GC-3′ (reverse primer); *Major allergen Pru p 1*:5′-ATG GGT GTC TTC ACA TAT GAG AG-3′ (forward primer) and 5′-GTT GTA GGC ATC GGG GTG GCC CT-3′ (reverse primer); *Type II SK2 dehydrin*: 5′-CTA TGA AGG GAA GGC TGG TGA AT-3′ (forward primer) and 5′-CTT CTC CTT CTC CTT GAT GGC CT-3′ (reverse primer). cDNA fragments of these genes were obtained by polymerase chain reaction (PCR) amplification. PCR products were cloned in the pGEM-T Easy vector (Tiangen, China) and sequenced.

For Northern blot analysis, aliquots of 30 μg of total RNA per lane were loaded in a 1.2% denaturing formaldehyde agarose gel. Hybridization with [^{32}P] dCTP-labeled PCR probe was carried out as described by Sambrook et al. (2001).

Statistical analysis

All statistical analyses were performed with SPSS 11.0. One-way ANOVA was used to compare means. After being analyzed by Levene's test, means with equal variance were tested by Duncan's test, whereas means with unequal variance were analyzed by Dunnett's T_3

test. Differences at $P < 0.05$ were considered as significant.

Results

Development of CI and changes of the relative electrolyte leakage

All fruits stored at 5 and 0°C did not show any CI symptoms during the first 14 days of storage. However, at 21 days of storage, the fruits stored at 5°C showed obvious pulp browning with 88% CI incidence and 46% CI index, but the fruits stored at 0°C showed no visible flesh browning at the same time (Fig. 1a, c). The relative electrolyte leakage, which has been proven an excellent indicator of cell membrane damage, gradually increased with storage time in peach fruit. No significant difference in the relative electrolyte leakage was found between fruits stored at 0 and 5°C for 14 days ($P > 0.05$). However, the relative electrolyte leakage in fruit stored at 5°C was above 30%, which was significantly higher than that at 0°C for 21 days ($P < 0.05$) (Fig. 1b).

Different expression of proteins in peach fruit

Comparative analysis of the proteome was performed to investigate the profiles of differentially expressed proteins in peach fruit stored at 5 and 0°C for 21 days (Fig. 2). Examination of the 2D gels revealed about 800 protein spots after exclusion of very faint ones with undefined shapes and areas. Protein spots were scored only when they were reproducibly observed in three independent replicates. A total number of 44 proteins whose expression was induced or repressed under chilling stress were detected in this study (Fig. 2). Protein identification was an analytical challenge because of the lack of genome sequence information for peach fruit. Tandem mass spectrometry (MS/MS), which has been reported to be the most successful technique to identify proteins correctly from organisms where DNA sequence is unknown (Jouber et al. 2001), was used to identify these proteins. MS/MS spectra were submitted for database searching in the Mascot search engine, and 32 proteins which scored greater than the threshold were identified with Mowse (Table 1).

The identified proteins were categorized functionally as energy, secondary metabolism, metabolism, defense, protein destination and storage, transcription, cell structure,

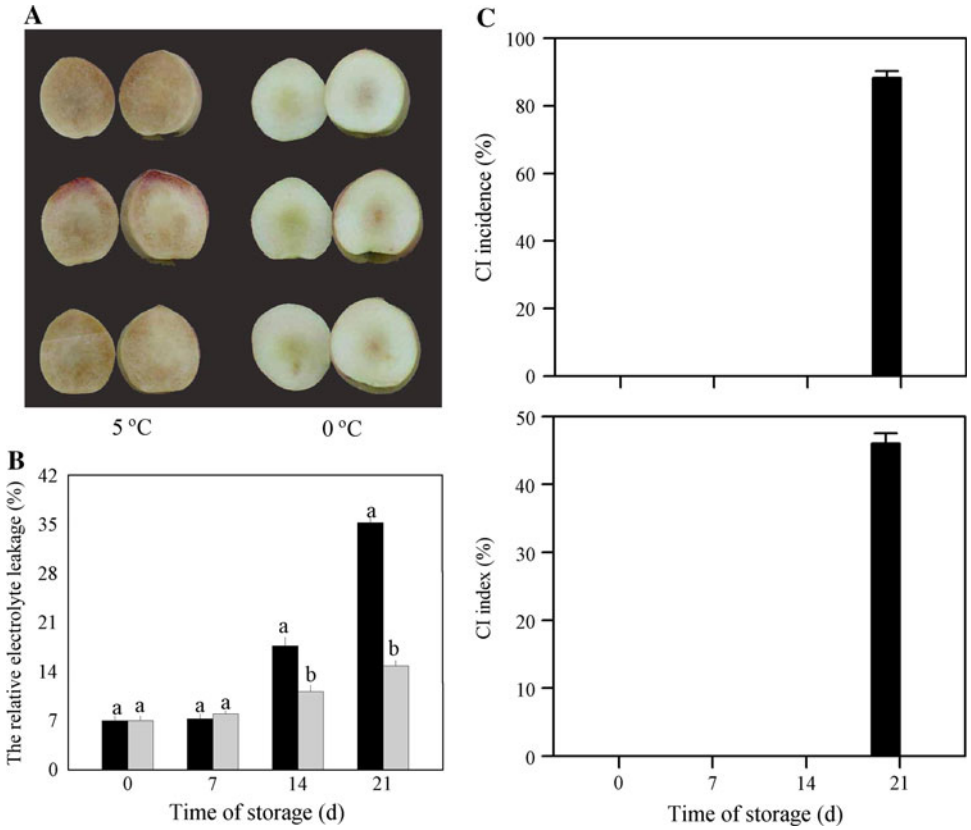

Fig. 1 Development of chilling injury in peach fruit stored at 5 and 0°C. **a** Images of fruit taken on day 21. **b** Changes of the relative electrolyte leakage in peach fruit during storage. **c** Measurement of CI incidence and CI index in peach fruit during storage. *Black column* and *gray column* represent 5 and 0°C storage, respectively. *Bars* with a *different letter* at a special time are significantly different at $P < 0.05$

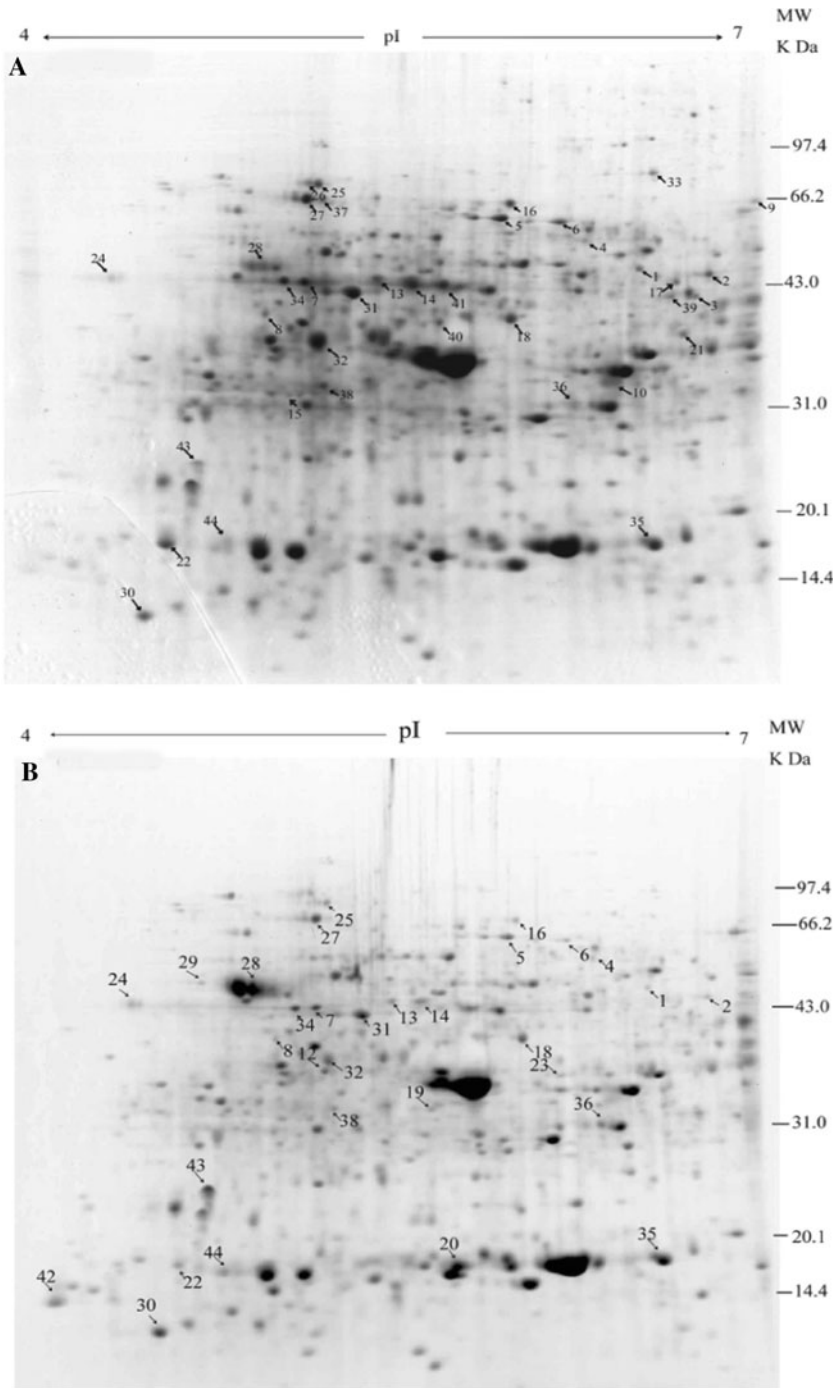

Fig. 2 Comparison of proteome patterns of peach fruit stored at 5°C (**a**) or 0°C (**b**) for 21 days. Protein extraction and 2D electrophoresis protocol were described in "Materials and methods". *Arrows* indicate proteins identified by MS/MS, which changed in abundance more than twofold between peach fruit stored at 5 and 0°C. The *numbers* indicate differentially expressed proteins and correspond to the numbers indicated in Table 1

and cell growth according to Bevan et al. (1998) and Schiltz et al. (2004). Of the differentially expressed proteins, 12 proteins were related to energy, including 6-phosphogluconate dehydrogenase (spot 1), NADP-dependent isocitrate dehydrogenase (spot 2), glutamate dehydrogenase B (spot 3), dihydrolipoamide dehydrogenase precursor (spot 4), NADP-dependent malic enzyme (spot 5), alpha-amylase (spot 6), secreted alpha-amylase (spot 7), alpha-galactosidase hydrolase (spot 8), Phosphoenolpyruvate carboxykinase (spot 9), thaumatin-like protein 1 precursor (spot 10), enolase (spot 11), and V-ATPase catalytic subunit A (spot 12). It was notable that the expressions of three proteins, i.e., 6-phosphogluconate dehydrogenase, NADP-dependent isocitrate dehydrogenase, and NADP-dependent malic enzyme, were apparently down-regulated when the fruit was stored at 0°C, and they were involved in NADPH production (Fig. 7). Of the remaining proteins, four were involved in metabolism, including gamma-aminobutyrate transaminase subunit precursor isozyme 3 (spot 17), glutamine synthetase

Table 1 Identification of proteins differently expressed in peach fruit stored at 5 or 0°C for 21 days

Spot[a]	Homologous protein	*Mr* (kDa)/*pI*	MASCOT score/threshold	Queries matched	SC[b] (%)	Organism	Accession number[c]
Energy							
↓ 1	6-phosphogluconate dehydrogenase	56.338/5.55	348/47	31	12	*Glycine max*	gil2529229
↓ 2	NADP-dependent isocitrate dehydrogenase	46.603/6.54	1,332/47	59	53	*Prunus persica*	gil15982950
↓ 3	Glutamate dehydrogenase B	44.48/6.38	161/47	14	8	*Nicotiana plumbaginifolia*	gil12643806
↓ 4	Dihydrolipoamide dehydrogenase precursor	53.933/6.71	97/47	8	7	*Bruguiera gymnorrhiza*	gil13873336
↓ 5	NADP-dependent malic enzyme	65.186/6.09	552/47	57	18	*Vitis vinifera*	gil1708924
↓ 6	Alpha-amylase	46.824/5.45	84/46	3	3	*Phaseolus vulgaris*	gil3769330
↓ 7	Secreted alpha-amylase	44.030/5.18	121/47	8	6	*Malus x domestica*	gil60652319
↓ 8	Alpha-galactosidase/hydrolase	72.297/8.94	63/47	2	1	*Arabidopsis thaliana*	gil30688284
↓ 9	Phosphoenolpyruvate carboxykinase	68.65/5.98	253/47	12	11	*Arabidopsis thaliana*	gil2827717
↓ 10	Thaumatin-like protein 1 precursor	25.748/8.29	436/47	23	38	*Prunus persica*	gil25091405
↑ 11	Enolase	15.848/5.13	184/48	7	31	*Prunus armeniaca*	gil63192024
↑ 12	V-ATPase catalytic subunit A	68.529/5.30	49/48	1	1	*Prunus persica*	gil15982954
Secondary metabolism							
↓ 13	Cinnamyl-alcohol dehydrogenase 5	38.718/5.42	109/47	7	9	*Arabidopsis thaliana*	gil15235295
↓ 14	Cinnamyl-alcohol dehydrogenase 1	38.474/5.73	165/47	15	9	*Striga asiatica*	gil109631192
↓ 15	Chorismate mutase	30.472/5.95	76/47	2	7	*Arabidopsis thaliana*	gil15238285
↓ 16	Polyphenol oxidase precursor	67.091/6.39	736/47	36	18	*Prunus armeniaca*	gil3282505
Metabolism							
↓ 17	Gamma-aminobutyrate transaminase subunit precursor isozyme 3	57.203/6.72	346/47	12	16	*Lycopersicon esculentum*	gil29837286
↓ 18	Glutamine synthetase cytosolic isozyme 1	38.82/5.46	89/47	5	10	*Glycine max*	gil121336
↑ 19	Temperature-induced lipocalin	21.45/5.6	463/47	31	41	*Prunus persica*	gil77744891
↑ 20	Major allergen Pru p 1	17.637/5.79	345/47	7	47	*Prunus persica*	gil82492265
Defense							
↓ 21	Beta-cyanoalanine synthase	38.204/6.38	223/47	14	12	*Betula pendula*	gil30840956
↓ 22	Pathogenesis-related protein PR-4	11.51/4.57	259/47	4	48	*Prunus persica*	gil25453225
↑ 23	Isoflavone reductase related protein	33.802/6.02	197/47	6	16	*Pyrus communis*	gil3243234
↑ 24	Putative ankyrin-repeat protein	37.918/4.53	89/48	5	7	*Vitis aestivalis*	gil37625031
Protein destination and storage							
↓ 25	Putative luminal-binding protein	73.595/5.11	860/47	45	20	*Isatis tinctoria*	gil74053614
↓ 26	Luminal binding protein	73.447/5.08	617/48	29	15	*Arabidopsis thaliana*	gil1303695
↓ 27	High molecular weight heat shock protein	71.171/5.17	276/33	6	11	*Malus x domestica*	gil6969976
↑ 28	Type II SK2 dehydrin	28.297/5.37	126/47	9	16	*Prunus persica*	gil73762178
Transcription							
↑ 29	RAD23 protein	41.483/4.67	92/47	4	4	*Solanum lycopersicum*	gil5640111
Cell structure							
↑ 30	Profilin	13.995/4.67	128/47	2	22	*Prunus persica*	gil27528310

Table 1 continued

Spot[a]	Homologous protein	*Mr* (kDa)/*pI*	MASCOT score/threshold	Queries matched	SC[b] (%)	Organism	Accession number[c]
↓ 31	Actin	41.707/5.44	778/47	27	50	*Gossypium hirsutum*	gil32186896
Cell growth							
↓ 32	1-aminocyclopropane-1-carboxylate oxidase	36.149/5.2	393/47	20	42	*Prunus persica*	gil7108577

↑ shows that the significantly increased spot intensity, and ↓ shows significantly decreased spot intensity in peach fruit stored at 0°C as compared to 5°C

[a] The spot numbers correspond to the numbers indicated in Fig. 2

[b] SC, amino acid sequence coverage for the identified proteins

[c] Accession number from the NCBI database of matched protein

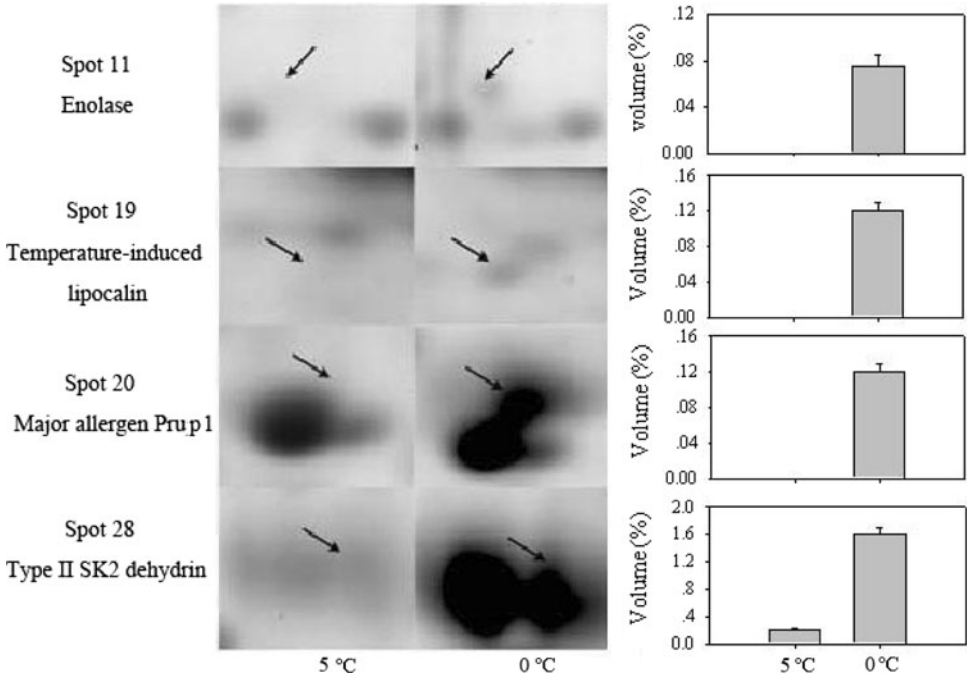

Fig. 3 Abundance variance of membrane stability related proteins in peach fruits stored at 5 or 0°C for 21 days. Protein extraction and 2D electrophoresis protocol were described in "Materials and methods". The number of each protein spot corresponds to its listing in Table 1. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The protein spots with significant changes in intensities ($P < 0.05$) were considered to be different. The *graph* represents an average of three spots from different gels. *Bars* represent standard errors of the mean

cytosolic isozyme 1 (spot 18), temperature-induced lipocalin (spot 19), major allergen Pru p 1 (spot 20); and four proteins, putative luminal-binding protein (spot 25), luminal binding protein (spot 26), high molecular weight heat shock protein (spot 27), and type II SK2 dehydrin (spot 28), were related to protein destination and storage. Most notably, we found that the abundance of enolase, major allergen Pru p 1, temperature-induced lipocalin, and type II SK2 dehydrin was obviously up-regulated in the fruit stored at 0°C for 21 days, and these proteins may be related to membrane stability (Fig. 3).

In addition, we indentified proteins involved in secondary metabolism (spot 13–16), defense (spot 21–24) and transcription (spot 29), cell structure (spot 30 and 31), and cell growth (spot 32), respectively (Fig. 2; Table 1). Among them, it was found that three proteins related to phenolic compound metabolization, i.e., cinnamyl-alcohol dehydrogenase 5, cinnamyl-alcohol dehydrogenase 1, and chorismate mutase, were repressed in peach fruit at 0°C as compared to that at 5°C (Fig. 6a).

Fatty acid (FAs) composition in membrane lipid

In peach fruit, five major FAs including two saturated fatty acids (SFAs; C16:0 and C18:0) and three UFAs (C18:1, C18:2 and C18:3) (Table 2) were identified and

Table 2 The fatty acid composition of total polar lipids extracted from peach fruit stored at 5 or 0°C for 21 days

Treatment (°C)	Fatty acid composition (mol %)					DBI
	C16:0	C18:0	C18:1	C18:2	C18:3	
5	83.57 ± 0.29	12.01 ± 0.33	3.88 ± 0.02	0.46 ± 0.03	0.09 ± 0.01	5.07 ± 0.08
0	83.20 ± 0.12	10.14 ± 0.05*	4.66 ± 0.04*	1.62 ± 0.02*	0.38 ± 0.02*	9.03 ± 0.13*

* Means significantly different from "5°C" at the $P < 0.05$ level

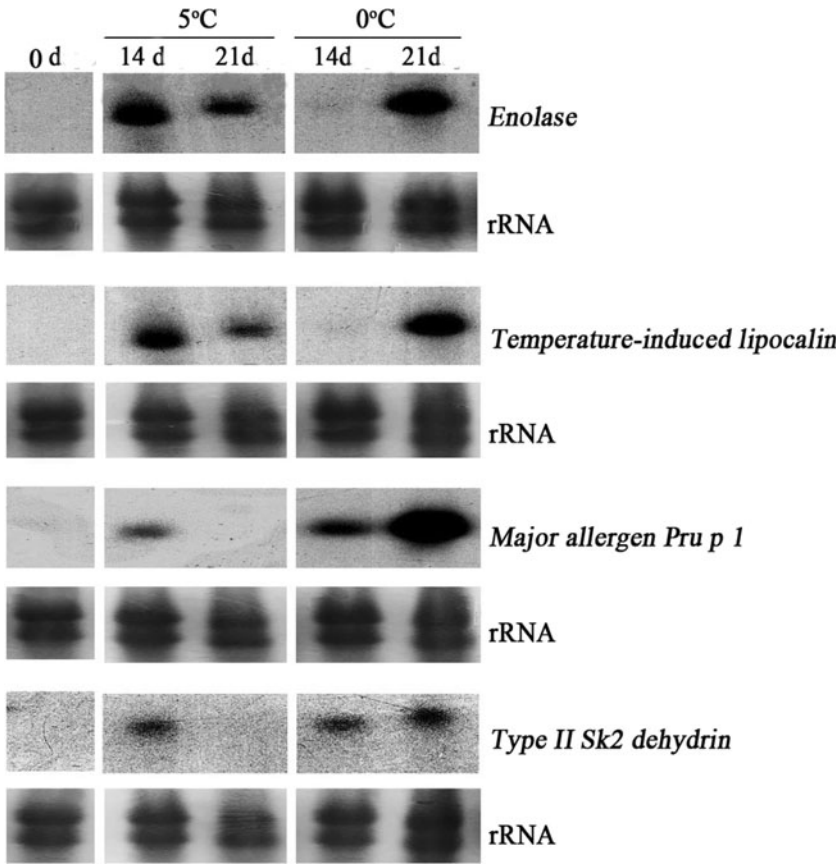

Fig. 4 Expression of membrane stability-related genes, i.e., *Enolase, Temperature-induced lipocalin, Major allergen Pru p 1,* and *Type II SK2 dehydrin* in peach fruit stored at 5 or 0°C for 0, 14 or 21 days

quantified. C16:0 was the most predominant FA and showed similar content between fruits stored at 0 and 5°C. The three UFAs showed significantly lower content in peach fruit stored at 5°C than that at 0°C. In addition, the DBI, a measure of the degree of fatty acid desaturation, was significantly lower in peach fruit stored at 5°C than at 0°C ($P < 0.05$) (Table 2).

The expression of membrane stability related genes by Northern blot analysis

On the basis of the results of Northern blot analysis, no transcripts of the four membrane stability related genes, *enolase*, *major allergen Pru p 1*, *temperature-induced lipocalin,* and *type II SK2 dehydrin* were observed in peach fruits stored in 5 and 0°*C* at 0 day. After 14 day's storage, the expressions of these genes from 5°C-fruits reached the maximum, and showed clearly higher levels than those from 0°C -fruits. The transcripts of these four genes from 0°C-fruits exhibited low levels at 14 days, but their expressions increased dramatically and were obviously stronger than that in 5°C at 21 days (Fig. 4).

Cytochemical localization and changes of H_2O_2 content

Electron-dense precipitates of cerium perhydroxides indicating the presence of H_2O_2, were not observed in controls with catalase or without $CeCl_3$ (Fig. 5a1, a2), whereas they were found at the interface of cell wall and plasma membrane in browning tissue (Fig. 5a3) or sound tissue (Fig. 5a4). The content of hydrogen peroxide changed dramatically between the 0 and 5°C samples during the

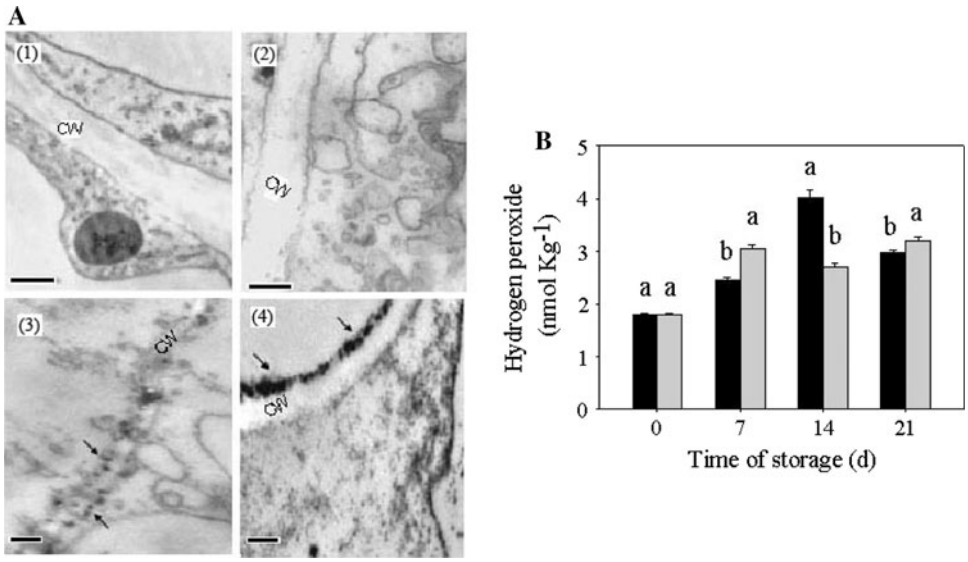

Fig. 5 Hydrogen peroxide localization (**a**) and its content (**b**) in peach fruit stored at 5°C (*black column*) or 0°C (*grey column*) by $CeCl_3$ staining and transmission electron microscopy after 21 days. *1* Control with catalase, *2* Control without $CeCl_3$, *3* peach fruit stored at 5°C and *4* peach fruit stored at 0°C. *CW* represents cell wall. *Arrows* indicates $CeCl_3$ precipitates. *Bars* represent 1 μm. Means with a different lowercase letter at a special time are significantly different at $P < 0.05$

storage periods, showing significantly higher level ($P < 0.05$) in the 5°C sample at day 14, but when storage time was up to 21 days, the fruits stored at 0°C had higher content of hydrogen peroxide as compared to that stored at 5°C ($P < 0.05$) (Fig. 5b).

The content of total phenolic compound

The content of total phenolic compound in peach fruit increased gradually with storage time, and also showed the same tendency in all treatments. But the content of total phenolic compound in peach fruit stored at 5°C was significantly higher than that at 0°C ($P < 0.05$) (Fig. 6b).

Discussion

Cell membrane is the first barrier that separates cells from their environment and also is a primary target for damage under environmental stress. Dysfunction of cell membrane at low temperature is considered to be the primary molecular events ultimately leading to the development of CI symptoms (Parkin and Kuo 1989). We previously found that controlled atmosphere (CA, 5% O_2 + 5% CO_2) storage maintained better membrane integrity of peach fruit compared to the control (Wang et al. 2005). In this study, we further proved that membrane integrity is an important indicator for chilling tolerance, as was illustrated by which peach fruit stored at 0°C showed lower electrolyte leakage (Fig. 1b), resulting in lower CI incidence and index than that at 5°C (Fig. 1c, d). In addition, there was a correlation between membrane stability and the level of UFAs and DBI in peach fruits (Table 2). The result was in accordance with review of Thomashow (1999), who considered that cold-tolerant plants contained high proportions of UFAs, which kept the membrane phase transition temperature below the applied chilling temperature. In this way, a membrane phase transition is avoided. Our recent study has also confirmed that the higher level of UFAs and DBI was beneficial for maintaining the appropriate membrane fluidity, whereby the membrane could perform its normal function (Zhang and Tian 2009).

Changes in the expression and activity of enzymes involved in several different metabolic pathways have been shown to occur in response to low temperature (Guy 1990; Hurry et al. 1995). Martin (1987) considered that regulation of metabolic processes depends on control of enzyme activity, and one of mechanism that regulates enzyme activity is the alteration of the concentration of enzyme protein in the cell. Therefore, to a large extent, protein expression level reflects enzyme activity. Proteomic provides a direct assessment of proteins involved in stress response pathways and consequently is a valuable link between the classical physiological approach and molecular tools (Renaut et al. 2004). In this study, we investigated that metabolic processes involved in membrane stability of peach fruit based on two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry. According to the results from proteome analysis, we found that the expression of enolase, one of the key enzymes that catalyzed the conversion of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway, was increased dramatically in peach fruit stored at 0°C (Fig. 3). Lee et al. (2009) have suggested that the glycolytic pathway is highly disrupted by chilling stress and that enolase can be used as a biomarker for cold stress in rice roots. A gradual up-regulation of the enolase in response to cold stress was reported in rice leaves (Yan et al. 2006), and

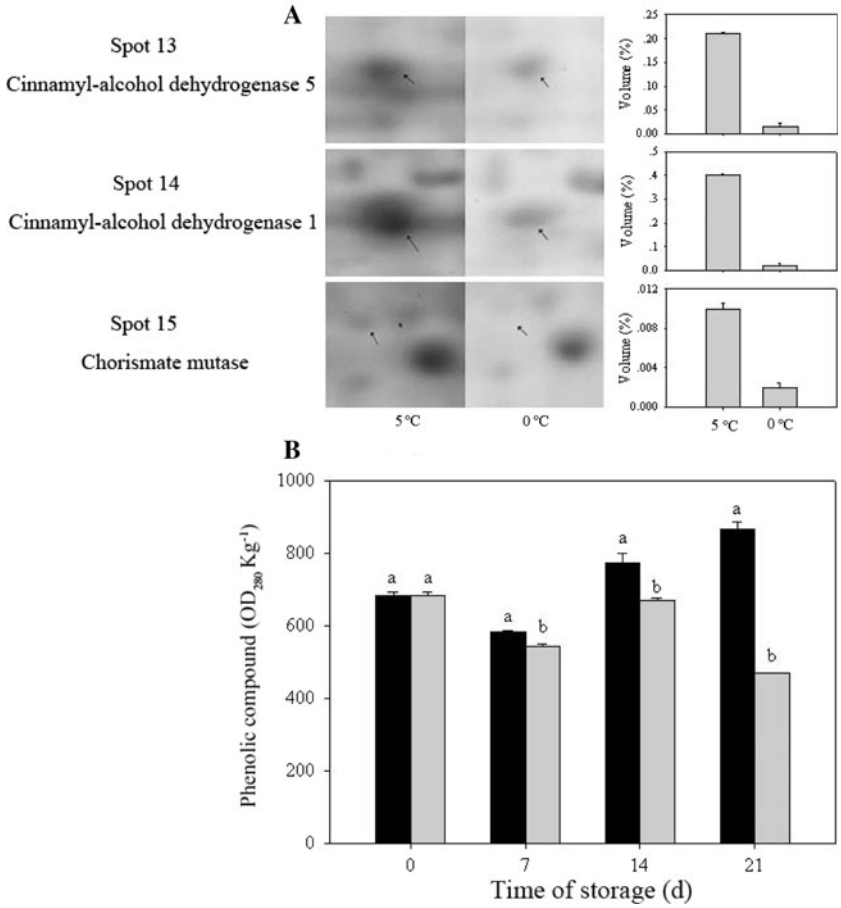

Fig. 6 Abundance variance of proteins involved in metabolism of phenolic compound (**a**) and the content of phenolic compound (**b**) in peach fruit stored at 5°C (*black column*) or 0°C (*gray column*) for 21 days. Protein extraction and 2D electrophoresis protocol were described in "Materials and methods". The *number* of each protein spot corresponds to its listing in Table 1. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The protein spots with significant changes in intensities ($P < 0.05$) were considered to be different. The *graph* represents an average of three spots from different gels. *Bars* represent standard errors of the mean

mutation of the enolase gene was proved to impair cold-responsive gene, such as the FAD gene transcription and then to result in the chilling sensitivity of *Arabidopsis* (Lee et al. 2002). Recently, we clarified that enhanced transcription level of the omega-3 FAD was positively related to the accumulation of C18:3, resulting in enhancing tolerance of peach fruit to chilling stress (Zhang and Tian 2009). The major allergen Pru p1 was significantly higher in peach fruits stored at 0°C than that at 5°C (Fig. 3). Pru p1 of peach (*Prunus persica*), as a lipid transfer protein (LTP), consisted of 91 amino acids with a calculated molecular mass of 9,178 Da (Pastorello et al. 1999). Both direct membrane stabilizing and destabilizing effects of LTPs have been reported by Hincha (2002). In vitro plant LTPs enhanced the transfer of phospholipids between membranes and could bind acyl chains. As a consequence, LTPs were thought to participate in membrane biogenesis and regulation of the intracellular fatty acid pools (Miquel et al. 1988). However, Kader (1996) considered that LTPs were extracellularly located and secreted, and a possible role for these proteins in intracellular lipid transfer was unlikely.

Temperature-induced lipocalin and Type II Sk2 dehydrin were significant higher in peach fruits stored at 0°C than at 5°C (Fig. 3). Bugos et al. (1998) first identified lipocalin-like proteins from plants, and considered those lipocalins to be key enzymes of the xanthophyll cycle responsible for the protection against photo-oxidative damage. Two cDNAs corresponding to a novel lipocalin were identified from wheat and *Arabidopsis,* respectively, and their transcripts were up-regulated during cold acclimation (Frenette Charron et al. 2002). Dehydrin is known as a group 2 late embryogenesis abundant (LEA) proteins, one of several ubiquitous water-stress-responsive proteins in plants (Ingram and Bartels 1996; Close 1997). LEA proteins were found in phylogenetically distant organisms and usually considered to be related to abiotic stress tolerance. However, no unifying concepts for their physiological roles and modes of action have been attained so far. Recently, Tunnacliffe and Wise (2007) presented several possible functions of the LEA proteins, these including as antioxidants and as membrane and protein stabilizers during water stress, either by direct interaction or by acting as molecular shields. The location studies of Hundertmark and Hincha (2008) just showed that some dehydrins are situated in the vicinity of membranes, but there was no proof of any partucular function. In addition, in numerous transgenetic studies where dehydrin genes have been

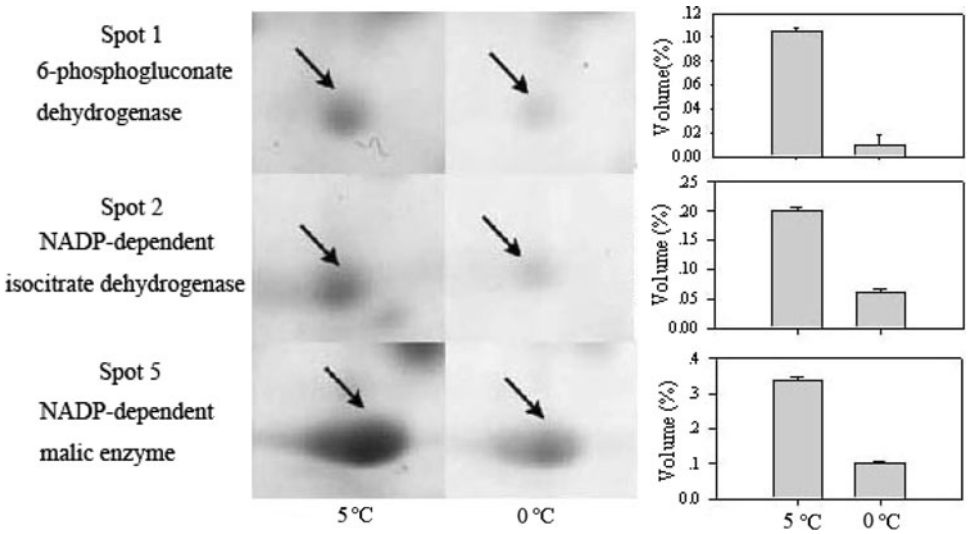

Fig. 7 Abundance variance of NADPH production-related proteins in peach fruits stored at 5 or 0°C for 21 days. Protein extraction and 2D electrophoresis protocol were described in "Materials and methods". The number of each protein spot corresponds to its listing in Table 1. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The protein spots with significant changes in intensities ($P < 0.05$) were considered to be different. The *graph* represents an average of three spots from different gels. *Bars* represent standard errors of the mean

introduced into plants, somewhat variable results were obtained. Some transgenetic plants showed better chilling tolerance at low temperature, but others had only slight or no tolerance to cold stress (Yin et al. 2006; Lång 1993). Coinciding with the relative levels of enolase, lipocalins, Pru p 1, and dehydrins (Fig. 3), the transcription levels of genes encoding these proteins were apparently higher in unchilled fruits (Fig. 4). The transcriptional expression of these genes might be activated by higher H_2O_2 level in peach fruits stored at 0°C for 21 days (Fig. 5). It is well known that H_2O_2 as a second messenger can directly regulate the expression of numerous genes, some of which are involved in plant defense and hypersensitive response (Desikan et al. 2000; Kovtun et al. 2000). Additionally, Kang et al. (2003) enucleated that exogenous H_2O_2 could activate many protective enzymes (such as SOD, CAT and APX), and thus enhance the tolerance of banana seedlings to low temperature stress.

The expressions of three phenolic compound metabolizing-related proteins were stimulated in the fruit at 5°C (Fig. 6a) and positively related with the higher content of phenolic compounds (Fig. 6b), thus leading to the serious flesh browning of peach fruit stored at 5°C (Fig. 1a). In general, flesh browning in fruits was considered to be the degradation of phenolic compounds into quinines by means of polyphenol oxidase (PPO), the quinones subsequently polymerizing into brown-coloured pigments (Kader 1994). The PPO and phenolic compounds are generally localized in separate compartments in the cell; therefore, breakdown of membrane integrity is apparently required for the enzyme to react the substrate (Veltmann and Peppelenbos 2001).

In addition, the abundance of three dehydrogenases related to sugar metabolism and energy pathways was found to decrease in peach fruit stored at 0°C (Fig. 7). These proteins, together with phosphoenolpyruvate carboxykinase (spot 9 in Table 1), are directly involved in glycolysis and tricarboxylic acid cycle. The lower abundance of these proteins leads to the lower assumption of sugars, which has several beneficial effects in protecting plants against stresses (Ingram and Bartels 1996). However, some authors considered that under chilling stress, plants required more energy production (Yan et al. 2006; Lee et al. 2009). Up-regulation of these proteins may be related to producing more energy in response to chilling stress in peach fruit. Therefore, further detailed researches will be needed to characterize the potential roles of these proteins in enhancing tolerance of peach fruit to chilling stress.

In summary, our results revealed that some proteins related to membrane stability and phenolic compound metabolization were involved in resistant of peach fruit to chilling stress. We also consider that 0°C low-temperature may regulate the endogenous H_2O_2 level, thus activating the transcriptional expression of genes encoding these proteins under chilling stress. These results provide further knowledge to explain why peach fruit stored at 0°C can acquire a higher resistance to CI as compared to that at 5°C.

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