Comparative transcriptomics and proteomics analysis of citrus fruit, to improve understanding of the effect of low temperature on maintaining fruit quality during lengthy post-harvest storage

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Abstract

Fruit quality is a very complex trait that is affected by both genetic and non-genetic factors. Generally, low temperature (LT) is used to delay fruit senescence and maintain fruit quality during post-harvest storage but the molecular mechanisms involved are poorly understood. Hirado Buntan Pummelo (HBP; Citrus grandis × C. paradisi) fruit were chosen to explore the mechanisms that maintain citrus fruit quality during lengthy LT storage using transcriptome and proteome studies based on digital gene expression (DGE) profiling and two-dimensional gel electrophoresis (2-DE), respectively. Results showed that LT up-regulated stress-responsive genes, arrested signal transduction, and inhibited primary metabolism, secondary metabolism and the transportation of metabolites. Calcineurin B-like protein (CBL)–CBL-interacting protein kinase complexes might be involved in the signal transduction of LT stress, and fruit quality is likely to be regulated by sugar-mediated auxin and abscisic acid (ABA) signalling. Furthermore, ABA was specific to the regulation of citrus fruit senescence and was not involved in the LT stress response. In addition, the accumulation of limonin, nomilin, methanol, and aldehyde, together with the up-regulated heat shock proteins, COR15, and cold response-related genes, provided a comprehensive proteomics and transcriptomics view on the coordination of fruit LT stress responses.

Key words: fruit quality, low-temperature storage, metabolism and metabolite transportation, proteomics, senescence, signal transduction, transcriptomics.

Introduction

The quality of fruit is a combination of attributes, properties, and characteristics that give each commodity value in terms of human food. Consumers are concerned primarily about the colour and flavour of dietary components, as well as aspects of nutritional quality that include energy, vitamins, minerals, dietary fibre, and the many bioactive compounds that enhance human health. During post-harvest storage, soluble sugars, organic acids, and the solid/acid ratio are important components of fruit quality that can be affected by both internal and external factors. Senescence is the most important internal factor that determines storage lifetime and the final quality of fruit. During senescence, cells undergo orderly changes in cell structure, metabolism, and gene expression. Metabolically, carbon assimilation is replaced by catabolism of macromolecules such as proteins, membrane lipids, and RNA (Xue-Xuan et al., 2010). However, no further phenotypic change is observed during post-harvest storage. Therefore physical characters are the main markers of fruit senescence.

Fruit senescence is a developmentally programmed degenerative process that constitutes the final step of fruit
development and is controlled by multiple developmental and environmental signals (Qin et al., 2009). Most studies of fruit senescence at the molecular level in climacteric fruit have been focused primarily on ethylene biosynthesis and its signal transduction (Botton et al., 2011), which suggest that fruit senescence is an oxidative phenomenon regulated by ethylene. However, study of the non-ethylene-mediated mechanisms of senescence physiology is of particular interest because it might create new opportunities for controlling fruit senescence (Qin et al., 2009). Citrus fruits are the most economically important subtropical fruits and citrus is becoming a key species in the study of fruit senescence. Unlike the climacteric tomato fruit, whose storage life is controlled by the phytohormone ethylene (Cara and Giovannoni, 2008; Botton et al., 2011), citrus fruits usually undergo gradual changes and no core substance regulating their senescence and post-harvest storage process has been identified. Therefore, analysis of senescence in citrus fruits might provide new insights into the fundamental pathways that contribute to the post-harvest properties of non-climacteric fruit.

Fruit senescence is naturally age-dependent and can be induced by abiotic and biotic factors, including low humidity, high temperature, oxidative stress, and pathogen infections (Alos et al., 2008). The internal signals (phytohormones), including abscisic acid (ABA), auxin, salicylic acid, ethylene, jasmonic acid, and brassinosteroid, also regulate fruit senescence (Ashraf et al., 2010; Sun et al., 2010; Yang et al., 2010). In addition, accumulation of hexoses through shifting cellular carbon flux from metabolism and biosynthesis could serve as signalling molecules that alter the biochemical or developmental processes in fruit (Fidelibus et al., 2008). Thus, studying fruit senescence will enhance understanding of a fundamental biological process and might provide the means to control senescence and improve agricultural traits of crop plants.

Low temperature (LT) has a positive effect on the post-harvest storage of fruit from harvest to human consumption, where lengthy storage for shipping, storing, and marketing is required. During that period, the main sources of damage to fruit are physiological disorders, such as senescence and water loss by evaporation, and pathogenic diseases. LT is usually chosen to extend storage life and maintain fruit quality during post-harvest storage, during which fruit quality declines gradually (Liu et al., 2011). Generally speaking, fruits taste good with a total soluble sugar/total organic acid ratio over 8 and LT is applied in inhibiting the ratio increase. Furthermore, fruit maintains greater flesh firmness and higher concentrations of organic acids and vitamin levels during cold storage. However, little information is available about cold signalling pathways and expression of cold-induced fruit-specific genes. Deciphering the mechanisms underlying LT-delayed fruit senescence will provide better understanding of the response to cold stress. Such knowledge is of great importance in developing breeding strategies designed to maintain fruit quality in post-harvest storage and improve LT tolerance in fruit.

In the past decade, gene expression and protein accumulation in fruit during post-harvest storage at LT has been studied by transcriptomic and proteomic approaches. Transcriptome analysis showed many genes change their expression (Maul et al., 2008; Vizoso et al., 2009) and proteomic analysis identified some differentially expressed proteins (Pan et al., 2009; Tanou et al., 2010; Yun et al., 2010). However, there is little information about fruit senescence and none about signal transduction or the regulation of gene expression in post-harvest fruit. Furthermore, cold-regulated genes have been estimated to represent up to 20% of the genome, and transcriptional responses to LT stress are quite variable, indicating that different sets of bio-physical and hormonal signals are integrated into a specific response for different species and tissues (Chinnusamy et al., 2007).

Hirado Buntan Pummelo (HBP; Citrus grandis × C. paradisi) is a popular variety of citrus fruit. Earlier, large differences of storage lifetime were found under different storage conditions, especially at different temperatures. Generally, HBPs are stored at ambient temperature (AT, 15–20 °C) for 2–3 months or at LT (8–10 °C) for 4–5 months. There are clear physiological differences between fruit stored under AT and LT, including concentrations of titratable acids, soluble solids, and other components of fruit quality and flavour (Yu et al., 2011). However, insufficient information is available for constructing a map of fruit senescence. The use of large-scale data for gene expression and protein accumulation will become available with the advent of proteomics and transcriptomics technology. LT-storage technology has been used for more than 100 years but little information is available concerning the transcriptional profiles and proteome associated with the maintenance of fruit quality during post-harvest storage under LT. In this study, fruit senescence was investigated at both the mRNA and protein levels to provide better understanding of the maintenance of the quality of citrus fruit during post-harvest storage at LT.

Materials and methods

Sample collection

HBP fruit at a commercially mature stage (ratio of total soluble sugar/total organic acid TSS/TOA greater than 8) was harvested in Changyang County, Hubei Province, China and was transported to Huazhong Agricultural University, Wuhan City, Hubei Province, China. Fruit weighing 150 kg was placed in a cold store at temperatures ranging from 8–10 °C and at a relative humidity of 85–90%. As a comparison, 150 kg fruit was also stored in storage room under AT (15–20 °C and 85–90% relative humidity). Juice sacs of two segments taken from each of ten fruits were sampled at 24, 48, 72, 96, and 120 days after harvest (DAH) and used directly for quality analysis or ground to powder in liquid nitrogen and stored at −80 °C.

Total protein extraction and two-dimensional gel electrophoresis

Proteins were extracted as described previously (Isaacson et al., 2006). Briefly, 6 g of frozen powder was suspended in 15 ml of homogenization medium [50% (v/v) phenol, 0.45 M sucrose, 5 mM EDTA, 0.2% (v/v) 2-mercaptoethanol, 50 mM TRIS–HCl, pH 8.8], shaken for 30 min at 4 °C, and then centrifuged for 30 min at
5000 g. The phenolic phase was collected and five volumes of 0.1 M ammonium acetate in 100% methanol was added to precipitate the proteins. The precipitate was collected by centrifugation for 30 min at 5000 g, washed twice with methanol, and then twice with acetone. After drying in a stream of dry nitrogen gas, protein pellets were dissolved in 0.6 ml of electrophoresis buffer (50 mM ammonium acetate, 0.5% CHAPS, 65 mM DTT, 0.2% ampholine, pH 4-7; Bio-Rad, Hercules, CA, USA). The protein concentration was measured with an RC DC protein assay kit (Bio-Rad) using BSA as the standard. The concentration of matrix solution (70% acetonitrile, 0.1% TFA, 10 mg ml⁻¹ α-cyano-4-hydroxy-cinnamic acid) and then spotted onto a stainless steel target plate. Tryptic peptides were analysed by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS) with a mass spectrometer (model 4800; Applied Biosystems, Framingham, MA, USA) to acquire MALDI and MS/MS spectra (Rizzarelli et al., 2006; Yamaguchi et al., 2010). The data were processed with Data Explorer (version 4.4, Applied Biosystems). The resulting peak lists were searched against the Viridiplantae non-redundant protein database at the NCBI homepage (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) using MASCOT v2.1.03 software (Matrix Science, London, UK). The search was performed using the following settings: green plants trypsin, one missed cleavage, fixed modifications of carboxamidomethyl, variable modifications of oxidation, peptide tolerance 100 ppm, fragment mass tolerance ±0.5 Da, peptide charge 1+. Only peptides with MS/MS ion scores significantly (P < 0.05) exceeding the MASCOT identity or extensive homology threshold were reported.

**Analysis of physiological characters**

Soluble sugars and organic acids were determined by gas chromatography (GC) using an Agilent instrument (model 6890N, Agilent, USA). Fruit flesh (3 g fresh weight) was ground into powder and suspended in 10 ml of 80% ethanol for 15 min at 75 °C in a water bath. It was then subjected to an ultrasonic bath for 45 min and centrifuged at 4000 g for 10 min. The supernatant was collected in a 50 ml volumetric flask. The precipitate was extracted twice with 80% ethanol, and each supernatant was recovered and added to the corresponding volumetric flask. After the addition of 1 ml of standard solution (0.025 g ml⁻¹ phenyl-β-D-glucopyranoside and 0.025 g ml⁻¹ methyl-α-D-glucopyranoside), the supernatant was diluted to 50 ml with 80% ethanol. A 1 ml sample of this stock solution was dried at 60 °C in a rotary evaporator (Concentrator 5301; Eppendorf, Hamburg, Germany) and the dry matter was dissolved in 0.8 ml of 0.3 M hydroxylamine hydrochloride in pyridine then heated at 75 °C for 1 h. Hexamethyl disilazane (0.4 ml) and chlorotrimethylsilane (0.2 ml) were added and the mixture was heated at 75 °C for 2 h. Finally, 0.5 ml of the supernatant was used for GC analysis (Bartolozzi et al., 1997) and three independent replicates were statistically analysed per sample using Student’s t-test.

The concentration of naringin was determined using high-performance liquid chromatography (HPLC). A sample (1 g fresh weight) was extracted in 10 ml of dimethyl sulfoxide/methanol solution (1:1) using an ultrasonic bath for 30 min. After dilution to 10 ml, the extract was filtered using 0.22 µm microporous film and then analysed by HPLC (Waters, USA) as described by Bailey et al. (1993). Ultraviolet absorbance was monitored at a wavelength of 285 nm, and the retention time was 2.6–2.8 min. The standard curve was linear within the range tested (0–100 µg ml⁻¹) and the coefficient of variation was <5% at 50 µg ml⁻¹. Three independent replicates were statistically analysed per sample using Student’s t-test.

Limonin and nomilin were measured by HPLC. Fruit flesh was ground into a powder in liquid nitrogen and then 3 g (fresh weight) was extracted with 15 ml of CH₃Cl₂ in a Soxhlet extractor (IKA Labortechnik, Staufen, Germany) over 15 cycles. The extract was evaporated to dryness at 60 °C in a rotary evaporator. The residue was dissolved in 1 ml of acetonitrile and filtered through microporous film (0.22 µm pore size). The concentration was determined by HPLC as described (Sun et al., 2005). Three independent replicates were statistically analysed per sample using Student’s t-test.

Ethanol, acetaldehyde, and methanol were extracted essentially as described (Mitcham and McDonald, 1993) but with minor modifications. Juice was collected from two segments of ten fruit (each sample) and stored at −80 °C. Juice samples were thawed, and a 4 ml sample was transferred to a 10 ml vial and capped with a septum. Samples were incubated at 65 °C for 30 min in a water bath. Using a glass syringe, 1 ml of headspace gas was collected.
and analysed for ethanol, methanol, and acetaldehyde using a GC instrument equipped with a Chromosorb 101 column and a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was 150 °C and the injection port temperature was 180 °C. Ethanol, acetaldehyde, and methanol were identified and quantified by comparison to peak areas from gas samples extracted after hydrolysis at 65 °C of 4 ml of a solution containing known concentrations of standards. Three independent replicates were statistically analysed per sample using Student’s t-test.

Total RNA extraction
Total RNA was extracted from the ten frozen samples corresponding to five different storage stages (24, 48, 72, 96, and 120 DAH) according to Liu et al. (2009) with moderate modification. A 3 g sample of powder was suspended in 20 ml of TRIzol reagent buffer (1 M guanidine thiocyanate, 1 M ammonium thiocyanate, 38% water-saturated phenol, 0.2 M sodium acetate, 5% glycerol). After gentle shaking and precipitation at room temperature for 15 min, 10 ml of chloroform was added to the suspension, shaken vigorously for 1 min then left to precipitate for 10 min. After centrifugation at 10,000 g for 15 min at 4 °C, an equal volume (15 ml) of cooled isopropanol was added to the supernatant, shaken gently, and left to precipitate for 10 min. After centrifugation at 10,000 g for 20 min at 4 °C, the pellet was recovered, washed with 5 ml of 75% ethanol, dried for 2–3 min, and then 800 μl of TESAR (10 mM TRIS–HCl, 1 mM EDTA, 1% N-laurylsarcosine sodium salt, pH 8.0), 800 μl of 0.07 M cetyltrimethylammonium bromide, and 800 μl of N-butanol were added and the mixture was centrifuged at 12,000 g for 6 min. The supernatant was recovered, 350 μl of 0.2 M NaCl was added, and the mixture was centrifuged at 12,000 g for 5 min. The precipitate was recovered, 50 μl of 3 M sodium acetate and 1 ml of ethanol were added, and the mixture was centrifuged at 12,000 g for 30 min at 4 °C. The precipitate was recovered, 20–50 μl of ultrapure water was added, and the solution was stored at −80 °C. Plastic consumables, ultrapure water, and reagents were treated with diethylpyrocarbonate to limit the degradation of mRNA.

Digital gene-expression profiling
Digital gene-expression (DGE) analysis was undertaken on fruit juice sacs at stage 72 DAH at both AT and LT. Samples (20 μg) of total RNA were sent to the Beijing Genomics Institute (Shenzhen) and prepared and sequenced using the Illumina Cluster Station and Genome Analyzer System (Solexa). Briefly, 6 μg of total RNA was used to purify mRNA with oligo(dT) adsorption beads and reverse transcribed, double-stranded cDNA was synthesized, and NlaIII was used to restrict at the CATG sites from the cDNA. cDNA fragments with the original 3′ ends were purified and Illumina adapter 1 was added at the 5′ ends and then restricted with MmeI at 17 bp downstream of the CATG site. Illumina adapter 2 was added at the 3′ ends, which finally acquired tags with different adapters at both ends to form a tag library. After 15 cycles of linear PCR amplification, 85 base strips were purified by TBE-PAGE in 6% polyacrylamide gels. The strips were digested and the single-chain molecules were fixed onto a Solexa Sequencing Chip (flow cell). Each molecule was grown into a single molecule cluster sequencing template through amplification in situ. Four types of nucleotides labelled by four different dyes at the 3′ ends were added for sequencing by synthesis. Each lane generated millions of raw reads with a sequencing length of 35 bp.

Analysis of DGE data
Clean tags were generated by removing the 3′ adaptor sequence, empty reads (reads with a 3′ adaptor sequence but no tag), low-quality tags (tags with unknown N′ sequences), tags that were too long or too short, and tags with a copy number of 1. To link the expressed signatures to known genes in the orange, the unique gene dataset for orange from The Institute for Genomic Research (TIGR) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=orange), the unigene dataset for Citrus clementina from TIGR (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=clementine), 597 orange transcription factors (http://plantfdb.cbi.pku.edu.cn/index.php?sp=ca), and 964 sweet orange cDNA sequences from the study laboratory were combined to form a reference gene dataset (Xu et al., 2009), and a preprocessed database of all possible CATG+17 nt tag sequences was created using reference gene sequences. All clean tags were mapped to the reference sequences and no more than 1 nt mismatch was allowed. Clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designated unambiguous clean tags and the number of these was calculated for each gene and then normalized to the number of transcripts per million (TPM) clean tags ( ’Hoen et al., 2008; Morrissy et al., 2009). Genes expressed differently in two samples were analysed as described (Audi and Claverie, 1997).

Determination of abscisic acid and ascorbic acid content
A 3 g sample (fresh weight) was ground into a powder in liquid nitrogen and extracted with 0.2 mM DTT in acetonitrile for 12 h at 0 °C. The supernatant was collected and condensed to dryness by rotary evaporation at 45 °C. Then 6 ml of 0.4 M phosphate buffer (pH 8.0), then washed twice with 2 ml of chloroform. Insoluble PVPP (150 mg) was added to the supernatant to remove polyphenol and the aqueous solution (3 ml) was acidified to pH 3.0 with formyl acid and extracted three times with 2 ml of ethyl acetate. The ester phase was collected and condensed at 45 °C and the precipitate was dissolved in 1 ml of chromatographically pure methanol. After passage through a microporous film (0.22 μm pore size), the ABA content was measured by HPLC analysis using a C18 column (150 × 3.9 mm). The analytes were separated using methanol/0.5% acetic acid (45:55, v/v) as the mobile phase and the flow rate was 1 ml min −1. The absorbance at a wavelength of 262.7 nm was measured and three independent replicates were statistically analysed per sample using Student’s t-test.

Ascorbic acid was extracted from a 0.5 g sample (fresh weight) using 4 ml of 0.2% metaphosphoric acid. The extraction was repeated twice and the supernatant was collected and made up to a volume of 10 ml using distilled water. A 1 ml sample of the ml solution was removed and filtered using 0.22 μm microporous filter, after which the contents were determined using HPLC. C18 reversed-phase column (Angelent Technology) was used with 0.2% metaphosphoric acid as the mobile phase and the column was eluted at 1 ml/min at 35 °C. UV detection was carried out at 243 nm. Three independent replicates were statistically analysed per sample using Student’s t-test.

Real-time quantitative reverse-transcription PCR verification
A sample of total RNA (~2 μg) was reverse transcribed for first-strand cDNA synthesis using the RevertAid First Strand cDNA synthesis kit (Fermentas, Lithuania) according to the manufacturer’s instructions. Real-time PCR used gene-specific primer pairs (Supplementary Table S1, available at JXB online) designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA) according to the sequence of the target gene in GenBank and was run on the ABI 7500 Real Time System (Applied Biosystems). Actin (GI:291419797) was used as the standard to normalize the content of cDNA as described (Liu et al., 2009). The primer pairs were: forward, 5′-CCACAGACAG-CATGAAGATCAA-3′; reverse: 5′-ATTCGTCGGAAGTCTG-GAG-3′; SYBR Green PCR Master Mix (Applied Biosystems) was used in the quantification of differential expression and 10 μl of the reaction mixture was added to each well. The thermal cycling programme was 50 °C for 2 min, 95 °C for 1 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Output data were
Results

Hirado Buntan pummelo (Citrus grandis × C. paradisi), a tropical and subtropical horticultural species with good LT tolerance, was used for LT-storage testing as part of an investigation designed to understand the mechanism of LT on maintenance of fruit quality during lengthy post-harvest storage. Fruits were chosen at random to be stored at LT or AT and were sampled at 24, 48, 72, 96, and 120 DAH. A proteome based on two-dimensional gel electrophoresis (2-DE) was used to monitor the overall difference in protein accumulation during storage at LT or AT. The physiological index was measured to show changes in fruit quality. Furthermore, to explore signal transduction pathways involved in the maintenance of fruit quality, comparative DGE profiling analysis was used at the 72 DAH stage (protein accumulation and physiological index in earlier studies indicated that 72 DAH is the crucial stage for maintenance of fruit quality). Endogenous hormones were assayed and the mRNA levels of selected genes were verified using quantitative reverse-transcription PCR.

2-DE gel analysis and identification of LT-storage stress-responsive protein in HBP juice sacs

To exploit the LT-storage stress-specific proteome in juice sacs of HBP fruit, 2-DE and MALDI-TOF MS/MS technology were used in this study. Spots were separated with pI 4–7 and a molecular mass of 10–100 kDa (Fig. 1). Representative gels are shown in Fig. 2 and >1000 spots were resolved reproducibly in each analysis gel. Comparative analysis and statistical analysis (Student’s t-test) revealed a total of 108 spots with significantly different accumulation (P < 0.05) and a difference of more than twofold at one or more time point(s) between AT and LT storage. The total number of LT down-regulated proteins was at a minimum (40) at 72 DAH; in contrast, the number of LT up-regulated proteins (total) was at a maximum (68) at 72 DAH (Table 1). The results suggest that 72 DAH is a crucial stage if fruit quality is to be maintained by LT for the whole duration of post-harvest storage.
information for the proteins is given in Table 2. To obtain functional information about the proteins identified, the literature and the annotated biochemical and biological functions based on the Blast2GO program was searched. Ten proteins were classified as unknown; the other 53 proteins were classified into seven functional categories. The results showed that the categories with considerable enrichment were metabolism, containing sugars and polysaccharide metabolism (5), secondary metabolism (5), other metabolism (8), protein destination and storage (12), and response to stimulus (12), indicating that these processes play a leading role in the maintenance of fruit quality at LT (Fig. 3A). Proteins with known functions were also categorized using the Blast2GO program based on biochemical activity or cellular position. Hydrolase activity (8), nucleotide binding (9), protein binding (10), and transferase activity (9) were the most abundant biochemical activity categories, which implied that hydrolysis and transportation of substrates are vital for the maintenance of fruit quality (Fig. 3B). For cellular positions, plasma membrane was the largest group of proteins (16), followed by plastid (15), mitochondrion (13), and nucleus (10) (Fig. 3C). These results showed that most of the differentially accumulated proteins are localized to the plasma membrane, plastid, mitochondrion, and nucleus.

**LT storage maintains fruit quality through decreased physiological index metabolism**

Fruit taste is determined by the content and type of soluble sugars and organic acids, which have an impact on the organoleptic quality of fruit. In ripe citrus fruit, the main soluble sugars are sucrose, fructose, and glucose. The main organic acid is citric acid, malic acid, and quinic acid. The trends in organic acid and soluble sugar contents in HBP juice sacs (fresh weight) during post-harvest storage are shown in Fig. 4. The malic acid concentration decreased gradually with increased storage time and obvious difference was observed between 24 DAH and 96 DAH (Fig. 4A). Between 72 DAH and 120 DAH, citric acid, the main organic acid (>90%) in this fruit, showed a greater increase under LT than under AT (Fig. 4B). Sucrose concentration increased during storage and was higher under LT than under AT at 72 DAH, whereas fructose and glucose concentrations were lower at 72 DAH under LT compared to AT (Fig. 4D-F). Taken together, LT maintained/increased the citric acid, sucrose, and TOA concentrations in the fruit but decreased the concentrations of fructose, glucose, TSS, and the ratio of TSS/TOA, especially at 72 DAH. This suggested that 72 DAH was the turning point in the accumulation of hexoses and citric acid under AT and LT, and that 72 DAH is a crucial time point for the levels of both protein and physiological characters.

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**Table 2. Number of proteins accumulated during storage**

A total of 108 differentially accumulated protein spots were detected during low temperature (LT) storage using two-dimensional gel electrophoresis. Protein accumulation regulated by LT was divided into four categories. The first category proteins were down-regulated more than two-fold (<0.5). Second category proteins were down-regulated less than two-fold (0.5–1). Third category proteins were up-regulated less than two-fold (1–2) and fourth category proteins were up-regulated more than two-fold (>2). DAH, days after harvest.

| Storage period | LT down-regulated proteins | LT up-regulated proteins |
|----------------|---------------------------|--------------------------|
|                | <0.5 | 0.5–1 | Total | 1–2 | >2 | Total |
| 24 DAH         | 14   | 41    | 55    | 30  | 23 | 53    |
| 48 DAH         | 10   | 43    | 53    | 33  | 22 | 55    |
| 72 DAH         | 15   | 25    | 40    | 44  | 24 | 68    |
| 96 DAH         | 18   | 29    | 47    | 44  | 17 | 61    |
| 120 DAH        | 22   | 31    | 53    | 27  | 28 | 55    |

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**Fig. 2.** Representative two-dimensional electrophoresis profiles of proteins from Hirado Buntan Pummelo during post-harvest under ambient temperature and low temperature. The juice sacs from two segments of ten individual fruits were sampled at 24, 48, 72, 96, and 120 days after harvest (DAH). A total of ten samples were harvested from the two storage conditions at 24 d intervals from 3 December 2008 to 1 April 2009. Proteins were extracted using phenol extraction protocol. Proteins (100 μg) were separated in the first dimension on IPG strip (17 cm, pH 4–7) and in the second dimension on a 15% SDS-PAGE gel. Gels were visualized by silver staining.
Table 2. Identities of LT-storage stress-responsive proteins in the juice sacs of Hirado Buntan Pummelo fruit

A total of 108 spots were identified using Applied Biosystems 4800 matrix-assisted laser desorption/ionization-time-of-flight tandem mass spectrometry and 63 proteins were successfully identified based on tryptic peptide sequences. As the citrus genome plan has not been completed, the peptide list was searched against Viridiplantae protein. The letter U in the protein identification number shows that the proteins were up-regulated by low temperature (LT) storage stress during storage, the letter D in the protein identification number shows that the proteins were down-regulated by LT, and the letter C represents proteins that had changed expression during storage. Protein accumulation is represented by the column configuration, and accumulation at 24, 48, 72, 96, and 120 DAH (days after harvest) is shown from left to right. Identification and accession numbers (protein name and GI number) are from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/guide/). Experimental molecular mass and pI (Exp. M/pI) were calculated by PDQuest, and mass is shown in kDa. Theoretical molecular mass and pI (Theo. M/pI) were available on NCBI. PC, peptide count; MS, mascot score. The protein accumulation under ambient temperature is shown as a white column and the protein accumulation under LT is shown as a gray column.

| No. | Protein accumulation | Protein name | Source organism | GI number | Exp. M/pI | Theo. M/pI | PC | MS |
|-----|----------------------|--------------|-----------------|-----------|-----------|-----------|----|----|
|     | Response to stimulus: 12 |              |                 |           |           |           |    |    |
| U1  |                      | Lactoylglutathione lyase, putative/ glyoxalase I, putative | Arabidopsis thaliana | 15220397 | 27.56/5.56 | 39.15/6.97 | 12 | 200 |
| U2  |                      | Cysteine protease inhibitor | Populus tremula | 52851070 | 21.55/4.54 | 15.59/6.51 | 3  | 81  |
| U3  |                      | Harpin binding protein 1 | Citrus paradisi | 38679311 | 26.32/6.00 | 31.28/9.61 | 7  | 300 |
| D1  |                      | ATARD2; acireductone dioxygenase [iron(II)-requiring]/metal ion binding | Arabidopsis thaliana | 18414289 | 22.16/6.06 | 23.35/4.99 | 5  | 104 |
| C1  |                      | Glyoxalase I | Glycine max | 4127862 | 19.35/5.63 | 20.95/6.51 | 4  | 63  |
| C2  |                      | Chitinase | Trifolium repens | 13560120 | 35.16/4.37 | 32.24/8.96 | 2  | 63  |
| C3  |                      | Basic chitinase class 3 | Vigna unguiculata | 871764 | 25.56/6.31 | 32.09/9.04 | 4  | 84  |
| C4  |                      | Cysteine synthase | Spinacia oleracea | 1066153 | 34.82/4.49 | 39.49/8.67 | 4  | 93  |
| C5  |                      | Cysteine protease inhibitor | Populus tremula | 52851070 | 21.73/4.51 | 15.59/6.51 | 3  | 79  |
| C6  |                      | Allyl alcohol dehydrogenase | Nicotiana tabacum | 6692816 | 35.41/4.38 | 38.07/6.56 | 5  | 91  |
| C7  |                      | Putative aldehyde dehydrogenase | Oryza sativa japonica group | 47900421 | 51.83/4.69 | 56.37/8.66 | 5  | 79  |
| C8  |                      | Mitochondrial aldehyde dehydrogenase | Sorghum bicolor | 20530129 | 51.13/4.87 | 58.76/6.65 | 6  | 88  |
|     | Protein destination and storage: 12 |              |                 |           |           |           |    |    |
| U4  |                      | Temperature-induced lipocalin | Citrus sinensis | 77744899 | 18.45/4.36 | 21.56/6.33 | 3  | 128 |
| U5  |                      | Putative membrane protein | Zea mays | 9998903 | 29.56/5.81 | 31.34/5.04 | 8  | 228 |
| U6  |                      | COR15 | Citrus clementina | 37524017 | 17.45/4.54 | 15.23/6.54 | 5  | 80  |
| No. | Protein accumulation | Protein name                                                                 | Source organism                  | GI number     | Exp. M/p | Theo. M/p | PC | MS |
|-----|----------------------|-------------------------------------------------------------------------------|----------------------------------|---------------|----------|-----------|----|----|
| U7  |                      | Multicatalytic endopeptidase complex, proteasome component, alpha subunit    | Arabidopsis thaliana             | 2511588       | 28.12/4.63 | 27.15/5.6 | 3  | 67 |
| U8  |                      | Small heat shock protein                                                      | Pseudotsuga menziesii           | 21068486      | 16.72/4.12 | 18.17/5.51 | 6  | 78 |
| D2  |                      | Chaperonin hsp60                                                             | Arabidopsis thaliana             | 16221         | 60.48/5.49 | 61.66/5.66 | 2  | 59 |
| C9  |                      | High-molecular-weight heat shock protein                                      | Malus domestica                  | 6969976       | 33.78/4.62 | 71.18/5.17 | 13 | 361|
| C10 |                      | 26S Proteasome regulatory complex ATPase RPT3                                 | Zea mays                         | 162462419     | 47.21/5.53 | 38.81/5.62 | 8  | 114|
| C11 |                      | 17.7 kDa Class I small heat shock protein                                     | Lycopersicon esculentum          | 4836469       | 15.15/4.12 | 17.73/5.84 | 5  | 85 |
| C12 |                      | 17.5 kDa Class I heat shock protein                                          | Carica papaya                    | 38639431      | 16.45/5.25 | 17.48/5.31 | 5  | 67 |
| C13 |                      | Heat shock protein 70                                                         | Saussurea medusa                 | 21327033      | 75.59/5.87 | 70.76/5.14 | 15 | 285|
| C14 |                      | HSP68 (68 kDa heat-stress DnaK homologue)                                    | Lycopersicon peruvianum          | 300265        | 66.59/5.54 | 62.35/5.2  | 11 | 270|

Sugars and polysaccharides metabolism: 5

| D3  |                      | UDP-o-glucuronate decarboxylase                                              | Hordeum vulgare                  | 50659026      | 36.98/4.37 | 38.95/7.1  | 13 | 282|
| C15 |                      | Caffeoyl-CoA-O-methyltransferase                                             | Broussonetia papyrifera          | 47680455      | 21.05/5.56 | 27.72/5.31 | 8  | 107|
| C16 |                      | Unnamed protein product                                                      | Arabidopsis thaliana             | 8953711       | 67.15/4.49 | 67.38/5.83 | 8  | 84 |
| C17 |                      | UDP-glucose dehydrogenase                                                    | Cinnamomum osmophloeum           | 40317278      | 48.12/4.52 | 52.9/5.99  | 6  | 180|
| C18 |                      | delta-1-Pyrroline-5-carboxylate dehydrogenase precursor                     | Arabidopsis thaliana             | 15383744      | 52.32/4.53 | 61.74/6.26 | 7  | 83 |

Secondary metabolism: 5

| D4  |                      | Putative thiamine biosynthesis protein                                       | Oryza sativa japonica group      | 27261025      | 29.48/5.50 | 36.93/5.44 | 5  | 67 |
| C19 |                      | Thiazole biosynthetic enzyme, chloroplastic                                  | Citrus sinensis                  | 6094476       | 32.16/5.92 | 37.58/5.4  | 7  | 162|
| C20 |                      | Plastid-lipid-associated protein, chloroplastic                             | Citrus unshiu                    | 62900641      | 28.26/6.51 | 35.2/5.24  | 7  | 197|
| No. | Protein accumulation | Protein name | Source organism | GI number | Exp. M/p | Theo. M/p | PC | MS |
|-----|----------------------|--------------|-----------------|-----------|----------|----------|----|----|
| C21 |                      | Isoflavone reductase-related protein | Pyrus communis | 3243234  | 33.12/4.37 | 33.81/6.02 | 3  | 83 |
| C22 |                      | Hypothetical protein, partial | Vitis vinifera | 225466585 | 18.26/5.62 | 40.60/7.08 | 3  | 51 |
|     | Other metabolism: 8  |              |                 |           |          |          |    |    |
| U9  |                      | Copia-type polyprotein, putative | Arabidopsis thaliana | 12321254 | 28.46/6.48 | 151.77/8.67 | 22 | 68 |
| D5  |                      | Protein disulfide isomerase | Zea mays | 162461925 | 34.92/3.68 | 40.04/6.29 | 8  | 79 |
| D6  |                      | Importin alpha 1b | Oryza sativa japonica group | 6682927  | 59.56/5.84 | 58.5/5.18 | 5  | 110 |
| C23 |                      | Seed maturation protein PM31 | Glycine max | 4838149  | 16.65/4.82 | 17.74/6.1  | 4  | 64 |
| C24 |                      | Importin alpha 2 | Capsicum annuum | 13752562 | 60.12/5.87 | 58.44/5.32 | 7  | 194 |
| C25 |                      | ATARD2; acireductone dioxygenase [iron(II)-requiring]/metal ion binding | Arabidopsis thaliana | 18414289 | 22.89/6.12 | 23.35/4.99 | 3  | 83 |
| C26 |                      | Transitional endoplasmic reticulum ATPase | Arabidopsis thaliana | 11265361 | 99.35/5.61 | 93.56/5.37 | 15 | 128 |
| C27 |                      | Kinetochore protein | Brassica juncea | 18958255 | 18.87/6.61 | 17.98/4.51 | 5  | 70 |
|     | Energy: 7             |              |                 |           |          |          |    |    |
| U10 |                      | Pyruvate dehydrogenase E1a-like subunit IAR4 | Arabidopsis thaliana | 23306664 | 39.15/4.32 | 43.29/7.62 | 7  | 68 |
| D7  |                      | NAD-malate dehydrogenase | Nicotiana tabacum | 5123836  | 33.45/5.29 | 43.28/8.03 | 8  | 321 |
| D8  |                      | Malate dehydrogenase | Glycine max | 53290964 | 71.21/5.82 | 36.12/8.23 | 4  | 90 |
| C28 |                      | Aconitase | Lycopersicon pennellii | 29027432 | 98.15/4.30 | 98.07/6.07 | 7  | 86 |
| C29 |                      | Malate dehydrogenase | Vitis vinifera | 7798706  | 34.56/4.67 | 36.86/8.79 | 2  | 188 |
| C30 |                      | Putative soluble inorganic pyrophosphatase | Oryza sativa | 15451551 | 24.18/5.21 | 22.26/5.71 | 6  | 74 |
| C31 |                      | Carbonic anhydrase | Solanum lycopersicum | 56662177 | 23.82/5.60 | 34.45/6.67 | 5  | 99 |
|     | Signal: 4             |              |                 |           |          |          |    |    |
| D9  |                      | Abscisic stress ripening-like protein | Glycine max | 38679405 | 14.40/5.02 | 25.34/5.58 | 2  | 118 |
Secondary metabolites are a vital indicator of fruit quality, especially with regards to human health. Fig. 5 shows the indexes of metabolites of secondary metabolism and anaerobic respiration during AT and LT storage. In this study, the concentrations of limonin and nomilin were higher when stored under LT than under AT at 48 DAH and 120 DAH (Fig. 5B, C). In addition, naringin content was higher under LT storage than under AT at 72 DAH and 120 DAH, but lower at 48 DAH (Fig. 5A). Alcohol metabolites, which were found to be volatile organic compounds, are important markers of fruit quality. In this present study, methanol and aldehyde concentrations were higher when fruit was stored under LT than under AT at 48, 96, and 120 DAH (Fig. 5D, E). However, no obvious change was detected in ethanol concentration between LT and AT storage (Fig. 5F). In summary, LT increased the concentrations of limonin, nomilin, methanol, and aldehyde.

**DGE profiling of specific genes in response to LT-storage stress**

To further understand the delay of senescence in the fruit at LT, regulation of gene expression was investigated using comparative DGE profiling analysis. According to the proteome data and the physiological index, 72 DAH is the inflection point in the maintenance of fruit quality under different storage conditions. Therefore, samples of 72 DAH at AT and LT were used for the construction of DGE libraries. The level of gene expression at LT and AT is highly correlated ($r = 0.999$). After removal of the 3’ adaptor sequence, empty reads, low-quality tags, tags too long or
too short (leaving tags of 21 nt long), and tags with a copy number of 1, there were 3,430,217 and 3,700,516 successful sequences (clean tags) produced for LT and AT, respectively, and from the clean tag sets, 57,898 and 77,460 distinct tags were observed for LT and AT, respectively (Supplementary Table S2). The distribution of total clean tags was quite similar at LT and AT. Tag copy numbers of 3–5 at AT and LT accounted for 3.33% and 2.86% of total clean tags and contributed 56.2% and 59.89% of total distinct clean tags, respectively, indicating that genes with low transcripts are abundant in both libraries.

To link the tags to known genes, a unigene dataset containing 26,826 contigs and 73,607 singletons was used for DGE tag mapping analysis. The results showed that 7916 and 10,003 genes were detected at LT and AT, respectively (Supplementary Table S2). Gene expression was calculated using the number of clean tags for each gene normalized to TPM according to Morrissy et al. (2009). Analysis of differential expression at LT coared to AT revealed 3133 down-regulated genes and 336 up-regulated genes. Gene ontology categories were assigned to the 3469 genes with significantly differential expression using the Blast2GO program (http://www.blast2go.org/start_blast2go#download_blast2go) to evaluate the potential functions of genes that showed significant transcriptional differences between AT and LT. The genes were finally classified into 13 categories on the basis of biological processes, as shown in Fig. 6A. Primary metabolic process (873), response to stimulus (328), nitrogen compound metabolic process (285), and transport (254) were the major categories, which is consistent with the data for the proteins. The significant enrichment categories classified on the basis of molecular function were protein binding (376), hydrolase activity (355), nucleic acid binding (297), transporter activity (159), and kinase activity (159), which correspond well to the protein data (Fig. 6B). The genes were classified on the basis of cellular components into cytoplasm (1204), plastid (500), mitochondrion (368), plasma membrane (263), and nucleus (253). Among them, plastid, mitochondrion, plasma membrane, and nucleus were the major subcellular organelles in the response to LT-storage stress, which is in accord with the protein data except the cytoplasm category (Fig. 6C).
ABA is active in fruit developmental processes and ABA-mediated signalling plays an important role in plant responses to cold stress. In this study, the concentration of ABA was lower under LT than AT (Fig. 7A). It decreased sharply under LT at 48 DAH and then maintained a low concentration range during later LT storage. That implied LT decreased ABA concentration during lengthy storage. In addition, ascorbic acid showed no obvious trend with LT-storage stress (Fig. 7B).

LT-stress-induced proteins and genes

In the present study, many proteins were shown to be regulated by cold stress, including cold-responsive (COR) genes, heat-shock proteins (HSPs), temperature-induced lipocalins (TILs), chitinase, cysteine synthase, cysteine protease inhibitor, allyl alcohol dehydrogenase, putative aldehyde dehydrogenase, and mitochondrial aldehyde dehydrogenase (Table 2). In particular, small HSPs and high-molecular-weight HSPs showed a large accumulation under LT (Fig. 8, Table 2). HSPs (CK701553, CN188563, TC14491, TC16014, TC15728, and CB290599) were up-regulated in response to LT stress, but 21 mRNA transcriptions (TC6871, TC16109, TC21784, EY655246, TC13533, TC19601, TC22565, TC11247, TC22289, TC14057, TC6139, TC13664, TC6325, TC19918, EY756065, TC2423, TC12712, EY705442, TC18120, TC14678 and TC14726) were down-regulated (Excel S1). Interestingly, calcineurin B-like proteins (CBLs) 1 and 4 were up-regulated, whereas CBLs 3 and 10 were down-regulated (Excel S1). Surprisingly, no C-repeat binding factor (CBF) was detected in either the protein data or the DGE data, but COR15, CBF regulated gene, was shown to be up-regulated, which might be because of a potential detection failure of the proteome and transcriptome technology or possibly incomplete citrus genome information.

LT storage delays fruit senescence by inhibiting expression of metabolic genes

A total of 1450 down-regulated genes were detected in DGE were analysed using the BINGO plugin in Cytoscape. The results showed 1036 genes were metabolism-associated genes, mainly concerned with carbohydrate metabolism,
nitrogen compound metabolism, lipid metabolism, macromolecule metabolism, secondary metabolism, and alcohol metabolism (Fig. S1).

A total of 117 genes were involved in carbohydrate metabolism identified in the differential test, 111 of which are downregulated and six are upregulated (Table 3). The suppressed genes were involved mainly in glycolysis, the citrate cycle, and other sugar metabolic pathways, including sucrose, starch, glucan, cellulose, and galactose metabolism. It is noteworthy that the mRNA levels of glycosyl-related genes, especially the glycosyl hydrolase family including members 2, 13, 17, 20, 28, and 38, were decreased in response to LT stress (Supplementary Excel S1). Downregulation of glycosyl hydrolases delays degradation of the plant cell wall (Lee et al., 2007), which is the main parameter of fruit senescence.

In this study, all 31 genes of secondary metabolism, mainly flavonoids, carotenoids, vitamins, and proteins, were down-regulated under LT stress (Table 3). In addition, 82 of 84 lipid metabolic genes, which encode phospholipases (TC16578, TC17789, EY686225, and TC13892) and acyl carrier proteins (TC4318, TC13606, TC13779, TC23479, TC6769, TC7499, TC6017, TC13708, and TC4898), were down-regulated by LT in DGE profiling (Supplementary Excel S1).

A total of 198 transporter genes were detected in DGE data, which were analysed using the BINGO plugin in Cytoscape (Fig. S2). Only eight genes, predicted to encode...
transporters for urea and water \((P < 0.05)\), were up-regulated by LT and involved in water loss. Many of the genes predicted to encode ion transport, vesicle-mediated transport, mitochondrial transport, protein transport, secretion, and intercellular transport were down-regulated by LT (high reliability, Fig. S2).

In summary, LT storage maintained fruit quality not only by inhibiting the degradation of macromolecules (such as polysaccharides, proteins, and lipids) and nutritional substances (such as sugars and organic acids), but also by inhibiting the transportation of substrates during lengthy storage.

**Signal transduction in citrus fruit senescence is regulated by sugar, auxin, and ABA**

Sugars, as crucial components in fruit quality, were also involved in signal transduction. In the present study, the fructose and glucose contents decreased under LT, especially at 72 DAH (Fig. 4). Furthermore, some genes which encode glucose- and fructose-binding proteins (such as TC11892, TC12351, TC13119, TC18748, TC3277, TC608, TC9110, CX046230, TC23285, TC25884, TC4031, and TC4180), which might play a role in signal transduction (Excel S1), were down-regulated by LT.

Auxin is involved in various aspects of growth and development. In the present study, all eight genes which encode auxin response factor proteins (TC13217, TC23352, TC4914, CK936688, TC25995, TC14087, TC10814, and TC19428) were down-regulated under LT and one auxin-repressed protein (TC22895) was up-regulated (Excel S1). It is interesting that all four genes which encode auxin-induced proteins (TC19324, TC19324, TC8111, EY714452, and TC1904) were down-regulated (Excel S1), and IAA9 (indoleacetic acid-induced protein 9) transcription factor decreased at the protein level during later LT storage (Table 2). This suggests that LT inhibits fruit senescence by down-regulation of auxin-induced proteins or up-regulation of auxin-repressed protein.

The phytohormone ABA is a major endogenous factor and the primary signal that regulates fruit development, maturation, and senescence. In this study, ABA content decreased sharply (by nearly 90%) from 24 DAH to 120 DAH under LT (Fig. 7). ABA-related protein and abscisic stress ripening-like protein was down-accumulated under LT (Table 2), and ABA-related genes, such as ABA-responsive like protein, ABA and ripening-inducible-like protein, ABA-responsive hva22 family protein, and ABA-responsive element-binding protein 2, were down-regulated under LT (Supplementary Excel S1). In addition, differentially expressed genes were detected by DGE (analysed using the CLUGO plugin in Cytoscape). This showed that ABA participated in the regulation of fruit senescence (Fig. S3). Moreover, no ABA-regulated genes or proteins were up-regulated by LT stress in this study, suggesting that ABA is not involved in the LT stress response in citrus fruit during post-harvest storage. Furthermore, 86 signal transduction genes detected in DGE were analysed using the
including COR genes, HSPs, and TILs. These genes and proteins are known to play a significant role in the response to cold stress, but their exact role in regulating citrus fruit senescence is still under investigation. Ascorbic acid (Fig. 7B) plays a crucial role in protecting plants against stress by re-establishing normal protein conformation, thereby helping to maintain cellular homeostasis (Wang et al., 2004).

In addition, TILs have been shown to be responsive to temperature stress, by suppressing sensitivity to oxidative stress, reducing the accumulation of lipid peroxidation products, and alleviating temperature-induced oxidative stress on plant membranes (Chi et al., 2009).

Other proteins are accumulated under LT, such as putative membrane protein, multicatalytic endopeptidase complex, proteasome component, alpha subunit, putative lactoylglutathione lyase, putative glyoxalase I, cysteine protease inhibitor, and harpin binding protein 1 (Table 2). However, little information is available concerning fruit protein conformation, thereby helping to maintain cellular homeostasis (Wang et al., 2004).

In summary, fruit endured LT during post-harvest storage through up-regulation of cold-resistant genes and accumulation of cryoprotective proteins.

**Ca^{2+}-dependent signal transduction of LT stress**

LT can reduce the fluidity and increase the rigidity of plant cell membranes. Plant cells can sense cold stress through LT-induced changes in membrane fluidity, protein and nucleic acid conformation, and/or concentration of metabolites (Chinnusamy et al., 2007). Ca^{2+}-decoding mechanisms within the plant cell appear to be more complex than those in other organisms (Luan, 2009). The CBL–CBL-interacting protein kinase (CIPK) network represents an example of significantly diverged Ca^{2+}-decoding paradigms that are unique to plants (Hirayama and Shinozaki, 2010). Moreover, a more detailed analysis of the Arabidopsis genome revealed a multigene CBL-encoding family with at least ten members (Luan et al., 2002), and CIPKs are most likely to represent targets of calcium signals sensed and transduced by CBL proteins (Kolukisaoglu et al., 2004). Distinct CBL–CIPK complexes play a role in a different signalling pathways.
pathway (Luan et al., 2002). It is reported that CBLs 1 and 4 were up-regulated under cold stress in Arabidopsis (Cheong et al., 2003) and CBLs might be correlated with senescence (De Michele et al., 2009). Therefore, CBL–CIPK complexes might be involved in the signal transduction of both LT stress and senescence. In addition, a family of Arabidopsis transcription factors, the CBF proteins, have been reported to control the expression of a regulator of cold-induced genes that increases freezing tolerance (Thomashow, 1999). Surprisingly, in this study, no CBF was detected in the differential expression profiles at the protein or mRNA level but the genes downstream of CBF were up-regulated, which might be the result of detection failure by the proteome and transcriptome technology or incomplete citrus genome information. Supplies of exogenous Ca\(^{2+}\) are no longer available for harvested fruit. However, Ca\(^{2+}\)-mediated signal
transduction might still be involved in both LT stress response and senescence, because the Ca\(^{2+}\) signal is presented not only by the concentration of Ca\(^{2+}\) but also by its spatial and temporal information (Allen et al., 2001).

**LT storage delays fruit senescence by inhibiting expression of metabolic genes**

During the post-harvest storage of fruit, although LT caused a decrease in most enzyme activity and inhibited the expression of many genes, fruit quality was still maintained (Jin et al., 2009a). Fruit quality is very complex and is affected by a mixture of sugars, acids, amino acids, minerals, volatile compounds, colour, and flavour, including major biochemical and sensory changes (Ogundiwun et al., 2009). Complex metabolic processes are involved in maximizing and maintaining fruit quality from production and processing through to marketing and consumption (Ogundiwun et al., 2009). In this study, LT storage decreased fruit metabolism, including reducing carbohydrate metabolism, nitrogen compound metabolism, lipid metabolism, macromolecule metabolism, and secondary metabolism. Interestingly, citric acid content was maintained under LT but glucose and fructose concentrations decreased significantly under LT after 72 DAH (Fig. 4). Hexose may be converted to citric acid during lengthy LT storage. Alternatively, glycosyl hydrolases, which catalyse the hydrolysis of glycosidic linkages to release smaller sugars, are extremely common enzymes with roles in nature including the degradation of biomass, such as cellulose and hemicellulose in the plant cell wall (Lopez-Casado et al., 2008). Down-regulation of glycosyl hydrolases delays degradation of the plant cell wall (Lee et al., 2007), and thereby inhibits fruit senescence.

Secondary metabolites are crucial components of fruit quality (Carli et al., 2009). Interestingly, most secondary metabolism genes were down-regulated under LT but the concentrations of naringin, limonin, and nomilin increased under LT. As limonin and nomilin have antioxidant capacity and might be involved in the cold stress response (Sun et al., 2005), the accumulation of naringin, limonin, and nomilin is likely to play a vital role in fruit response to LT stress.

Lipid metabolism has an important cold-tolerance role in plants (Badea and Basu, 2009). Plant membrane lipids have a tendency to change from a gel to a liquid-crystalline phase in response to LT stress by increasing the concentration of unsaturated fatty acids present (Badea and Basu, 2009). Membrane degradation is an early manifestation of senescence (Thompson et al., 1998) and the down-regulation of lipid-degrading genes would inhibit fruit senescence.

Alcohol metabolites, which were found to be volatile organic compounds, have been used for many years for sterilization and for their fungicidal and insecticidal properties (Pesis, 2005). Acetaldehyde and ethanol have been shown to retard senescence and inhibit ethylene production in plants, leading to fewer cold shock-related injuries in various fruits (Pesis, 2005). LT induced the accumulation of acetaldehyde and methanol, which not only inhibited fruit ripening and senescence by the inhibition of respiration and interference with normal biosynthesis (Podd and Van Staden, 1998), but also had an effect on fruit response to LT stress.

Fruit storage is a nutrient-maintaining and self-consuming process. Little information is available on substrate transportation during storage. Out of a total of 198 transporter genes, only eight genes were up-regulated by LT. The remaining genes were down-regulated by LT and are thought to encode for ion transport, vesicle-mediated transport, mitochondrial transport, protein transport, secretion, and intercellular transport. Therefore, the intercommunication of intercellular substrates is not unique. The communication between organelles, between cells, and between juice sac and pericarp is reduced in response to LT stress.

In summary, a distinct feature of fruit senescence is the substantial degradation of macromolecules (such as polysaccharides, proteins, and lipids) and nutritional substances (such as sugars and organic acids) during the latter stages of storage. This study suggests that LT maintained fruit quality mainly by inhibiting fruit senescence and inhibiting the transportation of substrates.

**Sugar-, auxin- and ABA-mediated fruit senescence during post-harvest storage**

Fruit senescence is the final stage in fruit development and maturation. Senescence is not simply an aging-dependent passive death, it is a tightly organized and controlled process in which cell components are degraded in a coordinated fashion (Xue-Xuan et al., 2010). To date, the ripening mechanisms of climacteric fruit, especially the effect of ethylene, have been well studied (Alexander and Grierson, 2002). Sugars are important signals in the regulation of plant metabolism and development, and sugar accumulation can

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**Table 3.** Functional categorization of chilling-responsive genes in Hirado Buntan Pummelo fruit

| Term                        | Total | Up  | Down | Graph score |
|-----------------------------|-------|-----|------|-------------|
| Primary metabolic process   | 873   | 61  | 812  | 430.5       |
| Response to stimulus        | 328   | 43  | 285  | 331.2       |
| Nitrogen compound metabolic process | 285 | 18 | 267  | 64.14       |
| Transport                   | 254   | 24  | 230  | 224         |
| Protein modification process| 200   | 21  | 179  | 200         |
| Catabolic process           | 166   | 13  | 153  | 166         |
| Transcription               | 127   | 11  | 116  | 127         |
| Carbohydrate metabolic process | 117 | 6  | 111  | 117         |
| Signalling                  | 100   | 5   | 95   | 21.98       |
| Translation                 | 88    | 4   | 84   | 88          |
| Lipid metabolic process     | 84    | 2   | 82   | 84          |
| Secondary metabolic process | 31    | 0   | 31   | 31          |
| Cell communication          | 10    | 2   | 8    | 9.6         |
induce senescence (Wingler and Roitsch, 2008). Moreover, senescence is influenced by plant hormones, including ethylene, ABA, and auxin.

Sugars, which are vital to fruit quality, apparently serve as signalling molecules that might alter the biochemical or developmental processes in the fruit (Jin et al., 2009b). In the present study, the fructose and glucose contents decreased under LT, and some glucose- and fructose-binding proteins were down-regulated by LT, which might play a role in signal transduction (Moore et al., 2003). This does not agree with the plant model, in which sugars are accumulated under LT in response to cold stress (Xue-Xuan et al., 2010). It is likely to be the intrinsic trait of post-harvest citrus fruit. LT decreased the glucose and fructose contents, then the hexose-transferred signal, and, finally, fruit senescence was inhibited.

Evidence suggests that auxin is involved in the senescence process (Lim et al., 2010); however, the role of auxin in senescence has been elusive, particularly because of its involvement in various aspects of growth and development. Most of these processes are initiated and/or mediated through auxin-regulated gene expression (Guilfoyle and Hagen, 2007). Auxin response factors are repressors of auxin signalling, which modulates early auxin-induced gene expression in plants (Tiwari et al., 2003). The decreased auxin response factors accelerated the auxin signal and then inhibited senescence (Lim et al., 2010).

ABA is essential for the induction and maintenance of fruit maturation and senescence (Cara and Giovannoni, 2008). In the present study, LT decreased ABA content, down-regulated ABA-related genes, and delayed fruit senescence. However, ABA is required for plant adaptation to environmental stress and the ABA-mediated stress response is the transcriptional regulation of stress-responsive gene expression (Knight and Knight, 2001). Many genes in vegetative tissues have been reported to be up-regulated under stress conditions (Xue-Xuan et al., 2010). ABA signal transduction might be a characteristic present in citrus fruit during post-harvest storage, which is not involved in LT stress. Ethylene and ROS are reported to be intracellular messengers and have been proven to have a crucial function in the signalling network operation of senescence (Xue-Xuan et al., 2010). As reported in tomato by Zhang et al. (2009), the ABA content possibly precedes ethylene production in fruit flesh. However, in this study no ethylene-related protein was found among the differentially accumulated proteins, and some ethylene-related genes changed their expression slightly. Additionally, endogenous ethylene is ineffective in inducing the ripening and senescence of non-climacteric fruit (Malladi and Burns, 2008), which means ethylene might not participate in ABA-mediated citrus fruit senescence. Moreover, fruit senescence has been reported to be an oxidative phenomenon in apple, which is regulated by ROS through changing the expression profiles of the mitochondrial proteins superoxide dismutase and oxidoreductase (Qin et al., 2009). However, no obvious change in the expression of the ROS-related genes for oxidoreductase, ascorbate peroxidase 2, or superoxide dismutase was found at the protein level or at the mRNA level in this study (Fig. 8), and ascorbic acid showed only a weak linkage with LT storage (Fig. S6). This suggests ROS may only have a small role to play in the regulation of citrus fruit senescence, which is a result that will need further verification.

Taking the results of this study together, LT-delayed fruit senescence is likely to be regulated by hexose-mediated ABA and the auxin signal pathway.

**Conclusion**

The results of this study provide a global picture of protein accumulation and changes of gene expression in HBP fruit during post-harvest storage at LT. Interpretation of the data for the proteins and mRNA revealed that some of them have been described in studies of cold-stress responses, indicating the comparability with cold-stress response in living plants and the validity of this work for further in-depth studies. This study has provided new insights into fruit post-harvest storage. During the response to LT stress, chitinase, cysteine synthase, cysteine protease inhibitor, allyl alcohol dehydrogenase, putative aldehyde dehydrogenase, and mitochondrial aldehyde dehydrogenase were up-regulated and there was an accumulation of limonin, nomilin, methanol, and aldehyde. Moreover, ABA does not appear to be involved in LT stress response, which was first discovered in fruit. Ca$^{2+}$ seems to be involved in signal transfer during LT stress and the CBL–CIPK pathway played a vital role in LT signal transduction. For fruit senescence, hydrolysis-related genes were down-regulated by LT, which delayed fruit softening. Genes involved in carbohydrate metabolism, nitrogen compound metabolism, lipid metabolism, macromolecule metabolism, and secondary metabolism were down-regulated, which maintains fruit quality, and transporter genes were down-regulated, which inhibited the exchange of substrate. All of the gene expression might be regulated by hexose, ABA, and auxin, and they might cross-communicate and work collaboratively in fruit senescence and the LT stress response.

**Supplementary material**

Supplementary data are available at *JXB* online.

**Supplementary Table S1.** Specific primers used in relative quantitative real-time reverse-transcription PCR.

**Supplementary Table S2.** Summary statistics of DGE tags in LT- and AT-stored HBP fruit juice sacs.

**Supplementary Fig. S1.** Graphical output of the LT-storage stress down-regulated metabolic genes found in HBP fruit juice sacs.

**Supplementary Fig. S2.** Graphical output of differentially expressed transporter genes in HBP fruit juice sacs.

**Supplementary Fig. S3.** Graphical output of differentially expressed signal transduction genes in HBP fruit juice sacs.

**Supplementary Fig. S4.** Graphical output of differentially expressed signal transduction genes in HBP fruit juice sacs.
Supplementary Excel S1. LT up-regulated and down-regulated genes.

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