Hot Water Treatment Delays Ripening-associated Metabolic Shift in ‘Okrong’ Mango Fruit during Storage

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ABSTRACT. Effects of hot water treatment (HWT) on metabolism of mango (*Mangifera indica* cv. Okrong) fruit during low-temperature storage (LTS) and subsequent room temperature fruit ripening (RTFR) were examined. Mature-green ‘Okrong’ mango fruit were treated by immersing in hot ($50 \pm 1^\circ\text{C}$) or ambient ($30 \pm 1^\circ\text{C}$) water for 10 min, stored either at 8 or 12°C for 15 days, followed by transfer to room temperature ($30 \pm 2^\circ\text{C}$) for 5 days. Rate of ethylene production was significantly reduced by HWT during LTS and RTFR in all treatments. HWT increased catalase activity, suppressed ascorbate peroxidase activity, and had no effect on glutathione reductase activity during the ripening phase but showed a slight stimulatory effect during LTS. HWT altered RNA transcripts of manganese-superoxide dismutase, pectate lyase, β -galactosidase, and β -1,3-glucanase, which exhibited increases during LTS. RTFR of LTS fruit caused reduction in transcript levels of these genes, except pectate lyase. Total protein patterns were altered by all treatments during LTS and RTFR, but HWT arrested loss of several proteins during RTFR. Taken together, results provide strong evidence that HWT increases the storage period of mango by extending fruit shelf life through the regulation of a myriad of metabolic parameters, including patterns of antioxidant and cell wall hydrolase genes and protein expression during storage at low and ambient temperatures.

Mango is one of the most important tropical fruit crops of Asia with a high demand in the world market (Tharanathan et al., 2006). Among mango cultivars presently grown in Thailand, ‘Okrong’, a desirable commercial cultivar, is mainly offered as fresh fruit. Mangoes are extremely perishable fruit and to extend their shelf life, especially during long-distance shipment, fruit are generally harvested at a physiologically matured stage, stored at low temperatures, and ripened at destination under favorable conditions (Arthachinta, 2000). Generally, HWT before LTS is required for insect disinfestation and disease control and to satisfy the quarantine requirements of some markets (Lurie, 1998; Paull and Chen, 2000).

Mangoes are chilling-sensitive and LTS can lead to development of physiological disorders resulting in uneven or impaired fruit ripening. To avoid chilling injury, mango fruit are generally stored at moderate temperatures ranging from 8 to 12°C and ripened at permissive temperature for marketing (Phakawatmongkol et al., 2004). The lengths of LTS have a significant effect on the

ability of fruit to ripen after it is moved to a permissive temperature. Longer LTS, even at the moderate temperatures, generally leads to inferior fruit quality attributes on ripening at permissive temperatures (Jacobi et al., 2001). Lipid peroxidation and the production of excess reactive oxygen species (ROS) leading to membrane degradation during HWT and LTS have been implicated in fruit inferior ripening or inability to ripen after LTS (Sala and Lafuente, 2004). Induction of ROS scavenging enzymes such as ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) has been suggested as a mechanism to protect cells under stress conditions by balancing ROS levels (Blokhina et al., 2003). The protective function of these enzymes against different stress conditions has been reported in fruit such as apple [*Malus ×domestica* (Ahn et al., 2007)], loquat [*Eriobotrya japonica* cv. Fuyang (Cao et al., 2009)], sweet orange [*Citrus sinensis* (Huang et al., 2008)], tomato [*Solanum lycopersicum* cv. Rhapsody (Yahia et al., 2007)], peach [*Prunus persica* (Zheng et al., 2007)], and mango (Kondo et al., 2005; Singh and Dwivedi, 2008; Wang et al., 2008, 2009; Zhao et al., 2009). Extended LTS also affects fruit textural changes because LTS fruit can exhibit either impaired or uneven fruit softening on ripening at permissive temperatures. Some of the proteins involved in mango fruit textural changes include expansin (Sane et al., 2005), pectate lyase (Chourasia et al., 2006), and endo- β -1,4-glucanase (Chourasia et al., 2008). However, the effects

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of HWT followed by LTS on expression patterns of these genes and on steady-state levels of protein and RNA have not yet been examined in detail.

Heat treatment has been reported to increase tolerance of fruit to chilling temperature (Fallik, 2011; Schirra et al., 2004). The role of HWT in enhancing postharvest shelf life of mango has been evaluated by a number of groups. Although some of the parameters have been individually evaluated in some cultivars, multiple biochemical pathways have not been investigated in 'Okrong' mango fruit. To understand the physiological and biochemical bases of enhanced chilling tolerance after heat treatment, we have evaluated a number of metabolic processes in 'Okrong' mango fruit during LTS and subsequent ripening at permissive temperature in fruit with and without previous HWT. The parameters examined included ethylene production, levels of antioxidant enzymes and lipoxygenase, transcript levels of cell wall hydrolases and manganese-superoxide dismutase (Mn-SOD), total RNA, and protein expression. We provide evidence, here, that in addition to modifying ethylene production, the HWT affects fruit shelf life by regulating several processes, including antioxidant enzymatic activities, cell wall hydrolase gene expression, and protein pattern both during LTS and subsequent ripening at permissive temperatures. Results support the hypothesis that the effects of HWT continued even after storage at low temperature during ripening at ambient temperature and increase shelf life of mango fruit during storage.

Materials and Methods

PLANT MATERIAL AND HEAT TREATMENT. Mature green mango 'Okrong' fruit were harvested 90 d after fruit set from a commercial orchard in Nakonratchasima province, Thailand, and transferred to the laboratory within 3 h. Fruit were selected for uniformity of shape, color, size as well as the absence of blemishes and disease symptoms. Furthermore, they were randomly divided into two groups; the treated group of 160 fruit was dipped in hot water at $50 \pm 1^\circ\text{C}$ for 10 min and the untreated control group of 160 fruit was dipped in tap water at $30 \pm 1^\circ\text{C}$ for 10 min. After HWT, fruit were placed at room temperature and allowed to dry before storage at 8 and 12°C [85% to 90% relative humidity (RH)] in temperature-controlled chambers and randomly sampled every 5 d. After 15 d, fruit were transferred to ripen at room temperature ($30 \pm 2^\circ\text{C}$) and sampled everyday until Day 20. In every sampling point, the pulp was excised from fruit, immediately frozen in liquid nitrogen, lyophilized, and stored at -80°C until used.

FRUIT FIRMNESS. Pulp firmness was evaluated using a hand-held penetrometer (Effegi, Alfonsine, Italy). The measurement was taken at three equatorial regions on the flesh of four mangoes and recorded as force in Newtons.

ETHYLENE PRODUCTION. Individual fruit were placed in airtight glass jars (2.4 L) fitted with a rubber septum and 1-mL gas samples were taken after 1 h of incubation with a syringe. Gas samples were analyzed using a gas chromatograph (8A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector fitted with a PoraPak Q 80/100 column (Restek Corp., Bellefonte, PA) held at 80°C using nitrogen as the carrier gas. Ethylene levels were determined and expressed as microliters per kilogram per hour.

ANTIOXIDANT ENZYME EXTRACTION AND ANALYSIS. Mango pulp (0.1 g freeze-dried weight) was ground in a mortar and pestle under liquid nitrogen and then homogenized in 1 mL of an extraction buffer containing (final concentrations) 45 mM

potassium phosphate buffer (pH 7.0), 0.5% (w/v) polyvinylpyrrolidone, 6.5 mM 1,4-dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $10,000 g_n$ for 25 min at 4°C and the supernatant collected to determine enzymatic activity. Protein concentration present in extracts from various samples varied from 0.04 mg to $0.33 \text{ mg}\cdot\text{mL}^{-1}$.

Catalase [electrical conductivity (EC) 1.11.1.6] activity was measured spectrophotometrically by determining a decrease in $A_{240 \text{ nm}}$ according to Zhao et al. (2006) with slight modifications. The reaction mixture contained (final concentrations) potassium phosphate buffer (40 mM, pH 7.0), H_2O_2 (40 mM), and 0.05 mL enzyme preparation in a total reaction volume of 2.05 mL. Enzyme solution containing hydrogen peroxide-free phosphate buffer was used as a control. CAT activity was expressed in units per milligram of protein; one unit is equivalent to use of H_2O_2 at $1 \text{ mmol}\cdot\text{min}^{-1}$. The changes in the hydrogen peroxide concentration were calculated based on its extinction coefficient at 240 nm of $43.6 \text{ M}^{-1}\cdot\text{cm}^{-1}$.

Ascorbate peroxidase (EC 1.11.1.11) activity was spectrophotometrically assayed by determining the decrease $A_{290 \text{ nm}}$ following Zheng et al. (2007) with slight modifications. The assay mixture contained (final concentrations) potassium phosphate buffer (40 mM, pH 7.0), L-ascorbic acid (0.5 mM), H_2O_2 (0.1 mM), and 0.05 mL of enzyme preparation in a total reaction volume of 2.01 mL. APX activity was expressed as units per milligram of protein; one unit is equivalent to the oxidation of ascorbic acid at $1 \text{ mmol}\cdot\text{min}^{-1}$. Changes in the concentration of ascorbic acid were calculated based on its extinction coefficient at 290 nm of $2.8 \text{ mM}^{-1}\cdot\text{cm}^{-1}$.

Glutathione reductase (EC 1.6.4.2) activity was measured spectrophotometrically by determining the decrease in the $A_{340 \text{ nm}}$ during 3 min according to the method of Shen et al. (2008) with slight modifications. The reaction mixture consisted of (final concentrations) potassium phosphate buffer (50 mM, pH 7.6) containing EDTA (1 mM), NADPH (0.15 mM), oxidized glutathione (0.5 mM GSSG), and 0.15 mL of enzyme preparation in a total reaction volume of 2.1 mL. GR activity was expressed as units per milligram of protein; one unit is equivalent to the oxidation of NADPH at $1 \text{ mmol}\cdot\text{min}^{-1}$. Changes in NADPH concentration were calculated using its extinction coefficient at 340 nm of $6.2 \text{ mM}^{-1}\cdot\text{cm}^{-1}$.

Protein content was measured according to the method of Bradford (1976) using bovine serum albumin as the standard.

PREPARATION OF PROBES FOR NORTHERN BLOTS. The mango probes for RNA blotting were obtained by reverse transcription-polymerase chain reaction (PCR) of total RNA isolated from freeze-dried pulp using specific primers for genes encoding the enzymes Mn-SOD, pectate lyase, β -galactosidase, β -1,3-glucanase, and pectate lyase. Primers used were designed based on the mango expressed sequence tags available in the NCBI database and are shown in Table 1. PCR products amplified with each set of primers were sequenced at the Purdue University genomic facility to establish their identity before using as probes. Briefly, 2 μg of mango pulp total RNA was reverse-transcribed using AMV reverse transcriptase with oligodT as primer as described by the manufacturer (Promega, Madison, WI). The resulting first-strand cDNA was treated with RNase H and PCR-amplified using gene-specific primers (Table 1). The PCR products were separated on 1% agarose gels, extracted, and cleaned using a gel extraction kit (Qiagen, Valencia, CA). The purified products were sequenced to establish gene identity and used as a probe for Northern hybridization.

Table 1. Primer sequences used to clone 'Okrong' mango homologs.

Accession no.	Gene	Primer ^a	Sequence
AY987389.1	Pectate lyase	F	5'-CGAAGCGGCAACAATGGCGG-3'
		R	5'-CCATCACGGCCACCAATGGCA
DQ366708.1	β -1,3-glucanase	F	5'-GCCGGGGGTGCACCAGAGAA-3'
		R	5'-CGTCAGCGGAGTAGGCCAGGA-3'
AJ505585.1	β -galactosidase	F	5'-GGACTGAGGCTTGGACCGGC-3'
		R	5'-AAAGCAGCACAGCCTCCCGA-3'
CD002002	Mn ²⁺ -superoxide dismutase	F	5'-ACCGAATCCGTCCTTGG-3'
		R	5'-CTTTGGTGGCTCCCCGCCTC-3'

^aF = forward; R = reverse.

RNA EXTRACTION AND NORTHERN GEL BLOT ANALYSIS. The total RNA was extracted from the lyophilized mango pulp essentially as described by López-Gómez and Gómez-Lim (1992). RNA levels were quantified by measuring absorbance at 260 nm with a spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was examined by determining the 260/280 nm ratio and electrophoresis on a 1% agarose gel. For a Northern blot analysis, 10 μ g of total RNA from each sample was electrophoresed on a 1% agarose-formaldehyde gel and blotted onto a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ) as described by Sambrook et al. (1989). The cDNA probes for Mn-SOD, pectate lyase, β -galactosidase, and β -1,3-glucanase were labeled using a random primer labeling kit (DECA PrimeII; Ambion, Austin, TX) and the radiolabeled probes purified on Sephadex G-50 columns (Sigma-Aldrich, St. Louis, MO). The membranes were sequentially hybridized with a ³²P-labeled probe of Mn-SOD, pectate lyase, β -galactosidase, β -1,3-glucanase, and ribosomal RNA at 37 °C in a solution containing 50% (v/v) formaldehyde, 5 $\times$ standard saline phosphate (SSPE)/EDTA, 0.1% (w/v) of sodium dodecyl sulfate (SDS), and 5 $\times$ Denhardt's solution. Hybridized membranes were washed two times in 5 $\times$ SSPE and 0.1% (w/v) SDS at 37 °C for 15 min followed by a 10-min wash in 2 $\times$ SSPE at 62 °C. The blots were exposed overnight on the phosphor-imaging cassette (Molecular Dynamics, Sunnyvale, CA), scanned in the Typhoon Phosphorimager (Amersham Biosciences) and the signal intensities quantified using ImageQuant 5.1 (Molecular Dynamics) as described by Srivastava et al. (2007).

SODIUM DODECYL SULPHATE-POLY ACRYLAMIDE GEL AND WESTERN BLOT ANALYSES. The freeze-dried samples were ground to a fine powder with liquid nitrogen and 6 mg of powdered tissue suspended in 200 μ L of Laemmli's buffer containing 1 mM phenylmethylsulfonyl fluoride and 2.5 mM Na₂EDTA (Biggs et al., 1986; Laemmli, 1970). The slurry was placed in a boiling water bath for 3 min, centrifuged at 14,000 g_n for 10 min in a microfuge, and the supernatant collected. Total protein concentrations in each sample were determined using the dye binding assay (Bradford, 1976). For sodium dodecyl sulphate-poly acrylamide gel (SDS-PAGE), 10 μ g of total protein from the supernatant of each sample along with pre-stained molecular weight markers (Bio-Rad, Hercules, CA) were separated on a 10% acrylamide gel (Sambrook et al., 1989). Gels were fixed, stained with 0.5% (w/v) Coomassie blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid, and destained in a solution of 40% (v/v) methanol and 10% (v/v) acetic acid. Gels were scanned and intensities of all strained bands were quantified using ImageQuant 5.1 (Molecular Dynamics).

WESTERN BLOT ANALYSIS. After separation of polypeptides by SDS-PAGE, gels were equilibrated with transfer buffer (25

mm tris, 250 mm glycine, 20% methanol, and 0.03% SDS) and then blotted onto nitrocellulose membrane using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) in the same buffer according to the manufacturer's recommendations. The membrane was blocked with tris-buffered saline (TBS) containing 3% non-fat dried milk for 1 h at room temperature. The membrane was incubated at 4 °C overnight with tomato LOX-specific antibodies

(1:200 dilution) in TBS containing 0.5% Tween-20 [TTBS (Sigma-Aldrich)] as described by Kausch and Handa (1997). After washing the membrane twice for 5 min each with TTBS, they were incubated for 1 h at room temperature with a goat antirabbit IgG (H+L) conjugated with alkaline phosphatase (Sigma-Aldrich) as the second antibody in 1:3000 dilution. The blot was washed two times for 5 min each with TTBS and once for 5 min with TBS and developed with 5-bromo-4-chloro-3-indolyl phosphate (6.5 mg·mL⁻¹) and nitro blue tetrazolium (3.3 mg·mL⁻¹) in 100 mM tris buffer, pH 9.5, containing 100 mM NaCl and 10 mM MgCl₂. The intensity of the immune-reactive signals was quantified by using ImageQuant 5.1 (Molecular Dynamics).

STATISTICAL ANALYSIS. The effects of the different treatments over the variables were evaluated by one-way analysis of variance at 5% of statistical significance based on a completely randomized design. Also, when the analysis of variance showed significant differences, the least significant difference test or Duncan's new multiple range test was carried out.

Results

MANGO FRUIT RIPENING DURING LOW-TEMPERATURE STORAGE AND ROOM TEMPERATURE FRUIT RIPENING. In initial investigations, the effect of a range of temperatures (45 to 55 °C) and time of exposure (5 to 10 min) was evaluated on mango fruit to determine the conditions to avoid development of heat injury symptoms. Based on these studies, a treatment at 50 °C for 10 min was found satisfactory and was used to evaluate the effects of HWT on various biochemical parameters, especially expression of antioxidant and cell wall hydrolases and protein patterns, during LTS and RTFR. Mature green 'Okrong' mango fruit were treated either with ambient temperature (30 $\pm$ 1 °C) or hot (50 $\pm$ 1 °C) water for 10 min and stored at 8 and 12 °C for 15 d followed by ripening at room temperature (30 $\pm$ 2 °C) to determine the effects of LTS and HWT on fruit quality and ripening.

Figure 1 shows that fruit maintained the external appearance during LTS storage at both temperatures. However, on transfer to room temperature, the untreated fruit began to show signs of shriveling, which were higher in fruit stored at 12 °C than those at 8 °C. During RTFR, the visible signs of shriveling were not apparent in HWT fruit previously stored at 8 °C and reduced in fruit previously stored at 12 °C. Collectively results indicate that HWT has beneficial effects on the appearance of fruit after their transfer from low temperature to room temperature. Both control and HWT fruit stored at 8 °C exhibited no change in fruit firmness (Fig. 2). At 12 °C, both control and HWT fruit did not exhibit any change in firmness until 10 d but showed some softening on 15 d of storage and the rate of fruit softening was

Fig. 1. Visual assessment of changes in fruit ripening and shrivelling of control and hot water-treated (HWT) 'Okroong' mango fruit after 5, 10, and 15 d low-temperature storage (LTS) at 8 and 12 °C and subsequent room temperature fruit ripening (RTFR) for 1 d (Day 16), 3 d (Day 18), and 5 d (Day 20) at 30 ± 2 °C. Two groups of 160 mature green fruit were treated by immersing in hot (50 ± 1 °C) or ambient (30 ± 1 °C) water for 10 min, stored either at 8 or 12 °C for up to 15 d followed by transfer to room temperature (30 ± 2 °C) for up to 5 d and sampled at indicated time intervals.

slower in the HWT fruit than the control fruit (Fig. 2). During RTFR, both the HWT and the control fruit exhibited rapid softening with little effect of storage temperature. Both the LTS 12 °C and the LTS 8 °C fruit exhibited similar firmness with and without HWT after 2 d of removal from the LTS (Fig. 2).

ETHYLENE BIOSYNTHESIS IN MANGO FRUIT. To examine the effects of LTS and HWT on fruit physiology, we followed changes in the rates of ethylene production in fruit during LTS and RTFR. During LTS, the rate of ethylene production was lower in both control and HWT fruit (Fig. 3). During RTFR, the rates of ethylene production increased severalfold in control LTS untreated fruit and were much higher in untreated fruit stored at 12 °C than 8 °C (Fig. 3). However, HWT eliminated this RTFR-associated rise in ethylene production in fruit previously stored at both at 8 or 12 °C. These results indicate that storage at lower temperature affects the fruit's ability to produce ethylene and HWT continues to suppress ethylene production even after their transfer to ambient temperature.

ANTIOXIDANT ENZYMATIC ACTIVITIES IN MANGO FRUIT DURING LOW-TEMPERATURE STORAGE AND SUBSEQUENT RIPENING AT AMBIENT TEMPERATURE. We examined the changes in the activities of CAT, APX, and GR to evaluate the effects of HWT on the production of ROS scavenging enzymes during LTS and RTFR in mango fruit. CAT activity was low in fruit throughout during LTS in untreated and HWT fruit. Transfer to ambient temperature increased CAT activity severalfold both in LTS 12 °C and LTS 8 °C untreated fruit but was two- to threefold higher in LTS 12 °C than LTS 8 °C fruit (Fig. 4A). HWT further enhanced CAT activity in LTS 12 °C fruit but not in LTS 8 °C fruit during RTFR (Fig. 4A).

Ascorbate peroxidase activity increased during LTS for most treatments except in HWT LTS 8 °C fruit (Fig. 4B). There was an approximate sixfold increase in APX activity in LTS 12 °C HWT fruit compared with approximately twofold for LTS 12 °C untreated fruit. After transfer to ambient temperature, APX activity in LTS 8 °C untreated fruit increased ≈ 18 -fold compared with approximately ninefold increase in untreated LTS 12 °C

Fig. 2. Changes in flesh firmness of control and hot water-treated (HWT) 'Okroong' mango fruit during low-temperature (8 and 12 °C) storage for up to 15 d and subsequent ripening at 30 ± 2 °C for up to 5 d. At indicated time intervals, the measurements were taken at three equatorial regions on the flesh of four fruit. Each data point represents the mean of 12 observations. SES are indicated with vertical bars. Other details are as in Figure 1.

Fig. 3. Changes in the rate of ethylene production of control and hot water-treated (HWT) 'Okroong' mango fruit during low-temperature (8 and 12 °C) storage for 15 d and subsequent ripening at 30 ± 2 °C for up to 5 d. Arrows indicated the day when fruit were transferred from low-temperature storage to room temperature for ripening. Each data point represents the mean of four observations. Vertical bars represent the SE.

fruit. In contrast, fruit exhibited only approximately fourfold increase in HWT LTS 8 °C and HWT LTS 12 °C fruit (Fig. 4B).

The patterns of GR activities were different from that of CAT and APX. The HWT itself increased the GR activity by two- to threefold in fruit immediately after the treatment and then declined thereafter (Fig. 4C). Untreated LTS 8 °C fruit showed an approximate fivefold increase in GR activity after

Fig. 4. Changes in catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities in the pulp of control and hot water-treated (HWT) 'Okrong' mango fruit during low-temperature storage (8 and 12 °C) up to 15 d and subsequent ripening at 30 ± 2 °C for up to 5 d. All enzymatic activities are expressed as units per milligram of protein. Arrows indicated the day when fruit were transferred from low-temperature storage to room temperature for ripening. Other details are as in Figure 1. Each data point represents the mean of four observations. Vertical bars represent the SE.

5 d that declined thereafter. This increase was not seen in untreated fruit stored at 12 °C or in HWT fruit stored at both low temperatures (Fig. 4C). On transfer to room temperature, there was a steady increase in the GR activity that continued to increase until Day 20 in all four treatments (Fig. 4C).

PROTEIN PATTERNS IN MANGO FRUIT DURING LOW-TEMPERATURE STORAGE AND SUBSEQUENT RIPENING AT AMBIENT TEMPERATURE. Total proteins from mango pulp from fruit with and without HWT and stored at 8 and 12 °C for increasing lengths of time and then ripened at room temperature were

extracted and analyzed by SDS-PAGE followed by image analyses to evaluate effects of HWT on changes in protein patterns during LTS and RTFR (Fig. 5A). After normalization for the total protein present in control fruit on Day 0, the 25 stained polypeptide bands ranging from 12 to 212 kD that exhibited quantifiable signal intensities in most pulp samples were selected and analyzed (Fig. 6). These analyses revealed several distinct patterns. Several proteins accumulated at 8 °C (13, 19, 31, 231, and 255 kD) and 12 °C (19 and 31 kD) storage with 19 and 31 kD exhibiting higher accumulation at both LTS. Accumulation of 231 and 255 kD proteins was delayed when HWT fruit were placed at low temperature. The proteins that accumulated only in HWT fruit while stored at 8 °C were 12, 23, 46, and 111 kD and at 12 °C were 83 and 111 kD. Among them, 12, 23, and 46 kD proteins accumulate on Day 5, whereas 83 and 111 kD exhibited increases during the latter part of storage at low temperature. The untreated fruit exhibited a dramatic decrease in most of these proteins, except 12, 17, and 46 kD polypeptides. During RTFR, untreated control fruit exhibited a dramatic decline in many proteins, including 13, 25, 27, 31, 37, 39, 53, 58, 62, 71, 79, 83, 91, 98, 111, 124, and 231 kD polypeptides, and this decline was generally arrested in the HWT fruit. Although RTFR-associated declines in the levels of these polypeptides were similar in control fruit previously stored at 8 or 12 °C, the HWT-associated arrest in their decline exhibited several patterns. The arrest in decline in the levels of many polypeptides (13, 15, 27, 58, 71, 83, 91, 93, 98, 111, 124, 231, and 255 kD) was higher in LTS 8 °C than LTS 12 °C fruit, but some polypeptides (17, 19, and 45 kD) exhibited lower decline rate in LTS 12 °C than LTS 8 °C fruit (Fig. 6), yet many other polypeptides (14, 25, 31, 37, 39, 53, 62, and 79) that showed a rapid decline in control fruit during RTFR exhibited similar rates of decline in LTS 8 °C and LTS 12 °C fruit on transfer to room temperature (Fig. 6). We interpret these results suggesting that HWT restricts degradation of many polypeptides in mango fruit and thus influences ripening process.

Because lipoxygenase has been implicated in initiating a lipolytic cascade in membrane deterioration leading to induction of chilling injury in chilling-sensitive crops, the changes in steady-state levels of this enzyme during LTS and RTFR in fruit with and without HWT were examined by immune blotting (Fig. 5B). The levels of the anti-LOX crossreactive band were quantified by image analyses. Western blot analysis of the mango pulp extracts showed the presence of two major bands of apparent molecular weights of 97 and 92 kD, which crossreacted with a tomato fruit ripening-specific lipoxygenase antibodies. These molecular weights of anti-LOX crossreactive proteins correspond with the general range of plant lipoxygenase molecular weight. The levels of 97 kD anti-LOX crossreactive protein increased approximately threefold during storage of control fruit at 8 °C and approximately twofold at 12 °C. A similar increase in the 92 kD crossreactive species was seen during storage of control fruit at 8 and 12 °C. The HWT increased levels of both LOX bands approximately fourfold and their levels remained elevated throughout LTS (Fig. 5B). During RTFR of control fruit, levels of the LOX protein remained elevated in LTS 8 °C but declined in LTS 12 °C fruit. This decline in LTS 12 °C fruit was especially pronounced for the 92 kD anti-LOX immunoreactive polypeptide. The levels of both 97 and 92 kD immunoreactive proteins remained elevated during RTFR for HWT fruit (Fig. 5B). The 92 kD LOX immunoreacting protein exhibited declined on Days 18 and 20 in untreated fruit

Fig. 5. Changes in the total (A) and lipoxigenase (B) protein patterns in the pulp of control and hot water-treated (HWT) 'Okrong' mango fruit during 5, 10, and 15 d storage at low temperature (8 and 12 °C) and subsequent ripening for 1 d (Day 16), 3 d (Day 18), and 5 d (Day 20) at room temperature (30 ± 2 °C). Total protein were extracted from the equal dry weights of lyophilized pulps and separated on 10% sodium dodecyl sulphate–polyacrylamide gel by electrophoresis. M represents the lane containing the molecular weight standard markers. Other details are as in Figure 1.

suggesting its instability during ripening at 12 °C. These patterns are similar to that observed for several other polypeptides using SDS-PAGE analyses (Figs. 5A and 6). Collectively, these data suggest that accumulation of lipoxigenase is regulated by low temperature with fruit at 8 °C exhibiting higher accumulation than those at 12 °C.

TOTAL RNA AND SPECIFIC TRANSCRIPT. The changes in steady-state levels of total RNA and transcripts of an antioxidant enzyme and three cell wall-metabolizing enzymes were determined to evaluate the effects of HWT on steady-state levels of RNA during extended storage of fruit at 8 and 12 °C and subsequent RTFR. LTS storage resulted in increases in total extractable RNA levels from 5-d LTS fruit, which was slightly higher at 12 °C than at 8 °C. The total RNA levels declined (25% to 40%) during the next 10 d of LTS (Fig. 7). During RTFR, fruit exhibited ≈50% reduction in total RNA within the first day of transfer to room temperature for fruit from all treatments except HWT 12 °C LTS fruit that showed ≈25% decrease in total RNA. On Days 18 and 20 at room temperature, the total RNA levels bounced back with HWT 8 °C LTS fruit exhibiting higher recovery (Fig. 7).

Figure 7 also shows the Northern blots for Mn-SOD, pectate lyase, β-galactosidase, and β-1,3-glucanase transcripts in fruit from various treatments. The steady-state levels of Mn-SOD transcript increase by Day 5 of LTS for all treatments. However, untreated LTS 8 °C fruit continued to accumulate higher levels of Mn-SOD until Day 15, whereas in the HWT fruit, the transcript level declined after peaking on Day 10 (Fig. 7). For 12 °C LTS fruit, the Mn-SOD levels were slightly higher in untreated fruit compared with HWT fruit on Days 5 and 10 of storage and declined thereafter. During the RTFR phase, the levels of Mn-SOD were higher in untreated fruit than HWT fruit (Fig. 7).

During LTS, pectate lyase and β-galactosidase exhibited large increases in HWT compared with untreated fruit and these increases were higher in fruit stored at 12 °C than at 8 °C (Fig. 6). However, pectate lyase exhibited a severalfold higher increase than β-galactosidase and these elevated levels were maintained throughout LTS (Fig. 7). The β-galactosidase transcript levels showed a decline in HWT fruit after 5 d under LTS. Transcript levels of β-1,3-glucanase also showed some increase at 8 °C during LTS, which was higher in HWT fruit. Moreover, fruit stored at 12 °C exhibited a slight change with or without HWT (Fig. 7). Differential patterns of transcript accumulation were also observed during the RTFR phase and the HWT fruit accumulated much higher levels of pectate lyase transcript than untreated control fruit with a higher increase in 8 °C LTS than 12 °C LTS fruit (Fig. 7). The transcripts of β-1,3-glucanase exhibited a higher increase during ripening only in untreated fruit stored at 12 °C. Overall LTS had a much stronger effect on the accumulation of pectate lyase than β-galactosidase transcripts with β-1,3-glucanase exhibiting the least increase in its transcript accumulation. Taken together these results suggest that

fruit continue to modify the regulation of some genes during LTS and RTFR periods with HWT influencing the transcript accumulation.

Discussion

We have shown that HWT greatly influences several metabolic processes, including oxidative processes, cell wall changes, and steady-state levels of protein and RNAs in mango fruit during LTS and subsequent ripening at room temperature. Among them, the effects on the total protein patterns during the ripening phase were most striking. Among the 25 discernible Coomassie blue-stained polypeptide bands, 19 exhibited dramatic decreases during the ripening phase in control fruit, whereas HWT stabilized their levels. At least three polypeptide bands at 31, 19, and 17 kD exhibited increases during the ripening phase. Stabilizing effects of HWT on metabolic processes were also evident because higher levels of Mn-SOD and pectate lyase transcripts were present in both 8 °C LTS and 12 °C LTS fruit during the ripening phase. We interpret these results suggesting that HWT significantly altered the overall metabolic processes likely by enhancing maintenance of homeostasis during LTS and the ripening phase. The external phenotype of the LTS fruit after HWT support this hypothesis because the untreated fruit began to show extensive signs of wrinkling after transfer to room temperature, whereas the HWT fruit exhibited noticeably reduced levels of fruit shriveling (Fig. 1). Based on our results, we propose that the residual effect of HWT on overall metabolism would likely extend the shelf life of fruit after HWT fruit are transferred from LTS to room temperature.

We have shown that HWT severely impaired recovery of ethylene biosynthesis in mango fruit on transfer of LTS fruit to ambient temperature for RTFR (Fig. 3). Similar results have

Fig. 6. Changes in protein patterns of the levels of indicated protein ranging from 37 to 255 kD (A) and 12 to 31 kD (B) in the pulp of 'Okrong' mango fruit during low-temperature storage (8 and 12 °C) for up to 15 d and subsequent ripening at 30 ± 2 °C for up to 5 d with (T) and without (C) hot water treatment (HWT) as shown in percentage of zero time. The stained gels shown in Figure 5 were scanned to quantify intensities of each band and expressed as the percentage of total stained intensity for the respective gel lanes on the y-axis. On x-axis, Days 0 to 15 represent low-temperature storage and Days 16 to 20 represent subsequent room temperature fruit ripening. Other details are as in Figure 1.

been reported previously in tomato, mango, apples, mandarin (*Citrus reticulata*), and peach (Biggs et al., 1988; Budde et al., 2006; Fallik et al., 2001; Ghasemnezhad et al., 2008; Ketsa et al., 1999). Depending on the length and temperature of treatment, some of these effects are at least partially reversible. Biggs et al. (1988) reported that tomato fruit show impaired levels of both 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) at 34 °C or above but their levels recovered on transfer to 25 °C. The ACS activity exhibited faster recovery than ACO activity and it was shown that both required *de novo* protein synthesis (Biggs et al., 1988). Ketsa et al. (1999) have reported that high-temperature treatments of mango fruit induced inhibition of ACS and ACO, but it recovered on transfer to permissive temperature. However, the ACO activity recovers faster than ACS activity. Reduced ethylene production after HWT has direct implications on mango fruit quality. Being a climacteric fruit, ethylene plays an important role in flavorful ripening of mango fruit. It is possible that heat treatment at lower temperatures coupled with LTS may allow acceptable ripening of mango fruit by reaching a threshold level of ethylene as observed by Ketsa et al. (1999). However, it would compromise the objective of meeting quarantine requirement for insect and fungal control in fruit destined for export. It would be of interest to determine if treatment with exogenous ethylene after removal of HWT fruit from LTS can improve both the shelf life and the organoleptic qualities of mango fruit.

Oxidative damage is considered to be an early response of sensitive tissues to low-temperature stress. Changes in levels of several antioxidant enzymes have been monitored in many fruit during LTS with and without heat treatment (Sevillano et al., 2009). Mango fruit stored at low temperature show an increase in the antioxidant enzyme activities with further increases with the advance of fruit ripening. Activities of SOD and

Fig. 6. (Continued).

CAT in the mango fruit with yellow color in the peel were markedly higher than those in the green and pre-yellow fruit after 9 d of the storage (Zhao et al., 2009). In the present investigation, the patterns of antioxidant enzyme activity production were different during LTS and subsequent RTFR phases. In the absence of HWT, the CAT and APX activities remained low, whereas the GR exhibited an increase in 8 °C LTS fruit. During RTFR, there was an early increase in CAT activity, a lower increase in APX activity, and similar increases in GR activity in the HWT fruit compared with control fruit (Fig. 4). The observed effects of HWT over the increase in antioxidant enzyme activities in mango fruit were in agreement with Yahia et al. (2007), who reported that hot water was a major factor leading to an antioxidant protective system in tomato. Exposing ‘Rhapsody’ tomatoes to mild heat treatment

at 34 or 38 °C and 95% RH for 24 h, which is recommended to ameliorate chilling injury and delay ripening, increased the activity of CAT and glutathione S-transferase enzymes (Yahia et al., 2007). The heat-treated (45 °C for 3 h) strawberry had higher SOD and APX activities than the respective control after 7 d of storage at 0 °C. Furthermore, the levels remained higher after 1 to 2 d of storage at 20 °C indicating that the heat treatment stabilized some protein after removal of low-temperature stress (Vicente et al., 2006).

Lipoxygenase along with phospholipase D have been implicated in initiating a lipolytic cascade in membrane deterioration leading to induction of chilling injury in chilling-sensitive plant tissues including fruit (Mao et al., 2007; Paliyath and Droillard, 1992; Pinhero et al., 1998). Our results show that LTS increased the levels of a fruit-specific lipoxygenase protein. This increase was higher at 8 °C than 12 °C. During the ripening phase, the levels of lipoxygenase protein in untreated fruit declined faster in fruit that were exposed to 12 than 8 °C suggesting a transitory effect on LOX protein levels. However, HWT fruit continued to retain higher levels of LOX protein in fruit exposed to both 8 and 12 °C. Induction in LOX activity has been reported during LTS in cucumber and loquat (Mao et al., 2007; Rui et al., 2010). However, results reported herein are different from that reported in the heat treated cucumber and loquat as those fruit exhibited a decrease in LOX activity after heat treatment (Mao et al., 2007; Rui et al., 2010). This discrepancy is likely the result of determination of LOX activity in

cucumber and loquat and LOX protein in the mango. It is possible that degradation of LOX protein was impaired in HWT fruit just like several enzymatic activities and many proteins present in HWT fruit. Alternatively, the higher LOX after HWT may help maintain the chilling tolerance in fruit by enhancing production of beneficial pharmacologically and biologically important compounds such as methyl jasmonate as reported earlier (González-Aguilar et al., 2004; Mizutani et al., 2002).

HWT had a noticeable effect on fruit firmness during storage at 12 °C than at 8 °C but exhibited little effect on fruit softening during RTFR (Fig. 2). Interestingly, despite a marked increase in the levels of transcripts of all cell wall hydrolase tested in HWT fruit, there was little change in the fruit firmness during LTS. These results suggest that LTS does not impair transcription but may inhibit synthesis or action of these cell wall hydrolases.

Fig. 7. Effect of hot water treatment (HWT) on steady-state levels of transcripts (A), quantitative changes in steady state levels of transcripts of indicated genes characterized (B), and changes in level of total extractable RNA (C) in 'Okrong' mango fruit pulp during low-temperature storage at 8 and 12 °C for up to 15 d and subsequent ripening for up to 5 d at 30 ± 2 °C. (A) Northern blots: 10 µg total RNAs were separated on a 1% agarose-formaldehyde gel and blotted onto a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The RNA blots were hybridized with ³²P-labeled probe of manganese-superoxide dismutase (Mn-SOD), pectate lyase (PL), β -galactosidase (bGal), β -1,3-glucanase (1,3-Glu), and ribosomal RNA. The bottom panel shows 18S ribosomal RNA (18S rRNA) to indicate RNA loading in each lane. (B) For relative gene expression, the signal intensities in the Northern blots were quantified and represented as fold Day 0 fruit. (C) Total RNA were quantified and normalized for per milligram of the dried pulp weight. The analysis was repeated three times. Other details are as in Figure 1.

Cold storage has been reported to influence protein and activity levels of several enzymes, including polygalacturonase and β -galactosidase, more than their transcript levels in ripening tomato fruit (Rugkong et al., 2010). Like mango (Fig. 6), grape (*Vitis vinifera*) has also been shown to increase accumulation of transcript of the β -1,3-glucanase gene (Romero et al., 2006).

Our results show that HWT greatly influenced protein patterns in mango fruit stored at 8 and 12 °C primarily by maintaining their steady-state levels on transfer to room temperature. However, some of these proteins also exhibited an increase during RTFR (Fig. 6). Both existing and newly synthesized protein in a living cell remain under constant risk of misfolding. Generally the misfolded proteins either undergo refolding to form an active protein or are directed to the ubiquitin-proteasome pathway for degradation (Moon et al., 2004). However, under a variety of conditions, the unfolded protein may aggregate in vivo and become a challenge to proteasomes. These aggregates persist in cellular milieu for a long time and may lead to physiological disorders. For example, protein aggregates have been reported to be associated with neurodegenerative diseases such as Huntington's and Parkinson's (Bukau et al., 2006). The molecular mechanisms leading to in vivo accumulation of protein aggregates are not yet understood, but chocking of the proteasome chamber by these aggregates has been implicated (Venkatraman et al., 2004). We propose that HWT of mango fruit results in unfolding of existing protein or misfolding of newly synthesized protein and the storage of HWT fruit at low temperature did not allow refolding or the ubiquitin-proteasome-based degradation resulting in aggregation of some proteins. We further suggest that some of these protein aggregates become resistant to degradation as observed in other organisms and an altered physiological state of fruit leading to slower fruit ripening. Present investigations provide only limited support to this hypothesis and further research is needed to substantiate this

premise. Also, the emerging transcriptome and proteomic technologies would help in establishing the identity of these proteins and the molecular mechanisms regulating effects of HWT on horticultural crops.

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