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Proteomic changes in mango fruit peel associated with chilling injury tolerance induced by quarantine hot water treatment

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ABSTRACT

The aim of this study was to identify proteins associated with the chilling injury (CI) tolerance induced by hot water treatment (HWT) in 'Keitt' mango fruit. A comparative proteomic analysis was performed between fruit with HWT (46.1 °C, 90 min) and non-treated (Control) after cold storage (0 and 20 d at 5 °C) and ripening (7 d at 21 °C); the expression of genes encoding some selected proteins was analyzed by real-time PCR. Twenty-six proteins were differentially expressed after HWT, 36 after 20 days of cold storage and 33 after ripening. Polypeptides with higher accumulation in HWT fruit included eleven heat shock proteins (HSPs), eight enzymes of the energetic metabolism (ACO1, ACO2, GAPDH-1, GAPDH-2, ADH, SDH, ADK, and ACAA) and seven of the secondary metabolism (PAL, CHS, CHI, PDS, HPPD, IsoCH, and PPO), four antioxidant enzymes (CAT, POD, Prx, and APX,), four proteins involved in hormone metabolism (P-gp2, ARF, ERF, and GA3ox1), two pathogenesis-related proteins (β-Glu and 2 s alb), four enzymes of cell wall metabolism (EGase, β-Gal, Rab11 and αMan), and three proteins involved in chloroplast metabolism (RuBisCo, PDX1, and rpl2). Non-treated fruit showed higher accumulation of polyphenol oxidase and alcohol dehydrogenase, suggesting a higher oxidation of phenols and lower efficiency in energy production. The CI tolerance induced by the quarantine HWT in mango fruit appears to be associated with the prevention of protein denaturation, the maintenance of the membrane functionality and energy efficiency, the activation of antioxidant and defense systems, the preservation of cell wall metabolism, and the synthesis of secondary metabolites.

1. Introduction

Mango (*Mangifera indica* L.) fruit is widely accepted due to its excellent organoleptic characteristics. Mexico is an important producer and exporter of mango (FAOSTAT, 2021), but its commercialization is limited by the susceptibility to chilling injury (CI) when is stored at temperatures below 13 °C (Tharanathan et al., 2006). CI symptoms in mango fruit include lenticel darkening, uneven color development, pitting, decay, and poor aroma (Sivankalyani et al., 2017; López-López et al., 2018; Vega-Alvarez et al., 2020). The development of these symptoms has been associated with membrane deterioration (Sivankalyani et al., 2016b; Cantre et al., 2017), an increased production of reactive oxygen species (ROS) (Sudheeran et al., 2018), and deficiency in energy production (Li et al., 2014). Transcriptomic analysis of chilled mango fruit showed the activation of metabolic pathways related to ROS production, glycerophospholipid catabolism and oxidation of α-linolenic acid, sugars metabolism, and biosynthesis of phenylpropanoids and aromatic metabolites (Sivankalyani et al., 2016b).

Quarantine hot water treatment (HWT) is mandatory for pest control in mango destined for exportation (USDA-APHIS, 2016). This treatment improves some fruit quality parameters (Luria et al., 2014; Dautt-Castro et al., 2018) and alleviates CI symptoms (López-López et al., 2018; Vega-Alvarez et al., 2020). The HWT-induced CI tolerance in 'Keitt' mango was associated with lower lipid peroxidation and membrane permeability; as well as an increase in the activity of antioxidant enzymes (*e.g.*, superoxide dismutase SOD and ascorbate peroxidase APX) and the maintenance of the cell wall metabolism (López-López et al., 2018; Díaz-Corona et al., 2020). Vega-Alvarez et al. (2020) analyzed the

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effect of HWT on the metabolome, respiration rate, and global antioxidant status in the peel of 'Keitt' mango after cold storage (20 d at 5 °C) and ripening (7 d at 21 °C). The authors associated the lower incidence of CI symptoms in HWT fruit with a decrease in respiration rate, higher levels of antioxidant compounds (galloylquinic acids, gallic acids ester, and gallotannins), increased content of simple sugars, and a high ratio of unsaturated/saturated fatty acids. However, there is no information about the changes in protein expression associated with the CI tolerance of mango 'Keitt'. Therefore, the aim of this research was to analyze changes in the proteome of 'Keitt' mango peel in response to HWT, cold storage, and ripening to improve the understanding of the biochemical basis of the CI tolerance induced by the HWT.

2. Materials and methods

2.1. Materials and postharvest treatment

Mature-green mango fruit (*Mangifera indica* L. cv. Keitt) were obtained from a local orchard located in Culiacan, Sinaloa, Mexico. They were selected based on pulp firmness (119 ± 8 N) (Mitcham and McDonald, 1992), size (400−600 g), color uniformity, and absence of physical damage. Fruit were disinfected with sodium hypochlorite solution (200 mg L^{-1}), and then divided randomly into two groups of 45 fruit each. The first group was used as control and the second received a quarantine HWT (46.1 °C, 90 min) according to the USDA-APHIS (2016). Fruit of both treatments were air-dried at 21 °C, stored for 20 d at 5 °C, and subsequently ripened for 7 d at 21 °C (80–90 % relative humidity) for visualization of chilling injury symptoms. Fifteen fruit per treatment (three replicates of five fruit each) were taken before cold storage (0 d at 5 °C), after cold storage (20 d at 5 °C), and after ripening (20 d at 5 ° C + 7 d at 21 °C). The peel was removed carefully, frozen in liquid nitrogen, and stored at −70 °C until use.

2.2. Chilling injury index (CII)

The CII was determined after cold storage and after the ripening period according to López-López et al. (2018) with some modifications. The symptoms evaluated corresponded to uneven color development (UCD), pitting (P), and decay (D). The injury level (IL) was calculated using a five-point scale (0–4) based on the percentage of damaged tissue: 0 = no tissue injury, 1 = 1 % to 25 % of tissue injury, 2 = 26 % to 50 % of tissue injury, 3 = 51 % to 75 % of tissue injury, and 4 = > 76 % of tissue injury. The CII was calculated per symptom using the following general formula: CII = SUM [(IL) x (Number of fruit at the IL)]/Total number of fruit in the replicate. The CII value reported for each treatment/storage condition is the average of 15 fruit (five per replicate).

2.3. Firmness

Peel firmness was evaluated according to Jha et al. (2010) with some modifications. A digital penetrometer Chatillon D-DFE-100 (Ametek, Berwyn, PA) equipped with a 2 mm diameter tip was used. Peel joined to the flesh was penetrated at a constant speed of 50 mm min^{-1} and three measurements were taken: two at the proximal ends and one at the center. Firmness was registered as the maximum force compression in Newtons (N) and the values reported are the average of 15 fruit (five per replicate).

2.4. Physiological parameters

Electrolyte leakage (EL) was evaluated using the method described by Chongchatuporn et al. (2013) with some modifications. For each replicate, thirty cylinders of mango peel (5 mm) were mixed with 30 ml of 0.3 mol L^{-1} mannitol, incubated under shaking (10 x *g*, 25 °C, 1 h) and the conductivity was measured using a manual conductivity meter (Hanna Instruments EC/TDS). The sample was autoclaved at 121 °C for 25 min and cooled to 25 °C, and the conductivity was measured again to obtain the total EL. The ratio of the initial and final conductivity values was used to calculate the percentage of EL. Lipid peroxidation in mango peel was estimated by measuring the malondialdehyde (MDA) content according to Hodges et al. (1999); it was expressed as nmol g^{-1} on a fresh weight basis. Three replicates of five fruit each were used.

2.5. Protein extraction and separation

Protein extraction was carried out following the methodology of Salazar-Salas et al. (2017) with some modifications. A mixture of frozen mango peel from five fruit per replicate was ground with liquid nitrogen and about 8 g of powered peel was homogenized with 10 ml of trichloroacetic acid in acetone (0.1 kg L^{-1}) containing 0.04 mol L^{-1} of dithiothreitol (DTT). The sample was centrifuged (11,000 *x g*, 15 min at 4 ° C), and the pellet was washed ten times using the same solution. Afterwards, the pellet was washed five times with 0.1 mol L^{-1} ammonium acetate in methanol (0.8 L L^{-1}) and then with acetone (0.8 L L^{-1}); it was transferred to 2 ml tubes and dried at room temperature. Proteins were extracted with a 1:1 mixture of phenol: SDS buffer [0.1 mol L^{-1} Tris-HCl (pH 8.0), 0.3 kg L^{-1} sucrose, 20 g L^{-1} SDS, 50 mL L^{-1} 2-mercaptoethanol]. After centrifugation (15,000 *x g*; 12 min), the proteins were precipitated from the phenolic phase with five volumes of 0.1 mol L^{-1} ammonium acetate in methanol (-20 ° C; 30 min). The protein pellet was recovered by centrifugation (15,000 *x g*, 12 min), washed with pure methanol followed by acetone (0.8 L L^{-1}), dried, and resuspended in 0.2 mL of rehydration buffer [7 mol L^{-1} Urea, 2 mol L^{-1} Thiourea, 20 g L^{-1} CHAPS, 0.01 mol L^{-1} DTT, 0.01 mL L^{-1} Bromophenol blue, 1 mL L^{-1} Ampholytes pH 3–10]. Proteins were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). First, 0.5 mg of protein was loaded by rehydration into 17 cm immobilized pH (5–8) gradient strips (Bio-Rad, Hercules, CA, USA). Isoelectrofocusing (IEF) was conducted with a Protean IEF system (Bio-Rad, Hercules, CA, USA) using the voltage and time conditions recommended by the manufacturer (250 V, 20 min; 10,000 V, 2.5 h; 10,000 V, 40,000 V). After IEF, the proteins were reduced with dithiothreitol [0.375 mol L^{-1} Tris-HCl (pH 8.8), 0.13 mol L^{-1} DTT, 6 mol L^{-1} urea, 0.2 L L^{-1} glycerol, 20 g L^{-1} SDS] and then alkylated with iodoacetamide [0.375 mol L^{-1} Tris-HCl (pH 8.8), 0.135 mol L^{-1} iodoacetamide, 6 mol L^{-1} urea, 0.2 L L^{-1} glycerol, 20 g L^{-1} SDS]. The proteins were separated by molecular weight with a Protean II XL system (Bio-Rad) using a 0.125 kg L^{-1} polyacrylamide gel running at constant current (40 mA) for approximately 14 h. The gels were stained with Coomasie blue R-250 and digitized with a ChemicDoc XRS photodocumentation system (Bio-Rad, Hercules, CA, USA). Qualitative and quantitative differences were analyzed using the PDQuest program (Bio-Rad); the number of pixels in each spot was measured as counts and represent the protein quantity. Spot densities were normalized against total density in-gel image to compensate for experimental variations in size and intensity. Normalized values were averaged for three different gels (three replicates).

2.6. Protein identification

Differentially accumulated proteins were identified by tandem mass spectrometry (MS/MS). Protein spots excised from the gels were washed with distilled water, followed by 500 ml L^{-1} acetonitrile, and vacuum-dried. Proteins were reduced with DTT (0.1 mol L^{-1}) in 0.1 mol L^{-1} ammonium bicarbonate (ABC) for 1 h at 56 °C and then alkylated with iodoacetamide (0.55 mol L^{-1}) in 0.1 mol L^{-1} ABC for 45 min at room temperature. Proteins were washed with 0.25 mol L^{-1} ABC and then were rehydrated with 0.045 mL of a digestion solution [0.005 mL of a trypsin solution [0.1 g L^{-1} (Promega, Madison, WI, USA)] plus 0.04 mL of 0.05 mol L^{-1} ABC] and incubated at 37 °C overnight. The peptides were desalted using C-18 zip-tips (Millipore, Billerica, MA, USA) and analyzed with a liquid chromatography system coupled to a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific, San Jose, CA, USA)

with a nanospray ion source (3.0 kV). Some of the peptides were sequenced from their collision-induced dissociation (CID) spectra using the Proteome Discoverer 1.3 software provided with the equipment.

2.7. RNA isolation

Total RNA was isolated from the same samples used for protein extraction as described by Chang et al. (1993). About 2 g of powdered tissue and 10 mL of extraction buffer [20 g L^{-1} CTAB, 20 g L^{-1} PVP K40, 0.1 mol L^{-1} Tris-HCl (pH 8.0), 0.025 mol L^{-1} EDTA, 2 mol L^{-1} NaCl, 20 mL L^{-1} β- mercaptoetanol] were mixed vigorously for 2 min and then incubated for 15 min at 65 °C. The mixture was extracted two times with 10 mL of chloroform: isoamyl alcohol (24:1) and the phases were separated by centrifugation (10,000 x *g*, 20 min, 25 °C). The supernatant was mixed with about 2.5 mL of 10 mol L^{-1} LiCl (one fourth of the volume recovered) and the RNA was precipitated overnight at −20 °C. After centrifugation (10,000 x *g*, 30 min, 4 °C), the pellet was resuspended in 700 µL of SSTE buffer [0.01 mol L^{-1} Tris-HCl (pH 8.0), 1 mol L^{-1} NaCl, 5 g L^{-1} SDS, 0.001 mol L^{-1} EDTA] preheated at 60 °C. The sample was extracted three times with 700 µL of chloroform: isoamyl alcohol (24:1); the supernatant recovered (10,000 x *g*, 10 min, 4 °C) was added with two volumes of pure ethanol and the RNA was precipitated for 2 h at −70 °C. The pellet was washed with ethanol (0.7 L L^{-1}), dried and resuspended in 25 µL of water free of ribonuclease. Contaminant DNA was removed using the DNase I kit (Sigma-Aldrich, St. Louis, USA).

2.8. Gene expression analysis

The transcript levels of genes encoding proteins associated with cold stress and chilling tolerance were determined by RT-qPCR. The RNA was quantified spectrophotometrically and 10 ng were used for the analysis using the kit SCRIPT One-Step RT-PCR (Jena Bioscience, California, USA). The amplification was performed using a real-time system model StepOnePlus™ (Applied Biosystems, Carlsbad, USA). The PCR primers (Suppl. Table 1) were designed using the Primer-BLAST program available at http://www.ncbi.nlm.nih.gov/tools/primer-blast; they were designed for an alignment temperature of 60−66 °C and to amplify a region of 100–250 bp. The amplification conditions were: 50 °C for 15 min for cDNA synthesis, then 95 °C for 5 min (denaturation), followed by 40 cycles at 95 °C (15 s) and 60 °C (1 min) with a ramp rate of 2.2 °C s^{-1}. The *Actin* gene was used as control and the expression values were calculated relative to the non-treated fruit.

2.9. Statistical analysis

Data corresponds to the mean of three biological replicates with three technical replicates. It was analyzed using two-way ANOVA, and the Fisher test ($p < 0.05$) was used for mean comparison between treatments and storage conditions. These analyses were performed using the statistical package STATGRAPHIC plus version 5.1 (Statistical Graphics Corporation™, Maryland, USA). Principal component analysis (PCA) was performed to investigate the grouping and relationship between chilling injury symptoms, physiological parameters, and protein accumulation; the open-source R studio program (Version 3.6.2) was used.

3. Results

3.1. Chilling injury Index (CII), firmness and physiological parameters

CII values showed that the CI symptoms were more severe in control than HWT mango fruit after cold storage and subsequent ripening (Table 1). The main CI symptoms observed were uneven color development, pitting, and decay (Suppl. Fig. 1); the HWT fruit showed lower CII values than those of control fruit (Table 1). Moreover, decay was only observed in control fruit after the cold storage (20 d at 5 °C). Peel firmness decreased significantly after cold storage only in HWT fruit and the value was lower compared to the control; this parameter decreased in both treatments after ripening but the values were not significantly different (Table 1). The HWT fruit showed less cold damage and their values for electrolyte leakage (EL) and malondialdehyde content (MDA) were lower than those of the control fruit (Table 1).

Table 1
Chilling injury index, firmness, and physiological parameters of control and HW-treated 'Keitt' mango fruit stored under chilling conditions and ripened.

Symptom/Parameter	Treatment	Storage Condition		
		0 d at 5 °C	20 d at 5 °C	20 d at 5 °C + 7d a 21 °C
Uneven color development (CII)	Control	ND	1.2 ± 0.4^{Aa}	1.9 ± 0.6^{Aa}
	HWT	ND	0.6 ± 0.2^{Aa}	1.0 ± 0.2^{Ab}
Pitting (CII)	Control	ND	0.8 ± 0.1^{Ba}	1.2 ± 0.2^{Aa}
	HWT	ND	0.5 ± 0.1^{Ab}	0.7 ± 0.2^{Ab}
Decay (CII)	Control	ND	0.7 ± 0.1^{Ba}	1.6 ± 0.2^{Aa}
	HWT	ND	0.0 ± 0.0^{Bb}	0.6 ± 0.1^{Ab}
Firmness (N)	Control	32.7 ± 0.4^{Aa}	29.8 ± 1.6^{Aa}	12.3 ± 0.6^{Ba}
	HWT	31.4 ± 1.8^{Aa}	23.6 ± 2.1^{Bb}	11.2 ± 1.7^{Ca}
Electrolyte leakage (%)	Control	19.4 ± 0.4^{Ca}	37.0 ± 4.2^{Ba}	42.6 ± 0.4^{Aa}
	HWT	19.6 ± 1.4^{Ca}	30.1 ± 3.7^{Bb}	36.5 ± 0.7^{Ab}
MDA content (nmol g^{-1})	Control	14.3 ± 1.5^{Ca}	41.2 ± 0.6^{Ba}	50.3 ± 2.2^{Aa}
	HWT	14.5 ± 1.8^{Ca}	30.9 ± 0.0^{Bb}	39.9 ± 1.4^{Ab}

Chilling injury index (CII) was determined for each symptom. HWT, hot water treatment; MDA, malondialdehyde. Values are the mean ± standard deviation of three replicates. Different letters in the same row (A, B, C) or column (a, b) indicate significant differences (Fisher, $P < 0.05$) between storage conditions and treatments, respectively.

3.2. Protein changes associated with cold stress and chilling tolerance in mango peel

The two-dimensional electrophoresis detected about 700 polypeptides in mango peel, and 43 of them were differentially accumulated among treatments and storage conditions (Fig. 1). The identified proteins are involved in heat stress response, antioxidant system, carbohydrate metabolism and energy production, hormone signal transduction, pathogenesis, cell wall metabolism, secondary metabolism, and photosynthesis (Table 2). The HWT before cold storage induced qualitative and quantitative changes in the abundance of several proteins in mango fruit peel. Compared with the levels of proteins in the control fruit, the HWT fruit showed higher accumulation of 23 proteins, including 10 small heat shock proteins (sHSP) (eight HSP17.6, spots 21, 27, 29, 32, 34, 36, 37, and 40; one sHSP class I, spot 33; one sHSP, spot 19), one high molecular heat shock protein (HSP 70 kDa, spot 1), one antioxidant enzyme (ascorbate peroxidase [APX], spot 39), one glycolytic enzyme (glyceraldehyde 3-phosphate dehydrogenase [GAPDH-2], spot 10), two Krebs cycle enzymes (two isoforms of aconitate hydratase [ACO1] and [ACO2], spots 4 and 5), three proteins related to hormone signaling (P-glycoprotein 2 [P-gp2], spot 2; auxin response factor [ARF], spot 28; gibberellin 3-oxidase [GA3ox1], spot 43), one pathogenesis-related protein (2S albumin [2S alb], spot 41), one enzyme related to cell wall metabolism (β-galactosidase [β-Gal], spot 7) and another related to the synthesis of secondary metabolites (chalcone synthase [CHS], spot 11), as well as two proteins involved in

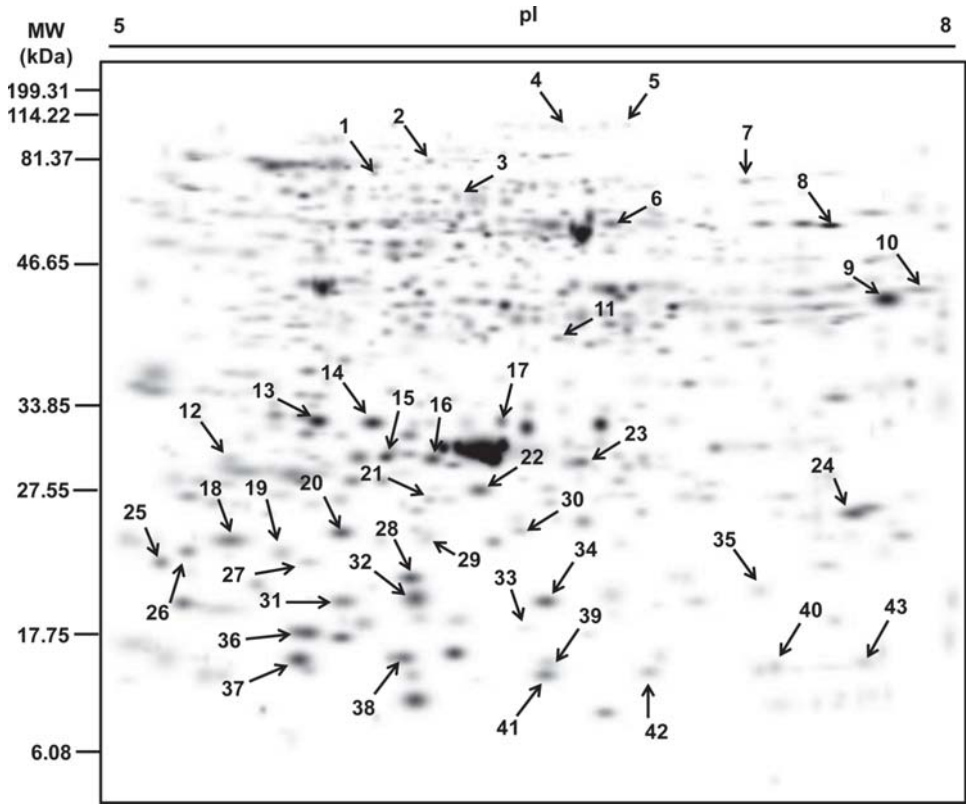

Fig. 1. Representative two-dimensional gel showing differentially expressed proteins (arrows) between control and hot water treated mangos stored for 0 and 20 days at 5 °C and after a ripening period of 7 days at 21 °C. Molecular weight (kDa) markers are shown to the left of the gel and the linear range of isoelectric points (pI) is shown above. Protein spots were identified by tandem mass spectrometry and listed in Table 2.

chloroplast metabolism (RuBisCo, spot 17; 50S ribosomal protein L2 chloroplastic [rpL2], spot 20) (Figs. 2–4; Suppl. Table 2). Ten of these proteins were only found in the HWT fruit: five sHSP (spots 19, 21, 27, 29, and 33) and APX (Fig. 2; Suppl. Table 2); ACO1, GA3ox1, and 2S alb (Fig. 3; Suppl. Table 2); and rpL2 (Fig. 4; Suppl. Table 2).

On the other hand, five proteins were down-regulated by the HWT: glutaredoxin-dependent peroxiredoxin (Prx, spot 35) (Fig. 2; Suppl. Table 2); succinate dehydrogenase (SDH, spot 15), adenylate kinase (ADK, spot 24), and ethylene response factor (ERF, spot 42) (Fig. 3; Suppl. Table 2); and polyphenol oxidase (PPO, spot 31) (Fig. 4; Suppl. Table 2).

Chilling stress and ripening also modified the abundance of proteins in mango peel. Thirty-six proteins showed differences ($P < 0.05$) between the control and HWT fruit after cold storage and 33 after the ripening period. The accumulation of all sHSP and HSP70kDa was higher in HWT fruit after chilling stress and after ripening; five sHSP (spots 19, 21, 27, 29, 33) were not detected in control fruit before and after cold storage, and three of them (spots 19, 27, 29) appeared in the control fruit only after the ripening period (Fig. 2; Suppl. Table 2). Analyzing the abundance of the antioxidant enzymes, APX was induced by the HWT and only detected in HWT fruit at the three storage conditions; moreover, the levels of APX, catalase (CAT, spot 8), and peroxidase (POD, spot 12) increased during cold storage in HWT fruit and their levels were higher compared to control fruit. After the ripening period, the accumulation of Prx and APX was also higher in HWT than control fruit (Fig. 2; Suppl. Table 2). Enzymes involved in carbohydrate metabolism and energy production like GAPDH-1, GAPDH-2, SDH, ADK, and acetyl-CoA acyltransferase (ACAA, spot 38) showed greater accumulation in HWT than control fruit after chilling stress and subsequent ripening. The levels of these proteins but GAPDH-1 increased in HWT fruit after cold storage (Fig. 3; Suppl. Table 2). On the other hand, the abundance of alcohol dehydrogenase (ADH, spot 14) increased significantly after ripening in chilled control fruit and its levels were greater than those of HWT fruit (Fig. 3; Suppl. Table 2). Regarding hormone signaling proteins, the expression of GA3ox1 was activated by the HWT, and this protein was observed in control fruit only after ripening but surprisingly its abundance was higher than that of HWT fruit whose level increased after cold storage; P-gp2 and ARF showed higher accumulation in HWT than control fruit after cold stress and ripening; P-gp2 maintained its levels in HWT fruit after cold storage but disappeared in control fruit. The content of ERF increased in HWT fruit after cold stress and was similar to that of the control even after the ripening period (Fig. 3; Suppl. Table 2). The pathogenesis-related protein 2S alb appeared in response to HWT and its expression in the treated fruit was upregulated by the cold storage and down regulated after ripening; this protein was detected in the control only after the ripening period and its levels were lower compared to HWT fruit. The expression of β-1,3-glucanase (β-Glu, spot 13) was downregulated in both treatments after cold stress but its levels were higher in HWT than control fruit; the abundance of this enzyme increased in both treatments after ripening and reached similar values (Fig. 3; Suppl. Table 2).

The abundance of the cell wall enzymes also changed significantly in response to HWT and storage conditions. α-Mannosidase (αMan, spot 25) was undetected before cold storage, upregulated after cold storage and downregulated after ripening with higher levels in HWT than control fruit. β-Gal was upregulated by the HWT and its content was affected by the storage conditions only in the control fruit whose values increased after cold storage and then decreased after ripening, reaching lower values than those of the HWT fruit. β-1,4-Endoglucanase (EGase, spot 6) was one of the most abundant proteins in mango peel before cold storage. HWT fruit had higher accumulation of EGase and Small GTP Rab11 (Rab11, spot 23) after cold storage and after ripening (Fig. 4; Suppl. Table 2). Regarding enzymes of the secondary metabolism, the levels of phenylalanine ammonia-lyase (PAL, spot 3) decreased in

Table 2
Identification by tandem mass spectrometry of proteins differentially accumulated between control and HW-treated mango fruit after cold storage and ripening.

Spot No.	ID	Description	Abbreviation	Experimental pI/ Mw (kDa)	Theoretical pI/ Mw (kDa)	Coverage (%)	Matching peptides
Heat stress response							
1	XP_004250958.1	Heat shock cognate 70 kDa protein 2-like *[Solanum lycopersicum]*	HSP70kDa	6.1/78.3	5.7/77.1	13.2	ATAGDTHLGGEDFDNR NALENYAYNMR QATKDAGVISGLNVMR
19	Q8L470	Small heat shock protein [*Solanum lycopersicum*]	sHSP	5.9/21.5	9.31/25.7	34.4	DGVLYITIPK EDVKVWLEEKMLVVK IALPENIDFEKIKAEVK SILWDNDIKSTK
21	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-1	6.5/15.4	6.1/17.5	52.6	FPQETSAFVSTR ETPEAHVFK MSLIPSFLGNQR
27	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-2	5.9/21.5	6.1/17.5	48.7	FKLPENVK VEVKKADVK ASMENGVLTVTVPK
29	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-3	6.4/22.7	6.1/17.5	51.9	ADLPGLKKEEVK DFSSLSTR NIEKEDK
32	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-4	6.3/19.1	6.1/17.5	53.2	ETPEAHVFK IDWRETPEAHVFK VEVKKADVK
33	XP_006447219.1	Class I heat shock protein [*Citrus clementina*]	sHSP Class I	6.7/23.3	6.05/15.6	21.7	VLQISGER EQEEKNDKWHR ASMENGVLTVIVPK
34	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-5	6.8/19.1	6.06/17.5	57.8	FPQETSAFVSTR VEVKKADVK ADLPGLK
36	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-6	6.0/17.0	6.1/17.5	56.5	FKLPENVK MDLLLLEK IDWRETPEAHVFK
37	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-7	6.1/16.1	6.1/17.5	61.7	VERSSGKFSR ADLPGLKKEEVK VEIEDNSVLQISGER
40	A0A0H3UZ51	Heat-shock protein 17.6 [*Mangifera indica*]	HSP17.6-8	7.4/13.3	6.1/17.5	37.7	FKLPENVK MSLIPSFLGNQR ADLPGLKKEEVK
Cellular detoxification							
8	K4BAE6	Catalase [*Solanum lycopersicum*]	CAT	7.7/59.2	7.2/56.9	9.35	FSTVIHER APGVQTPVIVR LNVRPSI
12	A0A2G3AAP9	Peroxidase [*Capsicum annuum*]	POD	5.7/27.81	9.1/29.8	15.4	GCDASLLLDSRGSIVTEK SMVKMGNISPLTGSR SNPNRNSAR
35	V4U3S4	Glutaredoxin-dependent peroxiredoxin [*Citrus clementina*]	Prx	7.4/19.6	7.3/12.9	19.5	YTHALGLELDLSEK VKAAN TFPK
39	B1A3K6	L-ascorbate peroxidase [*Litchi chinensis*]	APX	6.8/17.8	6.1/27.5	11.6	TGGPFGTIR LAWHSAGTFDLHSK LIEPLK
Carbohydrate metabolism and energy production							
4	A0A3Q7HBK4	Aconitate hydratase 1 [*Solanum lycopersicum*]	ACO1	6.7/107.4	7.6/108.1	10.2	GPMLLGVK TSLAPGSGVVTK GPMLLGVK
5	F2Y9D8	Aconitate hidratase [*Litchi chinensis*]	ACO2	7.0/107.4	6.1/96.5	7.3	TSLAPGSGVVTK GMTMSPPGPHGVK RDFNSYGSRR
9	A0A1B1FG09	Glyceraldehyde-3-phosphate dehydrogenase [*Mangifera indica*]	GAPDH-1	7.9/40.6	7.5/37.1	41.5	YDTVHGQWK

(*continued on next page*)

Table 2 (*continued*)

Spot No.	ID	Description	Abbreviation	Experimental pI/ Mw (kDa)	Theoretical pI/ Mw (kDa)	Coverage (%)	Matching peptides
							AAIKEESEGK
							AGIALNK
10	G8HI94	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (fragment) [*Citrus maxima*]	GAPDH-2	7.9/41.5	7.9/20.7	28.7	KVVISAPSK
							NAIKEESGGKLK
							KASYDEIK
14	D2XQZ9	Alcohol dehydrogenase 2 [*Mangifera indica*]	ADH	6.2/31.5	6.4/41.6	36.9	MSNTAGQVIR
							AFDYMLKGDSLR
							INTDRGVMINDGQSR
15	Q198M7	Succinate dehydrogenase (fragment) [*Mangifera indica*]	SDH	6.2/28.5	8.8/17.6	30.2	YVVARLHASEVDAQK
							VEPKASGGTK
							ASGGTKLK
24	XP_004237532.1	Putative adenylate kinase 6, chloroplastic [*Solanum lycopersicum*]	ADK	7.8/24.2	7.3/30.0	5.2	LLGVPHIATGDLVR
38	Q42918	Acetyl-CoA C-acyltransferase (3-ketoacyl-coa thiolase b) (fragment) [*Mangifera indica*]	ACAA	6.3/15.0	8.2/45.2	18.6	DCRFGVISMCIAPR
							CVATLLHEMK
							INVNGGAMAIGHPLGATGAR
Hormone transduction signal							
2	A0A0N6Z592	P-Glycoprotein 2 [*Mangifera indica*]	P-gp2	6.3/80.9	7.9/140.7	24.4	QRIAIARAMLK
							QTTKMRIK
							MFMQGFSGDLEAAHAK
28	Q7Y038	Auxin response factor [*Mangifera indica*]	ARF	6.3/20.0	7.3/37.1	29.5	RHLLQSGWSVFVSSK
							LVAGDAFIFLRCEK
							MMATASSELSIK
42	D3TIC3	Ethylene response factor [*Mangifera indica*]	ERF	7.1/15.0	4.6/16.4	11.5	NNNELELK
							DVATKEAAR
43	I2FFD6	Gibberellin 3-oxidase [*Mangifera indica*]	GA3ox1	7.7/9.6	6.3/9.4	37.4	KLAAKLMWLILGSLGIGK
							LAAKLMWLILGSLGIGK
							HCDVMEGYEKEMKK
Pathogenesis							
13	Q2ERX5	Glucan endo-1,3-beta-D-glucosidase [*Mangifera indica*]	β-Glu	6.0/31.0	6.1/19.5	45.9	MAVSSYSASEKLSR
							DKLQDLTDSAK
							MAVSSYSASEK
41	Q8H2B8	2S Albumin [*Anacardium occidentale*]	2S alb	6.7/14.2	5.7/16.3	18.8	ECCQELQEVDRRCR
							GGRYNQRQESLR
							ECCQELQEVDRR
Cell wall metabolism							
6	A6N4B9	Endoglucanase [*Mangifera indica*]	EGase	7.0/78.3	7.9/67.9	20.2	SGYLPHTQRVTWR
							YAGVQTLVAKFLMQGK
							YSTIITNKSDKTLK
7	Q4QYX3	Beta-galactosidase [*Mangifera indica*]	β-Gal	7.4/71.9	5.2/92.1	10.8	TEAYNTAKVNTQTSVIVK
							GDETVIKDLSCHK
							DLSCHK
23	W0IAJ5	Small GTP Rab11 [*Mangifera indica*]	Rab11	6.9/28.1	5.7/24.0	44.5	AMEAGDDGAGSAVPSKGEK
							VVLIGDSGVGKSNLLSR
							HSTFENVERWLR
25	A0A0K0MBQ3	Alpha-mannosidase (fragment) [*Mangifera indica*]	αMan	5.4/19.8	6.4/84.7	37.9	RLVR
							TFYSDLNGFQMSR
							MGFENMLIQR
Secondary metabolism							
3	A0A0G2REP6	Phenylalanine ammonia-lyase [*Mangifera indica*]	PAL	6.5/66.8	6.9/77.0	23.8	EELGTSFLTGEKVK
							TSPQWLGPQIEVIR
							EINSVNDNPLIDVAR
11	A0A060D9S4	Chalcone synthase 2 [*Mangifera indica*]	CHS	6.3/39.2	6.5/43.3	17.6	RFMMYQQGCFAGGMVLR
							FQRMCDKSMIK
							EVGLTFHLLKDVPGLIAK
16	M4PZ23	Chalcone-flavonone isomerase family protein [*Rhus chinensis*]	CHI	6.4/26.2	5.7/26.3	12.2	LLAESVLESIIGK
							THFLGGAGVRGLEIQGK
22	A0A0U2UEQ0	Phytoene desaturase [*Mangifera indica*]	PDS	6.5/26.4	5.2/20.9	32.3	EWMIKQGVPER
							DVLGGKVAAWK

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Table 2 (*continued*)

Spot No.	ID	Description	Abbreviation	Experimental pI/ Mw (kDa)	Theoretical pI/ Mw (kDa)	Coverage (%)	Matching peptides
26	E3VVJ0	4-Hydroxyphenylpyruvate dioxygenase [*Mangifera indica*]	HPPD	5.5/21.0	5.6/47.7	17.1	QGVPERVTTEVFIAMSK SIEEYEKTLEAR TLEARQIEAASVV
30	D3TIB2	Isochorismatase hydrolase (fragment) [*Mangifera indica*]	IsoCH	6.8/24.9	5.1/20.3	12.3	AFAVSHGLAVR EVIVSGVMTTLCCETTAR
31	D2XZ13	Polyphenol oxidase [*Mangifera indica*]	PPO	6.1/19.3	6.4/20.1	50.3	LCRR TISTVVPRPRK KVANALGVAHAAELK DTGVKFDMFINDED
Photosynthesis and cofactor synthesis							
17	Q5WPP8	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit [*Comocladia mollissima*]	RuBisCo	6.7/32.0	6.7/27.5	18.4	ARNEGRDLASEGNVIIR
18	XP_004242523.1	Putative pyridoxal 5′-phosphate synthase subunit PDX1 [*Solanum lycopersicum*]	PDX1	5.7/22.0	6.3/33.1	17.5	ALRMSGGDHIHSGTVVGK IHGMHFR NMDDDEVFTFAK
20	A9XFZ3	50S Ribosomal protein L2 [*Mangifera indica*]	rpL2	6.1/23.0	10.8/29.7	58.6	IPFVCGCR VGLAQMLR RPATPWGYPALGR RYILHPR GGQLARAAGAVAK GIITAGHRGGGHK

control fruit after cold storage and its abundance was lower than that of HWT fruit after cold storage and after ripening. The accumulation of CHS increased in both treatments after chilling stress and after ripening, but its content was higher in HWT than control fruit in both storage conditions (Fig. 4; Suppl. Table 2). The abundance of CHI and phytoene desaturase (PDS, spot 22) increased in HWT fruit after cold storage and in both treatments after ripening; the amount of CHI was higher in HWT than control only after cold storage, while that of PDS was higher in HWT than control after cold stress and after ripening (Fig. 4; Suppl. Table 2). The accumulation of 4-hydroxyphenylpyruvate dioxygenase (HPPD, spot 26) and isochorismate hydrolase (IsoCH, spot 30) changed during storage only in HWT fruit and their contents were higher in HWT than control fruit after cold storage, while this difference was only observed for HPPD after ripening (Fig. 4; Suppl. Table 2). The levels of PPO increased in control fruit and decreased in HWT fruit after the ripening period showing higher accumulation in control than HWT fruit. Other proteins differentially accumulated were 50S ribosomal protein L2 chloroplastic (rpL2, spot 20), which appeared only in the HWT fruit before cold storage, and probable pyridoxal 5′-phosphate synthase subunit (PDX1, spot 18) whose abundance was higher in HWT than control fruit during all storage conditions; rpL2 was upregulated after chilling stress and ripening in both control and HWT fruit, whereas PDX1 was upregulated after cold storage only in HWT fruit and after ripening only control fruit (Fig. 4; Suppl. Table 2). Finally, the levels of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCo, spot 17) increased almost three times in response to the HWT, then decreased after cold storage and remained without changes after ripening in treated fruit. By contrast, RuBisCo was upregulated by cold storage and ripening in control fruit, reaching higher values than those of HWT fruit (Fig. 4; Suppl. Table 2).

3.3. Changes in expression of genes encoding proteins associated with cold stress and chilling tolerance in mango peel

The relative expression of genes encoding some of the differentially expressed proteins was determined to better understand the molecular mechanisms related to CI tolerance induced by the HWT (Fig. 5). The gene for a small heat shock protein (sHSP) of 17.6 kDa increased its expression 66 times in response to the HWT and the transcript levels remained higher in HWT than control fruit after cold storage and subsequent ripening (Fig. 5), which corresponded with the greater accumulation of sHSP observed in treated fruit (Fig. 2). The transcriptional expression of the other selected genes did not change before cold storage (Fig. 5A). After cold storage, the transcript levels of *CAT*, *PAL*, and *PDS* were higher in HWT than control fruit (Fig. 5B) and corresponded with the protein accumulation (Figs. 2 and 4). After ripening, the transcriptional expression of *POD*, *β-GLU*, *β-GAL*, and *PDS* was higher in HWT than control fruit, but the opposite was observed for *PAL* (Fig. 5C); the protein levels of β-GAL and PDS were higher in HWT than control fruit (Fig. 4), but those of POD (Fig. 2) and β-GLU (Fig. 3) were not significantly different between the treatments. The enzyme PDS showed higher transcript and protein levels in HWT than control fruit after cold storage and subsequent ripening, which corresponded with the more uniform color development observed in treated fruit.

3.4. Principal component analysis

Principal component analysis was performed with the data of CII, physiological parameters, and differentially accumulated proteins identified by tandem mass spectrometry. The two principal components explained 63.5 % of the total variation and the samples and variables formed six clusters corresponding to the storage conditions and treatments (Fig. 6). Two large clusters were formed between the samples and variables analyzed. The first one included HWT fruit exposed to chilling stress (20 d at 5 °C) and ripened (20 d at 5 ° C plus 7 d at 21 ° C), HSP (sHSP17.6 kDa, sHSP Class I, sHSP, and HSP70kDa), antioxidant enzymes (POD, APX, and Prx), proteins involved in carbohydrate metabolism and energy production (GADPDH-1, GADPDH-2, SDH, and ACAA), hormone signaling (P-gp2, ARF, and GA3ox1), pathogenesis (β-Glu and 2S alb), cell wall metabolism (EGase, αMan, and Rab11), secondary metabolism (PAL, CHS, and CHI) and synthesis of carotenoids (PDS) (Fig. 6). The second cluster was formed by ripened chilled control fruit, chilling injury symptoms (pitting, decay, and uneven color development), physiological parameters (EL and MDA content) and the

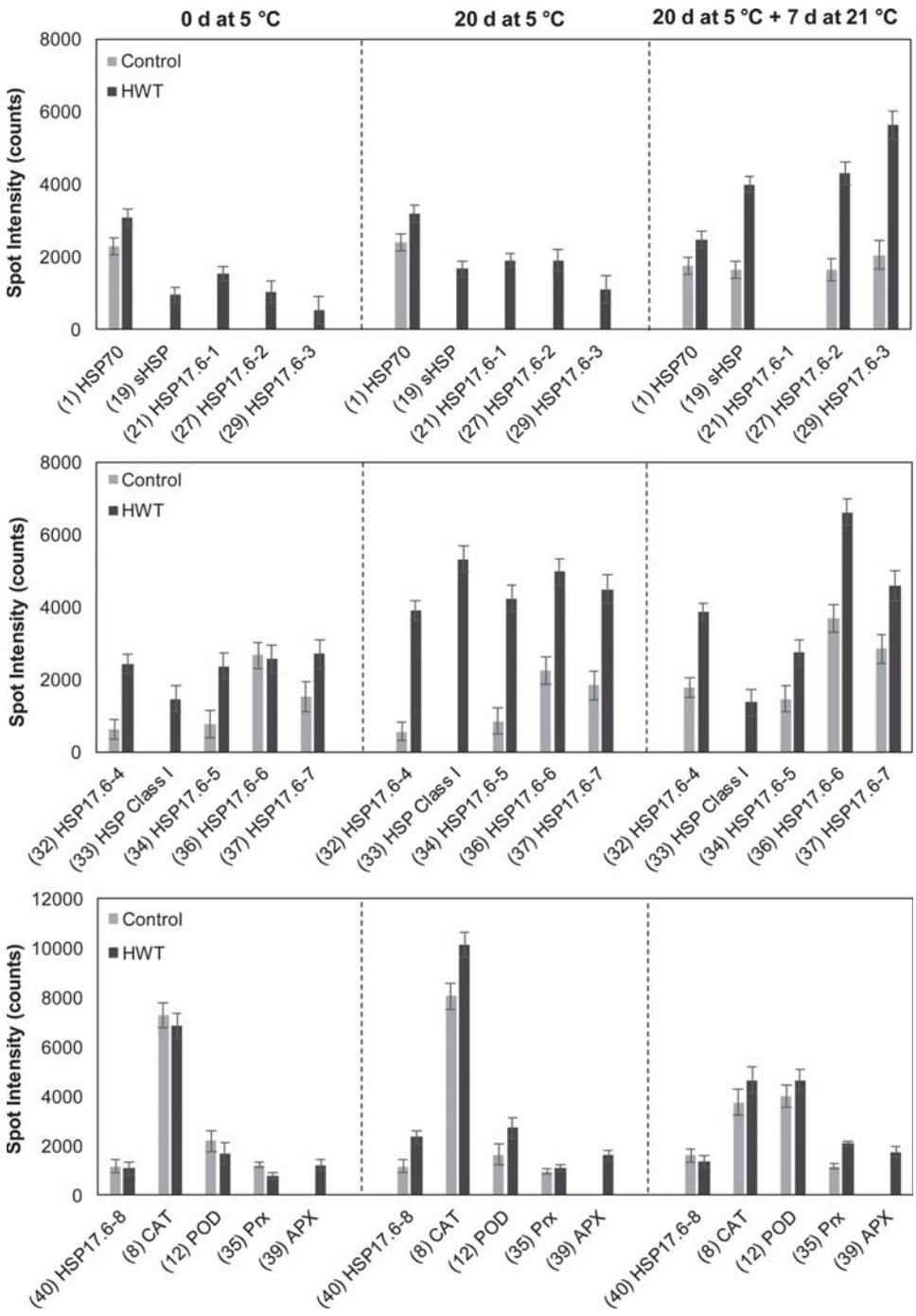

Fig. 2. Quantification of heat shock proteins and antioxidant enzymes differentially accumulated between control and hot water treated (HWT) mangos stored under chilling conditions and ripened. Spot intensity values (counts) are the mean of three replicates. Bars indicate the LSD ($\alpha = 0.05$); means are significantly different between treatments and storage conditions when the bars do not horizontally overlap. HSP, heat shock protein; CAT, catalase; POD, peroxidase; Prx, glutaredoxin-dependent peroxiredoxin; APX, ascorbate peroxidase.

enzyme ADH (Fig. 6). PPO was also located close to this group.

4. Discussion

4.1. Quarantine hot water treatment induces chilling injury tolerance in mango fruit

Chilling injury index (CII) values indicated lower incidence of pitting and decay and more uniform color development in HWT than control fruit (Table 1, Suppl. Fig. 1), confirming that this quarantine treatment provides CI tolerance in 'Keitt' mango as shown previously in this cultivar (López-López et al., 2018; Vega-Alvarez et al., 2020). In addition, EL values and MDA content were lower in HWT than control fruit (Table 1), suggesting a better integrity of the plastid membranes in the treated mangos that allows a more efficient synthesis of pigments (López-López et al., 2018) and energy production (Li et al., 2014; Vega-Alvarez et al., 2020). We investigated the mechanisms implicated in the HWT-induced CI tolerance in mango using a proteomic approach; it was assumed that differentially accumulated proteins between control and HWT fruit stored under chilling conditions are associated with this effect.

4.2. Heat shock proteins (HSPs) induction by HWT is associated with CI tolerance

The proteomic analysis revealed that HWT induced the expression of several HSPs in mango peel and their accumulation was higher in HWT than control fruit before and after cold storage, and after ripening

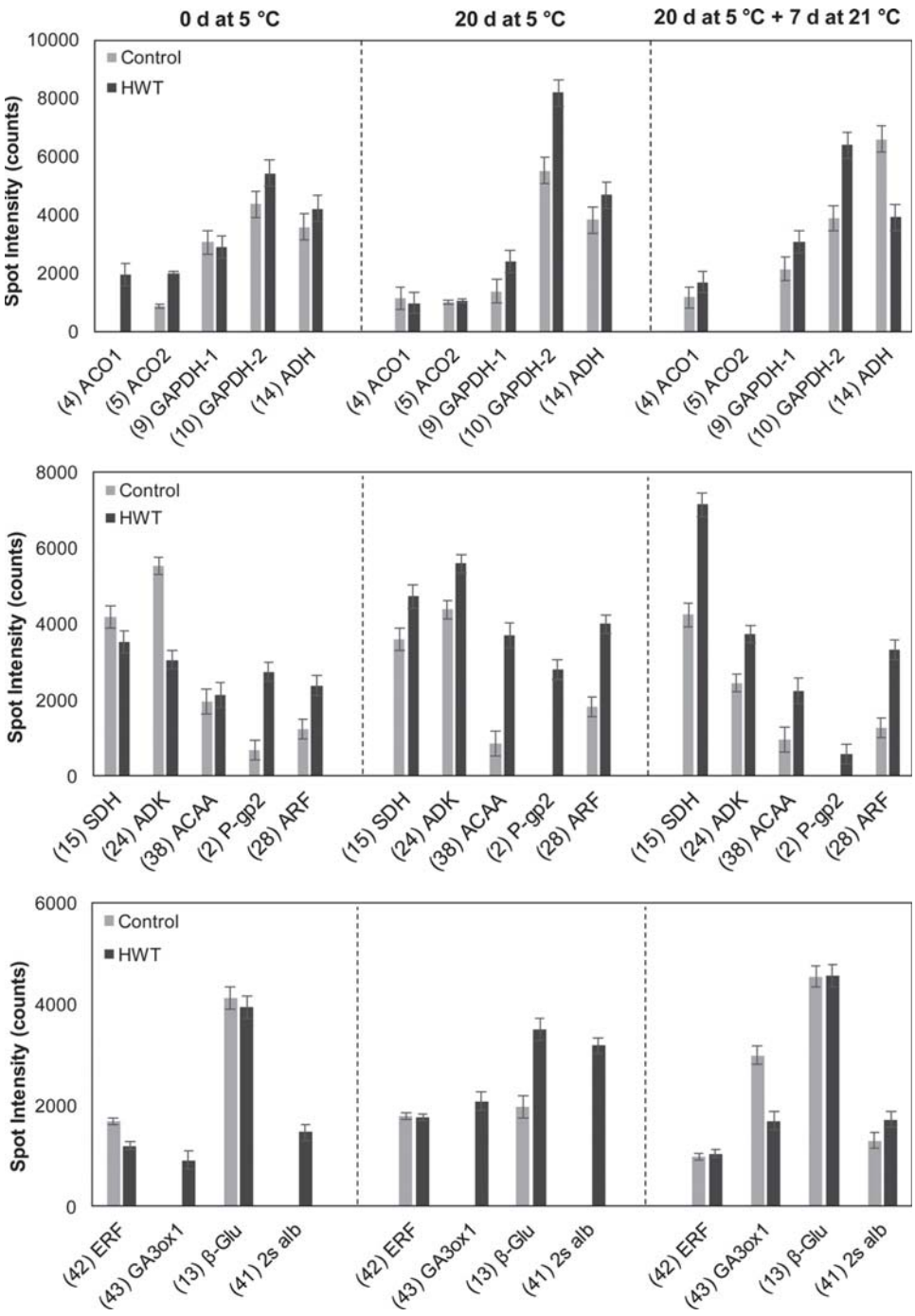

Fig. 3. Quantification of proteins involved in carbohydrate metabolism and energy production, hormone signal transduction and pathogenesis differentially accumulated between control and hot water treated (HWT) mangos stored under chilling conditions and ripened. Spot intensity values (counts) are the mean of three replicates. Bars indicate the LSD ($\alpha = 0.05$); means are significantly different between treatments and storage conditions when the bars do not horizontally overlap. ACO, aconitate dehydratase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; SDH, succinate dehydrogenase; ADK, adenylate kinase; ACAA, acetyl-CoA C-acyltransferase; P-gp2, P-glycoprotein 2; ARF, auxin response factor; ERF, ethylene response factor; GA3ox1, gibberellin 3-oxidase; β-Glu, β-1,3-glucanase; 2S alb, 2S albumin.

(Fig. 2; Suppl. Table 2), which corresponded with the transcript levels of the small HSP of 17.6 kDa (Fig. 5) and suggests an important role for the HSPs in CI tolerance. Heat shock proteins protect plant cells from different stresses since they participate in the refolding of partially denatured proteins, the degradation of irreversible denatured proteins, and the novo protein synthesis (Aghdam et al., 2013). Small heat shock proteins (sHSP) can modify the membrane properties to maintain its functionality; they can increase the fluidity of solid gel membrane structure under low temperatures or the rigidity of the liquid crystalline membrane state in the opposite scenery (Tsvetkova et al., 2002). Besides, organellar sHSP have antioxidant activity *in vitro* (Taylor et al., 2009) and play a role in pectin depolymerization (Ramakrishna et al., 2003). sHSP usually acts in association with high molecular weight proteins like HSP70 to prevent aggregation of unfolding proteins and to induce refolding (Löw et al., 2000). The accumulation of HSP70 occurs mainly in chloroplasts and mitochondria which are the main organelles affected by chilling stress (Horváth et al., 2008); therefore, the greater accumulation of this protein in HWT fruit may be supporting the normal chloroplast to chromoplast transition and energy production. The early accumulation of heat shock proteins, as it happened in mango peel of HWT fruit, could also be related to the induction of the enzymatic antioxidant system by promoting gene expression (Zhang et al., 2005). These results suggest that HSPs play a key role in CI tolerance acquisition by stabilizing membranes and proteins, increasing the activity of antioxidant enzymes, and decreasing the oxidative stress.

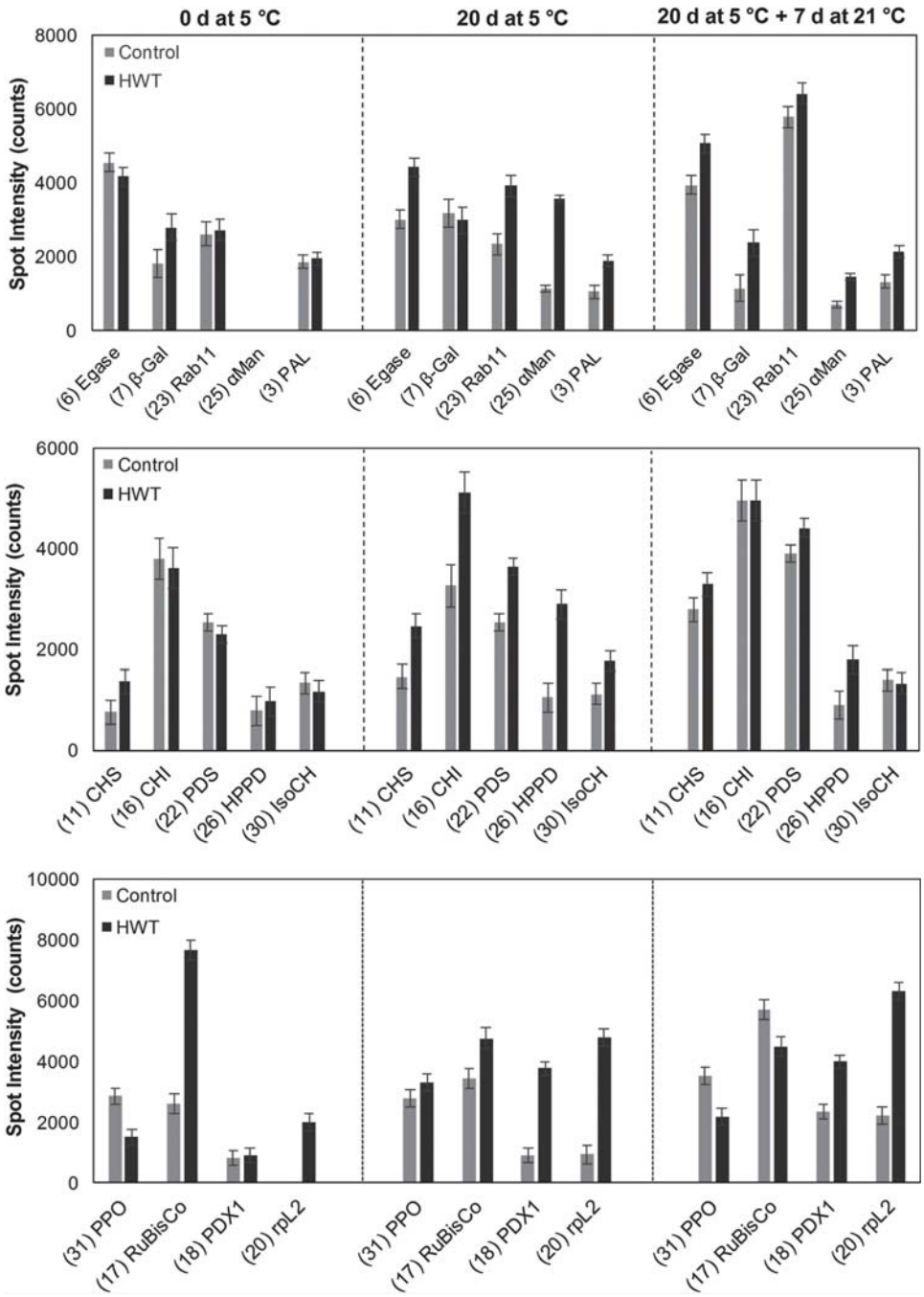

Fig. 4. Quantification of proteins involved in cell wall metabolism, secondary metabolism and photosynthesis that were differentially accumulated between control and HW-treated mangoes stored under chilling conditions and ripened. Spot intensity values (counts) are the mean of three replicates. Bars indicate the LSD ($\alpha = 0.05$); means are significantly different between treatments and storage conditions when the bars do not horizontally overlap. EGase, endoglucanase; β-Gal, beta-galactosidase; Rab11, small GTP Rab11; αMan, alpha-mannosidase; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone-flavonone isomerase family protein; PDS, phytoene desaturase; HPPD, 4-hydroxy-phenylpyruvate dioxygenase; IsoCH, iso-chorismatase hydrolase; PPO, polyphenol oxidase; RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; PDX1, probable pyridoxal 5′-phosphate synthase sub-unit; rpL2, 50S ribosomal protein L2.

4.3. HWT enhance the antioxidant enzymatic system as a mechanism to induce CI tolerance

The HWT induced the expression of APX and this enzyme was only detected in the peel of treated fruit at the different storage conditions. Moreover, the abundance of antioxidant enzymes (CAT, POD, Prx, and APX) increased in response to the cold stress only in HWT fruit and their levels were higher compared to control fruit (Fig. 2; Suppl. Table 2); the same was observed with the transcript levels of *CAT* (Fig. 5B). After ripening, only the contents of peroxiredoxin (Prx) and APX were higher in HWT than control fruit (Fig. 2; Suppl. Table 2). Interestingly, Prx decomposes a wide range of peroxides, including H_2O_2 and lipid peroxides in cytosol, chloroplast, mitochondria, and nucleus (Tripathi et al., 2009; Liebthal et al., 2018) while APX only eliminates H_2O_2 molecules. Both function in antioxidant defense by reducing a broad range of toxic peroxides and in the adjustment of cell redox and antioxidant metabolism (Baier et al., 2000; Finkemeier et al., 2005). H_2O_2 is a relative long-lived ROS with a dual role in stress conditions; at low levels can function as a signal molecule, regulating biological processes and triggering tolerance against various stresses, while at high levels leads to oxidative damage, inactivating enzymes and producing the OH radical that can cause lipid peroxidation, protein damage and membrane destruction (Sharma et al., 2012). The up-regulation of APX induced by the HWT may be a response to control the levels of H_2O_2 produced by the stress or to counter the decrease in Prx accumulation (Fig. 2; Suppl. Table 2); the second function was previously observed in *Arabidopsis thaliana* mutants where a PrxII F gene was suppressed (Finkemeier et al., 2005). Thus, the higher accumulation of antioxidant enzymes observed in HWT fruit after chilling stress and ripening may have prevented harmful levels of H_2O_2, avoiding the disruption of metabolic function,

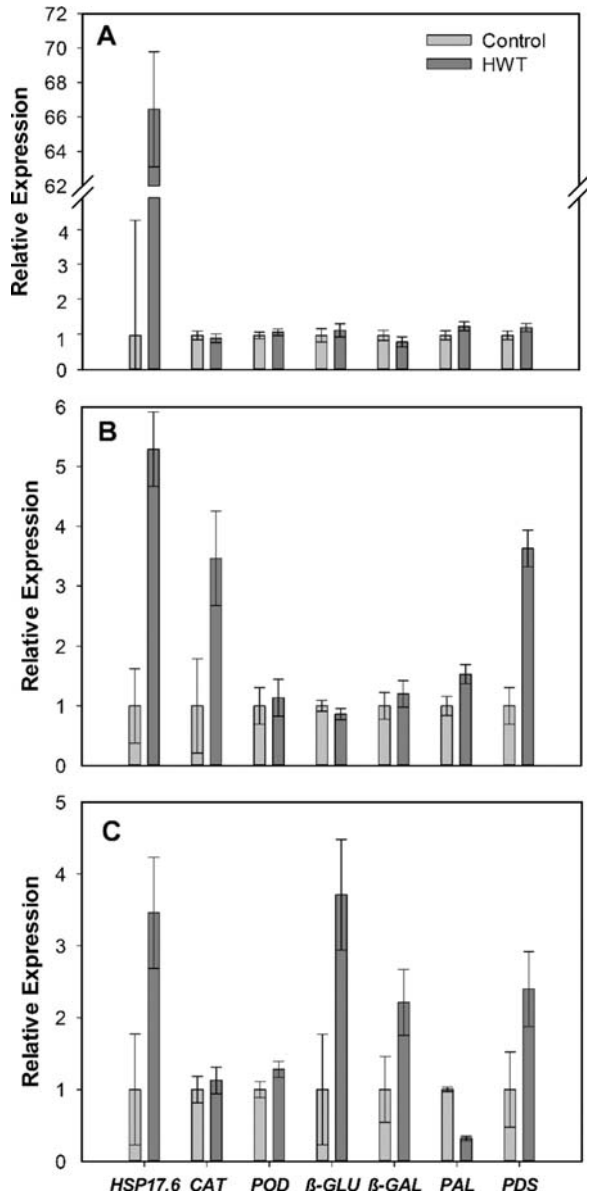

Fig. 5. Relative expression of genes encoding proteins differentially accumulated between control and hot water treated (HWT) fruit before cold storage (0 d at 5 °C) (A), after cold storage (20 d at 5 °C) (B), and after ripening (20 d at 5 °C + 7 d at 21 °C) (C). The actin gene (*ACT*) was used as reference and the expression values are relative to that of the control fruit. Means are significantly different between the treatments when the bars do not horizontally overlap. *HSP17.6*, small heat shock protein of 17.6 kDa; *CAT*, catalase; *POD*, peroxidase; *PAL*, phenylalanine ammonia-lyase; *PDS*, phytoene desaturase; *β-GAL*, β-galactosidase; *β-GLU*, glucan endo-1,3-β-D-glucosidase.

deterioration of the membranes, and the development of CI symptoms. In agreement with the detection of APX only in the HWT fruit in this study, Chongchatuporn et al. (2013) suggested that APX and POD activities increase the CI tolerance in mango fruit peel by counteracting the increment of ROS produced during chilling stress. Besides, CAT and POD were reported as the main enzymes that impact the chilling injury tolerance (Sevillano et al., 2009). The effect of the HWT on the accumulation of antioxidant enzymes corresponds with the increased enzymatic activity induced by HWT in the same mango cultivar (López-López et al., 2018; Díaz-Corona et al., 2020).

4.4. Changes in the energetic metabolism associated with chilling susceptibility and tolerance

The energy available to the cell plays an important role in the CI susceptibility and tolerance since the ATP levels have a direct impact in the membrane integrity, synthesis of macromolecules, and refolding of partially denatured proteins under chilling stress (Liu et al., 2011). Before cold storage, the HWT upregulated GAPDH2 and ADH, whereas the highest downregulation was observed for ADK, suggesting a temporary activation of anaerobic metabolism. After chilling stress and after the ripening period, the abundance of GAPDH1, GAPDH2, SDH, ADK, and ACAA were higher in HWT than control fruit (Fig. 3; Suppl. Table 2), suggesting a more efficient energetic metabolism in treated mangos. ADK catalyzes the reversible AMP phosphorylation to formation of ADP, compound required in ATP production (Igamberdiev and Kleczkowski, 2015). ADK also modulates the Calvin cycle, which is active in several fruit and is involved in sugar synthesis (Smillie, 1992). Vega-Alvarez et al. (2020) reported that the peel of HWT 'Keitt' mango had a higher accumulation of simple sugars after 20 days at 5 °C and ripening. Thus, ADK may play a role in CI tolerance by providing substrates for energy production and sugar synthesis that are vital for chilling acclimation and tolerance acquisition. ACAA catalyzes the final step of the β-oxidation producing acetyl-CoA and a CoA ester of a fatty acid two carbons shorter. Fatty acid oxidation is an important source of energy and provides precursors for the synthesis of molecules like sugars and hormones (Poirier et al., 2006), processes that could have been enhanced by HWT to induce CI tolerance. The results suggest that the HWT protected the machinery involved in energy production during chilling stress and ripening. On the other hand, ADH was the only protein up-regulated in chilled control fruit after the ripening period, suggesting damage in membrane components and low efficient energy production. These results are in agreement with the higher respiration rate reported by Vega-Alvarez et al. (2020) in chilled control mango fruit with more severe CI symptoms.

4.5. Hot water treatment activates hormone transduction signaling and pathogen resistance related mechanisms

A reduction in the accumulation of ethylene response factor (ERF) was observed immediately after HWT (Fig. 3; Suppl. Table 2), which corresponds with previous observations that HWT down-regulates the expression of ethylene responsive transcription factors (Dautt-Castro et al., 2018) and decreases the ACC oxidase and synthase activities (Ketsa et al., 1999; Bender et al., 2003). The enzyme involved in gibberellin metabolism GA3ox1 was detected only in the HWT fruit before and after cold storage, being upregulated by chilling temperatures, and detected in control fruit only after ripening (Fig. 3; Suppl. Table 2). Gibberellin 3-oxidase catalyzes the final step in the biosynthesis of active gibberellins and the greater accumulation of this enzyme in HWT fruit after chilling stress could be associated with higher gibberellin content and contribute to CI tolerance. Gibberellins also modulate the salicylic acid-dependent pathogen resistance by degrading DELLA proteins (De Bruyne et al., 2014). Ding et al. (2015) reported that exogenous gibberellin application in cherry tomato alleviated CI by enhancing proline content and the activity of SOD, CAT, and POD. In another study, the CI tolerance induced by gibberellin in tomato was associated with the up-regulation of *CBF1* and the down-regulation of *DELLA* (Zhu et al., 2016). In addition, higher levels of proteins involved in auxin metabolism (ARF and P-gp2) were observed in HWT fruit before and after cold storage and ripening; interestingly, P-gp2 was undetected in the control fruit after cold storage and ripening (Fig. 3; Suppl. Table 2). Luria et al. (2014) reported previously that HWT induced the up-regulation of genes involved in the metabolism of these hormones. ARFs modulate gene expression in response to auxin (Li et al., 2016) whereas P-gp2 is involved in auxin transport (Blakeslee et al., 2007); thus, auxin signaling and metabolism is negatively affected in control

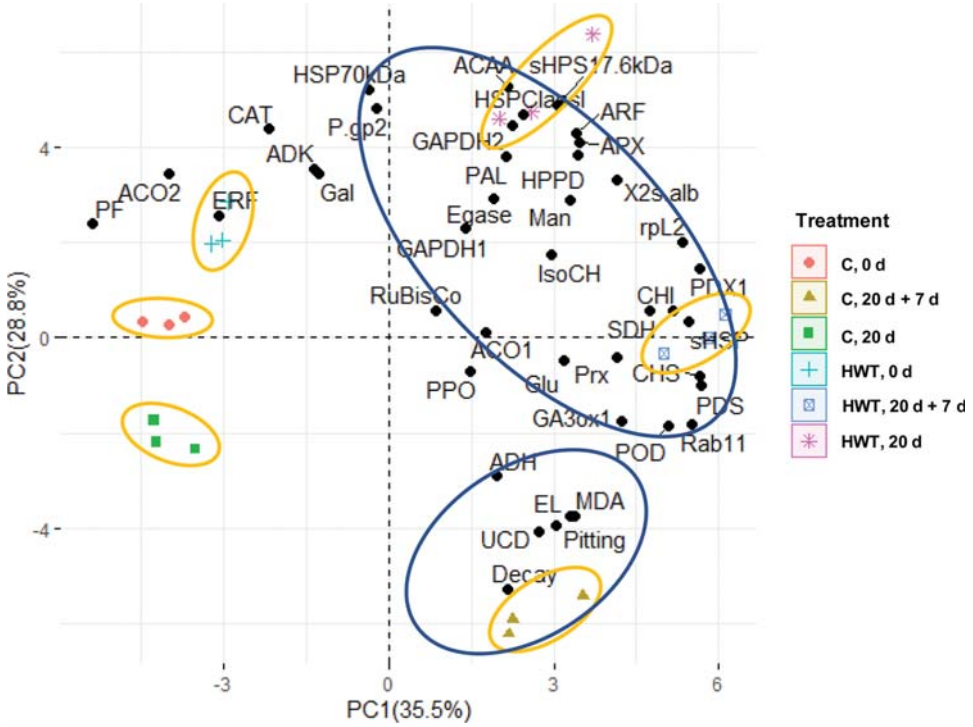

Fig. 6. Biplot based on principal component analysis of chilling injury symptoms, physiological parameters, and differentially accumulated proteins in control (C) and hot water treated (HWT) 'Keitt' mangoes stored for 0 d at 5 °C (0 d), 20 d at 5 °C (20 d), and 20 d at 5 °C plus 7 d at 21 °C (20 d + 7 d). Samples are represented by colored-shapes and the variables by black points. Chilling injury index (CII), peel firmness (PF), uneven color development (UCD), malondialdehyde content (MDA) and electrolyte leakage (EL). Protein abbreviations are listed in Table 2.

fruit, suggesting the importance of these processes in the induction of CI tolerance by the HWT. In this sense, Yang et al. (2013) concluded that the production of indole-3-acetic acid induced by low-temperature conditioning could be a key factor in the CI tolerance of kiwi fruit.

Chilling injury increases the fruit susceptibility to microbial growth because the cell suffers irreversible damage that facilitates pathogen proliferation (Sivankalyani et al., 2016b). HWT has been successfully applied to improve pathogen tolerance and in this study HWT fruit showed increased accumulation of 2S alb and β-1,3-glucanase (β-Glu) at different storage conditions (Fig. 3; Suppl. Table 2), which corresponded with the less incidence of decay in HWT fruit compared to that in control fruit. The antifungal activity of the storage protein 2S alb was associated with an increased membrane permeability in *Kluyveromyces marxiannus, Candida albicans, Candida parapsilosis, Candida tropicalis, Candida albicans, Saccharomyces cerevisiae*, *Fusarium oxysporum*, and *Colletotrichum lindemuthianum* (Agizzio et al., 2003; Ribeiro et al., 2012). β-Glu is a pathogenesis-related protein induced by salicylic acid (He et al., 2017) and shows antifungal and antifreeze activities. β-Glu over-expression leads to enhanced disease resistance against biotrophic and necrotrophic fungal phytopathogens (Ali et al., 2018). Interestingly, the higher accumulation of β-Glu in HWT fruit after chilling stress (Fig. 3; Suppl. Table 2) corresponded to the absence of decay (Table 1). The microbiological analysis of necrotic tissue in control fruit revealed the presence of the fungus *Colletotrichum gloeosporioides* based on morphological and molecular approaches (Suppl. Figs. 2 and 3). These results indicate that HWT activates pathogen defense mechanisms and decreases the development of fungal diseases in mango fruit.

4.6. Hot water treatment maintains normal cell wall metabolism in mango fruit

The HWT increased the accumulation of β-Gal and corresponded with the up-regulation observed previously for this gene in the pulp of mature green mangos dipped in hot water and ripened for 1–5 days (Yimyong et al., 2011; Dautt-Castro et al., 2018). However, the HWT did not affect significantly the peel firmness before cold storage (Table 1). Chilling stress affected negatively the expression of EGase in control fruit and in general the accumulation of the cell wall enzymes was higher in HWT than control fruit after cold storage and after ripening (Fig. 4; Suppl. Table 2), which corresponded with the higher loss of firmness observed in HWT than control fruit after cold storage. αMan and β-Gal remove mannosyl and galatosyl residues from cell wall polymers, EGase hydrolyzes cellulose and hemicellulose, while Rab11 is involved in the trafficking of cell-wall polymers and modifying enzymes. The expression and activity of these enzymes usually increases during ripening and softening of mango fruit (Zainal et al., 1996; Yashoda et al., 2007; Chourasia et al., 2008; Lawson et al., 2020), which corresponds with the upregulation observed for the *β-Gal* gene in HWT fruit after ripening (Fig. 5). Lawson et al. (2020) found a positive correlation between *Rab3* gene expression and the loss of firmness in mango fruit. A similar behavior was observed in the present study. It has been also reported that cell wall metabolism is modified by chilling stress, resulting in a firmer mango fruit due to the accumulation of lignin (Zhang et al., 2012; Sivankalyani et al., 2016b). Zhang et al. (2012) reported that the HWT maintains normal cell wall metabolism in mango fruit exposed to chilling stress by increasing the activity of polygalacturonase and β-Gal. The results of the present study also suggest that HWT preserved the functionality of the cell wall machinery in 'Keitt' mangos after cold storage and ripening.

4.7. Changes in the secondary metabolism induced by hot water treatment associated with CI tolerance

The secondary metabolism is crucial for the survival of plants to biotic and abiotic stresses (Jan et al., 2021). The HWT increased the accumulation of chalcone synthase (CHS) and corresponded with a previous report showing the up-regulation of this gene in the peel of 'Shelly' mangos in response to hot water (Luria et al., 2014); these authors reported higher expression of genes related to the biosynthesis of phenylpropanoids, anthocyanins, and flavonoids. In addition, Vega-Alvarez et al. (2020) observed a higher abundance of quercetin 3-*O*-rhamnoside and total phenolics in the peel of HWT 'Keitt' mango. On the other hand, HWT decreased the abundance of PPO and after ripening HWT fruit showed lower levels of this enzyme than the control

fruit (Fig. 4; Suppl. Table 2) that showed a higher prevalence of decay (Table 1, Suppl. Fig. 1). Thus, low PPO levels in HWT fruit may favor the cellular antioxidant capacity due to the reduced levels of oxidized phenolic compounds. Sivankalyani et al. (2016b) reported that the expression of the *PPO* gene was activated by chilling stress in the peel of 'Keitt' mango, resulting in lenticel discoloration and development of decay. Trejo-Marquez et al. (2010) reported a correlation between PPO activity and the browning of the peel and pulp in the same mango cultivar. This suggests that HWT prevents the cell death induced by chilling stress, avoiding the oxidation of phenolic compounds by PPO.

The enzymes PAL, CHS, and CHI showed higher accumulation in HWT than control fruit after chilling stress and ripening (Fig. 4; Suppl. Table 2), which could be associated with higher levels of phenolics and flavonoids. PAL catalyzes the first reaction of the phenylpropanoids pathway and CHS the step that compromise the flavonoids synthesis. Phenolics and flavonoids have been previously associated with CI tolerance (Sudheeran et al., 2018) and pathogen resistance against *Colletotrichum gloeosporioides* in mango peel (Sivankalyani et al., 2016a) due to their ROS scavenging capacity and antifungal activities. Recently, Vega-Alvarez et al. (2020) reported that HWT increased the phenolics content and antioxidant activity, as well as the content of gallotannins and quercetin 3-*O*-rhamnoside in the peel of 'Keitt' mango stored for 20 d at 5 °C and ripened for 7 d at 21 °C. The higher expression of *PDS* (Fig. 5), as well as the greater accumulation of HPPD and PDS in HWT than control fruit after cold storage and ripening (Fig. 4; Suppl. Table 2) corresponded with a more uniform color development and higher levels of carotenoids reported by López-López et al. (2018) in the pulp of HWT 'Keitt' mangos. HPPD catalyzes the conversion of p-hydroxyphenylpyruvate to homogentisate that can be used directly for the synthesis of tocopherol and indirectly for the synthesis of carotenoids by providing enough plastoquinone for phytoene desaturation (Norris et al., 1995). The increased level of HPPD may be also associated with an increase in tocopherol content which is a lipophilic antioxidant that protects the membranes from oxidative stress. Therefore, HWT appears to maintain fully functional the synthesis of pigments during cold storage and ripening. Another enzyme up-regulated in HWT fruit after chilling stress was IsoCH (Fig. 4; Suppl. Table 2), which catalyzes the hydrolysis of isochorismate to 2,3-dihydroxy-2,3-dihydrobenzoate and pyruvate during the synthesis of siderophores (Hubrich et al., 2021). Iron chelating siderophore molecules could prevent the Fenton reaction and thereby reduce the oxidative stress.

4.8. Hot water treatment maintains active the protein metabolism in chloroplasts and cofactor synthesis

Chilling stress negatively affects the chloroplasts, causing membrane deterioration and partial or complete loss of their functions (Liu et al., 2018). Chloroplasts are important for the fruit because they synthesize secondary metabolites necessary to hold up biotic and abiotic stresses. The integrity of the chloroplasts and their transcriptional machinery is required for the correct transition toward chromoplasts, which provide the external characteristic coloration in ripen fruit (Yang et al., 2009; Egea et al., 2010). Therefore, the up-regulation of rpL2 observed in HWT fruit (Fig. 4; Suppl. Table 2) could be associated with a greater synthesis of proteins necessary for the Calvin cycle, proteolysis, ATP synthesis, and ribosomal protein synthesis like RPS1, which has been associated with heat tolerance in *Arabidopsis* by functioning as a messenger and activating the nuclear heat shock factor and heat shock protein gene expression (Azim et al., 2014; Hu et al., 2020). The presence and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit has been already reported in tomato chromoplasts (Bravdo et al., 1977; Barsan et al., 2010). The up-regulation of RuBisCo in HWT fruit after hot treatment and chilling stress (Fig. 4; Suppl. Table 2) could be an indication of active CO_2 fixation or membrane integrity. After ripening, control chilled samples had a higher accumulation of RuBisCo (Fig. 4; Suppl. Table 2), corresponding with the higher values of CI index for the uneven color development symptom. The higher accumulation of PDX1 (Fig. 4; Suppl. Table 2) observed in HWT fruit after chilling stress and ripening could be related to the maintenance of cellular metabolism because pyridoxal 5′-phosphate is a coenzyme for many enzymatic processes, synthesis of biological molecules, and ROS scavenging; pyridoxal 5-phosphate enhances the activity of POD, APX, CAT, and glutathione peroxidase (Czégény et al., 2019). Titiz et al. (2006) demonstrated the importance of *PDX1* gene expression and vitamin B_6 in preventing the oxidation of photosynthetic components in *Arabidopsis*.

4.9. Protein clusters associated with chilling injury tolerance and susceptibility

The PCA analysis allowed to associate proteins with CI tolerance and susceptibility in 'Keitt' mango peel (Fig. 6). HWT fruit stored for 20 d at 5 °C and subsequently ripened (7 d at 21 °C) were located at the right quadrant close to all sHSP, glycolysis, and Krebs cycle enzymes (GAPDH1 GAPDH2 and SDH), antioxidant enzymes (APX, Prx, and POD), cell wall (Rab11, EGase and αMan) and hormone metabolism (AFR, Ga3ox1, and Pgp2), secondary metabolism (PAL, CHS, CHI, PDS, HPPD, and IsoCH), and pathogenesis-related proteins (2 s alb and Glu). The negative correlation found between CI symptoms, physiological parameters, and some of these proteins (Suppl. Table 3) supports the idea that CI tolerance induced by HWT is associated with the improvement of these metabolic pathways. These results correspond with previous studies demonstrating the importance of chaperone proteins, ROS scavenging enzymatic and non-enzymatic systems, as well as the maintenance of energy status and cell wall metabolism in the acquisition of CI tolerance (Sevillano et al., 2009; Aghdam et al., 2013; Li et al., 2014; Zhu et al., 2016; López-López et al., 2018; Vega-Alvarez et al., 2020). On the other hand, ripen chilled control fruit was located close to CI symptoms (UCD, pitting, and decay), physiological parameters (EL and MDA content), and ADH. These results provide further evidence that CI development is associated with the loss of membrane functionality and low energy levels as previously suggested for mango fruit (Li et al., 2014; Cantre et al., 2017).

5. Conclusions

The quarantine hot water treatment provided tolerance to chilling injury in 'Keitt' mango fruit. This tolerance was associated with the activation of heat shock proteins involved in the stabilization of proteins and membranes, enzymes involved in the control of redox state, energy production and cell wall metabolism, synthesis of phenylpropanoids and carotenoids, as well as the induction of hormone signaling and pathogenesis-related proteins. On the other hand, chilling injury susceptibility was related to loss of membrane functionality and lower efficiency in energy production.

CRediT authorship contribution statement

Nancy Y. Salazar-Salas: Investigation, Writing - Original Draft. **Dennise A. Chairez-Vega:** Methodology (Protein identification by MS/MS). **Milton Vega-Álvarez:** Methodology (Postharvest assays, quality and physiological parameters, RT-qPCR). **David G. González-Núñez:** Methodology (Protein separation by 2D-PAGE). **Karen V. Pineda-Hidalgo:** Data Analysis. **Jeanett Chávez-Ontiveros:** Methodology (Protein extraction, fungal identification). **Francisco Delgado-Vargas:** Writing-Reviewing and Editing. **José A. Lopez-Valenzuela:** Conceptualization, Supervision, Writing-Reviewing and Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2022.111838.

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