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Transcriptional changes associated with chilling tolerance and susceptibility in 'Micro-Tom' tomato fruit using RNA-Seq

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ABSTRACT

Tomato fruit are horticultural products of great economic and nutritional value, whose transportation and marketing at low temperature are limited due to their susceptibility to develop chilling injury (CI). Hot water (HW) pre-treatments have been shown to reduce the CI symptoms in tomato fruit, but the molecular mechanisms involved in the acquisition of CI tolerance remain unclear. In the present work, a comparative transcriptomic analysis between HW treated and non-treated fruit before and after cold storage was carried out. RNA-Seq analysis detected a large number of differentially expressed genes that ranged from 2235 (heat shock) to 5433 (cold storage). Three clusters of genes were identified after 2 weeks of cold storage: the chilling-response included the down-regulation of genes involved in photosynthesis, metabolism of cell wall, lipid and ethylene, as well as the up-regulation of genes for trehalose synthesis and transcription factors (DOF and MYB); the chilling-susceptibility was associated with the down-regulation of genes involved in carotenoid biosynthesis, which correlates with the main CI symptom of uneven ripening; meanwhile, the chilling-tolerance was related to the up-regulation of genes for heat stress (heat shock proteins and heat shock transcription factors) and detoxification (glutathione *S*-transferases). The induced tolerance to CI in tomato fruit seems to be related first with the protection of cell wall and membranes integrity, and second with the restoration of ethylene biosynthesis and signaling.

1. Introduction

Low-temperature storage is the most important method of reducing postharvest decay and maintaining the organoleptic and nutritional quality of fruits and vegetables; however, exposing tropical and subtropical crops to temperatures below 12 °C induces the development of a physiological disorder known as chilling injury (CI), which leads to a decrease in the postharvest quality and thus to important economic losses (Polenta et al., 2007; Aghdam et al., 2013). Remarkably, CI disorders usually become visible when fruits reach the consumers, turning fruits unpalatable and leading to consumer rejection (Lauxmann et al., 2012).

Physiological responses to CI have been comprehensively reviewed (Sevillano et al., 2009); the first response is related to alterations in cell membrane conformation and structure, caused by changes in the lipid composition of the membrane that results in a decrease of its fluidity and permeability; these changes affect the functionality of mitochondria and chloroplast, causing an overproduction of reactive oxygen species (ROS) and thus an increase in the oxidative stress, which is considered a secondary response to CI.

Tomato is the second most important horticultural crop worldwide; nevertheless, its transportation and marketing are limited by its susceptibility to develop CI, being 12 °C the minimum safe temperature for storage. The main CI symptoms in tomato fruit include uneven ripening, pitting and decay, and they usually appear when fruit are transferred to a ripening temperature (20–22 °C) after being stored at low temperature (2–6 °C) more than 2 weeks (Vega-García et al., 2010).

Among the existing tomato genotypes, the cultivar Micro-Tom is considered a model system because of its unique characteristics,

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such as small size, ability to grow in high densities, and short life cycle (Meissner et al., 1997). 'Micro-Tom' presents a very low frequency (0.06%) of nucleotide mismatch in exons with respect to the genome-sequenced cultivar Heinz 1706 (Aoki et al., 2010), and efficient genetic transformation protocols (Dan et al., 2006; Cruz-Mendívil et al., 2011) have been developed for this model system, making it a valuable tool for functional genomics. Furthermore, some studies have shown that 'Micro-Tom' fruit are susceptible to developing CI when exposed to low temperatures (Malacrida et al., 2006; Gómez et al., 2009; Luengwilai et al., 2012a, 2012b; Müller et al., 2013), but to a lower extent when compared to commercial tomato cultivars (Vega-García et al., 2010).

Omics-based approaches have allowed us to address the complex global biological systems that underlie various plant functions and responses (Mochida and Shinozaki, 2011). In this sense, proteomic studies in cold-stored tomato fruit have revealed that tolerance or defense mechanisms are related to the accumulation of heat shock proteins (HSPs), late embryogenesis abundant (LEA) proteins, molecular chaperones (GR-RBP) and antioxidant enzymes (TPxI); meanwhile, the susceptibility mechanisms include the uncoupling of photosynthetic processes (ATP synthase) and protein degradation machinery (26S proteosome) (Page et al., 2010; Vega-García et al., 2010; Sanchez-Bel et al., 2012).

Whereas proteomics enables the identification of posttranscriptional and posttranslational modifications, transcriptomic analysis allows the identification of genes coding for proteins that are difficult to determine by 2D gel electrophoresis (Seliger et al., 2009); in addition to stress-responsive transcription factors (TFs) which may be potential targets for crop breeding (Calderon-Vazquez et al., 2008). A transcriptomic analysis of 'Micro-Tom' fruit after a short cold storage (48 h at 6 °C) showed that 38 genes were up-regulated by cold, but only one coding for a dehydrin was related to previously identified cold-stress genes (Weiss and Egea-Cortines, 2009). Dehydrins belong to the group II of LEA family and protect proteins and membranes against unfavorable structural changes (Kosová et al., 2007). On the other hand, an expression analysis of tomato fruit after a long period of cold storage (4 weeks at 3 °C) showed that the CI visual symptom of uneven ripening was the outcome of alterations in genes from carotenoid biosynthesis, cell wall modification, ethylene biosynthesis and signaling (Rugkong et al., 2011). These transcriptomic approaches were focused on chilling response and susceptibility; therefore, studies focusing on CI tolerance mechanisms are still required.

Heat treatments (air, steam or water) have been previously used to alleviate the CI symptoms in tomato fruit, where hot water (HW) treatments have been the most efficient in terms of heat transfer and CI symptoms reduction (Lurie et al., 1997; Yang et al., 2009; Luengwilai et al., 2012a). These treatments are applied prior to cold storage, and consist in the immersion of fruit in HW (38–54 °C) for short times (1–15 min). It is thought that exposure to a high temperature triggers physiological responses that allow the tissue to cope in a better way with subsequent stress conditions (Lara et al., 2009); there is evidence that induced tolerance to CI in tomato fruit after a heat treatment is related to the accumulation of HSPs (Polenta et al., 2007), metabolites such as arachidic acid and 2-ketoisocaproic acid (Luengwilai et al., 2012b), and enhanced arginase activity (Zhang et al., 2013). However, the global transcriptomic changes in tomato fruit with induced tolerance to CI have not been analyzed yet, which may be useful in the understanding of the molecular network induced by a postharvest heat treatment.

Considering the above mentioned, in the present work a transcriptomic approach using RNA-Seq was undertaken to identify differentially expressed (DE) genes in tomato fruit after the heat shock, cold storage and subsequent ripening. DE genes were grouped in chilling-responsive regulons to identify common and unique responses to chilling, followed by a functional categorization to obtain a biological context of the chilling tolerance and susceptibility mechanisms in tomato fruit.

2. Materials and methods

2.1. Plant material and postharvest treatments

Tomato fruit (*Solanum lycopersicum* cv. Micro-Tom) were harvested at mature-green stage, and selected based on epidermis color (a^* = −13 to −10) and weight (2–3 g). The mature-green stage was selected due to their higher susceptibility to develop CI, and also to avoid the complex transcriptomic changes associated with ripening at breaker and later stages. After washing, tomato fruit were immersed for 7 min in water at 40 °C (HW) or at 20 °C (control), air-dried for 30 min at room temperature, and then stored for 14 days at 5 °C to induce chilling stress, followed by 14 days at 20 °C to allow ripening and symptoms development (Luengwilai et al., 2012a). Relative humidity was maintained above 90% during the storage.

2.2. CI index, physical and physiological parameters

CI index (Vega-García et al., 2010) and physical parameters of fruit quality (weight loss, color and firmness) were determined after 14 days at 5 °C + 14 days at 20 °C. The severity of the symptoms (U = uneven ripening, and W = wilting) was assessed visually as injury level (IL) using a five-point scale based on the percentage of tissue affected for each criterion (0 = no injury, 1 ≤10%, 2 = 11–25%, 3 = 26–40%, 4 ≥40%). CI index was calculated with the formula: (ILU + ILW)/2. The weight loss (%) was calculated using the values of initial weight (W_0) and final weight (W_f) with the following expression: $[(W_0 - W_f)/W_0] \times 100$. Color changes on the surface of tomato fruit were quantified by the values of L^*, a^*, b^* and Hue° using a colorimeter CR-200 (Konica Minolta Inc., Tokyo, Japan), and the total difference of color (ΔE) was calculated with the expression $\Delta E = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)}$. The pericarp firmness was evaluated with a digital force gauge Chatillon E-DFE-100 (Ametek, Berwyn, PA). Each fruit was cut into four segments and the locular tissue was removed; these segments were placed with the pericarp upward and penetrated in the equatorial region to a depth of 1 mm, using a 1 mm diameter tip and a constant speed of 0.83 mm s^{-1}. The maximum compression force was expressed in Newtons (N).

Ion leakage was measured in pericarp discs (1 cm diameter) after 0, 7 and 14 days of storage at 5 °C, and expressed as % (Zhao et al., 2009a). Respiration rate was assessed in chilled fruit (5 °C for 14 days) during reconditioning at 20 °C (0, 4, 8, 12, 16 and 24 h), and the production of CO_2 was expressed in μg kg^{-1} s^{-1} (López-Valenzuela et al., 2011).

For all postharvest assays, the experimental unit consisted in 10 fruit, and three replicates per treatment were used in each experiment. Data were subjected to analysis of variance and comparison of means with the Fisher's least significant difference test (LSD, $P \leq 0.05$), using the STATGRAPHICS Plus 5.1 software.

2.3. RNA isolation and high-throughput sequencing

Tomato fruit from both control and HW treatments were sampled after 0 h, 14 days at 5 °C, and 14 days at 5 °C + 14 days at 20 °C, and named C-0h, C-14d, C-14+14d, HW-0h, HW-14d and HW-14+14d, respectively. Five fruits were pooled for each condition, pericarp tissues were ground in liquid nitrogen and stored at −70 °C until use. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and purified using RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. RNA quality was monitored by gel electrophoresis, A_{260}/A_{280} ratio, and RNA integrity number (RIN). High quality RNA samples

(25 μg each) were used for cDNA libraries preparation and massive sequencing on a 5500 SOLiD System (with Exact Call Chemistry module), following the protocols from Applied Biosystems (Foster City, CA). Two technical replicates of sequencing (independent runs) were performed per pooled sample. In total, 5.44 Gb of single-end reads (75 bp length) were obtained from the six samples. Raw sequence data is available for download at NCBI Sequence Read Archive under the BioProject Accession: PRJNA229516.

2.4. Bioinformatic analysis

SOLiD reads were analyzed with the software CLC Genomics Workbench version 6.0 (CLC bio, Aarhus, Denmark). Raw reads were trimmed with the following parameters: phred quality score ≥20, ambiguous nucleotides ≤2, and read length ≥25 bp. Trimmed reads were mapped in color-space against the tomato reference 'ITAG2.3_cdna' (http://solgenomics.net/) consisting of 34,727 protein-coding genes, using default parameters (mismatches ≤ 2, length fraction ≥ 0.9, similarity fraction ≥ 0.8), and only those uniquely aligned reads were conserved. The number of uniquely mapped reads per gene were used as expression values and represented as unique gene reads (UGR). The resulting UGR values were subjected to quantile normalization to remove the bias of sequencing depth across all samples. Then, principal component analysis (PCA) was used to validate the repeatability of RNA-Seq data across technical replicates and also to compare the global expression patterns between the different conditions.

In order to have more certainty that the statistical differences between samples had a biological significance, genes with normalized UGR means <1 were discarded before statistical analysis with Baggerly's *t*-test, which compares the proportions of counts in a group of samples against those of another group of samples, and is suited for cases where replicates are available in the groups. Multiple testing corrections were performed by controlling the false discovery rate of *P* values and expressed as *Q*. Genes with $Q < 0.01$ and log2 fold change (FC) of at least ±1 were considered as DE.

In order to distinguish which DE genes were related to chilling tolerance and which to chilling susceptibility, the strategy proposed by Maul et al. (2008) was used. Common genes in control and HW samples were assigned to the 'chilling-response regulon', unique genes in control sample were assigned to the 'chilling-susceptibility regulon', and unique genes in HW sample were assigned to the 'chilling-tolerance regulon'.

2.5. Functional categorization

Lists of gene identifiers and log2 FC values from each condition (heat shock, cold storage, and cold storage + ripening) were imported into the MapMan software version 3.6 (Thimm et al., 2004), and assigned to functional categories (BINS) using the mapping tomato file 'Slyc_ITAG2.3'. Functional categories were tested for significance using the Wilcoxon rank sum test included in MapMan software, and significant ($P < 0.01$) BINS in at least one condition were displayed.

3. Results

3.1. Validation of HW treatment to induce CI tolerance in tomato fruit

After the cold storage + ripening period (14 days at 5 °C + 14 days at 20 °C), the main CI visual symptoms observed in 'Micro-Tom' fruit were uneven ripening and wilting (skin depressions), meanwhile fruit with HW treatment (40 °C for 7 min) reached a more uniform ripening and did not show wilting (Supplementary Fig. S1), showing a significant reduction (40%) in the CI index with respect to control fruit (Table 1). Because the CI index is a subjective evaluation, the visual symptoms were associated with measurable responses such as weight loss (for wilting), firmness and color parameters (for uneven ripening). At the end of the storage, HW treated fruit showed a reduction in weight loss and a more advanced ripening stage, as evidenced by color parameters closer to red (higher ΔE, higher a^* and lower Hue°) and lower pericarp firmness, with significant differences ($P \leq 0.05$) against control fruit (Table 1).

Table 1
Effect of a hot water (HW) treatment on chilling injury (CI) index and quality parameters in tomato fruit.

Sample	CI index	Firmness (N)	Weight loss (%)	Color parameters		
				a^*	Hue°	ΔE
Control	1.5 a	6.1 a	7.7 a	14.9 b	72.9 a	37.9 b
HW	0.9 b	4.7 b	6.7 b	21.2 a	65.7 b	42.6 a

Data recorded after 14 days at 5 °C + 14 days at 20 °C. Means with different letters within a column indicate significant differences (LSD, $P \leq 0.05$). ΔE = total difference of color.

Ion leakage and respiration rate are physiological markers of CI development; therefore, reductions in these parameters can be associated with CI tolerance mechanisms. In the present work, significant ($P \leq 0.05$) reductions in ion leakage of 32% and 22% were observed in HW treated fruit compared with control, after 7 and 14 days of cold storage, respectively (Supplementary Fig. S2). The respiration rate was 48% higher in control fruit compared with HW treated fruit immediately after their removal from cold storage (Supplementary Fig. S2). After 4 h at 20 °C, the respiration rate drastically decreased in both treatments, but HW treated fruit still showed a significantly ($P \leq 0.05$) lower value with respect to control; thereafter no significant differences were found between both treatments (Supplementary Fig. S2).

Taken together, the above results validate the effectiveness of the HW treatment (40 °C for 7 min) recommended by Luengwilai et al. (2012a), to induce CI tolerance in 'Micro-Tom' fruit harvested at mature-green stage. Two weeks of cold storage (at 5 °C) and two additional weeks of ripening (at 20 °C) were considered as suitable sampling points for the subsequent RNA-Seq analysis, since at these time points it was possible to detect CI development in control fruit and CI tolerance in HW treated fruit.

3.2. RNA-Seq analysis

In order to understand the regulation of the physiological and biochemical modifications described above, an RNA-Seq analysis was performed in pericarp tissue from both control and HW treated fruit after three time points: time zero (0 h), cold storage (14 days at 5 °C), and cold storage + ripening (14 days at 5 °C + 14 days at 20 °C). A total of 72,516,607 raw reads (75 bp length) were obtained from the six sequenced samples, and after trimming by quality and length, 48,012,815 reads (66.2%) were retained. From those, 32,254,475 reads (67.2%) were uniquely mapped in color-space format to the tomato reference transcriptome 'ITAG2.3_cdna' (http://solgenomics.net/), and conserved for further analyses. A summary of trimming and mapping statistics is provided in Supplementary Table S1.

The PCA (Fig. 1) shows that the two technical replicates of each sample were positioned closely together, which means that the sequencing results had an adequate repeatability; and four groups could be distinguished based on their positions: control (C-0h), heat shock (HW-0h), chilling (C-14d, HW-14d) and ripening (C-14+14d, HW-14+14d). Interestingly, after the application of the HW treatment, the expression profiles changed considerably and were separated from the control group. Meanwhile,

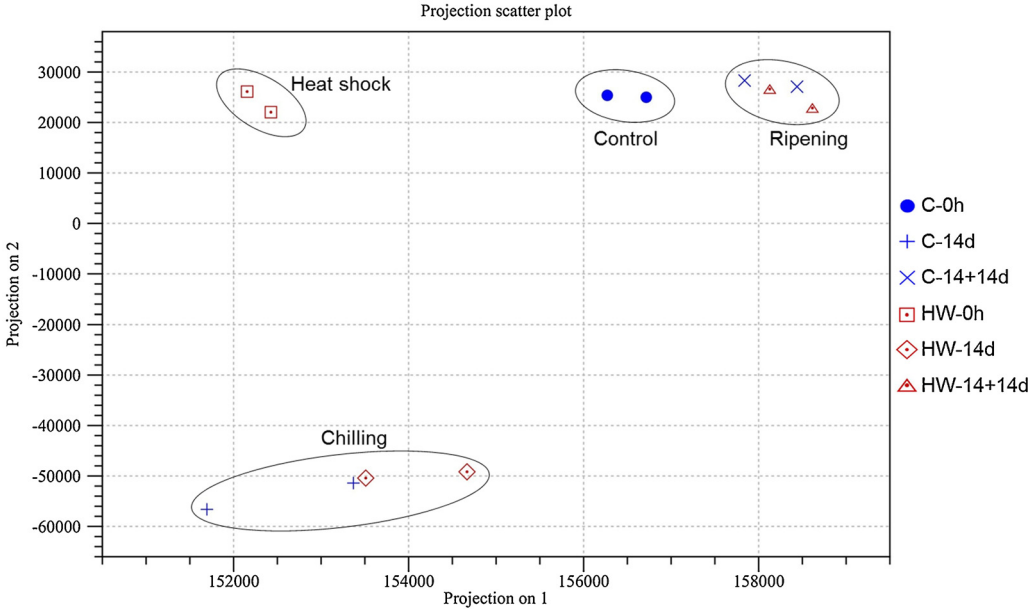

Fig. 1. Principal component analysis of tomato transcript profiles using normalized expression values of unique gene reads. Samples were obtained from C (control) and HW (hot water) treated tomato fruit after 0 h, 14 days at 5 °C, and 14 days at 5 °C + 14 days at 20 °C. Each symbol on the plot represents data from all genes in one sample reduced to the first and second principal components, and equal symbols represent the two replicates of sequencing. Samples that had similar expression profiles were grouped together, and four groups could be distinguished: control (C-0h), heat shock (HW-0h), chilling (C-14d, HW-14d) and ripening (C-14+14d, HW-14+14d).

the chilling samples formed the most distant group from the rest of samples, suggesting that major transcriptional changes associated with CI susceptibility and tolerance occur during cold storage, before the manifestation of visual symptoms at ripening temperature.

3.3. DE genes and chilling-responsive regulons

Gene expression values from each condition (heat shock, cold storage and ripening) were compared to those from control time zero (C-0h). The number of DE genes ranged from 2235 after the heat shock, to 5433 after the cold storage of HW treated fruit, with distribution of up- and down-regulated genes close to 50% in each comparison (Table 2). Complete details of DE genes from all comparisons are provided in Supplementary Table S2.

Until this point of the analysis, it was difficult to distinguish between common and unique responses to chilling, since most of the up- and down-regulated genes followed similar trends in both control and HW samples during cold storage and ripening (Supplementary Table S2); therefore, we proceeded to group the DE genes in chilling-responsive regulons as proposed by Maul et al. (2008). As observed in the Venn diagrams of Fig. 2, common genes in both control and HW samples were assigned to the 'chilling-response regulon', unique genes in control samples were assigned to the 'chilling-susceptibility regulon', and unique genes in HW samples were assigned to the 'chilling-tolerance regulon'. Detailed information of DE genes belonging to each regulon is

Table 2
Effects of hot water (HW) treatment, cold storage (CS) and ripening on the transcriptome of tomato pericarp.

Pairwise comparison	Differentially expressed genes		
	Up-regulated	Down-regulated	Total
CS/time zero	2757	2637	5394
CS + ripening/time zero	1992	1970	3962
HW/time zero	1045	1190	2235
HW + CS/time zero	2713	2720	5433
HW + CS + ripening/time zero	1712	1894	3606

Differentially expressed genes at $Q < 0.01$ (Baggerly's test) and log2 fold change ± 1.

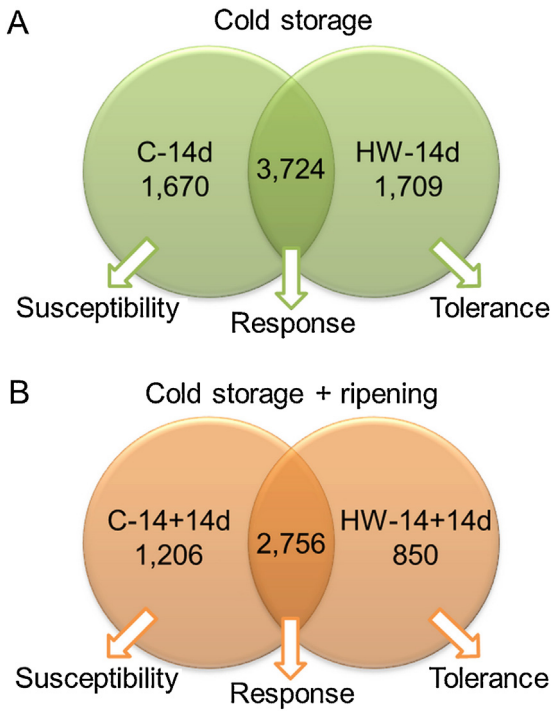

Fig. 2. Differentially expressed genes grouped in chilling-responsive regulons in tomato fruit after (A) cold storage (14 days at 5 °C) and (B) ripening (14 days at 5 °C + 14 days at 20 °C). Common genes in C (control) and HW (hot water) samples were assigned to the 'chilling-response regulon', unique genes in C sample were assigned to the 'chilling-susceptibility regulon', and unique genes in HW sample were assigned to the 'chilling-tolerance regulon'.

provided in Supplementary Tables S3 (cold storage) and S4 (cold storage + ripening).

3.4. Functional categorization of heat- and chilling-responsive genes

DE genes after heat shock and DE genes grouped in chilling-responsive regulons after cold storage and ripening were assigned to the functional categories included in the MapMan software (Thimm et al., 2004); and the Wilcoxon rank sum test was used to calculate the probability that the response of the genes assigned to a functional category was statistically different from that of all remaining genes. Fig. 3 shows the functional categories that were significant ($P < 0.01$) in at least one condition, and their average expression changes (log2 FC) are represented on a color scale (red = repression, blue = induction). Detailed information of these functional categories in all conditions is provided in Supplementary Table S5.

After heat shock, genes involved in metabolism of minor carbohydrates, protein targeting and folding, transport, heat stress and heat shock TFs (HSTFs) were up-regulated, while those related to secondary metabolism (phenylpropanoids and phenols), hormone metabolism, *N*-acetyltransferases, some TFs (bHLH, HB and MYB), DNA synthesis, posttranslational modification (kinase), protein degradation (subtilase) and signaling (receptor kinases) were down-regulated (Fig. 3).

After cold storage, the chilling-response regulon included the down-regulation of genes involved in photosynthesis, degradation of cell wall and lipids, metabolism of ethylene and jasmonate, biotic stress (signaling) and several enzymes (glycosidases, oxidases, nitrilases and lipases); this regulon also included the up-regulation of genes related with trehalose synthesis, abscisic acid-responsive proteins, RNA processing and binding, TFs (DOF and MYB-related), protein synthesis and degradation, and cell division. On the other hand, the chilling-susceptibility regulon after cold storage was associated with the down-regulation of isoprenoid biosynthesis and RNA transcription. Meanwhile, the chilling-tolerance regulon after cold storage showed the up-regulation of heat stress, glutathione *S*-transferases (GSTs) and RNA processing, as well as the repression of lipid metabolism and storage proteins (Fig. 3).

After cold storage + ripening, the chilling-response regulon included the down-regulation of photosynthesis, fermentation, biotic stress, auxin/IAA regulation, peroxidases, protease inhibitors, lipid transfer proteins and storage proteins, as well as the up-regulation of electron transport/ATP synthesis, heat stress, RNA transcription and protein synthesis. In the chilling-susceptibility regulon after ripening, it was observed the down-regulation of nitrilases and lipases. Finally, the chilling-tolerance regulon after ripening showed the up-regulation of electron transport/ATP synthesis and RNA transcription, and the down-regulation of signaling (receptor kinases) (Fig. 3).

The transcriptional changes of DE genes grouped in chilling-responsive regulons after cold storage and ripening (Fig. 2) were graphically represented into the MapMan pathways of cellular response overview (Supplementary Figs. S3 and S4), regulation overview (Supplementary Figs. S5 and S6) and metabolism overview (Supplementary Figs. S7 and S8). The final analysis consisted in the search of functional categories that had contrasting gene expression between the chilling-susceptibility and -tolerance regulons; for example, functional categories that were down-regulated in the susceptibility regulon and up-regulated in the tolerance regulon. The functional categories with contrasting expression after cold storage included the metabolism of minor carbohydrate, cell wall and lipid, biosynthesis of ethylene and carotenoids, heat and drought stress, as well as redox and detoxification (Table 3).

4. Discussion

4.1. Induction of HSPs and HSTFs during cold storage and ripening

HSPs constitute a stress-responsive family of proteins (15–115 kDa) that are rapidly accumulated in response to heat stress, besides other conditions such as fruit ripening, cold and oxidative stress (conditions involved in CI development); in addition, the HSTFs participate in regulating the gene expression of both HSPs and antioxidant proteins, increasing the protection against oxidative stress (Sevillano et al., 2009; Aghdam et al., 2013).

After cold storage, we observed that 91 DE transcripts were related to heat stress (Supplementary Table S5), which corresponded to 39.2% of the total genes in that BIN (20.2.1). Interestingly, six *HSPs* and two *HSTFs* were down-regulated in the chilling-susceptibility regulon, while 12 *HSPs* and one *HSTF* were up-regulated in the chilling-tolerance regulon (Table 3; Supplementary Fig. S3). Of these 12 up-regulated *HSPs*, six belong to the HSP20 family (small HSPs), four to the HSP70 family and two to the HSP90 family. It appears that the strong initial expression of HSPs after the HW treatment (Fig. 3; Supplementary Table S5) was sufficient to ensure the induction of several HSPs during cold storage of tomato fruit, exerting a protective effect against cold stress. These results are in agreement with Page et al. (2010), who observed that after cold storage of tomato fruits from two genotypes with contrasting CI tolerance, four small HSPs were up-regulated in the more tolerant genotype; furthermore, Sanchez-Bel et al. (2012) reported the up-regulation of two small HSPs as a first response of tomato fruit to cope with cold stress.

On the other hand, after cold storage + ripening we observed that the heat stress BIN was up-regulated in both control and HW treated fruit (Supplementary Table S5 and Fig. S4), which suggest that induction of HSPs at the end of storage, may not be related with thermal stress; instead, HSPs may be playing a role in tomato fruit ripening as previously reported by Neta-Sharir et al. (2005).

4.2. Induction of dehydrins and dehydration-responsive proteins by chilling

Dehydrins are expressed during late stages of embryogenesis, as well as in vegetative tissues subjected to drought, low temperature and salinity, suggesting their role in the protection of cells from damage caused by stress-induced dehydration (Caffagni et al., 2014). It has been reported that dehydrins are up-regulated in tomato when exposed to low temperature, both in leaf tissue (Caffagni et al., 2014) and fruit (Weiss and Egea-Cortines, 2009).

In the present work, 21 DE transcripts were related to drought/salt stress after cold storage (Supplementary Table S5), equivalent to 27.3% of the total genes in that BIN (20.2.3). We also observed that one dehydrin and two dehydration-responsive proteins were up-regulated in the chilling-susceptibility regulon; meanwhile three dehydration-responsive proteins were down-regulated in the chilling-tolerance regulon (Table 3; Supplementary Fig. S3). These results suggest that the up-regulation of dehydrins in the chilling-susceptibility regulon may be a response to cold-induced water loss in control fruit. Meanwhile, the HW treatment appears to be preventing water loss during cold storage, and thus the down-regulation of dehydrins in the chilling-tolerance regulon.

4.3. Induction of antioxidant enzymes by chilling

Oxidative stress occur when cell homeostasis is perturbed and there is a disproportionate increase in the production of ROS, if the plant cannot counteract them by the antioxidant system, then cell death occurs due to oxidative processes such as lipid peroxidation,

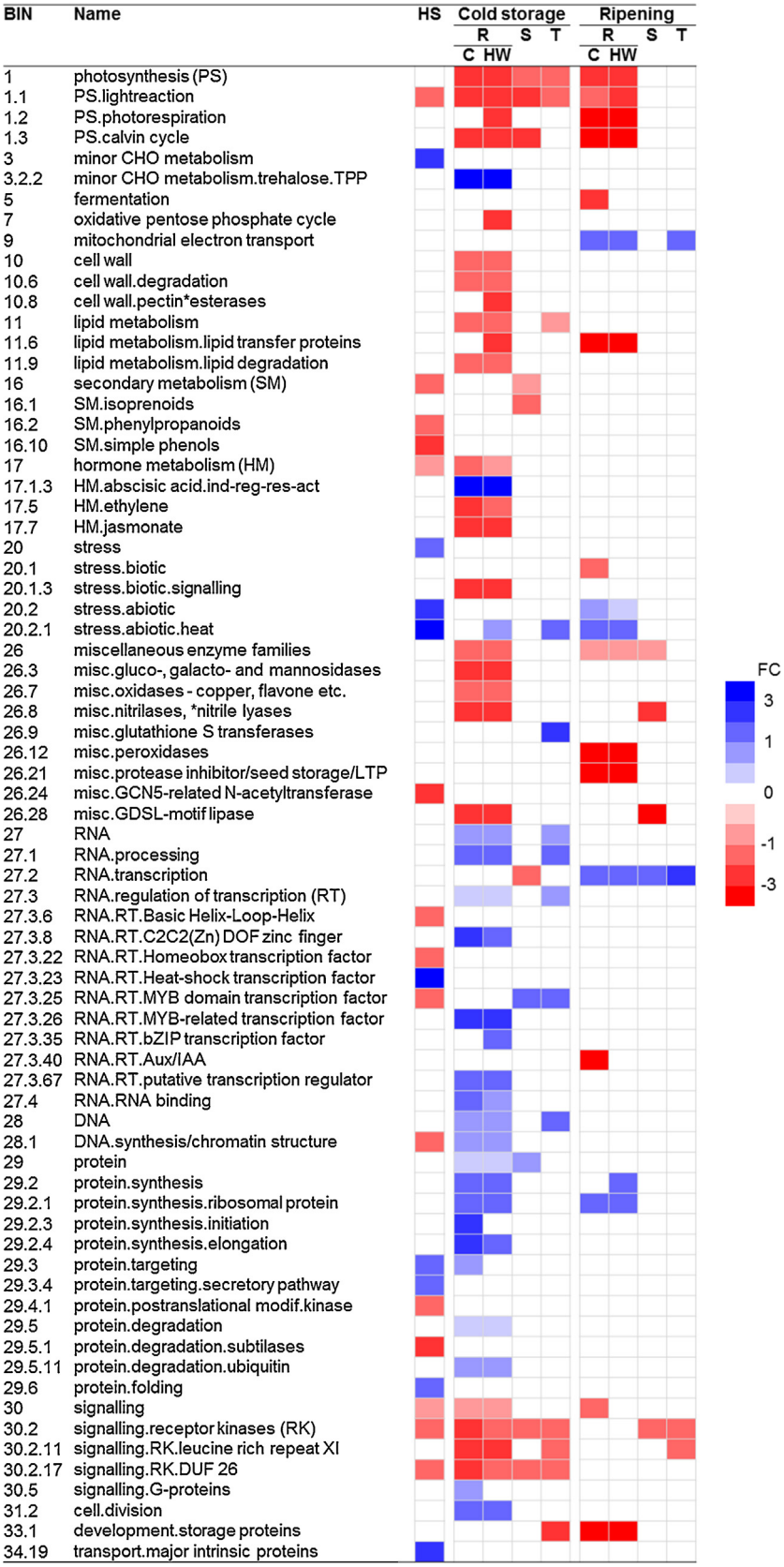

Fig. 3. Functional categorization and global gene expression changes in tomato fruit after heat shock (7 min at 40 °C), cold storage (14 days at 5 °C), and cold storage + ripening (14 days at 5 °C + 14 days at 20 °C). MapMan BINS that were significant ($P < 0.01$) in at least one condition are displayed. HS = heat shock, R = chilling-response, S = chilling-susceptibility, T = chilling-tolerance. R is composed of common genes in C (control) and HW (hot water) samples. The average expression changes (log2 FC) were represented on a color scale only in those conditions where the BIN was significant (for complete details, see Supplementary Table S5) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 3
Functional categories with contrasting gene expression between the regulons of chilling susceptibility (S) and tolerance (T) after cold storage (14 days at 5 °C).

BIN	ID	Description	FC (log 2)	
			S	T
Minor carbohydrate metabolism				
3.5	Solyc03g118650.2.1	Aldose 1-epimerase-like protein	−1.13	nd
3.5	Solyc10g011760.2.1	Aldose 1-epimerase family protein	nd	4.75
Cell wall metabolism				
10.6.1	Solyc06g073760.2.1	β-D-glucosidase	2.29	nd
10.6.2	Solyc12g013750.1.1	Endo-1,4-β-mannanase	1.40	nd
10.6.3	Solyc11g011300.1.1	Rhamnogalacturonate lyase	−2.45	nd
10.6.3	Solyc06g060170.2.1	Polygalacturonase family protein	3.47	nd
10.6.3	Solyc03g114240.2.1	BURP domain-containing protein	2.32	nd
10.6.3	Solyc05g014000.2.1	Pectate lyase	2.24	nd
10.6.3	Solyc12g009210.1.1	Polygalacturonase	1.36	nd
10.8.1	Solyc02g080210.2.1	Pectinesterase	−1.02	nd
10.8.2	Solyc03g025600.2.1	Pectinacetylesterase like protein	−1.04	nd
10.6.1	Solyc09g010210.2.1	Endoglucanase 1	nd	−5.82
10.6.2	Solyc10g008300.2.1	Endo-1,4-β-mannanase	nd	−1.30
10.6.2	Solyc02g084990.2.1	Endo-1,4-β-mannanase	nd	−2.02
10.6.2	Solyc01g111340.2.1	Endo-1,4-β-xylanase	nd	−2.92
10.6.3	Solyc12g009420.1.1	Polygalacturonase	nd	−3.67
10.8.1	Solyc05g047590.2.1	Pectinesterase	nd	−1.46
10.8.1	Solyc03g078100.2.1	Pectinesterase family protein	nd	−1.41
10.8.1	Solyc01g099940.2.1	Pectinesterase	nd	2.19
10.8.1	Solyc07g043240.2.1	Pectinesterase	nd	2.58
10.8.1	Solyc12g008530.1.1	Pectinesterase	nd	2.58
Lipid metabolism				
11.6	Solyc10g075050.1.1	Non-specific lipid-transfer protein	3.07	nd
11.9.2	Solyc06g083920.2.1	Lipase family protein	1.43	nd
11.9.3	Solyc07g014730.2.1	Phospholipase A2	1.98	nd
11.9.4	Solyc06g076680.2.1	Acyl-coenzyme A thioesterase 13	1.39	nd
11.9.4	Solyc12g099440.1.1	FA oxidation complex subunit α	1.34	nd
11.6	Solyc10g075150.1.1	Non-specific lipid-transfer protein	nd	−1.37
11.6	Solyc01g081600.2.1	Non-specific lipid-transfer protein	nd	−3.00
11.9.2	Solyc03g005020.2.1	Lipase	nd	−1.29
11.9.2	Solyc09g065890.2.1	Lipase	nd	−1.88
11.9.4	Solyc08g078390.2.1	Acyl-CoA oxidase	nd	−1.06
11.9.4	Solyc01g091520.2.1	Enoyl-CoA hydratase/isomerase	nd	−1.22
11.9.4	Solyc06g009160.2.1	FA oxidation complex subunit α	nd	−1.32
Carotenoid biosynthesis				
16.1.4	Solyc10g081650.1.1	Carotenoid isomerase, chloroplast	−1.40	nd
16.1.4	Solyc04g040190.1.1	Lycopene β-cyclase 1	−1.40	nd
16.1.4	Solyc06g074240.1.1	Lycopene β-cyclase, chromoplast	nd	−2.17
16.1.4	Solyc08g007440.1.1	Phytoene synthase	nd	3.96
16.1.4	Solyc02g081330.2.1	Phytoene synthase 2	nd	1.40
16.1.4	Solyc06g036260.2.1	β-carotene hydroxylase 1	nd	1.04
Ethylene biosynthesis				
17.5.1	Solyc11g072110.1.1	ACC oxidase-like protein	−1.53	nd
17.5.1	Solyc09g089580.2.1	ACC oxidase-like protein	−1.10	nd
17.5.1	Solyc08g079750.2.1	ACC synthase	1.94	nd
17.5.1	Solyc09g089730.2.1	ACC oxidase-like protein	nd	−2.36
17.5.1	Solyc02g071360.2.1	ACC oxidase 1	nd	1.04
17.5.1	Solyc07g049550.2.1	ACC oxidase	nd	3.62
17.5.1	Solyc02g091990.2.1	ACC synthase	nd	2.01
17.5.1	Solyc05g050010.2.1	ACC synthase	nd	2.78
17.5.1	Solyc08g081550.2.1	ACC synthase	nd	−1.10
Heat stress				
20.2.1	Solyc12g042830.1.1	Class I heat shock protein, HSP20	−1.61	nd
20.2.1	Solyc06g076570.1.1	Class I heat shock protein, HSP20	−2.79	nd
20.2.1	Solyc09g011710.2.1	Class I heat shock protein 3, HSP20	−1.68	nd
20.2.1	Solyc04g014480.2.1	Class I heat shock protein 3, HSP20	−2.28	nd
20.2.1	Solyc11g020330.1.1	Class IV heat shock protein, HSP20	−2.79	nd
20.2.1	Solyc06g076020.2.1	Heat shock protein, HSP70	−1.85	nd
20.2.1	Solyc06g053960.2.1	Heat stress transcription factor A3	−1.15	nd
20.2.1	Solyc09g059520.2.1	Heat stress transcription factor A3	−1.08	nd
20.2.1	Solyc06g076560.1.1	Class I heat shock protein, HSP20	nd	1.63
20.2.1	Solyc08g062450.1.1	Class II heat shock protein, HSP20	nd	2.22
20.2.1	Solyc03g123540.2.1	Class II heat shock protein, HSP20	nd	3.12
20.2.1	Solyc08g062340.2.1	Class II heat shock protein, HSP20	nd	2.30
20.2.1	Solyc03g113930.1.1	Class IV heat shock protein, HSP20	nd	4.55
20.2.1	Solyc05g014280.2.1	Heat shock protein, HSP20	nd	4.03
20.2.1	Solyc03g117630.1.1	Heat shock protein, HSP70	nd	2.73
20.2.1	Solyc11g066100.1.1	Heat shock protein, HSP70	nd	1.18
20.2.1	Solyc01g099660.2.1	Heat shock protein, HSP70	nd	2.13

Table 3 (*Continued*)

BIN	ID	Description	FC (log 2)	
			S	T
20.2.1	Solyc02g080470.2.1	Heat shock protein 4, HSP70	nd	1.27
20.2.1	Solyc06g036290.2.1	Heat shock protein, HSP90	nd	2.94
20.2.1	Solyc03g007890.2.1	Heat shock protein, HSP90	nd	3.06
20.2.1	Solyc08g080540.2.1	Heat stress transcription factor	nd	1.84
Drought stress				
20.2.3	Solyc02g084840.2.1	Dehydrin DHN1	1.69	nd
20.2.3	Solyc01g010870.2.1	Dehydration-responsive protein	1.07	nd
20.2.3	Solyc12g099390.1.1	Protein dehydration-induced 19	1.90	nd
20.2.3	Solyc01g091640.2.1	Dehydration-responsive protein	nd	−1.28
20.2.3	Solyc03g005600.2.1	Dehydration-responsive protein-like	nd	−2.00
20.2.3	Solyc04g063230.2.1	Dehydration-responsive protein	nd	−1.14
Redox				
21.1	Solyc02g078360.2.1	Thioredoxin family protein	3.70	nd
21.1	Solyc10g008390.2.1	Thioredoxin	1.02	nd
21.1	Solyc12g013810.1.1	Thioredoxin	1.37	nd
21.2.1	Solyc06g005160.2.1	Ascorbate peroxidase	1.08	nd
21.1	Solyc05g006870.2.1	Thioredoxin H	nd	−3.50
21.2.1	Solyc06g005150.2.1	Ascorbate peroxidase	nd	−1.03
21.5	Solyc07g020860.2.1	Peroxiredoxin	nd	−1.08
21.5	Solyc03g096040.2.1	Mitochondrial peroxiredoxin	nd	−2.48
Detoxification				
26.9	Solyc09g011600.2.1	Glutathione *S*-transferase-like protein	−1.67	nd
26.9	Solyc01g081310.2.1	Glutathione *S*-transferase	−1.55	nd
26.9	Solyc09g011590.2.1	Glutathione *S*-transferase-like protein	−1.24	nd
26.9	Solyc06g009020.2.1	Glutathione *S*-transferase	−1.16	nd
26.9	Solyc01g086680.2.1	Glutathione *S*-transferase	−1.16	nd
26.9	Solyc09g011510.2.1	Glutathione *S*-transferase-like protein	nd	2.60
26.9	Solyc09g011490.2.1	Glutathione *S*-transferase-like protein	nd	1.75
26.9	Solyc07g056480.2.1	Glutathione *S*-transferase-like protein	nd	2.62
26.9	Solyc07g056470.2.1	Glutathione *S*-transferase-like protein	nd	2.06
26.9	Solyc09g011550.2.1	Glutathione *S*-transferase-like protein	nd	2.19
26.9	Solyc07g056510.2.1	Glutathione *S*-transferase	nd	3.37

BIN = functional category code of MapMan software. ID = identifier of tomato reference 'ITAG2.3_cdna'. FC = fold change in gene expression compared to control time zero. nd = not differentially expressed.

protein oxidation, inhibition of enzymatic activity, and damage to DNA and RNA (Mittler, 2002). In our research group, Vega-García et al. (2010) reported the increased accumulation of an antioxidant enzyme thioredoxin peroxidase, in 'Imperial' tomato fruit since the initial stages of cold storage. Also, Cárdenas-Torres (2013) observed that 'Imperial' tomato fruit stored at 5 °C presented higher activity of the antioxidant enzymes superoxide dismutase, catalase and ascorbate peroxidase, compared to those fruit stored at safe temperature (12 °C).

In this report, four genes encoding antioxidant proteins (ascorbate peroxidase and three thioredoxins) were up-regulated in the chilling-susceptibility regulon; on the other hand, four antioxidant-related genes (ascorbate peroxidase, thioredoxin H, and two peroxiredoxins) were down down-regulated in the chilling-tolerance regulon after cold storage (Table 3; Supplementary Fig. S3). This evidence seems to indicate that the activation of antioxidant-related genes in control fruit may be a consequence of ROS accumulation caused by CI, and that cold storage did not disrupt the redox homeostasis in HW treated fruit, perhaps due to the protection exerted by HSPs and HSTFs.

4.4. Chilling blocks the second step of ethylene biosynthesis

The ethylene biosynthetic pathway comprises two steps, *S*-adenosylmethionine is converted to 1-aminocyclopropane-1-carboxylate (ACC) by the rate-limiting enzyme ACC synthase (ACS), and ACC is subsequently converted to ethylene by ACC oxidase (ACO); even though ACO activity is not limiting, certain ACO genes are ethylene inducible, and its silencing prevents ethylene synthesis and ripening (Klee and Giovannoni, 2011). Rugkong et al. (2011) reported that uneven ripening in cold-stored tomatoes was related to the down-regulation of genes associated with ethylene biosynthesis and signaling, which was reflected in a reduced ethylene production and lycopene accumulation.

Here, 68 DE transcripts related to ethylene metabolism were detected after cold storage (Supplementary Table S5), corresponding to 24.5% of the total genes in that BIN (17.5). Interestingly, *ACS* genes were up-regulated in both regulons, however, two *ACOs* were down-regulated in the chilling-susceptibility regulon, and two *ACOs* were up-regulated in the chilling-tolerance regulon (Table 3; Supplementary Fig. S5).

After cold storage + ripening, the chilling-susceptibility regulon was characterized by the down-regulation of two ethylene-responsive TFs (*ERFs*); in contrast, two *ERFs* were up-regulated in the chilling-tolerance regulon (Supplementary Fig. S6). These results may suggest that the HW treatment was able to restore the ethylene synthesis and signaling after cold storage and reconditioning to 20 °C (up-regulation of *ACOs* and *ERFs*).

4.5. Chilling alters the metabolism of sugars

Trehalose is a non-reducing disaccharide consisting of two units of glucose, and is accumulated in response to abiotic stresses in plants, by a two-step pathway involving trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP) (Iordachescu and Imai, 2008). Here, we observed a strong expression of three *TPPs* after 2 weeks of cold storage in both control and HW treated fruit (Fig. 3; Supplementary Table S5), suggesting that trehalose accumulation is part of the common response to chilling in tomato fruit, perhaps through its osmoprotectant property.

Expression profiling of *Arabidopsis* seedlings with high accumulation of trehalose-6-phosphate showed a correlation with the

expression of an aldose 1-epimerase gene (Schluepmann et al., 2004), which encodes for an enzyme that catalyzes the reversible conversion between α-D-glucose and β-D-glucose. Interestingly, the accumulation of an aldose 1-epimerase protein was decreased in tomato fruit stored at 2 °C compared to those stored at 12 °C (Sanchez-Bel et al., 2012). In the present work, we observed the down-regulation of an aldose 1-epimerase-like protein in the chilling-susceptibility regulon, and the strong up-regulation of an aldose 1-epimerase family protein in the chilling-tolerance regulon (Table 3). This seems to point out an important role of aldose 1-epimerase in the CI tolerance induced by a HW treatment.

4.6. Chilling induces the expression of cell wall degradation genes

The cell wall is largely composed of cellulose, hemicelluloses and pectins, these latter including homogalacturonans and rhamnogalacturonans. Pectin methylesterases catalyze the demethylesterification of cell wall homogalacturonans releasing acidic pectins and methanol, making the pectins more susceptible to the action of polygalacturonases, which catalyze the hydrolytic cleavage of galacturonide linkages (Brummell and Harpster, 2001; Micheli, 2001).

In the present work, the chilling-susceptibility regulon after cold storage was characterized by the up-regulation of six cell wall degradation genes: β-D-glucosidase, endo-1,4-β-mannanase, pectate lyase, and three polygalacturonases, as well as the down-regulation of two pectinesterases (Table 3; Supplementary Fig. S7). In contrast, the chilling-tolerance regulon showed the down-regulation of five cell wall degradation genes (endoglucanase, endo-1,4-β-xylanase, polygalacturonase and two endo-1,4-β-mannanases), and the up-regulation of three pectinesterases (Table 3; Supplementary Fig. S7). Biswas et al. (2014) observed that storage at 6 °C caused loss of turgor in tomato fruit, whereas storage at 2.5 °C resulted in loss of tissue integrity. In this sense, the down-regulation of cell wall degradation genes in the chilling-tolerance regulon may be contributing to the maintenance of cell wall integrity and turgor during cold storage, resulting in lower levels of water loss and wilting.

After cold storage + ripening, both categories of cell wall degradation and pectinesterases were up-regulated (Supplementary Fig. S8); however, the average FC of these categories in the chilling-tolerance regulon were 1.6 (degradation) and 2.8 (pectinesterases) fold higher than the chilling-susceptibility regulon (Supplementary Table S5). The up-regulation of pectinesterases in the chilling-tolerance regulon both during cold storage and ripening, could be associated with the lower pericarp firmness (Table 1) observed in HW treated fruit at the end of the storage.

4.7. HW treatment protects cell membranes from lipid degradation

Alterations in cell membrane conformation and structure are one of the first physiological responses to CI, which are caused by fatty acid (FA) peroxidation, increase in FA saturation index, degradation of phospholipids and galactolipids, and increase in sterol/phospholipid ratio (Sevillano et al., 2009). In this report, four genes related to lipid degradation were up-regulated in the chilling-susceptibility regulon after cold storage: triacylglycerol lipase, phospholipase A2, acyl-CoA thioesterase and FA oxidation complex subunit-α, while five genes within the same category were down-regulated in the chilling-tolerance regulon: acyl-CoA oxidase, enoyl-CoA hydratase/isomerase, FA oxidation complex α-subunit and two triacylglycerol lipases (Table 3; Supplementary Fig. S7).

The down-regulation of lipid-degradation genes seems to be one of the chilling-tolerance mechanisms by keeping the integrity of cell membranes in HW treated fruit, as indirectly evaluated here with the ion leakage assay (Supplementary Fig. S2). Supporting this information, Cárdenas-Torres (2013) observed reductions in lipid peroxidation (malondialdehyde content) of HW treated tomato fruit compared to control during cold storage.

4.8. HW treatment promotes color development

The most visually obvious change during ripening of tomato fruit is the transition from chloroplast to chromoplast organelles, which accumulate large quantities of carotenoids including the red pigment lycopene (Gapper et al., 2013). Lycopene biosynthesis in tomato fruit is composed of three functional units: phytoene synthase (PSY), phytoene desaturase (PDS)/ζ-carotene isomerase (ZISO), and ζ-carotene desaturase (ZDS)/carotenoid isomerase (CRTISO), and it is known that silencing of a desaturase (PDS or ZDS) results in the induction of the isomerase (ZISO or CRTISO) in the same functional unit (Fantini et al., 2013). Rugkong et al. (2011) reported that both *CRTISO* and *PSY* were down-regulated in cold-stored tomatoes, which led to a reduced accumulation of total carotenoids including lycopene.

In this work, a *CRTISO* was down-regulated in the chilling-susceptibility regulon after cold storage; meanwhile two *PSYs* were up-regulated in the chilling-tolerance regulon (Table 3; Supplementary Fig. S7). PSY catalyzes the conversion of geranylgeranyl diphosphate to phytoene, and is considered the main bottleneck in the carotenoid pathway (Ruiz-Sola and Rodríguez-Concepción, 2012); therefore, the up-regulation of this important gene in the chilling-tolerance regulon after cold storage, could be associated with a higher accumulation of lycopene in HW treated fruit during ripening, as evidenced here by fruit with color parameters closer to red (Table 1).

4.9. Transcriptional regulation of cold response

The best documented cold-response TFs are the *Arabidopsis* C-repeat/dehydration-responsive element (CRT/DRE) binding factors (CBFs), a small family of three transcriptional activators (CBF1-3) (Medina et al., 2011). Like *Arabidopsis*, tomato plants encode three *CBF* homologs (*LeCBF1–3*); however, only *LeCBF1* has been shown to be cold inducible in leaf tissue (Zhang et al., 2004) and in fruit (Zhao et al., 2009b) after 2 h of chilling stress. In the present work, a *CBF* (Solyc03g124110) was up-regulated after 2 weeks of cold storage in the chilling-susceptibility regulon (Supplementary Table S3), suggesting that *CBF* also plays a role during long-term chilling stress.

Although no contrasting expression patterns in TFs were found between the chilling-susceptibility and -tolerance regulons, the MYB and DOF TF families were significantly up-regulated during cold storage (Fig. 3; Supplementary Table S5). MYB proteins belong to the largest family of TFs in *Arabidopsis*, and are involved in the regulation of cold response in plants by binding to promoters of *CBF* genes (Chen et al., 2013). In this work, a total of 63 MYB TFs were DE after cold storage, which corresponded to 16.2% of the total transcripts in those BINS (27.3.25 and 27.3.26), of which, 50 MYB TFs were up-regulated by chilling in both control and HW treated fruit (Supplementary Table S6).

DOF domain proteins are plant-specific TFs with a highly conserved DNA-binding domain, which presumably includes a single C_2–C_2 zinc finger (Yanagisawa, 2004). In this work, 14 DOF TFs were up-regulated after 2 weeks of cold storage in both control and HW treated fruit (Supplementary Table S6). In agreement, the induction of a DOF TF has been reported in peach fruit after the application of a hot air treatment that induces CI tolerance (Lauxmann et al., 2012). Also, there is evidence that the DOF domain binds to the promoter of GSTs, whose primary function is the detoxification of xenobiotics

compounds and protection against oxidative damage (Chen and Singh, 1999), and its overexpression in tobacco seedlings resulted in enhanced tolerance to chilling stress (Roxas et al., 1997). Here, we observed that six GSTs were up-regulated in the chilling-tolerance regulon after cold storage (Table 3; Fig. 3), suggesting that GSTs play a role in the chilling-tolerance mechanisms, and their expression may be induced by DOF-type TFs. To our knowledge, this is the first report where the up-regulation of DOF TFs is associated with the cold-response mechanisms in tomato.

5. Conclusions

The induction of chilling tolerance in tomato fruit after the application of a HW treatment was validated by reductions in CI physiological markers (ion leakage and respiration rate) and visual symptoms (uneven ripening and wilting). Comparative RNA-Seq analysis allowed us to describe the molecular network associated with the overall response to cold stress in tomato fruit, as well as the unique transcriptional changes underlying the chilling susceptibility and tolerance, although they do not necessarily reflect changes in protein or metabolite levels. The chilling tolerance mechanisms included the up-regulation of genes related to heat stress (HSTFs and HSPs), detoxification (GSTs), and sugars metabolism (TPPs and aldose 1-epimerase), which may be acting synergistically to protect cell membranes from oxidative and osmotic stresses during cold storage. Also, the up-regulation of key genes in the biosynthesis of ethylene (*ACO*) and carotenoids (*PSY*) seems to be related to a more advanced ripening stage in HW treated fruit. On the other hand, the chilling susceptibility mechanisms included the up-regulation of genes related to degradation of cell wall and lipids, causing loss of cell turgor and a delayed fruit softening, as well as loss of cell membranes integrity and functionality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2014.08.009.

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