Physiologia Plantarum
An International Journal for Plant Biology

Small heat shock proteins and the postharvest chilling tolerance of tomato fruit

Martín D. Ré, Carla Gonzalez, Mariela R. Escobar, María Laura Sossi, Estela M. Valle and Silvana B. Boggio*

Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

Correspondence
*Corresponding author,
e-mail: boggio@ibr-conicet.gov.ar

Received 29 February 2016;
revised 13 June 2016

doi:10.1111/ppl.12491

Plants have the largest number of small heat shock proteins (*sHsps*) (15–42 kDa) among eukaryotes, but little is known about their function in vivo. They accumulate in response to different stresses, and specific *sHsps* are also expressed during developmental processes such as seed development, germination, and ripening. The presence of organelle-specific *sHsps* appears to be unique to plants. The *sHsps* expression is regulated by heat stress transcription factors (*Hsfs*). In this work, it was explored the role of *sHsps* in the chilling injury of tomato fruit. The level of transcripts and proteins of cytoplasmic and organellar *sHsps* was monitored in fruit during ripening and after cold storage (4 weeks at 4°C). Expression of *HsfA1*, *HsfA2*, *HsfA3*, and *HsfB1* was also examined. Two cultivars of tomato (*Solanum lycopersicum*) contrasting in chilling tolerance were assayed: Micro-Tom (chilling-tolerant) and Minitomato (chilling-sensitive). Results showed that *sHsps* were induced during ripening in fruit from both cultivars. However, *sHsps* were induced in Micro-Tom fruit but not in Minitomato fruit after storage at a low temperature. In particular, *sHsp 17.4-CII* and *sHsp23.8-M* transcripts strongly accumulated in Micro-Tom fruit and *HsfA3* transcript diminished after cold storage. These data suggest that *sHsps* may be involved in the protection mechanisms against chilling stress and substantiate the hypothesis that *sHsps* may participate in the mechanism of tomato genotype chilling tolerance.

Introduction

Tomato is one of the most important cultivated plants in the world. To extend the commercialization period, it is stored at low temperatures after harvest. However, the production yield and the fruit quality can be severely impaired by chilling. Chilling injury is a physiological alteration that becomes evident when the fruit is taken off from the fridge and reaches the consumers. The molecular basis of chilling injury and the signal transduction networks of the chilling response are poorly understood (Sapitnitskaya et al. 2006, Pedreschi and Lurie 2015). In addition, the diversity of chilling injury symptoms of tropical and subtropical fruits and vegetables suggests a multitude of responses to low temperature (Wang 2010). Membrane fluidity and permeability at low temperature (Nishida and Murata 1996), alteration in the cell-wall metabolism (Brummell et al. 2004), and oxidative stress (Malacrida et al. 2006) have been related to the chilling injury. Moreover, there is evidence of the existence of genetic variability in low-temperature sensitivity within Solanum species such as potato (Palta et al. 1993) and tomato (Gonzalez et al. 2015). Several reports have suggested that various genes related to different stress

Abbreviations – BCIP, 5-bromo-4-chloro-3-indolyl phosphate; *Hsfs*, heat stress transcription factors; NBT, nitro blue tetrazolium; *sHsps*, small heat shock proteins.

responses, including small heat shock protein (*sHsp*) genes, may have a role in the acquirement of chilling tolerance. For instance, it has been shown that fruit pre-storage treatments with jasmonate, salicylate (Ding et al. 2002), or heat shock (Polenta et al. 2007, Sevillano et al. 2010) induced *sHsp* synthesis and reduced chilling injury.

The *sHsps* are synthesized in response to high temperature and other stresses in the vegetative tissues of plants (Larkindale et al. 2005, Scarpeci et al. 2008). The *sHsps* can also be induced at a particular time during specific stages in plant life cycle such as embryogenesis (Almoguera et al. 1998), germination (Wehmeyer and Vierling 2000), anther and pollen development (Volkov et al. 2005, Giorno et al. 2010), and fruit development and ripening (Medina-Escobar et al. 1998, Neta-Sharir et al. 2005). The *sHsps* form large oligomers, ranging in size from 200 to 800 kDa, exhibiting a monomeric molecular mass of 12 to 42 kDa. Plant *sHsps* are encoded by nuclear multigene families and have been localized in the cytoplasm, nucleus, endoplasmic reticulum, mitochondria, chloroplasts and peroxisome (Waters 2013). Angiosperm *sHsps* have been classified in at least 11 subfamilies (Bondino et al. 2012, Waters 2013, Haslbeck and Vierling 2015). The mechanism of cell protection by *sHsps* is not clear, but it is well known that some *sHsps* act as molecular chaperones in vitro and in vivo (Sun et al. 2002), they do not require ATP, and they have a high capacity for binding denaturing proteins (Basha et al. 2012). Also, it has been reported that the association between *sHsps* and membranes may preserve membrane integrity during thermal fluctuations (Tsvetkova et al. 2002). Chilling-induced *sHsp* synthesis has been observed in cold-stored potato (*Solanum tuberosum*) (van Berkel et al. 1994). Also, winter-specific accumulation of *sHsps* associated with seasonal cold acclimation in perennial plants has been reported (Lubaretz and Zur 2002, Lopez-Matas et al. 2004).

The accumulation of *sHsps* is regulated by the large family of the heat stress transcription factors (*Hsfs*). The *Hsfs* bind to the heat shock elements present in the promoters of *sHsps*, and activate the transcription of *sHsps* and other heat stress genes (Kotak et al. 2007, von Koskull-Doring et al. 2007). In tomato (*Solanum lycopersicum*), *Hsfs* form part of a regulatory network that controls the expression of *sHsps* and other heat shock responsive genes (Schramm et al. 2008).

The role of *sHsps* during tomato fruit ripening and postharvesting is still unclear. Neta-Sharir et al. (2005) showed that the chloroplastic *sHsp 21-P* participates in the carotenoid accumulation while the fruit ripens. Also, the cytosolic *sHsp 17.7-CI* and *sHsp 17.3-CIII* transcripts were found in green and red fruits of tomato cv. Harzfeuer, while the proteins were only present in the red fruit (Low et al. 2000). In response to cold storage, Page et al. (2010) reported the increase of four *sHsps*, in tomato fruits from the more tolerant genotype while Sanchez-Bel et al. (2012) reported the up-regulation of two *sHsps* as the first response of tomato fruit to cope with cold stress. In another report, Ramakrishna et al. (2003) characterized a *sHsp* gene, viscosity 1 (*vis1*), from tomato providing evidence that high temperature and fruit ripening regulates vis1 production. More recently, Cruz-Mendívil et al. (2015) reported different changes in the expression of *sHsps* after hot treatment followed by cold storage of tomato fruit using RNA-Seq analysis.

Elucidating the molecular mechanisms of chilling tolerance is critical for maintaining fruit quality and diminishing the postharvest loss. To gain more insight into the molecular mechanisms involved in chilling tolerance, an investigation on the *sHsps* and *Hsfs* expression in tomato fruit while ripening on the vine, off the vine, and after cold storage was performed. A comparative approach was used employing two tomato varieties with contrasting postharvest chilling tolerance (Gonzalez et al. 2015): cv. Micro-Tom that is a model system to study postharvest chilling tolerance and cv. Minitomato that is susceptible to chilling injury.

Materials and methods

Plant material and treatments

Tomato (*S. lycopersicum*) plants (cvs. Micro-Tom and Minitomato) were grown in a controlled environment cabinet under a light intensity of 400 μmol s^{-1} m^{-2} at the top of the plants containing the fruit. The temperature ranged from 25°C during the light period (14 h) to 18°C in the dark, and the relative humidity was 70%. Plants were grown in soil, maintained under optimal irrigation, and supplied weekly with half-strength Hoagland solution (Malacrida et al. 2006).

Fruit ripening occurred in three different conditions: on the vine (fruits were allowed to ripen naturally on the plant), off the vine (fruits were picked at the mature green stage and directly placed on a shelf in the growing cabinet), and prechilled (fruits were harvested at the mature green stage, stored for 4 weeks at 4°C, and then transferred back to the growing cabinet). Fruits were collected at the mature green (G), yellow (Y), orange (O), and red (R) stages (Gonzalez et al. 2015). G_{ch} corresponds to green fruit conserved for 4 weeks at 4°C. G_{ch+n} corresponds to green fruit conserved for 4 weeks at 4°C and transferred during *n* days to 25°C. G_{off+n} corresponds to green fruit picked at the mature green stage and placed on the shelf at 25°C during *n* days. Each sample consists of four fruits of the same maturation stage and/or

treatment. Three replicates of each experiment were performed. Fruits between 2 and 3 g weight were selected and harvested 4 h after the light period began. Pericarp tissue of the harvested fruits was obtained by removing the locule tissues, skin, and seeds and was immediately processed or frozen in liquid nitrogen and stored at −80°C until analysis.

Color determination

Fruits were cleaned, dried, cut transversely through the center and placed on the scanner (Hewlett Packard) with the cut side down and a black background. The images obtained were analyzed employing 'Tomato Analyzer Color Test' (Rodriguez et al. 2010) designed to quantify the color parameters Red, Green, and Blue of the RGB color space. The average RGB values were employed to calculate *L**, *a**, *b** (numerical terms to express color from black to white, green to red, and blue to yellow axes, respectively) of the CIELAB color space and Hue and Chroma color descriptors. The scanner color calibration was achieved using Color Checker Munsell Color X-write.

Protein isolation and immunoblotting

For pericarp proteins extraction, 1 g of frozen tissue was ground in 0.6 ml of phosphate buffer (pH 7.8), 0.3 ml of glycerol, and 2% (w/v) polyvinylpolypyrrolidone, using mortar and pestle on ice. The extracts were centrifuged at 15 300 *g* for 10 min at 4°C. Following isolation, the protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard. One aliquot of each sample containing 40 µg of total protein was precipitated with 10% (v/v) trichloroacetic acid, resuspended in sample buffer and subjected to 15% SDS-PAGE and transferred to a nitrocellulose membrane. Equal loading control before immunoblotting analysis was achieved by Ponceau red staining of nitrocellulose membranes (Appendix S1, Supporting information). The membranes were blocked with 5% (w/v) milk powder in Tris-buffered saline for 1 h and incubated overnight at 4°C with primary antibody. The antibodies employed were rabbit polyclonal antibodies against *sHsps* (Polenta et al. 2007). The blots were rinsed and incubated with a secondary antibody against rabbit IgG conjugated to alkaline phosphatase, and processed for the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate system.

RNA isolation and real-time PCR (qRT-PCR)

Total RNA was isolated from fruit tissues using Trizol Reagent (Invitrogen Life Technologies, Karlsruhe, Germany). RNA quantity was measured spectrophotometrically, and RNA quality was checked by agarose gel electrophoresis. For reverse transcription, 0.75–1 µg of total RNA from each sample was incubated with RNase-free DNase RQ1 (Promega, Madison, WI). For cDNA synthesis, oligo(dT) primer and SuperScript III Reverse Transcriptase (Invitrogen) were used according to the manufacturer's instructions. qRT-PCR reactions were performed in a Mastercycler® ep realplex thermal cycler (Eppendorf, Westbury, USA) using the intercalation dye SYBR Green I (Roche Life Science, Buenos Aires, Argentina) as a fluorescent reporter to monitor dsDNA synthesis. A master mix for each qRT-PCR run was prepared. Final concentrations, in a total volume of 20 µl, were: 1 X qRT-PCR Buffer Minus Mg, 3 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.4 X SYBR Green I, and 0.5 U Platinum Taq DNA Polymerase (Invitrogen). 5 µl of 1:10 diluted cDNA was added. 1 µ*M* for a specific sense and anti-sense primers each was used. The gene specific primers used for qRT-PCR were designed using Primer3Plus Program (Untergasser et al. 2007) (Appendices S2 and S3). *rpl2* was used as a housekeeping gene.

Statistical analysis

Experimental data were subjected to statistical analysis of variance (ANOVA) with ripening stage and treatments as factors, followed by Fisher's least significant difference post hoc test. It was considered that a *P* value less than 0.05 was statistically significant. Model assumptions were tested by analysis of residuals. Each experiment was carried out at least three times.

Results

Expression of *sHsps* and *Hsfs* genes during tomato fruit ripening

Tomato fruits, cvs. Micro-Tom and Minitomato, differing in tolerance to postharvest chilling (Gonzalez et al. 2015), were selected for analysis of the *sHsps* expression during fruit ripening. The transcript level of the cytoplasmic *sHsps, sHsp 17.4-CII, sHsp 17.6-CI, sHsp 17.6-CII*, and *sHsp 17.7-CI* and organellar *sHsps, sHsp 21.5-ER* localized in the endoplasmic reticulum, *sHsp 21-P* in the chloroplast, and *sHsp 23.8-M* in the mitochondria were analyzed by qRT-PCR in the pericarp of mature tomato fruit. The results obtained are shown in Fig. 1. *sHsps* expression pattern was similar in the fruits of the two varieties while they ripened: the transcript level of the seven *sHsps* analyzed increased during the transition from the green fruit to the intermediate stages and then decreased in the red stage. In Micro-Tom fruit, the maximum values of *sHsps* expression were observed at

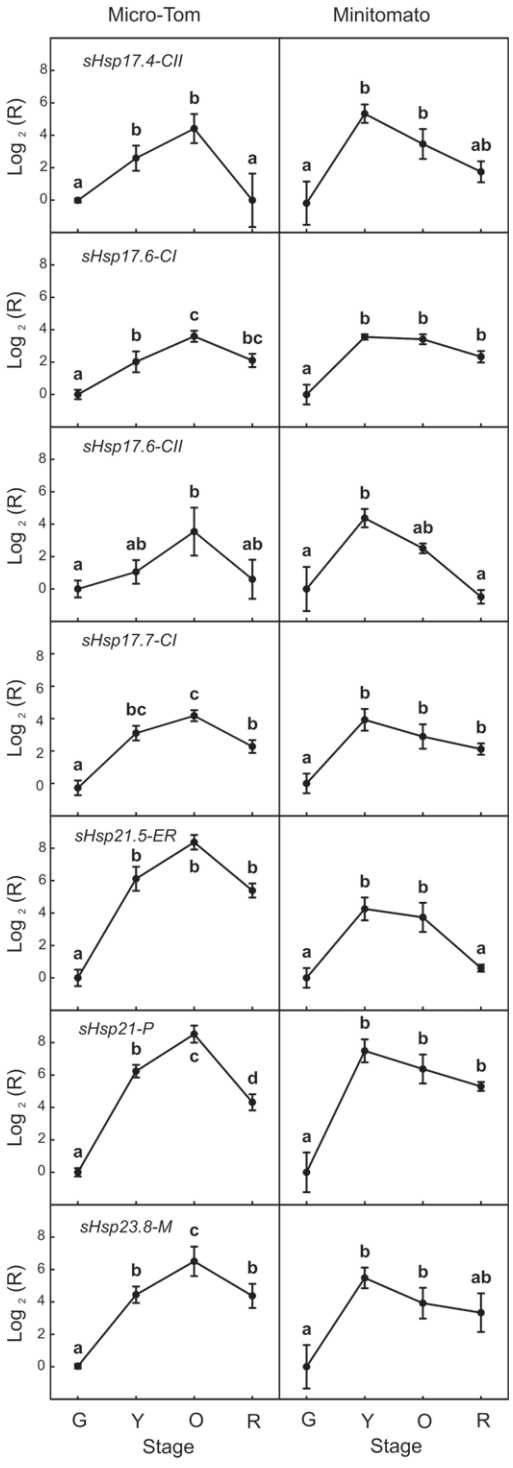

Fig. 1. *sHsps* expression throughout ripening in Micro-Tom (left panel) and Minitomato (right panel) fruits. Transcript abundance was evaluated using qRT-PCR. Values shown are the $\log_2$ fold change in mature green (G), yellow (Y), orange (O), and red (R) fruits, compared to G. Each value represents the average (± SE) of three independent biological replicates. Means with the same letter within a graph are not significantly different ($P \leq 0.05$).

the orange stage, while in Minitomato fruit the higher expressions were at the yellow stage. In Micro-Tom fruit, the organellar *sHsps* transcript level increased at orange stage about 100 and 500 times when compared to green fruit, while the four cytoplasmic *sHsps* increased their expression level only about 15–40 times in orange fruit compared to green fruit. On the other hand, in Minitomato fruit, transcripts of *sHsp 21-P* increased 180-fold, of *sHsp 23.8-M* increased 45-fold, and of the *sHsp* 21.5-ER increased only 20 times in the yellow stage compared to the mature green stage.

Transcription of *sHsps* is regulated upstream by *Hsfs*. The expression of a group of *Hsfs* was analyzed by qRT-PCR during ripening of Micro-Tom and Minitomato fruits (Fig. 2). *HsfA1* expression was statistically constant during ripening in the fruit of the two varieties analyzed. *HsfA3* expression of Micro-Tom fruit diminished to around 1/20th when ripened, but in Minitomato fruit, *HsfA3* expression decreased in the intermediate stages and partially recovered to the initial level in the red stage. *HsfA2* and *HsfB1* expression level increased during the ripening process in Micro-Tom fruit while it remained statistically constant in the case of Minitomato fruit.

Accumulation of *sHsps* during fruit ripening

sHsp protein abundance was analyzed by immunoblotting during ripening of Micro-Tom and Minitomato fruits in total protein extracts from fruit pericarp. A representative pattern of *sHsp* protein abundance is shown in Fig. 3. This analysis showed ripening-related changes in the *sHsp* protein abundance of the two fruit varieties. A main immunoreactive band was detected in all the fruits, and the intensity of this band increased during fruit ripening. Although Micro-Tom and Minitomato *sHsp* protein pattern during fruit ripening were very similar, the band intensity observed in Minitomato fruit extracts was clearly lower.

Micro-Tom and Minitomato fruit aspect during the first 3 days after cold storage

Micro-Tom and Minitomato fruits show different characteristic after postharvest chilling. While Micro-Tom fruit is chilling-tolerant, Minitomato fruit develops chilling injury symptoms after chilling storage (Gonzalez et al. 2015). The fact that chilling injury symptoms become evident after the product is returned to ambient temperature (Sharom et al. 1994) suggests that the physiological events that occur the first days after chilling are determinant for the subsequent fruit behavior. Therefore, a focus was put on the first days mature green fruit returned to ambient temperature after being stored 4 weeks at

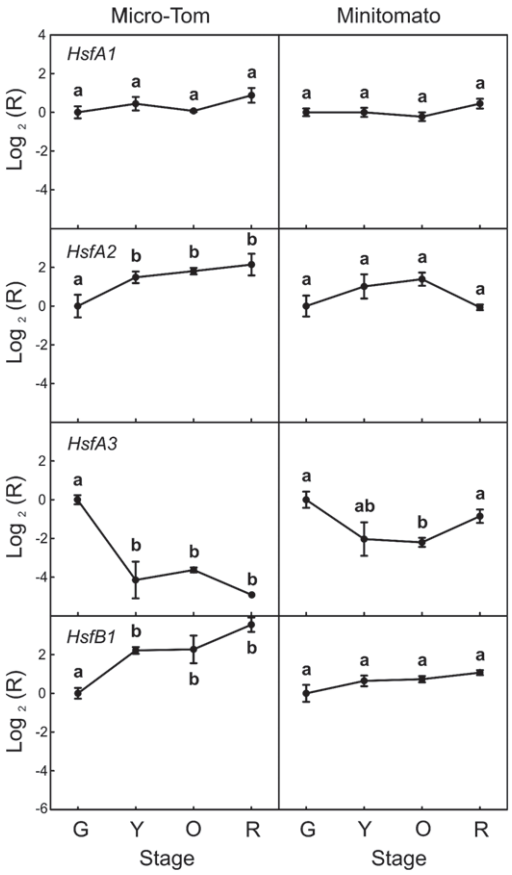

Fig. 2. *HsfA1*, *HsfA2*, *HsfA3*, and *HsfB1* expression throughout ripening in Micro-Tom (left panel) and Minitomato (right panel) fruits. Transcript abundance was evaluated using qRT-PCR. Values shown are the $\log_2$ fold change in mature green (G), yellow (Y), orange (O), and red (R) fruits, compared to G. Each value represents the average (± SE) of three independent biological replicates. Means with the same letter within a graph are not significantly different ($P \leq 0.05$).

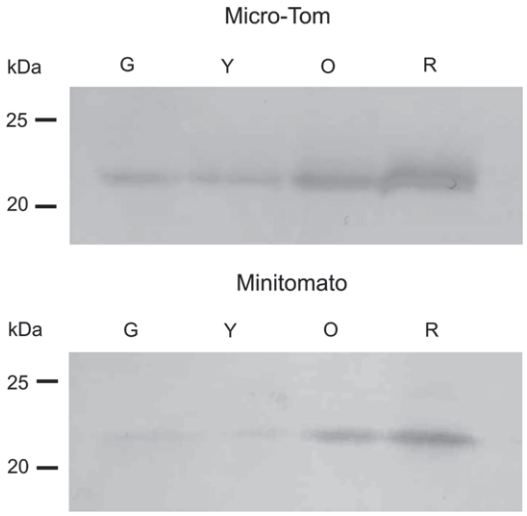

Fig. 3. Immunoblot analysis of *sHsps* expression in fruits of cv. Micro-Tom and cv. Minitomato ripened on the plant. The fruits analyzed were mature green fruit (G), yellow fruit (Y), orange fruit (O), and red fruit (R). A polyclonal antibody against tomato *sHsp* (Polenta et al. 2007) was used.

4°C (Fig. 4). When Micro-Tom and Minitomato fruits were taken from the cold camera, they were still green (Fig. 4). Three days later, Minitomato showed wet skin areas while Micro-Tom remained without visible alterations (Fig. 4). The *L** value (color brightness indicator) of the Minitomato mature green fruit when it was taken off the cold camera (G_{ch}) was significantly lower than the *L** value of mature green fruit before cold storage (G), and this value remained invariable during the 3 days analyzed (Table 1). On the other hand, no significant differences were found in the *L** Micro-Tom fruit values after chilling storage (G_{ch}) when compared to the mature green fruit that remained in the plant (G). Micro-Tom mature green fruit *a** value increased during and after chilling storage (G_{ch} and G_{ch+1d}, G_{ch+2d}, G_{ch+3d}) while Minitomato mature green fruit *a** value slightly increased the third day after cold storage (G_{ch+3d}). The *b** value of the mature green fruit of both tomato varieties diminished after the cold storage, but in Micro-Tom fruit, it was more pronounced (Table 1).

sHsps and *Hsfs* expression during the first 3 days after cold storage

The influence of postharvest chilling on *sHsps* expression was analyzed in the pericarp of Micro-Tom and Minitomato fruits immediately after the fruits were taken off from the cold storage, and during the three subsequent days. The results are shown in Fig. 5A. As a control, fruit harvested at the mature green stage and stored on the shelf at 25°C was used (Fig. 5B).

Transcript levels of *sHsp 17.4-CII* and *sHsp 23.8-M* increased in Micro-Tom mature green fruit after cold storage and remained higher during the first three days after cold storage (Fig. 5A). Expression of the *sHsp 23.8-M* gene in the Micro-Tom mature green fruit that was harvested and put on the shelf increased the first day (Fig. 5B) to the same value of the mature green fruit chilled (Fig. 5A). On the other hand, the transcript level of *sHsp 17.4-CII* in Micro-Tom mature green fruit diminished after postharvest on the shelf, remaining repressed during the following days evaluated (Fig. 5B). *sHsp 17.6-CI*, *sHsp 17.6-CII*, and *sHsp 21.5-ER* expression decreased after storage at 4°C of Micro-Tom mature green fruit (Fig. 5A), although the transcript level of *sHsp 17.6-CII* in Micro-Tom mature green fruit increased the first day after transferring the fruit to 25°C and reaching the same expression level as the fruit ripened at

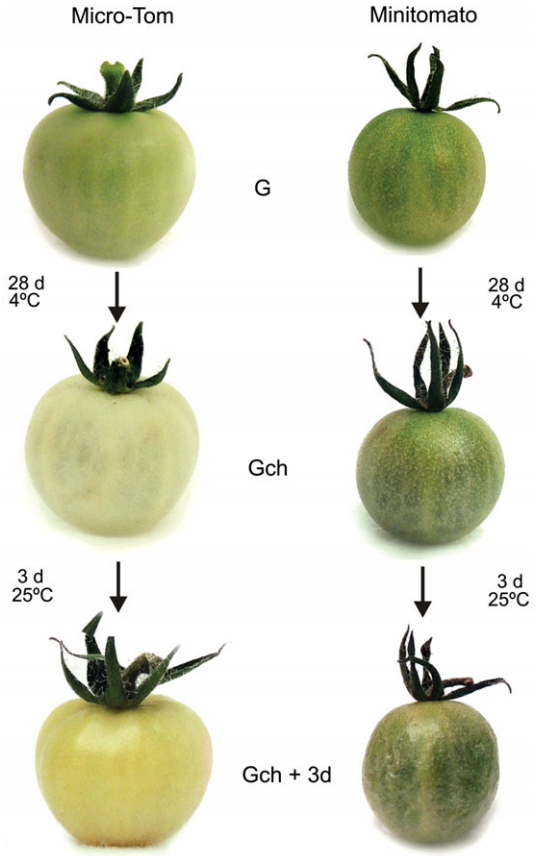

Fig. 4. Changes in the overall aspect of Micro-Tom and Minitomato green fruits stored at 4°C for 28 days and upon transfer to 25°C for 3 days. G: mature green fruit immediately after harvest, G_{ch}: mature green fruit after 28 days at 4°C. $G_{ch + 3d}$: mature green fruit 3 days at 25°C after 4°C storage.

25°C on the shelf (Fig. 5B). Instead, the *sHsp 17.6-CI* and *sHsp 21.5-ER* expression of the Micro-Tom chilled fruit were lower than the mature green fruit kept on the shelf at 25°C. *sHsp 17.7-CI* and *sHsp 21-P* expression levels of Micro-Tom mature green fruit were not altered by cold storage, but they increased their expression during the first days after cold storage. Nevertheless, transcript levels of *sHsp 17.7-CI* and *sHsp 21-P* in the chilled Micro-Tom fruit were slightly lower (Fig. 5A) than those of fruit kept on the shelf (Fig. 5B).

In Minitomato mature green fruit, the expression level of the analyzed *sHsps* diminished after 28 days at 4°C except for *sHsp 23.8-M* that remained statistically unchanged (Fig. 5A). Returning Minitomato mature green fruit to 25°C did not affect the expression level of all the *sHsps* analyzed. It is worth to mention that in Minitomato transcripts of *sHsps* were analyzed in mature green fruit only the first day at 25°C after the chilling storage because the fruit was severely injured and the RNA obtained the days after had poor quality for qRT-PCR analyses.

Micro-Tom transcript level of *HsfA1* did not change during chilling storage of mature green fruit but increased after 3 days at 25°C (Fig. 6A). When Micro-Tom fruit was harvested and put at 25°C on the shelf, the transcript level of *HsfA1* did not change significantly (Fig. 6B). Contrariwise, Minitomato transcript level of *HsfA1* in mature green fruit decreased during chilling storage and remained low after chilling storage (Fig. 6A).

The chilling storage of Micro-Tom and Minitomato mature green fruits caused a diminution in the *HsfA2* expression. Micro-Tom transcript level of *HsfA2* in mature green fruit increased at 3 days after the fruit was taken off the cold storage (Fig. 6A) and when the fruit was harvested and put on the shelf (Fig. 6B). On the other hand, the *HsAf2* expression in the Minitomato mature green fruit did not restore to the original value after chilling (Fig. 6A).

In the Micro-Tom mature green fruit, the transcript level of *HsAf3* was down-regulated by chilling storage although it increased after 3 days at 25°C on the shelf. In the Minitomato mature green fruit the transcript level of *HsAf3* was not altered by cold storage (Fig. 6A). *HsAf3* expression was not affected when Micro-Tom and

Table 1. Changes in color (*L**, *a**, *b**) of Micro-Tom and Minitomato mature green fruits before and after storage at 4°C during 4 weeks. G corresponds to green fruit from the vine and G_{ch+n} corresponds to green fruit conserved for 4 weeks at 4°C and transferred during *n* days to 25°C. Each value represents the average (± SE) of three independent biological replicates. Means in the same column with the same letter are not significantly different ($P \leq 0.05$).

	Micro-Tom			Minitomato		
Fruit	*L**	*a**	*b**	*L**	*a**	*b**
G	63.7 ± 0.5 ab	−22.0 ± 2.0a	52.0 ± 0.4a	58.5 ± 0.3a	−18.7 ± 1.3a	45.5 ± 0.3a
G_{ch}	65.1 ± 0.8 a	−16.6 ± 0.2b	44.7 ± 0.4b	55.8 ± 0.4b	−17.7 ± 0.3a	44.8 ± 0.2ab
G_{ch+1d}	63.8 ± 0.9 a	−16.7 ± 0.3b	45.7 ± 0.5bc	56.0 ± 0.3b	−17.7 ± 0.3a	43.8 ± 0.4bc
G_{ch+2d}	65.6 ± 0.8 a	−15.4 ± 0.4c	47.6 ± 0.5c	56.6 ± 0.4b	−18.4 ± 0.2a	45.9 ± 0.3a
G_{ch+3d}	61.2 ± 0.5 b	−13.3 ± 0.4d	44.3 ± 1.0b	55.4 ± 0.4b	−16.3 ± 0.4b	43.5 ± 0.3 c

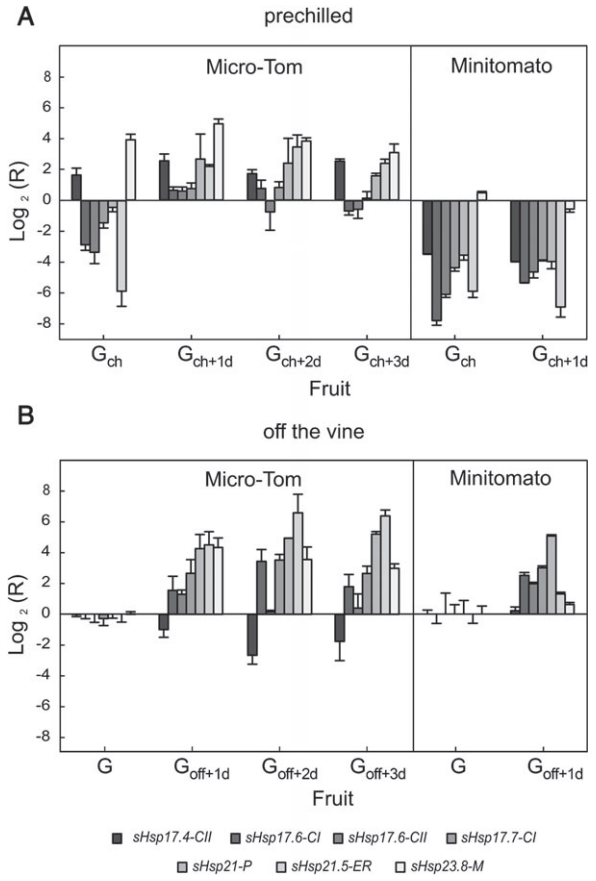

Fig. 5. *sHsp* gene expression after cold (A – prechilled) and ambient temperature (B – off the vine) storage of fruits cv Micro-Tom and Minitomato differing in chilling sensitivity. Green fruits were stored at 4°C for 28 days and transferred to 25°C for 3 days (A) or harvested and placed on a shelf at 25°C (B). Transcript abundance was evaluated using qRT-PCR after harvest (G), immediately after 4 weeks at 4°C storage (G_{ch}) and 1–3 days after being transferred to 25°C (G_{ch+n}). G_{off+n} corresponds to green fruit picked at the mature green stage and placed on the shelf at 25°C during *n* days. Values shown are the $\log_2$ fold change in fruits compared to G. Each value represents the average (± SE) of three independent biological replicates. See statistical analysis in Appendix S4.

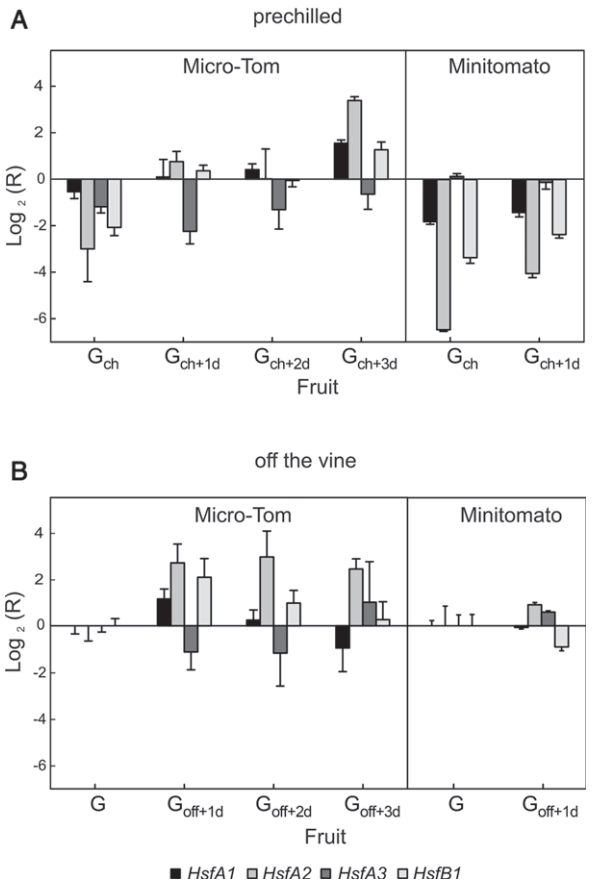

Fig. 6. *Hsf* gene expression after cold (A – prechilled) and ambient temperature (B – off the vine) storage of fruits cv. Micro-Tom and Minitomato differing in chilling sensitivity. Green fruits were stored at 4°C for 28 days and transferred to 25°C for 3 days (A) or harvested and placed on a shelf at 25°C (B). Transcript abundance was evaluated using qRT-PCR after harvest (G), immediately after 4°C storage (G_{ch}) and 1–3 days after being transferred to 25°C (G_{ch+n}). G_{off+n} corresponds to green fruit picked at the mature green stage and placed on the shelf at 25 °C during *n* days. Values shown are the $\log_2$ fold change in fruits compared to G. Each value represents the average (± SE) of three independent biological replicates. See statistical analysis in Appendix S4.

Minitomato fruits were harvested and put on the shelf without storage at 4°C (Fig. 6B).

The transcript level of *HsfB1* in the mature green fruit of the two tomato varieties diminished after chilling storage (Fig. 6A). Later, the transcript level of *HsfB1* in Micro-Tom mature green fruit rose while transcript of *HsfB1*in Minitomato mature green fruit level decreased (Fig. 6B).

Accumulation of *sHsps* after postharvest chilling

Immunoblotting analysis of *sHsps* was carried out on protein extracts from fruit at the end of cold storage (at 4°C during 4 weeks) and the first and second days after the fruit was taken from the 4°C storage and transferred to 25°C (G_{ch+1} and G_{ch+2}). The results obtained are shown in Fig. 7. A clear difference was observed in the *sHsp* protein pattern of Micro-Tom and Minitomato mature green fruits. The *sHsps* pattern of the Micro-Tom mature green fruit did not change after chilling storage (see Figs. 3 and 7), but it was markedly different when the fruit was rewarmed to 25°C, in fact, new *sHsp* bands of approximately 22–23 kDa were detected (Fig. 7). These new bands were not detected in fruits while ripening on the vine (Fig. 3). On the other hand, the chilling storage of the Minitomato mature green fruit reduced the *sHsp* protein level to almost undetectable levels,

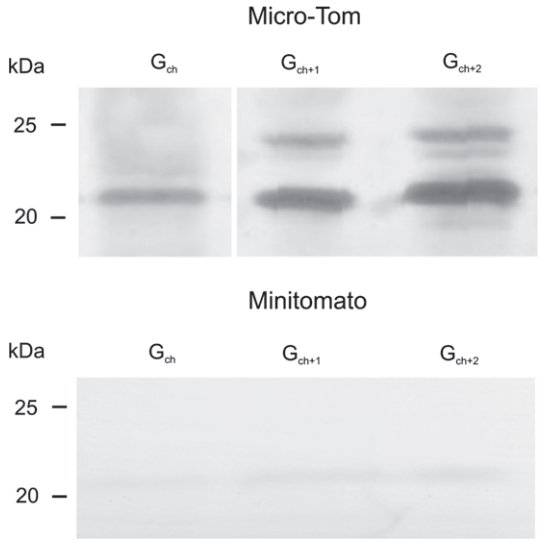

Fig. 7. Immunoblot analysis of *sHsps* expression in fruits cv. Micro-Tom and cv. Minitomato after postharvest chilling. The fruits analyzed were: green fruit after 28 days at 4°C (G_{ch}), green fruit after 28 days at 4°C + 1 day at 25°C (G_{ch+1d}) and green fruit after 28 days at 4°C + 2 days at 25°C (G_{ch+2d}).

although a slight induction in the main *sHsp* band could be observed when the fruit was rewarmed (Fig. 7).

sHsps and *Hsfs* expression during Micro-Tom fruit ripening after cold storage

Because Micro-Tom fruit tolerates chilling injury while Minitomato does not (Gonzalez et al. 2015), it was investigated if the *sHsps* expression level of ripening Micro-Tom fruit was affected by postharvest chilling. The results obtained by qRT-PCR are shown in Fig. 8.

Transcripts of *sHsp 17.4-CII* remained statistically invariant during ripening of the fruit prechilled while it increased during fruit ripening on and off the vine, showing a maximum at the orange stage (Fig. 8).

Transcript level of *sHsp 17.6-CI* and *sHsp 17.6-CII* (Fig. 8) increased at intermediate stages (Y and O) when ripening on the vine (Fig. 1). However, the expression of these *sHsps* was not altered when the fruit ripened off the vine or after cold storage.

The *sHsp 17.7-CI* expression of ripening fruit showed a maximum at intermediate stages keeping the same value at the R stage, in the three conditions analyzed. The level of *sHsp 17.7-CI* expression of fruit at intermediate ripening stages was lower in the prechilled fruit (Fig. 8).

Harvest and prechilling had no effect on the expression of *sHsp 23.8-M* gene in ripening fruit, except for the increase observed in the mature green fruit after storing at 4°C (Fig. 5). Orange fruit exhibited the highest *sHsp 23.8-M* expression in all the ripening conditions studied (Fig. 8). *sHsp 21-P* also showed an expression pattern with a maximum level at the O stage regardless of the ripening condition (Fig. 8).

Expression pattern of *sHsp 21.5-ER* during fruit ripening on the vine and prechilled showed a maximum at the O stage, while off the vine at Y, O, and R it remained statistically invariant. The prechilled R fruit expression level was lower than in non-chilled fruits (on and off the vine) (Fig. 8).

HsfA1 and *HsfA2* exhibited higher expression values when the fruit ripened off the vine with the previous chilling or not reaching similar values at the R stage (Fig. 9). *HsfB1* expression in the fruit is increased during ripening, in the three ripening conditions (Fig. 9). The *HsfA3* expression was decreased in fruit while ripening on the vine (Fig. 2), it was absent when the fruit was harvested and ripened off the vine, either with the previous chilling storage or not (Fig. 9).

Discussion

Ripening is the final phase of fruit development and involves profound changes in the biochemistry, physiology and gene expression of the fruit, which affect color, texture, flavor, and aroma (Carrari and Fernie 2006). Ripening is under the control of both external and internal factors, as the result of the operation of unique pathways, genes, and proteins. Additionally, in climacteric fruits, a peculiar burst in the ethylene evolution and the respiration rate at the onset of ripening occurs (Alexander and Grierson 2002), tuning the whole set of ripening-associated pathways (Liu et al. 2015). It is tempting to suppose that the metabolic changes that take place during ripening are a stressful endogenous condition for the fruits. Indeed, several authors have identified the defense and stress genes and proteins associated with the ripening process (Malacrida et al. 2006, Faurobert et al. 2007, Kesari et al. 2007, Palma et al. 2011). The results obtained herein, show that cytoplasmic and organellar *sHsps* and *Hsfs* have possible roles in the regulation of tomato fruit ripening. It is now clear that the *sHsps* are induced in response to most stresses, although not all *sHsps* respond in the same way (Waters 2013). Analysis of the expression profile of seven *sHsp* during fruit ripening in two tomato varieties (cvs. Micro-Tom and Minitomato) showed that the *sHsp* transcripts accumulated, peaked at intermediate stages (Y or O), and then declined. Similar transcription pattern was observed for class I *sHsp* genes *Sl17.6* and *Sl20.0* of tomato where they showed higher expression upon ripening (breaker and pink fruit) and then declined in the red fruit (Goyal et al. 2012) probably as a consequence

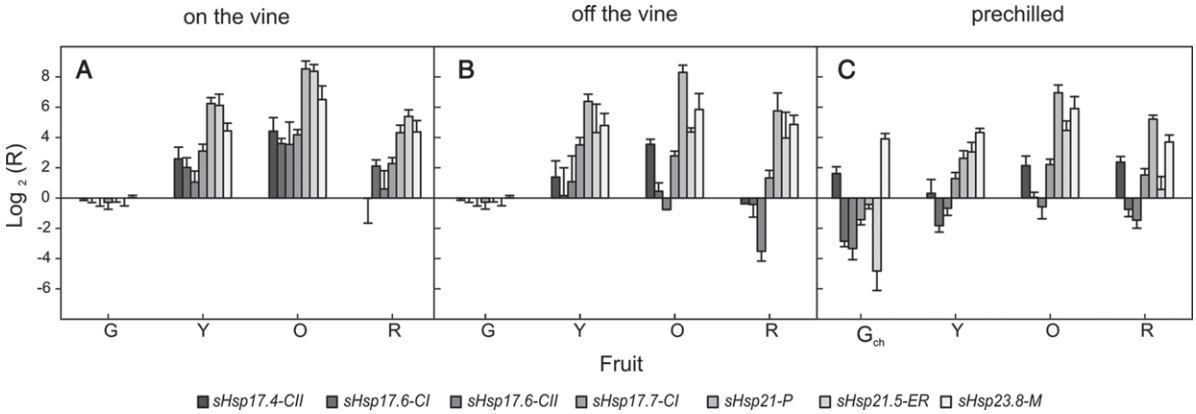

Fig. 8. *sHsps* expression throughout ripening in Micro-Tom fruits on the vine (A), off the vine (B), and after postharvest chilling (C). Transcript abundance was evaluated using qRT-PCR in green (G), green after 28 days at 4°C (G_{ch}), yellow (Y), orange (O), and red (R) fruits. Values shown are the $\log_2$ fold change compared to G. Each value represents the average (± SE) of three independent biological replicates. See statistical analysis in Appendix S4.

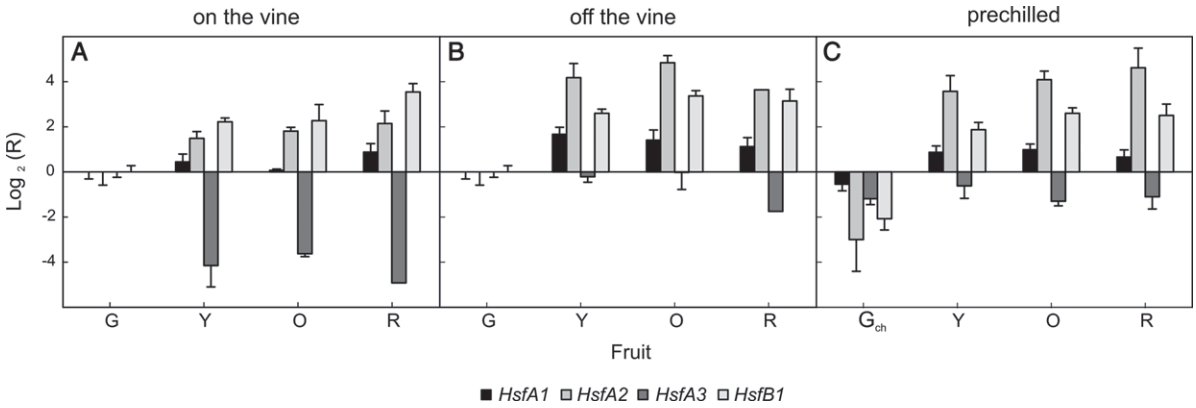

Fig. 9. *Hsfs* expression throughout ripening in Micro-Tom fruits on the vine (A), off the vine (B), and after postharvest chilling (C). Transcript abundance was evaluated using qRT-PCR. in green (G), green after 28 days at 4°C (G_{ch}), yellow (Y), orange (O), and red (R) fruits. Values shown are the $\log_2$ fold change compared to G. Each value represents the average (± SE) of three independent biological replicates. See statistical analysis in Appendix S4.

of the presence of physical clusters of *sHsps* genes in tomato. Also, Neta-Sharir et al. (2005) reported that the chloroplastic *sHsp 21-P* is induced during fruit ripening. In silico expression analysis of all members of *sHSP* identified in the tomato genome (Bondino et al. 2012) during fruit ripening using an RNA-seq database (TomExpress; http://gbf.toulouse.inra.fr/tomexpress) showed a clear peak of all transcripts at breaker stage in Ailsa Craig (Appendix S5). In a non-climacteric fruit such as strawberry, ripening expression of a cytoplasmic class I *sHsp* was observed (Medina-Escobar et al. 1998). It is worth to note that the highest transcript levels of *sHsp* at the intermediate stages (Fig. 1), occur together with the respiration burst (Alexander and Grierson 2002), denoting that *sHsps* may act as protecting proteins against denaturation as a result of the oxidative stress. Also, a protective role against oxidative stress for the accumulation of the mitochondrial *sHsp 22* was suggested by Banzet et al. (1998) in tomato cells. In Micro-Tom fruit, the organellar *sHsps* showed higher expression than cytoplasmic *sHsps* during ripening. On the other hand, in Minitomato fruit, the chloroplastic *sHsp 21-P* was the unique organellar *sHsp* that showed higher expression, suggesting that Micro-Tom fruit may cope better with organellar stress than Minitomato fruit.

The transcript profiles for *Hsfs* during fruit ripening were different in the two tomato varieties, except for *HsfA1*, the expression level of which was constant in both fruit types during fruit ripening. *HsfA1* is known as the master regulator for induced thermotolerance in tomato and cannot be replaced by any other *Hsf* (von Koskull-Doring et al. 2007). The expression of the other *Hsfs* (*HsfA2*, *HsfA3*, and *HsfB1*) was practically constant during fruit ripening in Minitomato but not in

Micro-Tom (Fig. 2). Probably a *Hsf* network plasticity is occurring in Micro-Tom fruit that explains the major accumulation of *sHsps* proteins during ripening in this fruit (Fig. 1). Under stressful conditions, it was reported that in rice *HsfA2a* generates different transcripts by alternative splicing (Wang et al. 2013) and in grapevine *VpHsf1* is involved in the response to biotic and abiotic stresses (Peng et al. 2013).

There exists abundant evidence showing a correlation between the accumulation of *sHsps* proteins induced by high temperatures, methyl jasmonate, methyl salicylate and other treatments, and the acquisition of chilling tolerance (Sabehat et al. 1998, Ding et al. 2002, Sevillano et al. 2010). Also, it has been shown that overexpression of a mitochondrial *sHsp* from tomato in tobacco can enhance heat tolerance (Sanmiya et al. 2004). In the present study, the tomato genotype advantage for the fruit to cope with the adverse conditions caused by refrigerated storage and the individual capacity of *sHsp* biosynthesis was investigated. Micro-Tom and Minitomato green fruits have shown contrasted sensitivity to postharvest chilling injury, being Micro-Tom more tolerant to cold storage (Gonzalez et al. 2015). This different sensitivity became evident during the first days at ambient temperature (18–25°C) after the chilling storage (4°C). Minitomato fruit showed rubbery texture, failed to develop color as Micro-Tom fruit did (minor a^* value) and was less luminous (minor L^* value) (Fig. 4 and Table 1). These macroscopic differences between varieties were also evident at the molecular level. Higher *sHsp* expression levels and new immunoreactive bands were detected in Micro-Tom fruit after chilling. Concomitantly, a clear distinction in the *sHsps* expression pattern of the fruit of the two varieties was observed (Fig. 5). Transcripts of all *sHsps* fell in Minitomato fruit during chilling storage while in Micro-Tom fruit transcripts of *sHsp 17.4-CII* and *23.8-M* increased 4- and 16-fold, respectively. In accordance with this observation, it has been reported that *sHsp 23.8-M* promoter is cold-inducible in tomato (Yi et al. 2006). Also, *sHsp 17.4-CII* has been associated with chilling resistance in tomato fruit pretreated with methyl jasmonate or methyl salicylate (Ding et al. 2001). Interestingly, transcript level of *sHsp 17.4-CII* in Micro-Tom fruit diminished during the first days after postharvest without chilling (Fig. 5B), indicating that *sHsp 17.4-CII* induction observed in the prechilled fruit (Fig. 5A) could be part of the response of Micro-Tom fruit to low temperature exclusively, not to the off the vine ripening condition. Other authors described up-regulation of *sHsp 17.7-CI* protein involved in the differential resistance to chilling conditions of two tomato lines (Page et al. 2010). Nevertheless, in ripening Micro-Tom fruit after cold storage, low transcription level of *sHsp 17.7-CI* was observed when compared with the on the vine ripening fruit (Fig. 8), suggesting that chilling tolerance can be achieved by induction of different *sHsps*.

In Micro-Tom fruit, the expression of the *sHsps* restored their level partially during the first days after the fruit was returned to ambient temperature and during ripening (Fig. 5). However, *sHsp* expression in Minitomato fruit did not change under the conditions described (Fig. 5). It has been reported (Neta-Sharir et al. 2005) that cold storage of a chilling-sensitive fruit variety inhibited the induction of *sHsp 21-P* expression. Constitutive *sHsp 21-P* synthesis (Neta-Sharir et al. 2005), or induced *sHsp 21-P* synthesis by preheat treatment (Sabehat et al. 1998) in fruit prevented some, but not all, symptoms of chilling injury, suggesting that *sHsp 21-P* could not be involved in protection against all chilling injury. The results obtained here show that the transcript level of *sHsp 21-P* was up-regulated during Micro-Tom fruit ripening at a higher level than in Minitomato regardless of the harvesting and chilling storage. This observation provides a new evidence of the correlation that exists between *sHsp 21-P* accumulation and amelioration of chilling symptoms in tomato fruit. It also points that *sHsp 21-P* natural induction could be involved in Micro-Tom fruit chilling tolerance. Despite the higher transcript level of *sHsp 21-P* observed in Micro-Tom fruit with respect to Minitomato after postharvest chilling (Fig. 5), a low expression value at the yellow stage was observed in the fruit that ripened after chilling when compared with the fruit that ripened on the plant (Fig. 8). This fact could be related to the longer ripening time of the prechilled fruit (Gonzalez et al. 2015) and the role of *sHsp 21-P* in the chloroplast to chromoplast conversion (Neta-Sharir et al. 2005). Additionally, the differences between the expression pattern of *sHsps* of the red fruit that was prechilled and the red fruit that ripened on the vine (Fig. 8) could explain, at least in part, the alteration in the fruit ripening process of the two cultivars (Gonzalez et al. 2015).

The cold storage response of tomato fruit from Micro-Tom and Minitomato was also different on the transcript level of *Hsfs*. The expression analysis of the four *Hsfs* showed that *Hsfs* were repressed by chilling storage in fruits from both varieties, except for *HsfA3* in Minitomato, which was not (Fig. 6). It has been stated that *HsfA3* functions in crosstalk with drought stress signaling (von Koskull-Doring et al. 2007). Also, *HsfA3* was induced by heat in tomato cells (Bharti et al. 2000), and it was up-regulated in maturing tomato microspore (Frank et al. 2009), showing that *HsfA3* is involved in the response to different stresses in different tomato tissues. The diminution of *HsfA3* transcript level observed in this work is probably associated with the tolerance to the

chilling of Micro-Tom fruit. *HsfA3* expression pattern in Micro-Tom fruit during ripening was different after chilling storage than in planta: when ripening on the vine, the *HsfA3* expression was significantly lower in ripening fruit than in green fruit, while when the fruit ripened off the vine with or without the previous chilling, *HsfA3* expression did not change (Fig. 9). Probably *HsfA3* does not develop a relevant role in fruit while ripening on the vine, but it is involved in the amelioration of chilling and harvesting effect on ripening off the vine.

In conclusion, this study shows that *sHsps* and *Hsfs* are part of the ripening program of tomato fruit and of the response to postharvest and chilling. In particular, the data suggest that as a result of the complex regulatory network of *sHsps* and *sHsfs* in tomato fruit, the accumulation of transcripts of *HsfA3*, *sHsp 17.4-CII* and *23.8-M* may be directly involved in the protection mechanisms to chilling stress.

Author contributions

S. B. B., E. M. V., and M. D. R. designed the experiments. M. D. R., C. G., M. R. E., and M. L. S. performed experiments. M. D. R. analyzed the data. S. B. B. and E. M. V. wrote the article.

Acknowledgements – This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (National Agency for the Promotion of Science and Technology, ANPCyT), Argentine (grants PICT2012-0482 y PICT2013-1015) and the Universidad Nacional de Rosario (National University of Rosario, UNR) (grant BIO263).

References

Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot 53: 2039–2055

Almoguera C, Prieto-Dapena P, Jordano J (1998) Dual regulation of a heat shock promoter during embryogenesis: stage-dependent role of heat shock elements. Plant J 13: 437–446

Banzet N, Richaud C, Deveaux Y, Kazmaier M, Gagnon J, Triantaphylides C (1998) Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells. Plant J 13: 519–527

Basha E, O'Neill H, Vierling E (2012) Small heat shock proteins and alpha-crystallins: dynamic proteins with flexible functions. Trends Biochem Sci 37: 106–117

Bharti K, Schmidt E, Lyck R, Heerklotz D, Bublak D, Scharf KD (2000) Isolation and characterization of *HsfA3*, a new heat stress transcription factor of *Lycopersicon peruvianum*. Plant J 22: 355–365

Bondino HG, Valle EM, Ten HA (2012) Evolution and functional diversification of the small heat shock protein/alpha-crystallin family in higher plants. Planta 235: 1299–1313

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Brummell DA, Dal CV, Lurie S, Crisosto CH, Labavitch JM (2004) Cell wall metabolism during the development of chilling injury in cold-stored peach fruit: association of mealiness with arrested disassembly of cell wall pectins. J Exp Bot 55: 2041–2052

Carrari F, Fernie AR (2006) Metabolic regulation underlying tomato fruit development. J Exp Bot 57: 1883–1897

Cruz-Mendívil A, López-Valenzuela JA, Calderón-Vázquez CL, Vega-García MO, Reyes-Moreno C, Valdez-Ortiz A (2015) Transcriptional changes associated with chilling tolerance and susceptibility in "Micro-Tom" tomato fruit using RNA-Seq. Postharvest Biol Technol 99: 141–151

Ding CK, Wang CY, Gross KC, Smith DL (2001) Reduction of chilling injury and transcript accumulation of heat shock proteins in tomato fruit by methyl jasmonate and methyl salicylate. Plant Sci 161: 1153–1159

Ding CK, Wang CY, Gross KC, Smith DL (2002) Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. Planta 214: 895–901

Faurobert M, Mihr C, Bertin N, Pawlowski T, Negroni L, Sommerer N, Causse M (2007) Major proteome variations associated with cherry tomato pericarp development and ripening. Plant Physiol 143: 1327–1346

Frank G, Pressman E, Ophir R, Althan L, Shaked R, Freedman M, Shen S, Firon N (2009) Transcriptional profiling of maturing tomato (*Solanum lycopersicum* L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response. J Exp Bot 60: 3891–3908

Giorno F, Wolters-Arts M, Grillo S, Scharf KD, Vriezen WH, Mariani C (2010) Developmental and heat stress-regulated expression of *HsfA2* and small heat shock proteins in tomato anthers. J Exp Bot 61: 453–462

Gonzalez C, Ré MD, Sossi ML, Valle EM, Boggio SB (2015) Tomato cv. micro-tom as a model system to study postharvest chilling tolerance. Sci Hortic 184: 63–69

Goyal RK, Kumar V, Shukla V, Mattoo R, Liu Y, Chung SH, Giovannoni JJ, Mattoo AK (2012) Features of a unique intronless cluster of class I small heat shock protein genes in tandem with box C/D snoRNA genes on chromosome 6 in tomato (*Solanum lycopersicum*). Planta 235: 453–471

Haslbeck M, Vierling E (2015) A first line of stress defense: small heat shock proteins and their function in protein homeostasis. J Mol Biol 427: 1537–1548

Kesari R, Trivedi PK, Nath P (2007) Ethylene-induced ripening in banana evokes expression of defense and stress related genes in fruit tissue. Postharvest Biol Technol 46: 136–143

Kotak S, Larkindale J, Lee U, von Koskull-Doring P, Vierling E, Scharf KD (2007) Complexity of the heat stress response in plants. Curr Opin Plant Biol 10: 310–316

Larkindale J, Mishkind M, Vierling E (2005) Plant responses to high temperature. In: Jenks MA, Hasegawa PM (eds) Plant Abiotic Stress. Blackwell Publishing Ltd, Oxford, pp 100–144

Liu M, Pirrello J, Chervin C, Roustan JP, Bouzayen M (2015) Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. Plant Physiol 169: 2380–2390

Lopez-Matas MA, Nunez P, Soto A, Allona I, Casado R, Collada C, Guevara MA, Aragoncillo C, Gomez L (2004) Protein cryoprotective activity of a cytosolic small heat shock protein that accumulates constitutively in chestnut stems and is up-regulated by low and high temperatures. Plant Physiol 134: 1708–1717

Low D, Brandle K, Nover L, Forreiter C (2000) Cytosolic heat-stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones in vivo. Planta 211: 575–582

Lubaretz O, Zur NU (2002) Accumulation of plant small heat-stress proteins in storage organs. Planta 215: 220–228

Malacrida C, Valle EM, Boggio SB (2006) Postharvest chilling induces oxidative stress response in the dwarf tomato cultivar Micro-Tom. Physiol Plant 127: 10–18

Medina-Escobar N, Cárdenas J, Muñoz-Blanco J, Caballero JL (1998) Cloning and molecular characterization of a strawberry fruit ripening-related cDNA corresponding a mRNA for a low-molecular-weight heat-shock protein. Plant Mol Biol 36: 33–42

Neta-Sharir I, Isaacson T, Lurie S, Weiss D (2005) Dual role for tomato heat shock protein 21: protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. Plant Cell 17: 1829–1838

Nishida I, Murata N (1996) Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Annu Rev Plant Physiol Plant Mol Biol 47: 541–568

Page D, Gouble B, Valot B, Bouchet JP, Callot C, Kretzschmar A, Causse M, Renard CM, Faurobert M (2010) Protective proteins are differentially expressed in tomato genotypes differing for their tolerance to low-temperature storage. Planta 232: 483–500

Palma JM, Corpas FJ, del Río LA (2011) Proteomics as an approach to the understanding of the molecular physiology of fruit development and ripening. J Proteomics 74: 1230–1243

Palta JP, Whitaker BD, Weiss LS (1993) Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of *Solanum* species. Plant Physiol 103: 793–803

Pedreschi R, Lurie S (2015) Advances and current challenges in understanding postharvest abiotic stresses in perishables. Postharvest Biol Technol 107: 77–89

Peng S, Zhu Z, Zhao K, Shi J, Yang Y, He M, Wang Y (2013) A novel heat shock transcription factor, *VpHsf1*, from Chinese wild *Vitis pseudoreticulata* is involved in biotic and abiotic stresses. Plant Mol Biol Rep 31: 240–247

Polenta GA, Calvete JJ, Gonzalez CB (2007) Isolation and characterization of the main small heat shock proteins induced in tomato pericarp by thermal treatment. FEBS J 274: 6447–6455

Ramakrishna W, Deng Z, Ding CK, Handa AK, Ozminkowski RH Jr (2003) A novel small heat shock protein gene, vis1, contributes to pectin depolymerization and juice viscosity in tomato fruit. Plant Physiol 131: 725–735

Rodriguez GR, Moyseenko JB, Robbins MD, Morejon NH, Francis DM, van der Knaap E (2010) Tomato analyzer: a useful software application to collect accurate and detailed morphological and colorimetric data from two-dimensional objects. J Vis Exp 37: 1856–1865

Sabehat A, Lurie S, Weiss D (1998) Expression of small heat-shock proteins at low temperatures. A possible role in protecting against chilling injuries. Plant Physiol 117: 651–658

Sanchez-Bel P, Egea I, Sanchez-Ballesta MT, Sevillano L, Del Carmen BM, Flores FB (2012) Proteome changes in tomato fruits prior to visible symptoms of chilling injury are linked to defensive mechanisms, uncoupling of photosynthetic processes and protein degradation machinery. Plant Cell Physiol 53: 470–484

Sanmiya K, Suzuki K, Egawa Y, Shono M (2004) Mitochondrial small heat-shock protein enhances thermotolerance in tobacco plants. FEBS Lett 557: 265–268

Sapitnitskaya M, Maul P, McCollum GT, Guy CL, Weiss B, Samach A, Porat R (2006) Postharvest heat and conditioning treatments activate different molecular responses and reduce chilling injuries in grapefruit. J Food Biochem 57: 2943–2953

Scarpeci TE, Zanor MI, Carrillo N, Mueller-Roeber B, Valle EM (2008) Generation of superoxide anion in chloroplasts of *Arabidopsis thaliana* during active photosynthesis: a focus on rapidly induced genes. Plant Mol Biol 66: 361–378

Schramm F, Larkindale J, Kiehlmann E, Ganguli A, Englich G, Vierling E, von Koskull-Doring P (2008) A cascade of

transcription factor DREB2A and heat stress transcription factor *HsfA3* regulates the heat stress response of Arabidopsis. Plant J 53: 264–274
Sevillano L, Mar Sola M, Vargas AM (2010) Induction of small heat-shock proteins in mesocarp of cherimoya fruit (*Annona cherimola* Mill.) produces chilling tolerance. J Food Biochem 34: 625–638
Sharom M, Willemot C, Thompson JE (1994) Chilling injury induces lipid phase changes in membranes of tomato fruit. Plant Physiol 105: 305–308
Sun W, Van MM, Verbruggen N (2002) Small heat shock proteins and stress tolerance in plants. Biochim Biophys Acta 1577: 1–9
Tsvetkova NM, Horvath I, Torok Z, Wolkers WF, Balogi Z, Shigapova N, Crowe LM, Tablin F, Vierling E, Crowe JH, Vigh L (2002) Small heat-shock proteins regulate membrane lipid polymorphism. Proc Natl Acad Sci USA 99: 13504–13509
Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35: W71–W74
van Berkel J, Salamini F, Gebhardt C (1994) Transcripts accumulating during cold storage of potato (*Solanum tuberosum* L.) tubers are sequence related to stress-responsive genes. Plant Physiol 104: 445–452
Volkov RA, Panchuk II, Schoffl F (2005) Small heat shock proteins are differentially regulated during pollen development and following heat stress in tobacco. Plant Mol Biol 57: 487–502
von Koskull-Doring P, Scharf KD, Nover L (2007) The diversity of plant heat stress transcription factors. Trends Plant Sci 12: 452–457
Wang CY (2010) Alleviation of chilling injury in tropical and subtropical fruits. Acta Hortic 864: 267–273
Wang H, Bian M, Yang Z, Lin C, Shi W (2013) Preliminary functional analysis of the isoforms of OsHsfA2a (*Oryza sativa* L.) generated by alternative splicing. Plant Mol Biol Rep 31: 38–46
Waters ER (2013) The evolution, function, structure, and expression of the plant sHSPs. J Exp Bot 64: 391–403
Wehmeyer N, Vierling E (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. Plant Physiol 122: 1099–1108
Yi SY, Sun AQ, Sun Y, Yang JY, Zhao CM, Liu J (2006) Differential regulation of Lehsp23.8 in tomato plants: analysis of a multiple stress-inducible promoter. Plant Sci 171: 398–407

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Equal loading control.

Appendix S2. Small heat shock proteins analyzed.

Appendix S3. Heat shock transcription factors analyzed.

Appendix S4. Statistical analysis for Figs. 5, 6, 8 and, 9.

Appendix S5. RNA-seq expression data of *sHsps* during ripening.

Edited by B. Huang