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Isolation and characterization of the main small heat shock proteins induced in tomato pericarp by thermal treatment

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In recent years, heat treatment has been used to prevent the development of chilling injury in fruits and vegetables. The acquired tolerance to chilling seen in treated fruit is related to the accumulation of heat shock proteins (HSPs). The positive effect of heat treatment has generally been verified for only a narrow range of treatment intensities and more reliable methods of determining optimal conditions are therefore needed. In this regard, quantitation of HSPs would seem to be an interesting tool for monitoring purposes. As a step toward the development of analytical methodology, the objective of this study was the isolation and characterization of relevant HSPs from plant tissues. Tomato fruits were exposed to a temperature of 38 °C for 0, 3, 20 and 27 h, and protein extracts from pericarp were analysed using SDS/PAGE. Analysis revealed the appearance of an intense 21 kDa protein band in treated samples. IEF of this band showed the presence of four major proteins (HSPC1, HSPC2, HSPC3 and HSPC4) with similar p*I* values. A monospecific polyclonal antiserum was raised in rabbits against purified HSPC1 protein, which cross-reacted with other small heat shock proteins. The major proteins were characterized by MS/MS analysis of tryptic peptides, all having blocked N-termini. The antiserum obtained proved suitable for detecting increased amounts of small heat shock proteins in tomatoes and grapefruits subjected to heat treatment for 24 and 48 h; these treatments were successful in preventing the development of chilling injury symptoms during cold storage. Our data are valuable for the future development of analytical methods to evaluate the optimal protection induced by heat treatment in different fruits.

Low-temperature storage is the most important method of reducing post-harvest decay and maintaining the organoleptic and nutritional quality of fruits and vegetables. However, exposing sensitive fruits to low temperatures induces the development of a group of symptoms collectively known as chilling injury, which lead to a significant change in overall quality [1,2]. Consumer concerns regarding the use of chemicals in food call for the use of physical techniques to preserve the original quality of fruits and vegetables after harvest. One useful strategy has been found to be the application of a particular stress, which then protects plant tissues against higher intensities of the same stress, or even against other types of stress [3]. Hence, the application of heat has been used to prevent the development of physiological alterations triggered by cold storage, consequently extending the shelf life of fruits and vegetables [4–7].

The effect of stress treatment on the physiological and biochemical parameters of fruits appears to be

Abbreviations
HSF, heat shock factor; HSP, heat shock protein; sHSP, small heat shock protein.

highly dependent on both the type and the intensity of the stress applied. Furthermore, beyond a certain level of stress, tissue changes could not be reversed, the damage thus becoming permanent [8]. This suggests that precise ascertainment of the 'limit of reversibility' is essential for the proper use of stress treatments. According to Saltveit [9], the development of chilling injury in sensitive tissues has two different components: the first is characterized by accumulated damage proportional to the duration of chilling. The second, which takes place after a threshold has been reached, is characterized by exponentially accumulated damage. It is this latter component that is believed to be potentially reversible by heat treatment.

Regarding the biochemical basis of heat protection, it has been reported that exposure of most live tissues to a transient temperature increment of 5–10 °C induces the synthesis of a specific group of proteins referred to as heat shock proteins (HSPs), which are usually present at low levels in nonstressed cells [10,11] Although the function of HSPs has not been fully established, they are believed to act in synergy with other mechanisms to deal with the cellular damage provoked by a stress [12]. In addition to high temperatures, HSP synthesis can be triggered by other types of stress, for example, the presence of heavy metals, nonionic detergents, exposure to low pH, microbial infections, tissue rupture and genetic lesions [13]. Anaerobic stress is particularly important in plants, and evidence of a genetic response common with heat stress has been found. Thus, by studying the transcriptome of *Arabidopsis* seedlings kept under anoxia, Loreti *et al.* [14] found that in addition to the expected induction of genes encoding enzymes involved in alcoholic fermentation, different genes coding for HSPs were also affected.

The most relevant HSPs in plants have molecular masses ranging from 15 to 40 kDa, and are therefore known as 'small heat shock proteins' (sHSP) [15]. Plant sHSPs belong to at least six different gene families, the production of each family being closely related to a particular cell compartment. Thus, two forms (class I and II) are cytosolic, whereas the others are found in the chloroplast, mitochondrion and endoplasmic reticulum [16]. Owing to the physiological importance of HSP expression as a mechanism for dealing with stress, previous studies have focused on the regulatory aspects of this response. It has been shown that heat-stress-inducible genes share conserved promoter elements (heat shock elements) representing the recognition site of heat stress transcription factors (heat shock factors; HSF). It is believed that HSFs are the terminal component of a signal-transduction chain leading to the activation of genes responsive to heat and other stresses [17]. Unlike yeast and *Drosophila*, which have a single HSF [18,19], plants have a complex HSF family, with at least 17 members in tomato [20]. It is believed that this complex system represents a link between cell stress and other genetic networks. In tomatoes, a cooperative mechanism between two main HSFs, Hsf1 and Hsf2, has been reported. Hsf1 has a unique function as a key regulator for induced thermotolerance and is responsible for the subsequent synthesis of Hsf2, which then becomes the dominant Hsf [21]. Interestingly, Hsf2 activity is controlled in part by a network of HSPs, which influence its solubility and intracellular localization [22]. In addition to this important transcriptional control, other regulatory mechanisms, such as the phosphorylation of sHSPs, have also been mentioned [23].

Despite the potential practical importance of HSPs, no precise quantitation methods have been reported in plants. Given that, in the case of fruits, the success of stress treatment is highly dependent on precise application at optimal intensities, we proposed that the evaluation of HSPs may be an interesting tool to monitor this intensity. Therefore, an in-depth study of this group of proteins is a prerequisite for the optimization of stress treatments. The objective of this study was to accomplish a detailed physicochemical and immunological characterization of the more prominent HSPs accumulated in tomato pericarp in response to heat treatment. The information generated will be valuable in the future development of analytical methods to evaluate optimal protection induced by heat treatment in different fruits, considering the cross-reactivity shown by antibodies with other commodities of economic importance such as grapefruit.

Results and Discussion

Biochemical characterization of sHSP

Figure 1A, shows the SDS/PAGE pattern of protein extracts obtained from control (C) and heat-treated tomatoes (27 h at 38 °C; HS). Heat-treated samples showed a distinct protein band with an apparent molecular mass of 21 kDa (arrow). The accumulation of proteins of mass 15–30 kDa seems to be an important component of the physiological response of plant tissues to a transient increase in environmental temperature [24]. In particular, proteins with molecular masses around 21 kDa are of interest in the response of individual plant organs to heat stress. For example, the entire *Nicotiana attenuata* plant reacted to high temperatures by overexpressing a set of proteins in the

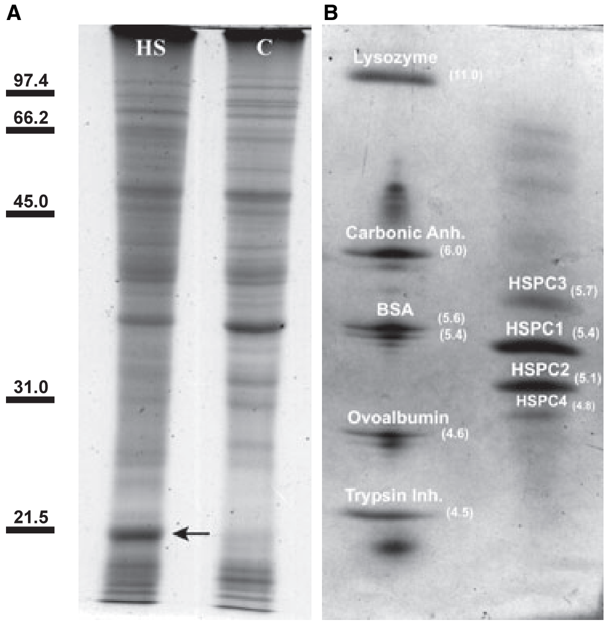

Fig. 1. (A) SDS/PAGE of protein extracts from tomatoes untreated (Control, lane C) or treated for 27 h at 38 °C (lane HS). (B) IEF pattern of the 21 kDa band excised from the HS lane of the SDS/PAGE shown in (A) (indicated by an arrow). The isoelectric points of proteins are indicated in between brackets and major bands are labelled (HSPC1, HSPC2, HSPC3 and HSPC4).

range 16–21 kDa, whereas only one band of 21 kDa was accumulated when a single leaf was heated [25]. In a genetic study, Sabehat *et al.* [26] succeeded in cloning two heat-shock-related genes, one showing perfect homology with the chloroplast tomato gene *tom111*. This gene corresponds to a HSP21 and was triggered by high temperature, but not by other types of stress such as drought or anaerobiosis. This evidence showed the importance of the 21 kDa protein as an early biochemical indicator of heat stress in plant tissues.

To better characterize the overexpressed 21 kDa protein, the band was excised from the SDS/PAGE (Fig. 1A) and resolved by IEF analysis. Figure 1B shows the presence of a set of proteins in the original 21 kDa band. Estimated p*I* values of the four main proteins (herein termed HSPC1, HSPC2, HSPC3, HSPC4) are shown in brackets in Fig. 1. The occurrence of several HSPs with similar p*I* values has been reported previously. Ritenour *et al.* [24] studied apples that had been heated and detected the accumulation of up to 17 new proteins with estimated molecular masses of 15–29 kDa. Most of the proteins accumulated in the 20 kDa region and had acidic p*I* values. Similarly, Nover & Scharf [27] studied protein extracts from tomato cells and found three 20-kDa HSPs with p*I* values of 7.0–7.3, and five 21-kDa proteins with p*I* values of 5.1–6.0. Iwahashi & Hosoda [28] studied the pericarp of heated tomatoes and found a mitochondrial sHSP with a molecular mass of 23.9 kDa and p*I* of 4.99. This HSP was one of the 14 proteins that appeared after exposure to heat stress, among a total of 963 proteins detected by bidimensional electrophoresis.

The purification protocol used in this study included two electrophoretic steps: SDS/PAGE followed by IEF, which modifies the classical 2D IEF–SDS/PAGE protocol. Reversal of these steps had two positive effects: first, the amount of starting material used in the electrophoretic run could be increased markedly (800 μg of total protein); and second, the resolution of the protein isoforms was improved due to the concentration effect exerted by the use of IEF as the final step. The purity grade of the isolated HSPC1 and HSPC2, as well as the reproducibility attained after the IEF step during the purification of HSPC1, HSPC2, HSPC3 and HSPC4, was confirmed by electrophoretic analysis (data not shown). In addition to being suitable for obtaining large amounts of purified proteins, the described protocol is also useful for analytical purposes. For example, the increased concentration of individual stress proteins induced by the exposure of tomato fruits to extended heat treatments is shown in Fig. 2A.

Immunological characterization of sHSP

The main overexpressed sHSP (HSPC1) was used as an immunogen to produce polyclonal antibodies for further characterization of the heat response. The antiserum obtained is a valuable tool for identifying heat shock-induced proteins and/or developing reliable quantitative methods. Interestingly, western blot analysis (data not shown) revealed that the obtained antibodies cross-reacted with the four main proteins detected by IEF in heat-treated tomatoes (Fig. 1B). This is probably due to the presence of a primary domain (known as an α-crystallyn domain) that is shared by different sHSPs [15]. Previously, other authors have used an immunological approach to detect tomato HSPs. Among them, Sabehat *et al.* [4] showed that antibodies raised against pea or cereal HSPs were also able to detect HSPs from tomato fruit, this being evidence of the cross-reactivity among sHSPs from different plant species. Even more, antibodies against a synthetic sHSP-oligopeptide antigen were also able to recognize sHSP from tomato leaf [29].

From a biotechnological point of view, the obtained antibodies are useful for developing immunological

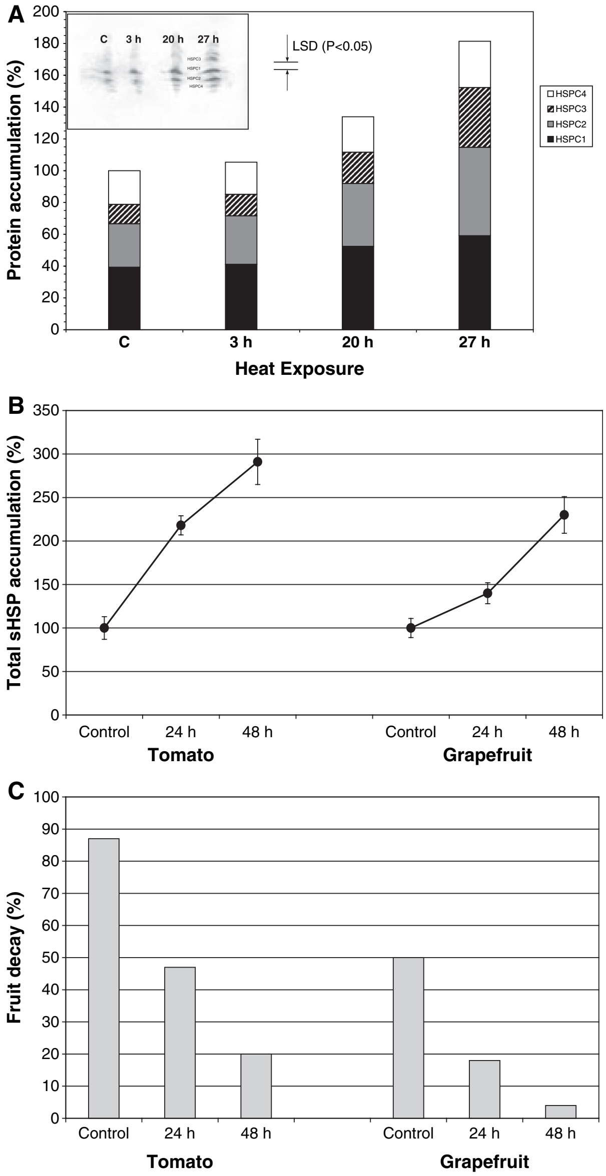

Fig. 2. (A) Densitometric analysis of bands after IEF analysis of protein extracts from tomato fruit exposed for 0 (C), 3, 20 and 27 h at 38 °C. Major bands are labelled (HSPC1, HSPC2, HSPC3 and HSPC4). Total constitutive amounts present in control fruits were considered as 100%. The least significant difference (LSD) was calculated from densitometric values to compare treatments and individual proteins means. (B) Total sHSP accumulation (%) after the application of heat treatments in Fortaleza tomatoes and Marsh grapefruits. Percentages were calculated by densitometric analysis of blotted membranes revealed with anti-HSPC1, with constitutive amounts present in control fruits being considered as 100%. Bars = standard deviations. (C) Fruit decay (%) after cold storage of Fortaleza tomatoes (21 days at 2 °C) and Marsh grapefruits (60 days at 2 °C). After storage, fruits were exposed to 20 °C until chilling injury symptoms were evident.

methods (western blot, dot blot, ELISA, etc.) to monitor the onset of the sHSP protection induced by the application of stress treatments. Because accumulation of these proteins has been reported in several economically important species, such as soybean, pea, sunflower, wheat, rice, maize [30], strawberry [31], apple [24] and papaya [32], the development of such methods would have a wide application in different areas. However, to the best of our knowledge, no commercial antibodies against fruit sHSPs are available. To overcome this, the described electrophoretic method proved successful for purifying individual sHSPs at amounts sufficient to raise an immunological response in laboratory animals.

This study shows that the augmented synthesis of proteins reactive to anti-HSPC1 serum reflects the increased intensity of the treatments applied (Fig. 2B). Increased accumulation of HSPs in treated fruits has been linked to the acquisition of a tolerance to low temperatures [4,11]. Interestingly, the conditions required for maximum HSP induction in avocado fruits correlated well with the temperature that provided maximal protection against chilling injury [33]. In addition, in tomato leaves, a positive correlation has been found between sHSP accumulation and photosynthetic thermotolerance [26]. In other produce like lettuce, a close relationship between HSP synthesis and the prevention of browning has been noted [34].

Sequencing the main sHSP from heat-treated tomatoes

In order to identify and characterize inducible tomato sHSPs, the IEF-separated HSPC1, HSPC2, HSPC3 and HSPC4 bands were submitted to in-gel trypsin digestion, and the peptide mixture was analysed by nanoelectrospray MS. All the sequences matched from product ion spectra of doubly and triply charged tryptic peptide ions had probability-based Mowse scores > 42. Both, the Mascot assigned and nonmatched MS/MS spectra were checked manually. On the whole, MS/MS-derived amino acid sequences from HSPC1 peptide ions matched residues 2–154 of entry AAD30454 in the NCBI databank, a 17.6 kDa class I sHSP from *Lycopersicon esculentum* (Fig. 3). MS/MS analysis of the doubly charged ion, *m/z* 314.7, showed that the N-terminal residue corresponded to an *N*-acetylated serine (Fig. 3). The sequenced peptides represent $> 99.9\%$ of the 17.6 kDa class I sHSP, and the calculated isoelectric point of this protein is 5.8. The product ion mass spectra of tryptic peptides from HSPC2 unambiguously identified this protein as entry AAD30453 in the NCBI databank, a 17.8 kDa class I

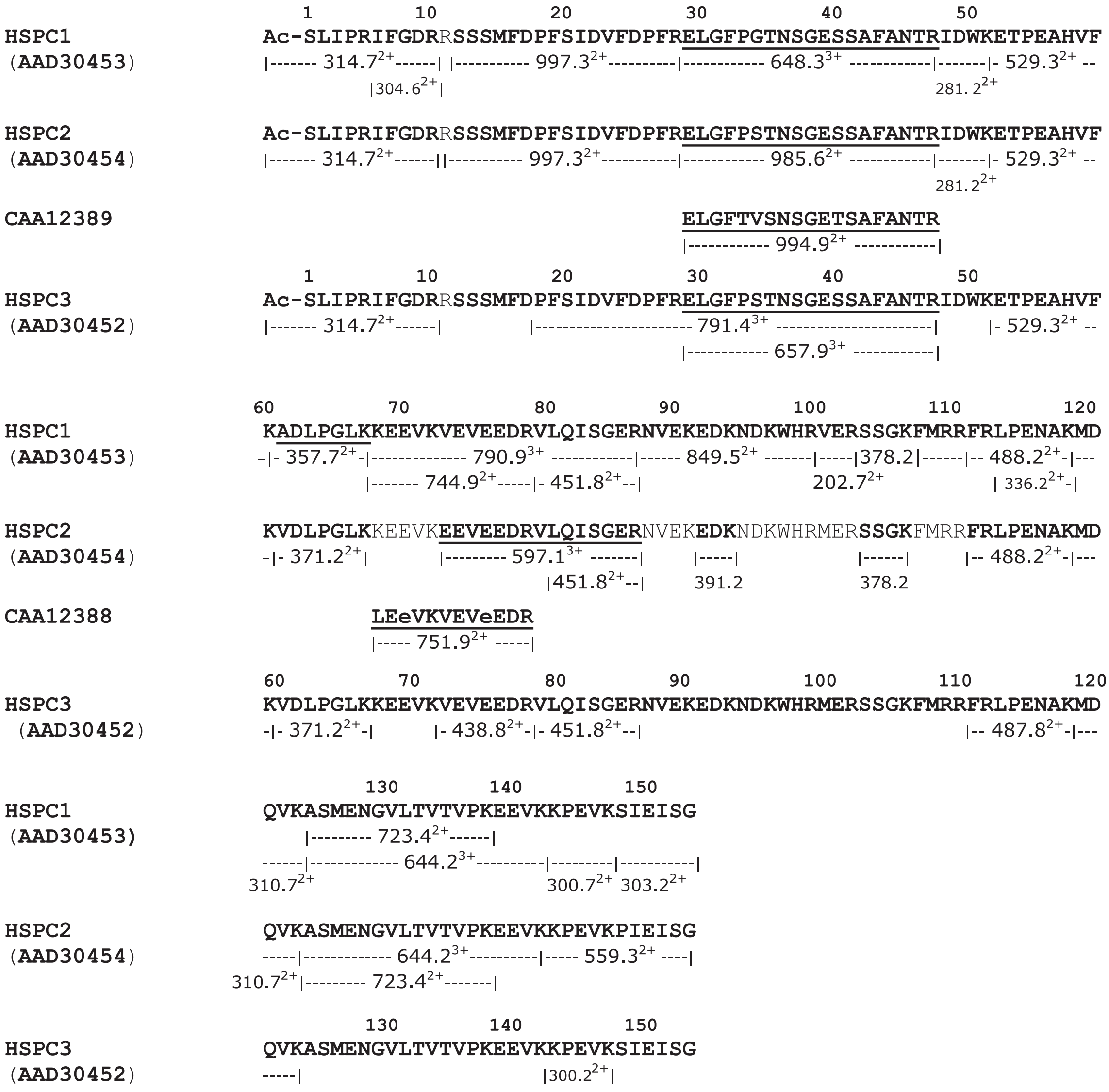

Fig. 3. Assignment of the CID-MS/MS-derived amino acid sequences of tryptic peptide ions HPSC1, HSPC2 and HSPC3 to the amino acid sequences of the class I sHSPs 17.6 kDa (AAD30453), 17.8 kDa (AAD30454) and 17.7 kDa (AAD30452) from *L. esculentum*, respectively. Protein-specific amino acid sequences are underlined. The sequences of the minor HSPC2 tryptic peptide ions at *m/z* 994.9 (2+) and 751.9 (2+), which matched, respectively, the 20 and 19.9 kDa sHSPs from *L. peruvianum*, are shown below the HSPC2 sequence.

sHSP from *Lycopersicon esculentum* with a calculated p*I* of 5.6. The amino acid sequence coverage was 86% (Fig. 3). For HSPC1, the N-terminal residue of HSPC2 was an *N*-acetylated serine (Fig. 3). In addition, the sequences of ions 751.9 (2+) (LeEVKVEVeEDR, where e = *O*-methylated glutamic acid) and 994.9 (2+) (ELGFTVSNSGETSAFANTR), also found in the HSPC2 tryptic peptide mixture (Fig. 3), matched, respectively, the sHSPs 19.9 kDa (p*I* 5.4; NCBI entry CAA12388) and 20 kDa (p*I* 5.2; NCBI entry CAA12389) from *L. peruvianum* (*Solanum peruvianum*). This result might not be surprising bearing in mind that members of the genus *Lycospersicon* have become tolerant to a wide range of environmental and nutritional conditions through cross-breeding. Among the attributes found in some of the commercial varieties of tomato currently cultivated, it has been documented that some pest- and disease-resistance characters have been incorporated from *L. peruvianum* [35]. However, the possible causal relationship between sHSP expression level and acquired resistance deserves further investigation.

HSPC3 mass fingerprinting followed by MS/MS analyses identified this protein as the sHSP 17.7 kDa (p*I* 5.7; NCBI entry AAD30452) (Fig. 3). In particular, the sequence at position 29–47 was used to distinguish between the different highly related tomato sHSPs reported. The triply charged ion at *m/z* 657.9 found in the tryptic digest of HSPC3 corresponds to an AAD30452-sHSP-specific marker (Fig. 3).

MS/MS-derived amino acid sequences of the doubly charged peptide ions at *m/z* 314.7 (acetyl-1SLIPR5) 487.7 (112FRLPENAK119), 451.8 (80VLQISGER87) and 529.3 (52ETPEAHVFK60) from the tryptic peptide mixture of HSPC4 are conserved in tomato sHSP. Hence, although these sequences identified HSPC4 as a sHSP, they did not allow us to assign it unambiguously among the different family members.

Previously, Iwahashi & Hosoda [28] conducted a detailed study comparing bidimensional electrophoresis profiles of control and heated tomatoes, sequencing the N-terminal peptides of heat-induced proteins. However, the N-terminal sequence reported for a 23 kDa sHSP (FNTNTQMTAYDQDR) does not correspond to any of the sHSPs reported here.

Relationship between sHSP accumulation and damage prevention in cold-stored fruits

Figure 2 shows the relationship between the increase in sHSP concentration induced by different heat treatments (Fig. 2B), and the damage suffered by fruits subjected to cold storage (Fig. 2C). To asses the applicability of HSP-based methods in different types of fruit, experiments were carried out with two species: tomato and grapefruit.

In the case of tomato, the variety Fortaleza was used and showed high sensitivity to cold storage (87% damage in untreated fruit after 21 days at 2 °C). Despite the high susceptibility to chilling, Fig. 2C shows that application of both 24 and 48 h treatments was able to successfully prevent the development of chilling-associated symptoms in a significant percentage of fruits (47 and 20% of damaged fruits, respectively). In a previous experiment carried out in our laboratory using another variety (*Colt 45*; data not shown), we found similar results for mild treatments (30 and 60 min immersion at 42 °C). However, fruits subjected to an excessive intensity (72 h at 39 °C) were more susceptible to decay (55% more than control fruits), despite the induction of higher concentrations of sHSPs (670% compared with control).

In the case of grapefruit, fruits exposed to 38 °C for 24 and 48 h showed, respectively, increased accumulations of proteins reactive with anti-HSPC1 serum (Fig. 2B), and the development of chilling-associated symptoms was also prevented (Fig. 2C). Grapefruit is a well-known chilling-susceptible commodity, and the effect of heat treatment on the tolerance to cold storage has been previously evaluated by analysing different quality parameters [36]. The biochemical basis of protection was initially linked to increased levels of the polyamine putrescine [37]. Pavoncello *et al.* [38] reported that hot water treatments were able to induce resistance to *Penicillium digitatum*, along with a concomitant overexpression of different groups of HSPs. In this study, we show quantitative evidence for the relationship between increasing sHSP accumulation and the induction of tolerance to chilling.

Results obtained with tomato and grapefruit show that heat treatment is effective in preventing chilling injury when applied at optimal intensities. In the commodities studied here, it was shown that sHSP accumulation is a suitable parameter for monitoring treatment intensity, and the antiserum obtained was able to quantify the extent of this accumulation. However, more research is needed to ascertain the precise relationship between treatment intensity, sHSP accumulation and treatment performance, which seems to be a promising approach to determine the 'limit of reversibility' of heat treatments.

Concluding remarks

Heat treatments applied to tomatoes induced the synthesis of two major (HSPC1 and HSPC2) and two minor (HSPC3 and HSPC4) class I sHSPs. The antiserum obtained against HSPC1 cross-reacted with other members of this family, and the increased amounts of sHSP detected in heat-treated tomatoes and grapefruits showed a positive correlation with the tolerance to the development of chilling injury after cold storage. Thus, we proposed the quantification of sHSPs as a tool to predict treatment performance. In this regard, the described electrophoretic method may be a helpful tool to purify individual sHSPs and obtain specific antiserum in laboratory animals considering that, to the best of our knowledge, there is no available commercial antibodies against fruits sHSPs. In addition, other information generated in this study provides lays the groundwork for genetic studies on the protection induced by heat treatment in plants.

Currently, our group is working on the development of an ELISA to monitor the accumulation of HSPs in fruits subjected to different treatment intensities. By using robust analytical techniques, it should be possible to measure more precisely the sHSP concentration induced by exposure to different time–temperature combinations, and to establish optimal intensity ranges to prevent the development of chilling injury in susceptible commodities like tomato and citrus fruit.

Experimental procedures

Chemicals

Hybond nitrocellullose membrane (pore size = 0.45 μm) were purchased from Amersham (Little Chalfont, UK), goat anti-(rabbit IgG) coupled to alkaline phosphatase from Bio-Rad (Hercules, CA), pharmalyte ampholytes from Amersham and bovine pancreas trypsin from Roche (Basel, Switzerland).

Fruit treatments

Mature green tomatoes (*L. esculentum*) of uniform size were obtained from our greenhouse. Fruits were surface-sterilized for 3 min with a chloride solution (150 mg·L^{-1}), rinsed thoroughly with tap water for another 3 min, and left on filter paper to drain. Thermal treatment was applied as described previously [8]. In the purification experiment, 60 fruits of the Cardenal variety were divided into four lots, and three of them heat-treated at 38 °C in a chamber for 3, 20 and 27 h, respectively; the remainder received no treatment and were regarded as the control group (C).

In the case of Fortaleza tomatoes and Marsh grapefruits, 90 fruits were divided into three lots, two lots being heat-treated at 38 °C in a chamber for, respectively, 24 and 48 h; the remainder received no treatment and were used as a control group. To induce chilling injury, fruits were stored at 2 °C (21 days in the case of tomatoes; 60 days in the case of grapefruits), and then exposed to 20 °C until symptoms were evident.

Protein extraction

Proteins were extracted from tomato pericarp (three pools of five fruits) following the method of Hurkman & Tanaka [39] with some modifications. Briefly, 1 g of tissue was ground with a pestle and mortar in liquid nitrogen. The homogenate was mixed thoroughly in the presence of 1 mL of extraction buffer (100 mM Tris/HCl pH 8.0, containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride and 2% v/v β-mercaptoethanol) and 4 mL of phenol saturated with 100 mM Tris buffer (pH 8.0), and centrifuged at 21 000 ***g*** for 10 min at 4 °C. The phenolic phase was recovered, mixed with 4 vol of 0.1 M ammonium acetate in methanol, and incubated overnight at −20 °C. Proteins were pelleted by centrifugation at 21 000 ***g*** for 20 min at 0 °C. The pellet was washed twice with ammonium acetate in methanol, once with cold acetone (80% v/v), and dried at room temperature. The dried residue was resuspended directly in sample buffer (25 mM Tris pH 6.8, 1% w/v SDS, 10% v/v glycerol, 5% v/v β-mercaptoethanol and 0.002% w/v bromophenol blue) and boiled for 2 min before electrophoresis. The protein concentration was determined by the Lowry method [40].

Electrophoretic analysis

SDS/PAGE was carried out according to the procedure of Laemmli [41]. For analytical purposes, 15 μg of protein were loaded onto each well of a 0.75-mm-thick gel, whereas for preparative use, 800 μg of protein were loaded in a 1.5-mm-thick gel.

Proteins were separated by using 12% homogeneous polyacrylamide slab gels. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue solution. IEF was carried out in a vertical system: gel composition was 5% polyacrylamide, 0.4% pH 3–10 ampholyte, 2% pH 4–6.5 ampholyte, and 8 M urea. The bands of interest from previous SDS/PAGE analysis were excised, soaked in 20 mM NaOH for 20 min and loaded onto the IEF gel. Electrophoresis was run in a Protean II electrophoresis system (Bio-Rad) at the following voltage steps: 150 V for the first 30 min, 200 V for the subsequent 60 min, and 250 V for the last 90 min. Calibration proteins (p*I* = 4.5–11) were used to estimate the p*I* values of the different protein bands. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue.

Antigen preparation and immunization protocol

Protein bands of interest were excised from IEF gels, rinsed several times with NaCl/P$_i$ and homogenized in the same buffer. Rabbit immunization for the production of polyclonal antibodies was carried out as described previously [42]. Animals were maintained under conditions that fulfilled all ethical and scientific requirements for animal use included in Directive 86/609/EEC and Recommendation 2007/526/EC. Preimmune serum (day 0) was considered as the negative control. Protein extracts (0.4 μg·mL^{-1}) were injected on days 1, 4 and 14. Serum containing the polyclonal antibodies against HSPC1 protein was aliquoted and stored at −80 °C until use.

Immunoblotting

For western blot analysis, separated polypeptides were transferred (50 min at 100 V) onto a Hybond nitrocellulose membrane (0.45 μm) using the Mini Protean II Electrophoresis System. For dot blot, 3 μg of proteins at a concentration of 3 μg·mL^{-1} was poured onto the nitrocellulose membrane. The polyclonal antiserum raised against HSPC1 (diluted 1 : 750) was used as primary antibody. The secondary serum was goat anti-(rabbit IgG) coupled to alkaline phosphatase (Bio-Rad, dilution 1 : 1500). Membranes were revealed with Nitro Blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Image analysis

Gels were analysed with a GS-800 Imaging Calibrated Densitometer (Bio-Rad). Images were captured and processed by QUANTITY ONE 1-D ANALYSIS software (Bio-Rad). Lane and band-based functions were used to determine apparent molecular masses, p*I* values and relative and absolute amounts of proteins. A known amount of BSA was used as protein standard for lane-based protein quantification. Analysis of variance was performed using the General Linear Model procedure from SAS software and least significant difference ($P < 0.05$) was calculated to compare treatments and individual proteins means.

Protein enzymatic digestion and MS/MS peptide sequencing

Protein bands of interest were excised from a Coomassie Brilliant Blue-stained SDS/PAGE and subjected to automated reduction, alkylation with iodoacetamide, and digestion with sequencing grade bovine pancreas trypsin using a ProGest digestor (Genomic Solutions, Ann Arbor MI) following the manufacturer's instructions. The tryptic peptide mixtures were loaded in a nanospray capilar and subjected to ESI-MS analysis using a QTrap mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a nanospray source (Protana, Denmark). Doubly or triply charged ions selected after enhanced resolution MS analysis were fragmented using the enhanced product ion [43] with Q0 trapping option. Enhanced resolution was performed at 250 $\text{amu}\cdot\text{s}^{-1}$ across the entire mass range, a scanning mode that enables mass accuracy of $< 20\ \text{mg}\cdot\text{L}^{-1}$ making charge state identification reliable up to charge state 5. For MS/MS experiments, Q1 was operated at unit resolution, the Q1-to-Q2 collision energy was set to 35 eV, the Q3 entry barrier was 8 V, the linear ion trap Q3 fill time was 250 ms and the scan rate in Q3 was 1000 $\text{amu}\cdot\text{s}^{-1}$. CID spectra were interpreted manually or using the online form of the MASCOT program (version 2.2) at http://www.matrixscience.com against the MSDB database (taxonomy: mammals). MS/MS ion-search parameters include allowance for a tryptic miscleavage, an MS/MS tolerance of ± 0.5 Da, carbamidomethylation of cysteine residues as fixed modification, methionine oxidation and N-terminal pyroglutamic acid as variable modifications.

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