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Protein Changes in the Albedo of Citrus Fruits on Postharvesting Storage

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In this work, major protein changes in the albedo of the fruit peel of Murcott tangor (tangerine x sweet orange) during postharvest ageing were studied through 2D PAGE. Protein content in matured on-tree fruits and in fruits stored in nonstressing [99% relative humidity (RH) and 25 °C], cold (99% RH and 4 °C), and drought (60% RH and 25 °C) conditions was initially determined. Protein identification through MS/MS determinations revealed in all samples analyzed the occurrence of manganese superoxide dismutase (Mn SOD), actin, ATP synthase β subunit (ATPase), citrus salt-stress associated protein (CitSap), ascorbate peroxidase (APX), translationally controlled tumor protein (TCTP), and a cysteine proteinase (CP) of the papain family. The latter protein was identified in two different gel spots, with different molecular mass, suggesting the simultaneous presence of the proteinase precursor and its active form. While Mn SOD, actin, ATPase, and CitSap were unchanged in the assayed conditions, TCTP and APX were downregulated during the postharvest ageing process. Ageing-induced APX repression was also reversed by drought. CP contents in albedo, which were similar in on- and off-tree fruits, were strongly dependent upon cold storage. The active/total CP protein ratio significantly increased after cold exposure. This proteomic survey indicates that major changes in protein content in the albedo of the peel of postharvest stored citrus fruits are apparently related to the activation of programmed cell death (PCD).

KEYWORDS: TCTP; APX; cysteine proteinase; oxidative stress

INTRODUCTION

The main damages to citrus fruits after harvest come from losses of water. To prevent fruit dehydration, different treatments such as cold storage or wax coatings are commercially used (*1–3*). However, these postharvest treatments may also provoke damage and injuries, mostly affecting the fruit peel of susceptible varieties (*4, 5*). The peel of citrus fruit is composed of two major tissues with different properties and anatomical structures: the colored external layer, called flavedo, and the colorless inner spongy tissue termed albedo (*6*). Research on ripening and the effects of postharvest treatments and storage conditions on citrus fruit quality is mostly focused on the flavedo, the most visible tissue of the fruit (*7, 8*), and also on the pulp (*9*). However, the albedo, which supports the vessel network connecting the pulp with the flavedo and hence with the rest of the plant and possesses in addition many other fundamental biological roles such as protection, for example, has been scarcely studied. Furthermore, the albedo is an important source of pectin (*10*) with several applications in the food industry. Peeling feasibility (*11*) and major physiological disorders such as "creasing" are also dependent upon albedo structure and performance.

Mature fruit detachment apparently triggers a sequence of physiological and signaling events accelerating fruit senescence and regulating cell death. Although there is no direct evidence in citrus, it is reasonable to propose that programmed cell death (PCD) may be involved in the regulation of these late developmental stages. There is wide physiological evidence in citrus, on the other hand, that abscission of ovaries after pollination (*12*) and of fruit during development is certainly induced at the metabolic (*13, 14*) and hormonal (*15–17*) level by signals that have often been related to PCD. Among these regulatory molecules, carbon starvation (*18, 19*), ABA (*20*), and ethylene (*21*) are thought to play pivotal regulatory roles during abscission, senescence, and programmed cell death (*22–24*). It is also known that cold and osmotic stresses that also activate

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programmed cell death (*25–27*) may alter growth and development of citrus by affecting the functionality of fruit tissues (*28, 29*).

In this work a proteomic survey was carried out in postharvest citrus fruits under several storage conditions to elucidate the involvement of PCD-related proteins in the ageing processes of the albedo.

MATERIALS AND MEHTODS

Plant Material and Treatments. Mature fruits of Murcott tangor (tangerine x sweet orange) (*30*) were harvested at random from trees grown at IVIA (Moncada, Valencia, Spain). After harvesting, albedo from a set of fruits were sampled with a razor blade while other fruit sets were stored in three different conditions: (a) 99% RH and room temperature (CFT), (b) 99% RH and 4 °C (CS), or (c) 60% RH and 25 °C (WS). Albedo from these fruits was sampled 13 days later. In an additional experiment Murcott fruits were first stored at 4 °C and then exposed to room temperature. Albedo from both conditions (cold and room temperature) were sampled at days 3 and 15. Albedo tissue was frozen in liquid nitrogen, ground to a fine powder, and stored at −80 °C until analysis. For every sample, aliquots of fresh tissue were dehydrated at 60 °C until a constant water content was registered.

Total Protein Content Quantifications. Albedo tissue was lyophilized and extracted with 50 mM Tris-HCl, pH 7.4, and 1% protease inhibitor cocktail (Sigma-Aldrich) on ice. The mixture was centrifuged at 13000 rpm for 15 min at 4 °C. Protein content of the supernatant was quantified with the BCA assay kit from Pierce (Rockford, IL). An extraction was carried out using three independent samples of each type.

Sample Preparation for 2D PAGE. For micropreparative gels, about 200 mg of albedo tissue was extracted with rehydration buffer [8.5 M urea, 4% (w/v) CHAPS, 0.2% IPG buffer, pH 3–10, 0.2% bromophenol blue, and 1% protease inhibitor cocktail for plant cells (Sigma-Aldrich)], vortexed, and sonicated briefly 2–3-fold alternatively. Samples were centrifuged at 15300 rpm for 30 min at 4 °C. The supernatant was transferred to fresh tubes and again centrifuged at 15300 rpm for 30 min at 4 °C. An aliquot of 200 μL from the upper phase to avoid pectins was precipitated at −20 °C overnight with 1.6 mL of cold acetone. The precipitated protein was pelleted by centrifugation and redissolved in rehydration buffer. The protein content was quantified using the BCA assay kit from Pierce.

For analytical gels, extractions were carried out as described by Saravanan and Rose (*31*) with some modifications. Basically, 300 mg of frozen albedo tissue was ground in liquid nitrogen using a pestle and mortar. The resulting powder was homogenized in 1.5 mL of cold extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, pH 7.5, 50 mM EDTA, 0.1 M KCl). The mixture was vortexed and sonicated shortly for three times. To remove cell debris, the sample was centrifuged at 13000 rpm for 1 h at 4 °C. The supernatant was transferred to fresh tubes and centrifuged again in the same way. The clarified supernatant was then mixed with an equal volume of Tris-buffered phenol, pH 8 (Sigma), and centrifuged at 13000 rpm for 30 min at 4 °C to separate the aqueous from the phenol phase. The upper phenolic phase was reextracted twice with 1 volume of extraction buffer. Protein was then precipitated from the separated phenol phase by incubation with 5 volumes of 0.1 M ammonium acetate in methanol at −20 °C overnight. A pellet was precipitated by centrifugation at 5000 rpm for 5 min at 4 °C and then washed three times with cold methanol and three more times with cold acetone. Finally, the pellet was air-dried for 2 h and resolubilized in lysis buffer [7 M urea, 2 M thiourea, 4% CHAPS, 0.2% IPG buffer, pH 4–7, 0.2% bromophenol blue, and 1% protease inhibitor cocktail for plant cells (Sigma-Aldrich)]. The protein content was quantified using the Bradford assay (Sigma).

2D Protein Gel Electrophoresis. Isoelectric focusing: For micropreparative gels, sample aliquots of 100 μg of protein were diluted with rehydration buffer [8.5 M urea, 4% (w/v) CHAPS, 0.2% IPG buffer, pH 3–10, 0.2% bromophenol blue (Sigma-Aldrich)] to yield 200 μL. This mixture was then loaded onto 11 cm IPG strips, pH gradient 3–10 (Bio-Rad). For analytical gels, proteins were loaded onto 7 cm IPG strips, pH gradient 4–7 (Bio-Rad). Isoelectric focusing, including passive rehydration at 20 °C overnight, was performed on a PROTEAN IPG cell unit (Bio-Rad). The following voltage programs were applied: linear increase to 8000 V (11 cm strips) or 4000 V (7 cm strips) and then the voltage was kept to yield 30000 or 10000 V·h, respectively. Following IEF, the strips were equilibrated for application to the second dimension (SDS–PAGE) for 15 min in equilibration buffer [1.5 M Tris-HCl, pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS] plus 2% (w/v) DTT and for another 15 min in equilibration buffer plus 2.5% (w/v) iodoacetamide. Subsequently, 11 cm strips were placed on top of self-cast SDS–polyacrylamide gels (13.3 × 8.7 cm; Criterion System, Bio-Rad) while 7 cm strips were on top of hand-cast SDS–polyacrylamide gels (12% T, 2.6% C). The strips were sealed with warm agarose solution (1.5% agarose in 45 mM Tris–borate/1 mM EDTA). Electrophoresis was carried out with a Tris/glycine running buffer system either in a Criterion Dodeca cell (Bio-Rad) or a Mini-PROTEAN 3 cell (Bio-Rad), both at a constant voltage of 120 V.

Gels were fixed in 40% (v/v) methanol/20% (v/v) acetic acid shaking overnight, stained with Sypro Ruby (Bio-Rad, Hercules, CA) overnight, and destained with 10% acetic acid (10 min × 3) followed by three more 10 min washes with 5% acetic acid.

Image Analysis. Stained gels were imaged using PDQuest with a Bio-Rad Molecular Imager FX or Quantity One with a Bio-Rad Gel Doc 2000 system. Quantification was performed in at least one gel per sample with three independent samples and extractions per experimental condition. Proteins were selected on the basis of their abundance and variation reproducibility (threshold level >2-fold). All selected proteins were present as single spots in both types of gel used. These proteins were quantified on nonsaturated gels and normalized for the total protein content in each gel. The molecular weight of citrus proteins was experimentally determined by comparison with known 1D molecular markers (broad range; Bio-Rad) run along with the samples during the SDS–PAGE.

Protein Identification. Candidate protein spots were manually picked from gels and prepared for tryptic digestion. Gel pieces were washed with 100 mM NH_4HCO_3 and dehydrated with ACN. The cysteine residues were reduced by 100 μL of 100 mM DTT at 56 °C and alkylated by 100 μL of 55 mM iodoacetamide at room temperature in the dark. After gel dehydration with ACN, proteins were digested overnight at 37 °C in 40 μL of a solution containing 18 ng/μL modified porcine trypsin (Promega, Madison, WI) prepared on 100 mM NH_4HCO_3. A second peptide extraction was performed with 60% (v/v) ACN in 1% (v/v) TFA. Nano-LC-MS/MS analysis of the resulting tryptic peptides was performed using a CapLC capillary LC system (MicroMass) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Ultima) or an Agilent 1100 series capillary LC system (Agilent Technologies, Palo Alto, CA) coupled directly online with a LTQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). In both cases chromatographic separations were conducted on a 10 cm × 75 μm New Objectives Picofrit column (Woburn, MA) packed with the C18 phase and eluted for 50 min with a gradient of 5% B to 90% B for 30 min (mobile phase A = 0.1% formic acid in water; mobile phase B = 0.1% formic acid in 95% acetonitrile). The top four ions in each survey scan were then subjected to automatic low-energy CID, and the resulting uninterpreted MS/MS spectra were searched against NCBInr or SwissProt protein databases using the Mascot 2.0 search algorithm (Matrix Science, London, U.K.). For positive identification, at least two peptides of the protein with a significant score ($P \leq 0.05$) were obtained. Each protein was identified in two independent analyses from different gels.

Determination of Protein Sequences. Significantly sequenced peptides by MS/MS were used as queries in BLASTs against the EST Citrus database of the Citrus Functional Genomics Project (CFGP) (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) (*32*). The retrieved protein sequences were used to estimate their theoretical values of p*I* and MW using the ExPASy (*33*) Web site tools (http://www.expasy.org/tools/).

Statistical Analysis. Protein identifications were obtained by MS/MS analyses of the same spot in two different gels that provided the same results. In general (except for Mn SOD), two peptides for each protein with significant MASCOT score (at $P \leq 0.05$) were required for positive identification. Quantitative measurements are indicated as average values and standard errors of at least three independent

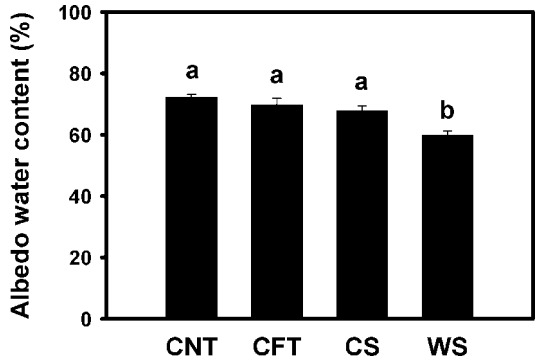

Figure 1. Albedo water content (%) in fruits of cv. Murcott exposed to the following storage conditions: (a) none, day 0, fruits on tree (control treatment, CNT), (b) 13 days storage at 99% RH and 25 °C (ageing treatment, fruits off tree, CFT), (c) 13 days storage at 99% RH and 4 °C (cold stress, CS), and (d) 13 days storage at 60% RH and 25 °C (water stress, WS). Error bars are the SE from four to six independent analyses. Different letters indicate significant differences according to the LSD test ($P \leq 0.05$).

determinations from biological replicates. Data were analyzed with ANOVA and the LSD test to discriminate significant differences between averages at $P \leq 0.05$. Linear correlations between parameters in **Table 3** were analyzed at three levels of significance ($P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$).

RESULTS AND DISCUSSION

The major goal of this work was to reveal protein changes in the albedo of the peel of mature citrus fruit either nondetached or detached from the tree. Detached fruits were stored in a nonstressful postharvest environment at high relative humidity (99%) and room temperature (25 °C) or exposed to cold (99% RH, 4 °C) or drought (60% RH, 25 °C) conditions. After 13 days of storage, only the albedo of fruits under low RH showed a significant reduction of water content. These values were 72% in albedo of fruits sampled at day 0 (control on tree), 70% in fruits stored at room temperature and 99% RH (control off tree, CFT), 68% in fruits stored at 4 °C and 99% RH (cold stress treatment, CS), and only 60% in fruits stored at room temperature and 60% RH (water stress treatment, WS) (**Figure 1**).

Criteria for protein selection were (i) unequivocal identification by MS/MS, (ii) presence in all three gels of each experiment, and (iii) reproducible and significant expression differences and/or high abundance. The results presented below were based on positive identification of single protein spots by MS/MS. Two different spectrometric methods (Q-Tof and LTQ analyzers) applied to spots from two different gels were used. Relative quantifications were statistically analyzed by ANOVA and LSD tests to determine significant differences ($P \leq 0.05$).

Identification of Proteins. Eight proteins were identified in all samples and treatments in the albedo of citrus fruits. Four proteins out of these eight showed reproducible and significant change in their abundance (estimated as gel spot intensity). Two of them apparently corresponded to the precursor and active form of the same cysteine proteinase, according to the MS/MS analysis and database search (**Table 1**; Supporting Information, Table 1). The difference in mass between both isoforms (approximately 7 kDa, **Table 1**) was certainly compatible with a posttranslational modification leading to protease activation as described in cysteine proteinases of the papain family (*34*). Besides these two cysteine proteinases [putative precursor (CPp) and active (CPa) forms], a translationally controlled tumor protein (TCTP) and an ascorbate peroxidase (APX) (Supporting Information, Figure 1; **Table 1**), showing also a significant change among storage conditions (**Table 2**), were identified. The other four identified proteins showed a difference among treatments lower than 2-fold. These proteins were actin (function category of cellular structure), ATP synthase β subunit (ATPase) (energy of cellular metabolism), and two proteins related to the response against oxidative stress, a manganese superoxide dismutase (Mn SOD) and a citrus salt-stress associated protein (CitSap) (Supporting Information, Figure 1; **Table 1**). Identifications were confirmed twice in independent samples. In general, two or more significant peptides were obtained for protein identification (Supporting Information, Table 1). Because the citrus genome is not yet sequenced, initial database searches retrieved homologues from other plant organisms, except for CitSap (Supporting Information, Table 1). Further searches by means of MASCOT with amino acid substitutions were then performed. Citrus sequences were obtained using the best scored peptidic sequences as queries in a BLAST search against the CFGP citrus EST database (*31*). The most significant hit was taken as the most likely sequence of the identified protein (Supporting Information, Table 2).

Protein Changes after Treatments. Soluble protein content in the albedo of fruits on tree was 164 mg/g DW and increased significantly to 199, 220, and 202 mg/g DW in CFT, CS, and WS treatments, respectively (**Figure 2**). Increase in total protein content was previously shown to occur also after nutritional (*35*) and cold stresses (*36*) in other experimental models.

Fruit Detachment. Stress induced by fruit detachment was likely related to starvation of substances, particularly photoassimilates, mineral nutrients, hormones, and water. Two proteins reduced at least 2-fold their content in the albedo after 2 weeks at 99 % RH and 25 °C: TCTP and APX (**Table 2**). TCTP has been postulated to be an antiapoptotic protein (*37*, *38*). In plants, it has been previously found in roots of Al-tolerant soybean cultivars during Al toxicity response (*39*), and although its role in plants is not completely understood, it is believed that TCTP performs essential functions in cells (*40*). TCTP appears to be a cytoplasmic calcium-binding protein, with a role in maintenance of Ca^{2+} homeostasis. In animals, TCTP is downregulated after Ca^{2+} stress (*41*) that, along with H_20_2, is a main PCD-inducing factor (*22*). In the experiments reported here, a strong reduction of TCTP content (0.5-fold in comparison with CNT; **Table 2**) in the albedo of detached fruits was observed, although evidence of a hypothetical relationship with calcium starvation was not obtained. However, TCTP has also been detected interacting with microtubules (*42*), an observation of particular relevance also to PCD since it has been demonstrated that tubulin is a direct target of antiapoptotic and proapoptotic polypeptides that apparently act through the assembly and disassembly of microtubules, respectively (*43*). Furthermore, there was a tough negative correlation ($P \leq 0.001$) between TCTP and β-actin protein levels (**Table 3**), another main component of the cellular cytoskeleton. Actually, this correlation might be anticipated in view of the fact that depolymerization of tubulin molecules is strongly linked to increases of β-actin gene expression (*44*). Taken together, the data suggest an antiapoptotic activity for TCTP in the albedo of citrus fruits likely related to microtubule stabilization. This function would be reduced after fruit detachment of the tree.

The other protein with altered levels in detached fruits was APX, a polypeptide reported to be downregulated or dramatically inhibited in programmed cell death activation (*45*, *46*). The balance between ROS detoxification enzymes such SODs,

Table 1. Protein Identification: Number of Peptides Significantly Matched for Each Protein[a]

spot no.	protein	no. of peptides significantly matched		p*I*		MW (kDa)	
		Q-Tof	LTQ	experimental	theoretical	experimental	theoretical
1	CPp	2	2	4.7	5.7	47.1	50.2
2	CPa		3, 1[b]	4.7		40.0	
3	APX	2	4	4.9	5.6	24.0	30.0
4	TCTP	3	4	4.7	4.5	26.0	19.0
5	Mn SOD	1		6.3	6.6	27.8	29.6
6	actin	4, 2[b]		5.7	5.3	43.0	41.7
7	ATPase	10		5.8	5.5	56.7	60.8
8	CitSap	2	5	6.2	9.0	24.0	29.3

[a] In general, each protein was analyzed twice in individual gels with two kinds of mass spectrometer techniques (Q-Tof and LTQ). Experimental and theoretical (ExPASy tools) measurements of protein p*I* and MW are shown. For peptide sequences and further details, see Supporting Information, Table 1. [b] Two independent analyses. Abbreviations: CPp, precursor cysteine proteinase; CPa, active cysteine proteinase; APX, cytosolic ascorbate peroxidase; TCTP, translationally controlled tumor protein; Mn SOD, manganese superoxide dismutase; ATPase, ATP synthase β subunit; CitSap, citrus salt-stress associated protein; p*I*, isoelectric point; MW, molecular weight; Q-Tof, quadrupole time of flight; LTQ, linear ion trap.

Table 2. Relative Abundance of Albedo Proteins in the Peel of Control Citrus Fruits on Tree (CNT) and after Storage for 13 Days at 99% RH and 25 °C (CFT), 99% RH and 4 °C (CS), or 60% RH and 25 °C (WS)[a]

protein	fold induction			
	CNT	CFT	CS	WS
CPp	1.0 b	0.9 b	1.5 a	1.0 b
CPa	1.0 b	1.1 b	2.9 a	1.7 b
CPtot	1.0 b	1.0 b	2.0 a	1.3 b
APX	1.0 a	0.5 b	0.9 ab	1.2 a
TCTP	1.0 a	0.5 b	0.6 ab	0.7 ab
Mn SOD	1.0 a	1.1 a	0.8 a	1.2 a
actin	1.0 a	1.5 a	1.3 a	1.2 a
ATPase	1.0 a	1.1 a	1.1 a	1.5 a
CitSap	1.0 a	0.9 a	0.7 a	1.0 a

[a] CNT values are normalized to 1 for proper comparisons. In each row, different letters indicate significant differences ($P \leq 0.05$, $n = 3$). Abbreviations: CPp, precursor cysteine proteinase; CPa, active cysteine proteinase; CPtot, CPp plus CPa; APX, cytosolic ascorbate peroxidase; TCTP, translationally controlled tumor protein; Mn SOD, manganese superoxide dismutase; ATPase, ATP synthase β subunit; CitSap, citrus salt-stress associated protein.

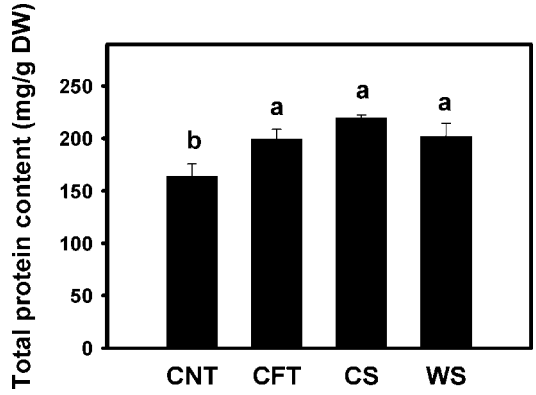

Figure 2. Total protein content in the albedo of fruits exposed to various storage conditions. See legend to Figure 1 for explanation of storage conditions. Soluble proteins were extracted from 100 mg of lyophilized albedo and quantified by BCA assay. Error bars are the SE from three independent analyses. Different letters indicate significant differences according to the LSD test ($P \leq 0.05$).

APX, catalase, or glutathione peroxidase is crucial for the steady-state level of superoxide ions and H_2O_2 (*47*). In the experiments reported in **Table 2**, levels of Mn SOD and CitSap were similar in all cases, although APX was strongly reduced. Since H_2O_2 is a powerful inductor of PCD in plant cells (*48*), the reduction of APX levels has been interpreted as a defensive response, allowing the operation of additional signals to trigger further mechanisms of cell defense (*45*). These data suggest that APX downregulation along with apoptosis may play an important role in the ageing processes associated with fruit detachment.

Drought and Cold Stresses. It has also been shown that APX is induced by drought (*49*, *50*) and cold (*51*) stresses and that a high content of APX enhanced tolerance to both (*51*, *52*). In our study, APX was highly responsive to drought stimuli (≥2-fold), a treatment that reversed the effects of CFT ageing raising APX protein levels to CNT values (**Table 2**). This observation suggests that highly stressing situations like those generated with WS may limit the regulatory role of APX in PCD.

We have also identified a cysteine proteinase (CP) of the papain family (CP1 family), as deduced from the sequence retrieved from the CFGP EST database (Supporting Information, Table 2). Protease activity can be regulated at different levels: through transcription/translation and posttranslational processing and also by specific protease inhibitor proteins (*53*). On the basis of mass determinations, the identified protein was present in two different forms, very likely the prepropeptide and the active form. This analysis suggested a posttranslational modification of the prepropeptide following a process previously described in the CPs of the papain family (*34*).

Cysteine proteinases are an extended group of enzymes with many cellular functions (*34*). In the flavedo of citrus fruit, for instance, a CP with a role in processing of proteins targeted to the vacuole was suggested (*54*). Cysteine proteinases of the papain family and protease inhibitors also play an essential role in the modulation of PCD triggered by oxidative stress, i.e., H_2O_2 in soybean cells (*55*). According to this report, inhibition of CPs resulted in the reversion of the PCD process. Several CP groups also show differential response to external stimuli as cold (*56*) or drought (*57*) stresses. In our study, the identified CP protein showed a significant increase in albedo of fruits exposed to 4 °C (2-fold as prepropeptide and total content and 3-fold as the active form; **Table 2**). However, drought did not induce a significant increase in protein expression, and their levels in CNT and CFT samples were pretty similar for both forms (**Table 2**). The cold effect was further confirmed when fruits conditioned to 4 °C were transferred for an additional 2 weeks to room temperature (**Table 4**). Both protein forms, CPp (precursor) and CPa (active), had similar high levels in fruits at 4 °C, 3 and 15 days after the beginning of the experiment. However, CP values dropped significantly in fruits that were exposed to room temperature. The amounts of the proteins were 116 (day 3) and 39 (day 15) for CPp and 172 (day 3) and 47

Table 3. Correlation Coefficients between Expression of Identified Proteins, Ratio CPa/CPtot, Total Protein Content (TP), and Water Content (% W) in the Albedo of Mature Citrus Fruits[a]

	CPa/CPtot	CPp	CPa	CPtot	TP	APX	ATPase	TCTP	SOD	actin	CitSap	% W
CPa/CPtot	1.00	0.78	0.93	0.89	0.95	0.15	0.35	−0.59	−0.32	0.52	−0.82	−0.54
CPp	ns	1.00	0.96	0.98	0.59	0.32	−0.08	−0.11	−0.73	0.03	−0.74	−0.11
CPa	*	*	1.00	0.99	0.79	0.25	0.10	−0.34	−0.59	0.27	−0.83	−0.30
CPtot	*	*	***	1.00	0.73	0.28	0.04	−0.25	−0.65	0.19	−0.81	−0.23
TP	*	ns	ns	ns	1.00	−0.13	0.30	−0.81	−0.23	0.77	−0.85	−0.48
APX	ns	ns	ns	ns	ns	1.00	0.58	0.62	0.21	−0.68	0.34	−0.56
ATPase	ns	ns	ns	ns	ns	ns	1.00	−0.10	0.73	0.06	0.22	−0.98
TCTP	ns	ns	ns	ns	ns	ns	ns	1.00	0.04	−0.99	0.70	0.22
Mn SOD	ns	ns	ns	ns	ns	ns	ns	ns	1.00	−0.03	0.69	−0.59
actin	ns	ns	ns	ns	ns	ns	ns	***	ns	1.00	−0.67	−0.17
CitSap	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	1.00	−0.02
% W	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	1.00

[a] Correlations were made with data from fruits on tree and from fruits stored under the following conditions: detachment, cold, and drought. Significances are shown at $P \leq 0.05$ (*), $P \leq 0.01$ (**), or $P \leq 0.001$ (***) levels. Abbreviations: CPa, active cysteine proteinase; CPp, precursor cysteine proteinase; CPtot, CPa plus CPp; TP, total protein content in albedo; APX, cytosolic ascorbate peroxidase; ATPase, ATP synthase β subunit; TCTP, translationally controlled tumor protein; Mn SOD, manganese superoxide dismutase; CitSap, citrus salt-stress associated protein; % W, albedo water content in weight percentage; ns = not significant.

Table 4. Effect of Cold Induction Storage on Expression of the Cysteine Proteinase Precursor and Active Proteins and Total Amount (OD Units) in the Albedo of Mature Citrus Fruits[a]

		day 3	day 15	significance
CPp	CS	179.4	167.6	ns
	RT	116.3	38.5	*
		ns	*	
CPa	CS	238.6	180.9	ns
	RT	172.3	47.2	*
		ns	*	
CPtot	CS	418.0	348.6	ns
	RT	288.6	85.6	*
		ns	*	

[a] Sets of 4 °C conditioned fruits (CS) were transferred to room temperature (RT) for 3 or 15 days and analyzed for protein expression. Values are the means of two to five repetitions. Statistical significances according to the LSD test are shown for time and treatment comparisons ($P \leq 0.05$). Abbreviations: CPp, precursor cysteine proteinase; CPa, active cysteine proteinase; CPtot, CPp plus CPa; * = significant difference at $P \leq 0.05$; ns = no significance.

(day 15) for CPa (**Table 4**). Therefore, a role for CP in the regulation and recycling of proteins during cold acclimation can be suggested on the basis of the increase of its activity, estimated as the rate of conversion of the prepropeptide into the active form (**Figure 3**). The activation of this proteinase might be a direct "per se" consequence of cold stress, or alternatively, it might be due to an indirect effect related to the increase of cold-induced protein synthesis. This last alternative appears to be supported by the significant correlation ($P \leq 0.05$) existing between total protein content and the rate of proteinase conversion into active form (**Table 3**). Moreover, recycling of damaged proteins seems to be coordinated with synthesis of new proteins (*34*).

Another identified protein, an ATPase, did not show a statistically significant variation between the different storage conditions at sampling times (**Table 2**) although their average values correlated significantly with albedo water content ($P \leq 0.01$) (**Table 3**).

In conclusion, the proteomic survey performed in this work comparing nondetached and detached citrus fruits indicates that the major changes in protein content in the albedo of the postharvest stored citrus fruits are related to the activation of programmed cell death (PCD). Fruit detachment repressed ascorbate peroxidase (APX) and translationally controlled tumor protein (TCTP) whereas ageing-induced APX repression was reversed by drought. Cold storage induced expression of a cysteine proteinase.

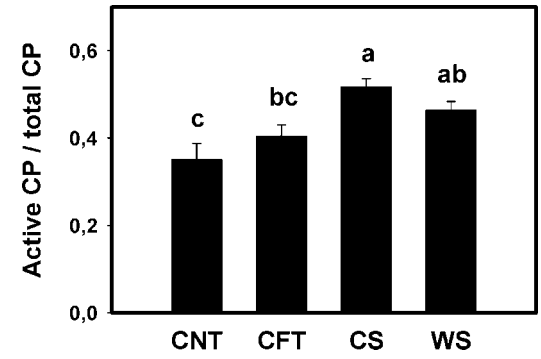

Figure 3. Conversion rate of the cysteine proteinase precursor in its putative active form in the albedo of fruits exposed to various storage conditions. Total protein is the sum of the quantifications of precursor and active spots on each 2D gel. Values are averages of the ratio between the amounts of active form and total protein. Error bars are the SE from three independent determinations. Different letters indicate significant differences according to the LSD test ($P \leq 0.05$). See legend to Figure 1 for explanation of storage conditions.

ABBREVIATIONS USED

CFGP, Citrus Functional Genomics Project; CNT, control on tree; CFT, control off tree; CS, cold stress; WS, water stress; CP, cysteine proteinase; APX, ascorbate peroxidase; TCTP, translationally controlled tumor protein; Mn SOD, manganese superoxide dismutase; CitSap, citrus salt-stress associated protein; ATPase, ATP synthase subunit β; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Supporting Information Available: Table 1 listing peptides matched for protein identification by ESI-MS/MS, Table 2 listing ORF protein sequences retrieved from the CFGP EST database, and Figure 1 showing annotated nonsaturated images of representative Sypro Ruby-stained two-dimensional gels for all storage conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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