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Anthocyanins Accumulation and Related Gene Expression in Red Orange Fruit Induced by Low Temperature Storage

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The aim of this work was to study the impact of moderately long storage periods at 4 °C upon red orange [*Citrus sinensis* (L.) Osbeck] anthocyanins production and the expression of structural genes involved in their biosynthesis such as phehylalanine ammonia lyase (PAL), chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR), and UDP-glucose flavonoid glucosyl transferase (UFGT). Our results showed that low temperature-induced anthocyanins accumulation in red orange juice vesicles after 75 days reached values eight times higher than those kept at 25 °C. Furthermore, real-time polymerase chain reaction showed that expression of PAL, CHS, DFR, and UFGT was strongly induced during low temperature exposure since levels of all transcripts increased at least 40-fold with respect to control samples. Interestingly, in orange fruits subjected to a brief exposure at low temperature (45 days) and subsequently kept at 25 °C, the anthocyanins content dropped although samples still maintained higher levels of these pigments than those registered in control oranges. Concordantly, the expression of *chs*, *dfr*, and *ufgt* declined upon return to control conditions, but it was always much higher in samples subjected to brief cold induction than in the control samples. On the contrary, the amount of PAL transcripts became negligible immediately after the temperature change from 4 to 25 °C, thus indicating that "early" and "late" genes, respectively, implicated in the first and in the last steps leading to the anthocyanins, might be affected by different regulation mechanisms.

KEYWORDS: Anthocyanin; red orange; *Citrus sinensis*; juice vesicles pigmentation; cold temperature storage; RT-real time PCR

INTRODUCTION

Anthocyanins are water soluble pigments belonging to the flavonoids compound family involved in nature in a wide range of functions such as flowers, fruits, and seeds pigmentation to attract pollinators, to disperse seeds, to protect against UV light damage, and in plant defense to protect against pathogen attack (*1*, *2*). Because anthocyanins impart much of the color and flavor of fruits and vegetables, they are usually components of the human diet and are not only considered exclusively as food products but also as therapeutic agents; in fact, anthocyanins have been suggested to protect against oxidative stress, coronary heart diseases, certain cancers, and other age-related diseases (*3*). At least part of these presumed health-promoting features can be attributed to the antioxidant properties of these compounds whose chemical structure appears ideal for free radical scavenging. Nowadays, the anthocyanin's biosynthesis pathway has been almost completely elucidated and most of the structural genes encoding the enzymes responsible for each step have been isolated from different sources (*4*). The general phenylpropanoid pathway is initiated by phenylalanine ammonia lyase (PAL), which catalyzes the elimination of ammonia from L-phenylalanine to form *trans*-cinnammate; then, the metabolic pathway branches to give rise to a thousand compounds, many of which are specific to particular plant species (*5*). The first committed step for anthocyanins biosynthesis is catalyzed by chalcone synthase (CHS), which condenses malonyl-CoA and 4-coumaroyl-CoA to form tetrahydroxy chalcone. This last compound is isomerized to flavanone naringenin, which is subsequently converted to the dihydroflavonol dihydrokaemferol by hydroxylation. The other two dihydroflavonols, dihydroquercetin and dihydromyricetin, are formed from dihydrokaemferol by further hydroxylation reactions. Then, anthocyanin 3-O-glycosides are synthesized from dihydroflavonols by the consecutive reactions catalyzed by dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose flavonoid glucosyl transferase (UFGT) (*5*). The activity of the anthocyanins biosynthetic genes is largely regulated at the transcriptional level, and

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consequently, the pigmentation pattern must be specified by the expression patterns of the regulatory genes (*4*, *6*). Moreover, several environmental stimuli such as light, osmotic, and cold temperature stresses can activate the transcription of anthocyanins biosynthetic genes (*7*). The cold induction of pigmentation has been studied in flower development and related to the activation of the expression of anthocyanin biosynthetic genes, including PAL, CHS, DFR, and ANS (*8*). As regards tree fruits, studies on low temperature-induced anthocyanin accumulation have only been carried out on apple (*9*) and grape skins (*10*). It has been shown that postharvest storage temperature can affect anthocyanin levels in small fruits, such as strawberries (*11*), cranberries (*11*), and several blueberry cultivars (*12*); in this last case, the authors showed that the anthocyanins contents of fully ripe blueberry fruits (100% degree of maturity) belonging to most of the cultivars tested did not change significantly once they were subjected to a postharvest low temperature exposure for periods ranging between 3 and 7 weeks (*12*); they also found that, depending on cultivar type, pigments accumulation might occur but only in fruits with other degrees of maturity (ranging between 50 and 75%) (*12*). However, the molecular basis of anthocyanins accumulation due to low temperature exposure concerning the fruits edible portion has not yet been established. Several cultivars of red orange [*Citrus sinensis* (L.) Osbeck], such as Tarocco, Moro, and Sanguinello, are characterized by the presence of anthocyanins in both the rind and the juice vesicles (*13*). It has been shown that in the postharvest phase, oranges fruits can be stored at low temperatures for different long periods (*14*). The storage temperature as well as the duration turned out to be critical for orange fruits as prolonged storage, over 3 months, at 4 °C negatively influences the sensory quality of blood orange juices due to the increase of the malodorous substance vinylphenol (*15*, *16*). Therefore, because the anthocyanins content of blood oranges represents a very high quality marker, it is desirable to find a convergence point, for temperature and storage duration, to obtain the maximum of anthocyanins accumulation and a high fruit's acceptability by consumers, trying also to limit the cost of too prolonged thermocontrolled storage. In this work, we studied the impact of a low temperature exposure (4 °C) during a moderately long storage period (75 days) on orange [*C. sinensis* (L.) Osbeck], cv. Tarocco, anthocyanins production and on the expression of structural genes involved in their biosynthesis such as *chs*, *dfr*, and *ufgt* whose partial cDNA clones have been previously isolated in our laboratory (*17*). Moreover, considering the crucial role of PAL in the phenylpropanoid biosynthesis as an initial enzyme of the pathway, we cloned a *pal* partial cDNA clone from Tarocco orange flesh and monitored its expression during the experimental period along with the previously mentioned anthocyanins biosynthetic enzymes. To test the effects of a brief cold exposure, the anthocyanin contents and gene expression were also assayed in orange samples in which the cold treatment was extended only for 45 days and then control storage conditions (25 °C) were resumed.

MATERIALS AND METHODS

Plant Material and Storage Conditions. Pigmented oranges (Tarocco) [*C. sinensis* (L.) Osbeck] were harvested in January 2004 from approximately 15 year old trees grown at a private farm in the territory of Francofonte (Italy). Freshly harvested oranges were washed with distilled water, gently dried with paper towels, and then left to dry at room temperature for 3 h. Subsequently, orange fruits were randomly placed in two boxes (150 fruits per box), one of them stored in a ventilated cold room at 4 °C and 90−95% relative humidity (RH) ("cold-stored" samples). The remaining box was placed in a temperature-controlled device kept at 25 °C and 90−95% RH ("control" samples). After 45 days, 50 fruits from the cold-stored samples were transferred to the temperature-controlled chamber and kept at 25 °C for a further 30 days, representing the "ex-cold-stored" samples. Samplings were carried out before storage (time 0) and every 15 days for a total storage period of 75 days. During each sampling, 18 fruits per box were collected and divided into three subgroups of six fruits each. The orange fruits of each subgroup were then peeled, chopped, and mixed to constitute three independent mean samples in triplicate; orange fleshes were then immediately frozen with liquid nitrogen and stored at −80 °C until used.

Extraction of Total RNA and cDNA Synthesis. The total RNA from orange fruit juice vesicles was extracted using TriZol (Invitrogen, Carlsbad, CA) as an extracting solution. RNAs were routinely quantified using RNA Quant-it assay kit (Molecular Probes, Carlsbad, CA). Reverse transcription was achieved by using 2 μg of total RNA as the starting material and the Superscript RNAse H^- First-strand synthesis kit (Invitrogen).

PCR Amplification of PAL Partial Clone from Tarocco Orange Juice Vesicles. The orange [*C. sinensis* (L.), Osbeck] PAL partial cDNA clone was isolated by polymerase chain reaction (PCR) amplification using for/rev primers designed on the basis of the *Citrus clementina* × *Citrus reticulate* PAL cDNA sequence (AJ238754): for-PAL, 5′-CGGTTTGCAACCAAGTCTATTG-3′; rev-PAL, 5′-GCAGGGGT-CATCAATGTATGC-3′. The PCR reaction was carried out using the following conditions: 94 °C × 2 min, (1 cycle); 94 °C × 1 min, 55 °C × 1 min, 72 °C per 1 min (25 cycles); 72 °C per 20 min (1 cycle). The PCR experiment produced an amplification product of 605 bp, whose identity was confirmed by sequencing.

PCR Products Analysis, Cloning, and Sequencing. PCR products were separated by 1.1% agarose gel containing 0.5 μg/mL ethidium bromide. The Qiaquick PCR purification kit (Qiagen, Valencia, CA) was used to extract PCR fragments from the PCR amplification reaction mixture. The amplified fragment was cloned into pCR 4- TOPO vector (Invitrogen) and sequenced by the fluorescence detection method using both T7 and T3 as primers (MWG-Biotech, Ebersberg, Germany).

The nucleotide sequences reported in this paper were submitted to Genbank under accession numbers: DQ088064 [PAL, partial coding sequence (cds)], AB009351 (CHS, complete cds), AY519363 (DFR, complete cds), AY519364 (UFGT, partial cds), and AY498567 (EF-1α partial cds).

Measurement of *pal*, *chs*, *dfr*, and *ufgt* Expression by Real-Time Quantitative RT-PCR. Real-time PCR, Taqman assays, were performed using the SuperScript III Platinum two-step qRT-PCR kit (Invitrogen) in a Smart Cycler II (Cepheid, Sunnyvale, CA). Primers and probes, whose sequences are shown in **Table 1**, were designed using the MWG-Biotech "PCR Primer Design Service" and there obtained. The relative quantitation of genes expression between orange samples was calculated using the comparative threshold (C_T) method (*18*). Three independent triplicates of quantitative PCR experiments were performed for each gene to generate an average C_T and to calculate standard deviation. The housekeeping gene elongation factor (EF-1α), which was reported to be constitutively expressed (*19*), was used as an endogenous reference, and ΔC_T was calculated by subtracting the average EF-1α C_T from the average C_T of the gene of interest. This value was calculated for each sample, and then, the comparative expression level of the single genes was given by the formula $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ was calculated by subtracting the baseline's ΔC_T to the sample's ΔC_T and where the baseline represents expression level at the time 0. The dynamic range of PAL, CHS, DFR, UFGT, and EF-1α was determined by monitoring the variation of ΔC_T with template dilution; efficiency was very similar for each primer/probe systems. For each triplicate, 5 ng of cDNA was added to a final volume of 25 μL with a final concentration of 1× Platinum two-step qRT-PCR master mix, 100 nM each primers, and 100 nM probe. The cycling program was as follows: 50 °C × 2 min (1 cycle), 94 °C × 2 min (1 cycle), 94 °C × 15 s, 55 °C × 30 s, and 72 °C × 30 s (45 cycles). Negative controls without reverse transcriptase were routinely included. Experiments were repeated at least three times on independently isolated RNA preparation.

Table 1. Primers and Taqman Probes Sequences

	primers	Taqman probes
PAL	forward 5′-CAGCACAACCAAGATGTAAAC-3′ reverse 5′-CAAATCAATAGCCTGGCAAAG-3′	5′-FAM-CCTGAAGCTCATGTCTTCCACATTCTTGGT-3′-TAMRA
CHS	forward 5′-TCTATCGACGGGCATCTTC-3′ reverse 5′-TGCCTCGGTTAGGCTTTTC-3′	5′-FAM-CCTTACCTTTCACCTCCTCAAGGATGTTCC-3′-TAMRA
DFR	forward 5′-GCTGTTCGTGCTACTGTTC-3′ reverse 5′-GTTTCCCTCTTCGGCTAAATC-3′	5′-FAM-AAGGCAAGCACTCACCTGACTTTATGGAAA-3′-TAMRA
UFGT	forward 5′-TCTTCAGCACTCCGCAATC-3′ reverse 5′-TCCATCGGATACGTCGTAAG-3′	5′-FAM-CCAACAGCGTCATCTTCCCAGCAATGT-3′-TAMRA
EF	forward 5′-CACCACCCCCAAGTACTC-3′ reverse 5′-GTTGTCACCCTCGAAACC-3′	5′-FAM-AAGGTTGGATACAACCCCGAGAAAGTCCCA-3′-TAMRA

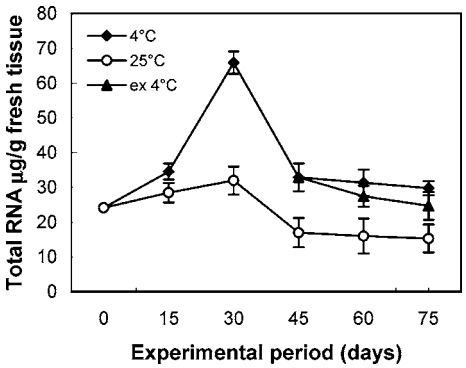

Figure 1. Total RNA content in orange juice vesicles subjected to different storage temperatures. Total RNA was extracted from each sample and quantified by fluorometer assay using the RNA Quant.it kit (Molecular Probes) as detailed in the Materials and Methods. Each point represents the mean value of three replications ± SE (or SD). Each replication was composed of six fruits.

Total Anthocyanins Content. Anthocyanins determination was performed by pH differential spectrophotometry according to a slight modification of the method described in Rapisarda et al. (*20*). Briefly, aliquots (1 g) of orange flesh were frozen in liquid nitrogen, powdered by mortar and pestle, and successively extracted with 1 mL of water by vigorous shaking for 1 h at 4 °C. Samples were centrifuged at 12000*g* × 20 min, and then, the surnatant was recovered and analyzed for anthocyanins content by dissolving aliquots of fresh orange flesh extract, respectively, in buffer 1 (55 mM KCl, 0.145 N HCl, pH 1.0) and buffer 2 (0.2 M CH_3COONa, pH 4.5) and measuring the absorbance of samples at both 510 and 700 nm. The anthocyanins content was calculated by the following formula: $p/p = (\Delta Abs/\epsilon \times L) \times MW \times DF \times (V/W_t) \times 100\%$, where $\Delta Abs = [Abs_{510nm}\ (pH\ 1.0) - Abs_{700nm}\ (pH\ 1.0)] - [Abs_{510nm}\ (pH\ 4.5) - Abs_{700nm}\ (pH\ 4.5)]$; ϵ = cyanidin-3-O-glucoside molar absorbance coefficient (26900); MW = cyanidin-3-O-glucoside molecular weight (449.2); DF = dilution factor; V = final volume (mL); W_t = sample weight (mg); and L = cell path length (usually 1 cm).

RESULTS

Effect of Low Temperature Storage on Total RNA Content. Total RNA levels were analyzed in orange fruits exposed to different storage temperatures. As described in the Materials and Methods, orange fruits were subjected to a postharvest storage at two different temperatures (4 °C, cold-stored samples, and 25 °C, control samples) for 45 days. Subsequently, a subgroup of cold-stored oranges was transferred to 25 °C (ex-cold-stored samples) and monitored for a further 30 days along with cold-stored and control samples. As described in **Figure 1**, the total RNA content in cold-stored samples increased during the first phase of storage reaching values three times higher with respect to the starting amount. In contrast, oranges kept at 25 °C showed only a slight increase of RNA. After 30 days, the RNA levels decreased in the control as well as in the cold-stored samples, but in the latter case, the amount of total RNA was always higher than in the control fruits. Moreover, the total RNA content in the ex-cold-stored samples kept at 25 °C during the last 30 days of the experimental period exhibited values slightly lower than in the cold-stored fruits, never reaching, however, the low levels registered for the control samples.

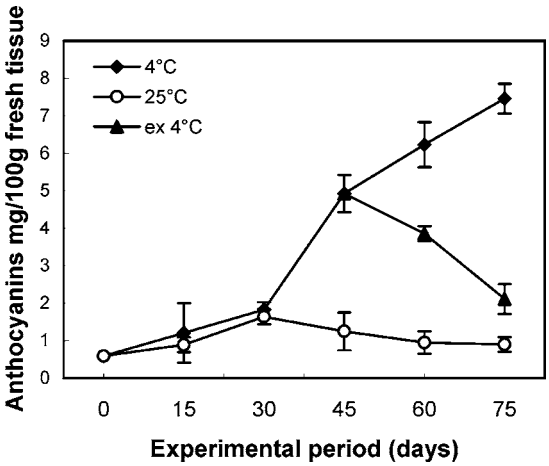

Figure 2. Time–course accumulation of anthocyanins in orange juice vesicles during low temperature exposure. Orange flesh anthocyanins were quantified from tissue extracts as described in the Materials and Methods. Each point represents the mean value of three replications ± SE (or SD). Each replication was composed of six fruits.

Anthocyanins Content in Cold-Treated Oranges. The effect of temperature storage upon the anthocyanins content of orange fruits has been described in **Figure 2**. The amount of anthocyanins in control samples increased slightly during the first 30 days of storage and then decreased showing values close to the time zero samples. On the contrary, despite the overlapping patterns showed by both the cold-stored and the control samples during the first 30 days of storage, the anthocyanins content of the low temperature exposed fruits dramatically increased throughout the remaining storage period (**Figure 2**). Pigment levels, in fact, rose from an initial value of 0.59 mg/100 g (time 0) to 7.46 mg/100 g (after 75 days storage). For the ex-cold-stored samples, we observed, during the last 30 days storage at 25 °C, a decay of the anthocyanins content even though, at the last sampling, they maintained about the 28% pigment level with respect to the cold-stored samples.

Sequence Analysis of *pal* cDNA Clones and the Encoded Proteins. Partial cDNA clones putatively coding for PAL were isolated from the Tarocco orange juice vesicles [*C. sinensis* (L.) Osbeck] by RT-PCR. The PCR experiment generated an amplification product whose length was close to the expected

value for the partial sequences (PAL, ca. 605 bp). The partial nucleotide sequences showed 98.0% identity with the available *C. clementina* × *C. reticulata* PAL cDNA (data not shown).

Expression Pattern of the Anthocyanins Biosynthesis Related Genes in Orange Fruits: *pal, chs, dfr,* and *ufgt*. The expression profile of *pal*, *chs*, *dfr*, and *ufgt* was investigated in orange fruits exposed to different storage temperatures using the real time RT-PCR. Results are illustrated in **Figure 3**, which reports the relative transcript levels of considered genes standardized to the constitutive EF-1α gene expression level and normalized to the time 0 $2^{-\Delta\Delta C_T}$ (see the Materials and Methods for details). As shown in **Figure 3A**, *pal* was expressed rather constantly along the whole experimental period during the 25 °C storage. Conversely, after the first 15 days of low temperature exposure, the levels of *pal*'s transcripts started to sharply increase reaching the maximum values (54-fold with respect to time 0) after 75 days of storage. It is worthwhile to emphasize that *pal* mRNA rapidly decreased to basal levels in correspondence to the storage temperature change from 4 to 25 °C (**Figure 3A**). The transcripts of *chs*, *dfr*, and *ufgt* were also expressed at almost constant levels at 25 °C; similarly, in the cold-treated samples, they all enhanced up until the end of the experimental period reaching at that stage expression levels ranging between 35 and 40 times higher than the time 0 samples (**Figure 3B**−**D**). Surprisingly, mRNA of *chs*, *dfr*, and *ufgt* was also detected in the ex-cold-stored sample even after the storage temperature switched to 25 °C. In fact, prior to the last sampling, we observed that all of the investigated genes were still highly expressed with *ufgt*, as the most abundant followed by *dfr* and *chs*, respectively (**Figure 3B**−**D**, 60 days of storage), whereas at the end of the storage period, *chs*, *dfr*, and *ufgt* expression was substantially similar ranging between 8- and 12-fold higher than the time 0 samples (**Figure 3B**−**D**, 75 days of storage). It has to be underlined that the EF-1α partial cDNA clone was amplified in all studied samples with any significant difference in the C_T values between the control and the cold-exposed samples thus demonstrating that the strong increase of genes expression observed in the cold-stored orange fruits did not reflect a general enhancement in RNA production due to low temperature, as shown in **Figure 1**. Moreover, a negative control represented by RNA samples in which reverse transcriptase was omitted always showed a negative C_T.

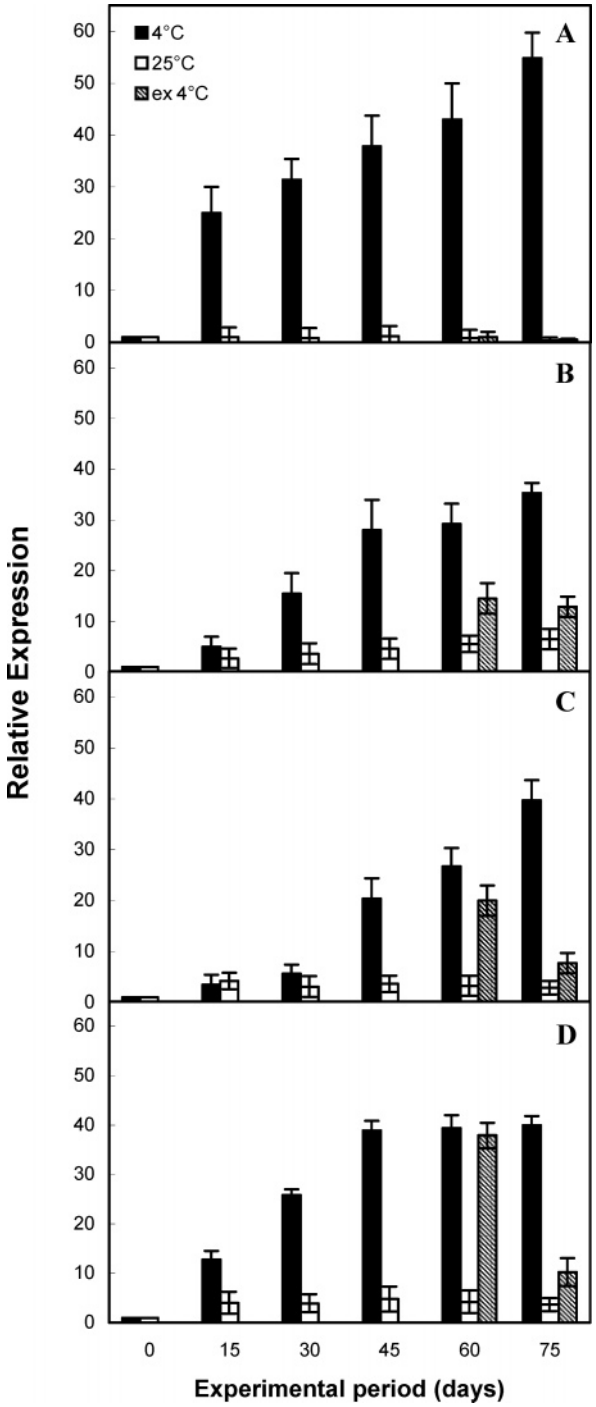

Figure 3. Expression pattern of anthocyanins biosynthetic genes in orange juice vesicles during low temperature exposure. (**A**) PAL, (**B**) CHS, (**C**) DFR, and (**D**) UFGT. The relative quantitation of genes expression between samples oranges was calculated by real time RT-PCR using the comparative threshold (C_T) method, as described in the Materials and Methods. The housekeeping gene EF-1α was used as an endogenous reference, and ΔC_T was calculated by subtracting the average EF-1α C_T from the average C_T of the gene of interest. The comparative expression level of the single genes was given by the formula $2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T$ was calculated by subtracting the baseline's ΔC_T to the sample's ΔC_T where the baseline represents expression level at the time 0. Each point represents the mean value of three replications ± SE (or SD). Each replication was composed of six fruits.

DISCUSSION

The anthocyanins of fruits and vegetables have been considered as therapeutic agents due to their beneficial health effects because of their supposed protection against certain cancers, cardiovascular diseases, and aging (*3*). Moreover, they have also been deemed a source of colors for food products (*21*) in alternative to synthetic dyes whose harmful effects upon human health have often been assumed and, in some cases, demonstrated. The major anthocyanins of blood oranges are cyanidin-3-O-glucoside and cyanidin 3-glucoside-6″-malonate present in almost equal proportions and representing about 85% of total pigment contents (*22*). Because of its very negative redox potential ($E_{1/2}$ = −405 mV) (*23*), cyanidin-3-O-glucoside participates along with ascorbic acid and hydroxycinnamates in conferring to red orange fruits distinctive antioxidant properties (*24*). The storage of oranges at low temperature is necessary to extend their commercial life, although prolonged storage to low temperatures (8 °C) has been shown to induce increases in free *p*-coumaric and ferulic acids whose high levels might represent the first step to the formation of undesirable vinylguaicol and vinyphenol (*16*). Flavor alteration of blood oranges juices due to the increase in vinylphenol concentrations has been demonstrated during a long storage at 4 °C (*15*). In fact, freshly squeezed blood orange juice did not contain vinylphenols and

they also were not detected within the first 2 months. Conversely, they were present in traces at 3 months and in significant amounts at 4 months of storage (*15*). As regards anthocyanins, it has been shown that the pigment contents of blood orange juice rose during a prolonged storage (86 days) at 8 °C (+500%); this is a positive parameter of fruit quality (*16*). Consequently, the aim of this work was to study the impact of a moderately long storage period at 4 °C upon orange anthocyanins production and on the expression of structural genes involved in their biosynthesis such as PAL, CHS, DFR, and UFGT, which are located at key points of the phenylpropanoid biosynthesis pathway: at the beginning of the general phenylpropanoid biosynthetic pathway (PAL), at the first reaction specifically leading to anthocyanins (CHS), at the last branch point leading to both colored anthocyanins and colorless flavonols (catechins and proanthocyanidins) (DFR), and, finally, at the last reaction of anthocyanidin glicosylation (UFGT). The above-mentioned parameters have also been monitored in orange samples in which cold treatment was extended only for 45 days and then they were subsequently placed at 25 °C for a further 30 days. Our results showed that low temperature-induced anthocyanin accumulation in orange juice vesicles reached, after 75 days, values eight times higher as compared with those kept at 25 °C (**Figure 2**). In this respect, the induction was much higher (+1240%) than that previously obtained by storing oranges fruits at 8 °C for 86 days (*16*). Moreover, the maximum of anthocyanins accumulation has been gained after 75 days of storage during which it has been shown that vinylphenols had not yet formed (*15*). Interestingly, orange fruits subjected to a brief low temperature exposure (45 days) still maintained higher levels of anthocyanins than those registered in control samples, thus suggesting that 45 days of exposure was sufficient to get at least a 2-fold increase even after a subsequent 30 day storage period at 25 °C. Besides, we also monitored the general "wellness" of orange fruits by determining the pattern of total RNA content (**Figure 1**). Our finding showed that at 4 °C the amount of total RNA was higher than in fruits stored at 25 °C, thus suggesting that low temperature storage, at least in the first 30 days of storage, likely preserves processes linked to RNA biosynthesis, such as transcription, and related to RNA function such as synthesis of proteins. RT-PCR showed that expression of PAL, CHS, DFR, and UFGT was strongly induced during low temperature exposure since levels of all transcripts increased at least 40-fold with respect to control samples; the PAL was the most induced transcript (54-fold). With regard to citrus fruits, induction of PAL activity was found to be related to peel damage induced by γ-irradiation in *C. clementina* (*25*) and to the healing process occurring after mechanical wounding in Valencia oranges (*26*); more recently, PAL mRNA accumulation was correlated with chilling resistance during Hernandina mandarin low temperature storage (*27*). Because all of the above-mentioned results have been obtained in nonproducing anthocyanins fruits, we assume that PAL accumulation might reflect a general demand for the whole phenylpropanoid pathway dedicated to the synthesis of protective compounds during abiotic stress exposure in Citrus genus, as supposed also by Sanchez-Ballesta et al. (*27*). This hypothesis is supported by our data indicating that *pal* mRNA, among the genes tested, profoundly increased during cold storage in order to provide with substrates all of the enzymes located downstream in the pathway. Moreover, concordantly with the anthocyanins levels, we found that expression of CHS, DFR, and UFGT was always much higher in samples subjected to brief cold induction than in the control samples (**Figure 3B−D**, IV and V samplings). Only PAL transcripts became nearly undetected upon return to control conditions probably due to both a rapid kinetic of degradation and a strong inhibition of the mRNA biosynthesis process. The reasons for the above-mentioned distinct behavior of various transcripts are presently unknown although it indicates that "early" and "late" genes involved in anthocyanins biosynthesis may undergo different regulation mechanisms. However, the rapid removal of the PAL transcripts upon a temperature change from 4 to 25 °C while CHS, DFR, and UFGT mRNA are still fully detected may also be differently explained. PAL may represent the key enzyme for general control of stress-induced plant response and therefore strictly controlled by environmental conditions. In this respect, the absence of PAL mRNA might represent a cellular signal indicating the end of stress imposition to which cells respond preventing the formation of others phenylpropanoid compounds. This progressive change in intermediate metabolites could sequentially modulate the expression levels of specific genes downstream in the pathway such as CHS, DFR, and UFGT (**Figure 3**), as well as their activity. In conclusion, our study suggests that genes of the anthocyanin biosynthesis pathway in oranges can be considered *cor* (cold-regulated) genes, and because this pathway is well-defined, it is an excellent subject for characterizing plant molecular responses to low temperature. Moreover, as far as we know, this is the first report in which the accumulation of anthocyanins due to cold exposure is related to the expression of their key biosynthetic enzymes in the edible portion of fruits.

ABBREVIATIONS USED

Cds, coding sequence; FAM, 6-carboxyfluorescein; TAMRA, tetramethylrhodamin; PAL, phehylalanine ammonia lyase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; UFGT, UDP-glucose flavonoid glucosyl transferase; EF, elongation factor.

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