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Short Cold Storage Enhances the Anthocyanin Contents and Level of Transcripts Related to Their Biosynthesis in Blood Oranges

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ABSTRACT: The health benefits associated with the consumption of anthocyanin-containing foods are extensively documented. Mature fruits of blood oranges and their hybrids are characterized by the presence of these bioactive pigments, the abundance of which can be enhanced by storing fruit at cooling nonfreezing temperature. In this work the effects of short low-temperature exposure (4 °C × 15 days) upon orange anthocyanin content and the expression of structural genes belonging to the pigment biosynthesis pathway were investigated. The results highlight that anthocyanin levels of fruit exposed to cold sharply increase, reaching, after 6 days of storage, a value 8 times higher than that observed in the time zero samples, thus suggesting that fruit with enhanced health-related attributes might be obtained at this storage stage. The analysis of gene expression shows that the amount of transcripts of all considered genes (CM1, PAL, CHS, DFR, ANS, UFGT, and GST) sharply increased after 3−6 days of cold storage, confirming previous data showing that the biosynthesis of anthocyanins is a cold-regulated pathway. By comparing the expression of selected genes (PAL, DFR, and UFGT) between blood and common oranges, it turns out that those genes strictly involved in anthocyanin biosynthesis are not cold responsive in common oranges. Moreover, the data highlight that the EST encoding the transcription factor NAC domain protein is selectively induced by cold in blood oranges but not in common oranges, thus proposing it as a candidate gene specifically involved in blood orange response to cold exposure.

KEYWORDS: *Citrus sinensis, blood orange, anthocyanin, cold storage, NAC transcription factor*

INTRODUCTION

Sweet orange is an economically important crop grown in tropical and subtropical areas of the world. In particular, Italy produces around 4% of the world orange crop and 37% of that of the European member states. In the marketing year 2009−2010, Italian citrus fruit production reached 3.8 million MT, a large part consisting of the "blood" varieties, planted mainly in Sicily and used almost entirely for fresh consumption.[1] Blood oranges [(*Citrus sinensis*) L. Osbeck] such as Tarocco, Moro, and Sanguinello are characterized by the presence, in the rind but mainly in the edible fruit portion, of anthocyanins,[2] representing the most abundant flavonoid constituents of fruits and vegetables. Among the flavonoids, anthocyanins have been intensively studied with special regard to their biofunctional properties, which may be helpful in the prevention of certain degenerative diseases including cancer, aging, neurological diseases, inflammation, and diabetes as well as bacterial infections.[3−5] At least part of these presumed beneficial effects can be driven by the antioxidant properties of these food bioactives having chemical structures that appear to be ideal for free radical scavenging.[6,7] Nowadays, the anthocyanin's biosynthesis pathway has been completely elucidated and the structural genes encoding the enzymes responsible for each step have been isolated from different *Citrus* species.[8−10] Most interestingly, the pigment content of freshly harvested fruits may significantly increase throughout cold storage due to ongoing biosynthesis of anthocyanins.[11,12] Therefore, the enhancement of anthocyanin levels due to cold postharvest storage might be exploited to improve the healthy attributes of oranges during some of the standard presale procedures that normally require 10−15 days of refrigerated preservation. In this respect, cooling to nonfreezing temperature is by far the most important practice in achieving shelf-life extension of fresh fruits to at least 12 days.[13] Moreover, oranges have suitable morphological (easily divisible in segments) and physiological (nonclimacteric fruit) characteristics for the preparation of ready-to-eat products, the consumption of which is widely increasing as a consequence of their convenience and freshness. Refrigerated storage is mandatory for this kind of minimally processed product.[14] In addition, cold quarantine treatments, which involve the exposure of fruit to low temperature for a period of 10−16 days, represent the accepted procedure for Medfly disinfestations of citrus fruits required by the regulatory agencies of most importing countries.[15] With regard to the length of the cold storage period, a convergence point, for temperature and storage duration, was previously found to obtain the maximum of anthocyanin accumulation and still high fruit acceptability by consumers (4 °C for 77 days).[11] At those storage conditions, as part of an effort to identify target genes for engineering tolerance to low temperature, a collection of ESTs specifically induced by cold was generated and analyzed by suppressive subtractive hybridization.[12] Interestingly, an EST encoding the chloroplastic isoform of chorismate mutase (CM1) involved in phenylalanine synthesis was retrieved in the subtracted library along with genes involved both in the early step of flavonoid biosynthesis, such as phenylalanine ammonia-lyase (PAL), and in the successive reactions leading to anthocyanins (chalcone synthase (CHS) and anthocyanidin synthase (ANS)).[12]

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Among the regulatory proteins operating in the signal transduction pathways, transcription factors belonging to the plant-specific NAC family were also found in the subtractive library (clone 378, FK826627).[12] The NAC transcription factors are multifunctional proteins with various roles in the plant life cycle as well as in plant stress response.[16] Stress inducible NAC transcription factors mediate transcriptional regulation through a sequence-specific DNA binding site, which contains the CATGTG motif within the promoter region of target genes.[17] Although a portion of the NAC genes has been largely characterized in model plants such as *Arabidopsis* and rice,[16,18] knowledge of the *Citrus* NAC proteins is less exhaustive except that two NAC genes have been characterized in common orange pulp.[19,20] More recently, analysis of the *Citrus* ESTs database (*Citrus* HarvEST, http://harvest.ucr.edu/) led to the identification of 45 citrus NAC genes, most of them induced by abiotic stress such as wounding, cold, and drought.[21] Interestingly, the EST encoding a NAC-like transcription factor specifically induced in the cold-exposed blood oranges (clone 378, accession number FK826627) has never been associated with cold stress response in *Citrus*; these data suggest that it might perform specific roles during cold stress signaling in blood oranges.[12] In this work, in the attempt to both limit the cost of prolonged thermocontrolled storage and respond to the increasing demand of cold shortly stored fruits still characterized by high-quality attributes, we studied the impact of low-temperature exposure during a short storage period (4 °C × 15 days) upon orange [*C. sinensis* (L.) Osbeck Tarocco Sciara] anthocyanin content and on the expression of structural genes belonging to the pigment biosynthesis pathway. Moreover, we measured the expression level of the aforementioned NAC-like encoding EST, in both blood and common oranges, to assess its possible involvement in the response to cold exposure.

MATERIALS AND METHODS

Plant Material and Storage Conditions. Blood [(*C. sinensis*) L. Osbeck Tarocco Sciara] and common [(*C. sinensis*) L. Osbeck Navel] oranges were harvested in January 2010 from an approximately 15-year-old tree grown at the orchard of the Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee in the territory of Palazzelli (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, the oranges were randomly placed in two boxes (75 fruits per box), one of them stored in a ventilated cold room at 4 °C and 90−95% relative humidity (RH) ("cold stored" samples) in darkness. The remaining box was placed in a temperature-controlled device kept at 25 °C and 90−95% relative humidity (RH) in darkness ("control" samples). Samplings were carried out before storage (time 0) and every 3 days for a total storage period of 15 days. During each sampling, 12 fruits per box were collected and divided into three subgroups of four fruits each. Oranges of each subgroup were then peeled, chopped, and mixed to constitute three independent mean samples; the orange flesh was then immediately frozen with liquid nitrogen and stored at −80 °C until RNA isolation and anthocyanin content determination.

Measurement of Gene Expression by Real-Time Quantitative RT-PCR. Real-time PCR was performed using the SuperScript III Platinum two-step qRT-PCR kit. To minimize mRNA loss and avoid DNA contamination, isolated polyA$^+$ RNA was used as a template for first-strand synthesis before RT-PCR. Pure mRNA was prepared from orange flesh using the Quickprep mRNA purification kit (GE Healthcare, Piscataway, NJ). Reverse transcription of mRNA (1 μg) was achieved by following the manufacturer's protocol. The relative quantitation of gene expression between cold-treated and control orange samples was calculated using the comparative threshold (C_T) method[22] as detailed in Crifò et al.[12] The housekeeping gene elongation factor (EF-1α), which has been reported to be constitutively expressed,[23,24] was used as an endogenous reference. The comparative expression level of the single genes was given by the formula $2^{-\Delta\Delta CT}$, where $-\Delta\Delta CT$ was calculated by subtracting the baseline's ΔCT from the sample's ΔCT, where the baseline represents expression level at time 0. To each sample, 25 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 and 100 nM per primer (Table 1). Negative controls without reverse transcriptase were routinely included. The nucleotide sequences reported in this paper are in Genbank under the following accession numbers: EY747178 (CM1, partial cds), DQ088064 (PAL, partial cds), AB009351 (CHS, complete cds), AY519363 (DFR, complete cds), AY581048.1 (ANS, complete cds), AY519364 (UFGT, complete cds), EF597102.2 (GST, complete cds), FK826627.1 (clone 378, NAC domain protein FK826627), AY498567 (EF-1α partial cds).

Table 1. Sequences of Primers Used for the RT Real Time PCR Experiments

	primer sequence
CM1	*forward* 5′ AAC TGA AAA ACT TCA CTC CAA 3′
	reverse 5′ CTG CAT AGG TGG CAA CA 3′
PAL	*forward* 5′ GAT TTG AGA CAT TTG GAG GA 3′
	reverse 5′ ATG GAT GAA GCT CTC CAC TA 3′
CHS	*forward* 5′ TCT ATC GAC GGG CAT CTT C 3′
	reverse 5′ TGC CTC GGT TAG GCT TTT C 3′
DFR	*forward* 5′ GCT GTT CGT GCT ACT GTT C 3′
	reverse 5′ GGC TAA ATC GGC TTT CCA TA 3′
ANS	*forward* 5′ GGG TGA CTG CTA AAT GTG TT 3′
	reverse 5′ CAA GTC CCC TGT GAA GAA TA 3′
UFGT	*forward* 5′ TCT TCA GCA CTC CGC AAT C 3′
	reverse 5′ TCC ATC GGA TAC GTC GTA AG 3′
GST	*forward* 5′ GCA GCA AAG TAT GCA AAC C 3′
	reverse 5′ GTC ATT GAA ATT GTG TGC TTC 3′
NAC domain protein	*forward* 5′ TGG ATT TGC AAG CAT ATA ATC 3′
	reverse 5′ CAT TTT TGG AAC ACT ACA TTT AA 3′
EF-1α	*forward* 5′ GGC TAG GTA CGA TGA AAT TG 3′
	reverse 5′ GTT GTC ACC CTC GAA ACC 3′

Measurement of Total Anthocyanin Content. Anthocyanin determination was performed by pH-differential spectrophotometry according to a method of Lo Piero et al.[11] 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* for 20 min; then, the supernatant was recovered and analyzed for anthocyanin 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. Anthocyanin content was calculated by the following formula: $p/p = (\Delta Abs/\varepsilon \times L) \times MW \times DF \times (V/W_t) \times 100\%$, where $\Delta Abs = (Abs_{510\ nm}\ pH\ 1.0 - Abs_{700\ nm}\ pH\ 1.0) - (Abs_{510\ nm}\ pH\ 4.5 - Abs_{700\ nm}\ pH\ 4.5)$, 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).

Statistical Analysis. For anthocyanin determination, data sets are reported as average values ± standard deviation (SD) of three independent experiments performed upon sample triplicates each consisting of four fruits. With regard to the analysis of gene expression, for each gene, three independent experiments of quantitative PCR were performed upon sample

triplicates to generate an average C_T and to calculate SD, each triplicate resulting from mRNA sample independently isolated from four fruits.

RESULTS AND DISCUSSION

Anthocyanins are water-soluble vacuolar pigments responsible for coloring from red to purple many fruits, vegetables, and cereals. Available experimental and epidemiological evidence suggest that anthocyanins possess bioactive properties.[4] Among the *C. sinensis* varieties, only blood oranges and their hybrids contain these healthy compounds, the abundance of which might be increased by exposing orange fruit to postharvest cold storage.[11,12] Previous reports have investigated the effect of prolonged refrigerated storage upon blood oranges (77 days);[11,12] however, the results of that analysis might not be applicable to some of the common postharvest procedures (shelf-life extension, preparation of ready-to-eat products), which normally take 12−15 days.[13−15] Therefore, the blood orange fruit have been exposed to short cold storage (4 °C × 15 days) to adjust the length of storage and to obtain therapeutic value-added fruits for human consumption.

The effect of storage temperature on the anthocyanin content of orange fruit has been described in Figure 1. The amount of anthocyanins in control samples does not significantly change during the whole experimental period, showing slight fluctuations around values between 0.08 and 0.40 mg/100 g of fresh tissue. In contrast, the anthocyanin content of fruit exposed to low temperature sharply increases, reaching, after 6 days of storage, a value 8 times higher (2 mg/100 g) than that observed in the time zero samples (Figure 1). Afterward, the level of pigments decreases until the end of the experimental period, maintaining values higher than the control samples (Figure 1). These results suggest that the refrigerated storage of the blood oranges for 6 days after fruit harvest leads to fruit with added nutritional value, which can be consumed either fresh or after minimal processing. They also provide a novel strategy augmenting the abundance of pigments while they are subjected to common postharvest procedures involving cool-temperature preservation, thereby avoiding prolonged and expensive thermocontrolled storage. To correlate the observed enhancement of the anthocyanin content (Figure 1) with the expression of genes involved in the pigment biosynthesis, RT-real time PCR experiments were performed upon orange fruit stored at both 4 and 25 °C for 15 days. We focused the interest upon CM1 catalyzing the chemical reaction for the conversion of chorismate to prephenate in the pathway to phenylalanine biosynthesis. Afterward, the analysis was carried out on enzymes located at key points of the flavonoid 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) (dihydroflavonol-4-reductase (DFR)), at the consecutive steps involving anthocyanidin synthesis and glycosylation (ANS and UDP-glucose flavonoid glucosyl transferase (UFGT), respectively) and, finally, to the last step preceding the pigment vacuolar storage and represented by the conjugation of anthocyanin to glutathione catalyzed by glutathione transferase (GST).[10] In detail, Figure 2 shows the expression pattern of CM1 and PAL, which are expressed rather constantly during the whole experimental period of 25 °C storage. Conversely, after 3−6 days of low-temperature exposure, the levels of CM1 and PAL expression start to sharply increase, reaching the maximum value after 15 days of storage, the PAL being the most induced transcript. The induction of PAL activity was also found to be related to both

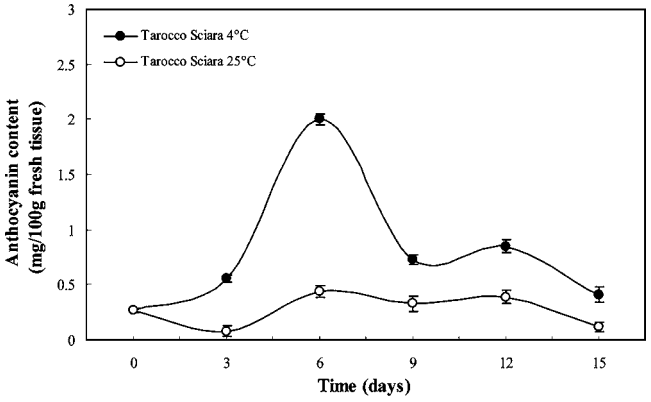

Figure 1. Effect of low-temperature exposure (4 °C × 15 days) upon the anthocyanin content in Tarocco Sciara orange flesh. Anthocyanin content was determined in blood orange flesh as described under Materials and Methods. Each point represents the mean value of three independent experiments carried out upon sample triplicates ± SE (or SD), each triplicate consisting of four fruits.

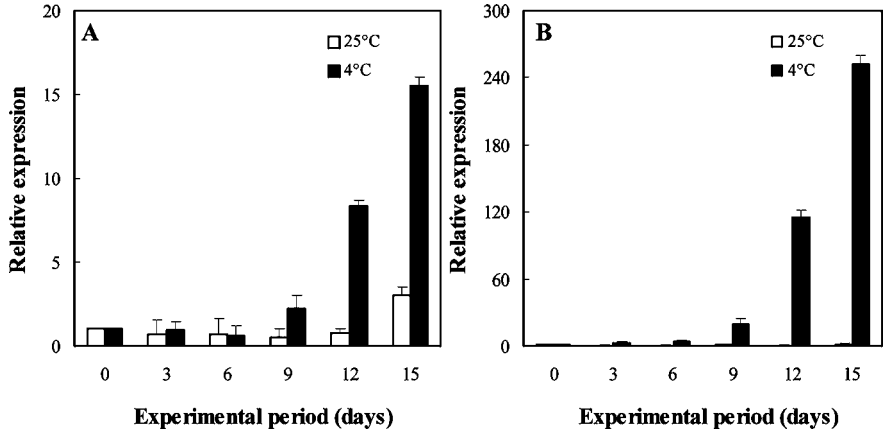

Figure 2. Analysis of the expression of (A) chorismate mutase (CM1) and (B) phenylalanine ammonia-lyase (PAL) in response to cold storage in blood orange flesh (Tarocco Sciara). The relative quantitation of gene expression between orange samples was calculated by real time RT-PCR using the comparative threshold (CT) method; each value represents the mean value of three independent experiments performed upon sample triplicates ± SD, each triplicate resulting from mRNA samples independently isolated from four fruits.

biotic[25] and abiotic stress responses[26,27] in different *Citrus* species and probably reflects a general demand of protective compounds, thereby fulfilled by providing with substrates the enzymes located downstream in the pathway.[28]

The expression profiles of CHS, DFR, ANS, UFGT, and GST were also investigated in both cold-exposed and control samples (Figure 3). The level of transcripts coding the aforesaid genes remains nearly unchanged in samples stored at 25 °C, in some cases showing a decrement with respect to time zero samples. On the contrary, in cold-stored samples the amount of transcripts of all considered genes sharply increases after 3−6 days of storage, reaching at the end of the experimental period expression levels ranging between 10- and 100-fold higher than the time 0 samples (Figure 3). The comparison between the profile of anthocyanin content and the expression of genes involved in their biosynthesis suggests that the anthocyanin peak obtained after 6 days of exposure (Figure 1) seems to be not logically linked with the sharp increase in gene expression observed later, between 9 and 15 days of storage (Figures 2 and 3), this being most likely necessary to resume the pigment content that is observable during prolonged cold storage.[12] Therefore, as the increase of gene expression might not be uniquely responsible for the enhancement of anthocyanin content, the pigment peak registered at 6 days of storage at 4 °C might be also explained with a major stability status of the existing enzymes rather than with induction of gene transcription. In regard to this aspect, it has been shown that the enzyme molecule in aqueous systems normally exists in its native state N that is in equilibrium with the partially denatured and enzymatically less active U state, and, that higher temperature tends to unfold the enzyme in a cooperative process.[29]

It has been shown that the expression of genes involved in anthocyanin biosynthesis in common (not pigmented) oranges is not missing but down-regulated during fruit ripening.[2] Therefore, in the attempt to assess whether the pathway is cold regulated also in common (not pigmented) *C. sinensis* varieties, Navel oranges have been cold stored (4 °C × 15 days) similarly to the blood variety, and the expression of selected genes (PAL, DFR, and UFGT) has been monitored by RT-real time PCR. As shown in Table 2, which reports the expression fold change at 15 days of storage normalized to zero time sample, the expression of PAL, DFR, and UFGT is, as expected, strongly induced by cold in blood oranges, whereas no enhancement of transcripts has been observed at 25 °C. In the not pigmented Navel oranges the transcript levels of PAL increased in response to cold up to 11-fold higher than zero time samples, thus confirming the key role of this enzyme in the synthesis of protective flavonoid compounds.[26,27] However, the increase of gene expression is much less pronounced than that observed in cold-exposed blood oranges, in which a >250-fold change was registered. Furthermore, the expression levels of DFR and UFGT, which are more strictly implicated in anthocyanin biosynthesis, do not rise in response to low-temperature exposure and remained at negligible values in samples stored at both 4 and 25 °C. Consequently, these data emphasize that the expression levels of DFR and UFGT cannot be regulated by cold in the common Navel orange and also indicate that induction of anthocyanin biosynthetic genes in orange fruits is closely determined by their genetic background.[2]

Analysis of the EST collection comprising clones specifically induced by cold in blood orange led to the identification of clone 378 (FK826627) homologous to *Populus trichocarpa*

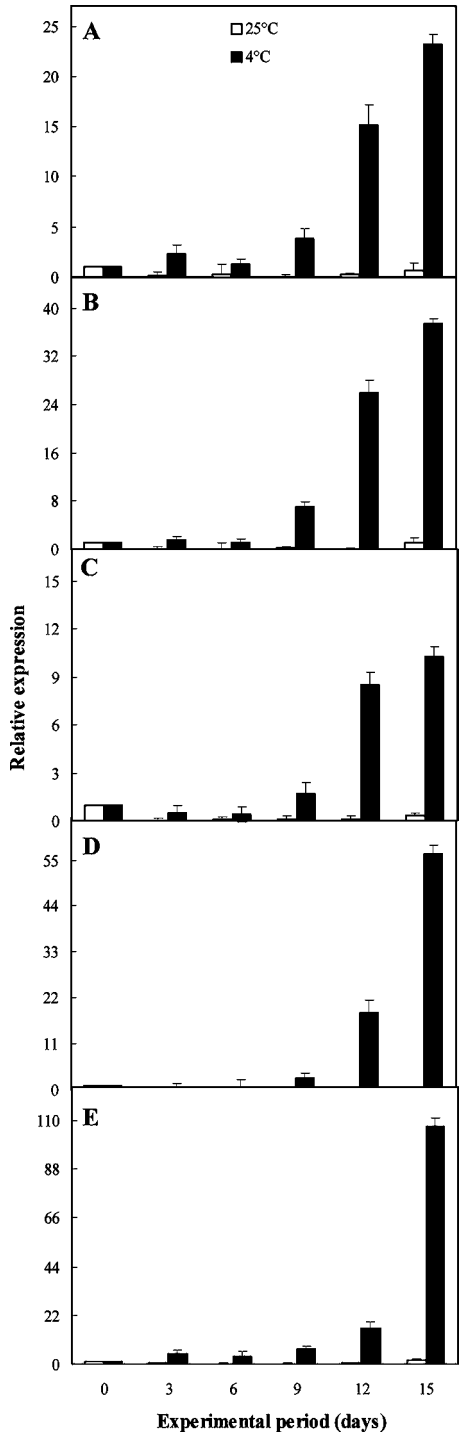

Figure 3. Expression pattern of anthocyanin biosynthetic genes in blood orange during low-temperature exposure. The relative quantitation of gene expression between orange samples was calculated by real time RT-PCR using the comparative threshold (CT) method: (A) chalcone synthase (CHS); (B) dihydroflavonol-4-reductase (DFR); (C) anthocyanidin synthase (ANS); (D) UDP-glucose flavonoid glucosyl transferase (UFGT); (E) glutathione *S*-transferase (GST). Each point represents the mean value of three independent experiments performed upon sample triplicates ± SD, each triplicate deriving from mRNA samples independently isolated from four fruits.

NAC domain protein (IPR00344). It turned out to be different from that found up-regulated during the chilling exposure of control nonacclimated grapefruit[30] and, as far as we know, it has

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Table 2. Effect of 15 Days of Cold Storage upon Expression of PAL, DFR, and UFGT in both Pigmented (Tarocco Sciara) and Nonpigmented (Navel) Orange Flesh[a]

	normalized amount relative to time 0 samples stored at 4 °C $2^{-\Delta\Delta CT}$		normalized amount relative to time 0 samples stored at 25 °C $2^{-\Delta\Delta CT}$	
gene	Tarocco Sciara	Navel	Tarocco Sciara	Navel
phenylalanine ammonia-lyase (PAL)	252.5 ± 14.1	11.63 ± 0.7	1.5 ± 0.2	0.38 ± 0.15
dihydroflavonol-4-reductase (DFR)	32.0 ± 1.0	0.37 ± 0.1	1.0 ± 0.5	0.09 ± 0.01
UDP-glucose-flavonoid glucosyltransferase (UFGT)	56.5 ± 1.3	0.19 ± 0.1	1.7 ± 0.6	0.11 ± 0.1

[a]Each value represents the mean value of three independent triplicates ± SE (or SD).

never been associated with cold stress in *Citrus*. As its specific characterization is lacking, the expression profile of this gene was monitored by RT real-time PCR during the short cold storage in both blood and common oranges. NAC protein family members are highly conserved at the N-terminal NAC binding domain, whereas they have a widely variable C-terminal domain, which plays a major role in the regulation of transcription, generally acting as a transcriptional activator or a repressor functional domain.[16,17,31] To discriminate among the different NAC transcription factors that might be putatively induced by cold, specific primers have been designed within the C-terminal functional domain which identifies the single NAC domain proteins. Therefore, RT real-time PCR experiment monitoring the expression profile of clone 378 (FK826627) was performed on blood oranges, and the results are in Figure 4A.

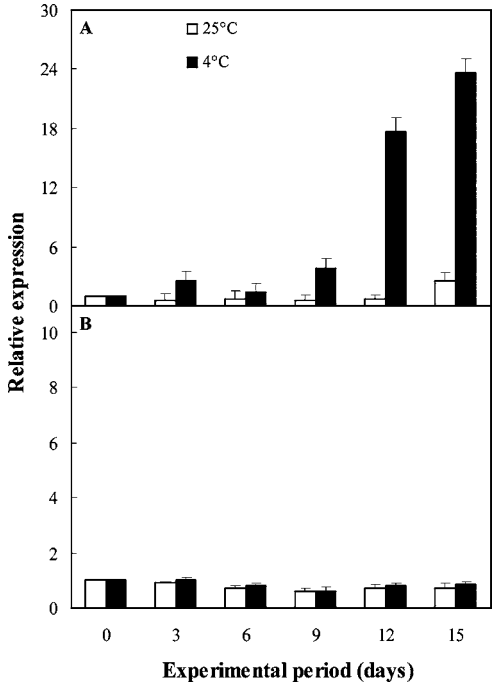

Figure 4. RT real time analysis of NAC domain protein (FK826827) in (A) blood (Tarocco Sciara) and (B) common (Navel) oranges subjected to cold storage. Experimental details are under Materials and Methods. Each value represents the mean value of three independent experiments performed upon sample triplicates ± SD, each triplicate arising from mRNA samples independently isolated from four fruits.

Analysis clearly reveals that the transcripts increase during cold storage from 6 days of storage until the end of the period under investigation, showing a >20-fold change. Conversely, the experiments carried out upon common oranges highlight that the EST encoding the clone 378 is induced neither in control samples nor in cold-exposed samples (Figure 4B), as observed in the case of DFR and UFGT (Table 2). Therefore, the data suggest that *C. sinensis* EST FK826627 is connected to the cell signaling cascade leading to the response to cold in blood oranges but not in common oranges, thus proposing it as candidate gene specifically involved in blood orange response to cold exposure. Future work will be undertaken to assess the functional linkage between the NAC domain protein and the response to cold of blood oranges, which doubtless involves the increase of the anthocyanin content. Interestingly, the CATGTG motif, binding the NAC transcription factors to the promoter region of the target genes, has been found in the DFR promoter region previously isolated from blood oranges.[9]

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ABBREVIATIONS USED

CM1, chorismate mutase; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; ANS, anthocyanidin synthase; DFR, dihydroflavonol-4-reductase; UFGT, UDP-glucose flavonoid glucosyltransferase; GST, glutathione transferase; EF, elongation factor.

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