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# Dehydrin from *Citrus*, Which Confers in Vitro Dehydration and Freezing Protection Activity, Is Constitutive and Highly Expressed in the Flavedo of Fruit but Responsive to Cold and Water Stress in Leaves

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A cDNA encoding a dehydrin was isolated from the flavedo of the chilling-sensitive Fortune mandarin fruit (*Citrus clementina* Hort. Ex Tanaka × *Citrus reticulata* Blanco) and designed as *Crcor15*. The predicted CrCOR15 protein is a $K_2S$ member of a closely related dehydrin family from *Citrus*, since it contains two tandem repeats of the unusual *Citrus* K-segment and one S-segment (serine cluster) at an unusual C-terminal position. *Crcor15* mRNA is consistently and highly expressed in the flavedo during fruit development and maturation. The relative abundance of *Crcor15* mRNA in the flavedo was estimated to be higher than 1% of total RNA. The high mRNA level remained unchanged during fruit storage at chilling (2 °C) and nonchilling (12 °C) temperatures, and it was depressed by a conditioning treatment (3 days at 37 °C) that induced chilling tolerance. Therefore, the expression of *Crcor15* appears not to be related to the acquisition of chilling tolerance in mandarin fruits. However, *Crcor15*, which was barely detected in unstressed mandarin leaves, was rapidly induced in response to both low temperature and water stress. COR15 protein was expressed in *Escherichia coli*, and the purified protein conferred in vitro protection against freezing and dehydration inactivation. The potential role of *Citrus* COR15 is discussed.

**KEYWORDS: Citrus; dehydrin; chilling injury; freezing; late embryogenesis abundant (LEA); maturation; water stress**

## INTRODUCTION

Low temperature is one of the major environmental factors limiting plant growth and survival. Cold-sensitive plants or specific plant organs may become injured when exposed to temperature below that for optimum growth, leading to important losses in productivity (*1*). Many tropical and subtropical plant species, such as *Citrus*, are susceptible to chilling injury when exposed to low nonfreezing temperatures. Fortune mandarin (*Citrus clementina* Hort. Ex Tanaka × *Citrus reticulata* Blanco) is a late-ripening *Citrus* cultivar greatly appreciated for the quality of its fruit, but it is very sensitive to chilling injury, developing necrosis and pitting in the flavedo tissue (outer colored part of the peel) when stored at temperatures below 5 °C (*2*).

In recent years, genes and metabolic pathways involved in the perception and signal transduction of plant responses to extreme temperatures have been identified (*3*). Most of our knowledge in this field arises from studies conducted in the chilling-tolerant *Arabidopsis thaliana* during cold-induced freezing acclimation (*4*), but little is known about the molecular basis of cold responses in agronomically important chilling-sensitive plants. Recent results, where heterologous expression of the C-repeat/dehydration-responsive element binding factor 1 (CBF1) from *Arabidopsis* enhanced tolerance to chilling and oxidative stress in chilling-sensitive tomatoes (*5*), indicate that certain conserved mechanisms may still operate between chilling-sensitive and chilling-tolerant plants and even between freezing and chilling tolerance. Several studies have led to the identification of scores of cold-responsive genes (COR), many of which encode apoplastic antifreezing proteins (AFPs), late embryogenesis abundant (LEA) proteins, or other novel polypeptides (*1*). In citrus fruits, little is known about the molecular mechanisms operating during cold stress. In Fortune mandarin, we have shown that the induction of phenylalanine-ammonia lyase (PAL) gene expression is concomitant with the development of chilling symptoms (*6*) and that a pretreatment of 3 days at 37 °C, which prevented cold-induced damage (*2*), abolished the cold-induced PAL mRNA accumulation (*7*). Furthermore,

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we have shown that heat-induced chilling tolerance stimulates the expression of genes encoding proteins involved in secondary metabolism, cell wall modification, oxidative damage and other stress-responsive proteins, illustrasting the complex molecular mechanisms operating in the acquisition of chilling tolerance in citrus fruits (*8*).

Dehydrins, a subgroup of LEA proteins also known as LEA-D11 or LEA type 2, comprise an immunologically distinct family which are among the most prevalent plant proteins induced during periods of water deficit imposed by drought, salinity, low temperature, and freezing (*9*, *10*) and as a part of the developmental program activated during seed maturation (*11*). Dehydrins present common features such as extreme hydrophilicity, solubility at high temperature, and the presence of a conserved lysine-rich 15-amino-acid motif (K-segment) often present in one or more copies (*10*). The K-segment is predicted to form a class A amphipathic α-helix with the potential for both water binding and hydrophobic interaction. Other structural domains of most dehydrins include a tract of Ser residues (S-segment), a conserved amino acid sequence (DEYGNP) in the N-terminus, similar to nuclear localization sequences, and a $\phi$-segment rich in polar amino acids and either Gly or a combination of Pro and Ala. According to the presence of different domains and tandem repeat motifs, diverse sub-classes of dehydrins have been proposed (*10*).

The function of dehydrins remains speculative, but there are a number of lines of evidence supporting their role in protecting macromolecules or plant cellular structures from the water deficit-induced damage (*10*, *12*). Functional in vitro analyses of dehydrins isolated from several plant species (*Arabidopsis*, wheat, spinach, peach, *Citrus*, and birch) have been conducted. Dehydrins protected lactate dehydrogenase enzyme activity from freeze−thaw inactivation (*13*−*15*) and improved recovery of α-amylase activity under low water availability (*16*). In vivo studies have been also conducted, demonstrating that heterologous expression of dehydrins is able to confer freezing tolerance (*17*, *18*). In addition, it has been reported that a 35 kDa dehydrin from cowpea cosegregated with chilling tolerance (*19*).

In *Citrus*, a number of dehydrin genes have been isolated and their expression patterns in vegetative and reproductive tissue have been analyzed. Two dehydrin genes, *cor11* and *cor19*, were first isolated from leaves of the cold-hardy *Citrus* relative *Poncirus trifoliata* (*20*). In the flavedo of *Citrus unshui* mandarin fruit, *Cucor19* was showed to have cold-responsive expression (*21*). More recently, a *cor15* gene from the peel of grapefruit (*Citrus paradisi*) has been characterized and its involvement in the acquisition of fruit chilling tolerance was suggested (*22*). These dehydrins belong to a distinct subfamily, which possesses an unusual K-segment, a nuclear localization signal, and a Ser tract at the C-terminus of the protein (*22*). In vitro assays with the recombinant $K_3S$ dehydrin CuCOR19 protected catalase and lactate dehydrogenase against freezing inactivation, thus suggesting the involvement of this gene family in the acquisition of low-temperature tolerance (*15*).

In the present study, we report on the isolation of a new member of the *Citrus* dehydrin family from the flavedo of the chilling-sensitive Fortune mandarins. The Fortune dehydrin shares high sequence homology with *cor15* from grapefruit. Accumulation of the corresponding mRNA was very high and constitutive in the flavedo of Fortune fruit throughout maturation and after cold storage, but it was induced in leaves after water and cold stress. To understand the functional role of this *Citrus* dehydrin, in vitro assays of the protection conferred by the recombinant CrCOR15 protein against dehydration and freezing enzyme inactivation were performed.

## MATERIALS AND METHODS

**Plant Material and Treatments.** Fruits of the hybrid Fortune (*Citrus clementina* Hort. Ex Tanaka × *Citrus reticulata* Blanco) and of Hernandina mandarins, a spontaneous mutation of one of the Fortune parents (*Citrus clementina* Hort. Ex Tanaka), were harvested at random from trees grown at Sagunto, Valencia, Spain. After harvesting, fruits were immediately stored at either 2 °C (chilling temperature) or 12 °C (control temperature) and 80−85% relative humidity (RH). Another group of both Fortune and Hernandina mandarins were subjected to a 3 day heat-conditioning treatment (37 °C and 90−95% RH) and then stored at 2 °C and 80−85% RH for up to 28 days. For each experiment, fruits were randomly divided into two lots. The first lot was subdivided into three replicates of 20 fruits each to visually estimate chilling damage. The second lot contained three replicates of 5 fruits per temperature and storage period and was used to study changes in gene expression. Chilling injury index was estimated on a rating scale from 0 (no damage) to 3 (severe injury) (*23*). Flavedo samples were periodically collected from total surface of fruits stored, in constant darkness, at 2 and 12 °C for up to 28 days. To study changes in gene expression during fruit maturation, Fortune mandarins were periodically harvested (October to March) from the same orchard. Color of the peel was measured with a Minolta CR-330 and is expressed as changes in color index (*a/b* Hunter ratio), a reference of fruit maturity in citrus fruits (*24*). Flavedo tissue was excised from 30 fruits per maturation stage, frozen in liquid nitrogen, ground to a fine powder, and stored at −80 °C until analysis.

To study the effect of low temperature on the expression of *Crcor15* gene in vegetative tissues, 2-year-old Fortune mandarin trees grafted onto Cleopatra mandarin (*Citrus reshi* Hort. Ex Tanaka) were used, following the experimental conditions described by Sanchez-Ballesta et al. (*6*). Water stress experiments were performed in detached mature leaves of Fortune mandarins. Leaves were incubated on filter paper at 20 °C for 1−6 h and the rate of weight loss was determined after each dehydration period. Three replicates of 10 leaves per time were collected, frozen in liquid nitrogen, and stored at −80 °C. Experiments were repeated at least twice with similar results.

**RNA Extraction, Screening of a cDNA Library, and Sequencing.** Total RNA and poly$(A)^+$ RNA were isolated from flavedo of fruits and from leaves according to Sanchez-Ballesta el at. (*6*). To isolate cold-stress responsive genes from citrus fruit, a cDNA library constructed from poly$(A)^+$ RNA of flavedo of Fortune fruits stored for 21 days at 2 °C (*6*) was differentially screened. As positive and negative probes, $^{32}P$-labeled single-stranded total cDNA synthesized from poly-$(A)^+$ RNA from fruits stored at 2 °C for 21 days and from freshly harvested fruits, respectively, were used. The cDNAs isolated were sequenced and searching and alignments were performed by use of the GCG package (Genetic Computer Group, Madison, WI) and the BLAST program at the NCBI (Bethesda, MA).

**RNA Gel Blot Analysis.** RNA gel blot was performed as described in Sanchez-Ballesta et al. (*6*). In the experiment in which a high mRNA resolution was required to discriminate the RNA hybridization signal of *Crcor15* from other closely related mRNAs, denatured total RNA (8 μg) with 8 M urea and 1 mM EDTA, pH 8, were separated on a 5% polyacrylamide−urea gel. A high-resolution gel (20 × 37 × 0.1 cm) was used at constant power (10 mA) during 15 h and a restrictive voltage of 680 V. RNA molecular weight marker type III (Boehringer, Mannhein, Germany) was used. Likewise, 0.001 μg of in vitro synthesized *Crcor15* transcript (as described below) was used as control. Once electrophoresis was completed, RNA was stained with ethidium bromide and incubated for 30 min with 1× transfer buffer ($Na_3PO_4$ 25 mM, pH 6.5). The RNA was transferred onto Hybond-N membrane by use of an LKB 2005 Transphor and 1× transfer buffer for 16 h at constant power (25 V) and cross-linked to the membrane with a UV Stratalinker 800.

The DNA probe was randomly primer labeled with [α-$^{32}P$]dCTP. Filters were prehybridized and hybridized at 65 °C in 7% SDS, 0.33 M phosphate buffer, pH 7.2, and 1 mM EDTA and then washed twice

in 2 × SSC/0.1% SDS at room temperature and twice in 0.1 × SSC/0.1% SDS at 65 °C and exposed to Kodak X-Omat SX film at −80 °C.

**Estimation of Abundance of the *Crcor15* mRNA.** To estimate the relative abundance of *Crcor15* mRNA in the flavedo and leaves of Fortune mandarin, in vitro transcription of *Crcor15* cDNA was performed. Two micrograms of *Crcor15* cDNA in Bluescript KS was linearized with *Eco*RI and purified by incubation with 1 volume of proteinase K (18 $\mu g^{-1}$ $\mu L$) for 30 min at 37 °C. Then, two phenol/chloroform extractions were carried out and the linearized cDNA was precipitated, washed with 70% ethanol, and resuspended in 20 μL of water. One microgram of the linearized cDNA was in vitro transcribed to RNA in the sense orientation with 2.5 mM ribonucleotides, RNA guard (20 units), and T7 RNA polymerase (40 units) for 1 h at 37 °C. Then, the sample was treated with DNase I, RNase-free (20 units), for 15 min at 37 °C. The transcript was precipitated with 0.1 volume of 4 M LiCl and 3 volumes of ethanol, washed, and resuspended in water. The RNA concentration was measured spectrophotometrically. Denatured poly$(A)^+$ RNA samples from flavedo of freshly harvested fruits and from leaves of Fortune, and increasing concentrations of the in vitro synthesized transcript, accounting for 0.001%, 0.01%, 0.1%, and 1% of the total mRNAs, were separated on 1.2% (w/v) agarose−formaldehyde gels. Northern blot analysis and hybridization with *Crcor15* probe was performed as above.

**Production of the Recombinant CrCOR15 Protein in *Escherichia coli*.** The open reading frame encoded by the gene *Crcor15* was cloned as a C-terminal fusion with an amino acid His tag in pQE vectors (QIA express, Qiagen, Germany) and transformed in *E. coli* strain M15 [pREP4]. Recombinant protein was induced by adding 2 mM (final concentration) isopropyl β-D-thiogalactopyranoside (IPTG). The soluble recombinant His tagged CrCOR15 protein was affinity-purified on Ni−NTA agarose resin (QIA express, Qiagen, Germany) under native conditions. Purified protein was dialyzed against 10 mM potassium phosphate buffer, pH 7.5, concentrated by precipitation with 2.5 volumes of acetone, and resuspended in 50 mM potassium phosphate buffer, pH 7.5. Concentration of recombinant CrCOR15 was determined by the Bradford protein assay, and homogeneity was estimated by SDS−PAGE (*25*) followed by CBB staining. Protein was stored at 4 °C until used for dehydration and cryoprotection assays.

**Dehydration and Cryoprotection Assays.** Malate dehydrogenase (MDH) activity was used to test the effect of CrCOR15 on enzyme protection against dehydration, and lactate dehydrogenase (LDH) activity was used for cryoprotection assay. All experiments were performed in polypropylene tubes to avoid protein adsorption to glass. For MDH activity, the enzyme and the corresponding protein were dissolved in 50 mM potassium phosphate buffer, pH 7.2, and for LDH activity, in 50 mM Tris-HCl, pH 7.5. In both cases, final enzyme concentration was 250 nM (monomer), corresponding to 10 $\mu g\ mL^{-1}$ MDH and 8.3 $\mu g\ mL^{-1}$ LDH. In dehydration and cryoprotection assays, the protein:enzyme molar ratio ranged from 0.5 to 4.

Dehydration assay was carried out as follows. Aliquots of 75 μL of the protein−enzyme mixture were placed in a Speed-Vac concentrator (Savant Instruments) and dehydrated to 99.0% ± 0.5%, with respect to the initial weight. Evaporating cooling prevented heating and did not freeze the sample during vacuum-drying. Immediately after dehydration, samples were rehydrated to the initial volume with Milli-Q water (Millipore), ensuring adequate dissolution, and residual MDH activity in the mixture was determined.

Cryoprotection assay was performed in aliquots of 50 μL of LDH and protein mixture subjected to two cycles of freeze and thaw. Samples were immersed in liquid nitrogen for 30 s and then allowed to thaw for 5 min at room temperature. After the process was repeated, residual LDH activity was measured.

MDH and LDH enzymatic activities were determined in aliquots of 8 and 15 μL of the mixtures, respectively, in a final volume of 600 μL of the reaction assay buffer. MDH enzymatic activity was assayed in 150 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM oxalacetate (Sigma) and 0.2 mM NADH (Boehringer). LDH activity was assayed in 25 mM Tris-HCl, pH 7.5, containing 2 mM pyruvate (Sigma) and 0.15 mM NADH (Boehringer). MDH and LDH activities were monitored as the decrease of absorbance at 340 nm for 1 min due to the conversion of NADH to NAD at 25 °C. Results are means of three replicate samples ± SE.

**Chemicals and Proteins.** Bovine serum albumin (BSA; initial fraction by cold alcohol precipitation, globulin-free) was purchased from Sigma Chemical Co. (St. Louis, MO). RNase A from bovine pancreas, malate dehydrogenase (MDH, EC 1.1.1.37) from pig heart, and lactate dehydrogenase (LDH, EC 1.1.1.27) from hog muscle were purchased from Boehringer (Mannhein, Germany). The commercial stocks of MDH and LDH were supplied in 50% glycerol. Unless otherwise indicated, other chemicals and reagents were obtained from Sigma.

## RESULTS

**Isolation and Sequence Analysis of *Crcor15 G*ene.** During the course of a differential screening study designed to identify genes regulated by chilling injury in fruits of the chilling-sensitive Fortune mandarin, a cDNA showing a strong hybridization signal with both the positive and negative probe was isolated. Sequence analysis of the protein showed that it shared 97.8% identity with the COR15 dehydrin recently identified from grapefruit (*22*); the clone was designed as *Crcor15* (GenBank Accession Number AY327515) (**Figure 1**). The full-length sequence of the *Crcor15* cDNA consists of 736 bp with an open reading frame of 414 bp and encoded a polypeptide of 138 amino acids with a predicted molecular mass of 15.2 kDa and a p*I* of 6.54. Hydrophilic and neutral amino acids residues in CrCOR15 account for up to 55.8% of total residues.

The Fortune mandarin CrCOR15 protein contains two large tandem repeats of 31 and 39 amino acids, respectively, including a Gln cluster and a conserved dehydrin Lys-rich motif known as K-segment. A similar structure to the nuclear localization signal sequences at the N-terminus and S-segment are also present. According to the different domains and the nomenclature designated by Close (*10*), CrCOR15 belongs to the dehydrin type $K_2S$ (**Figure 1**).

***Crcor15 mRNA* Is Specifically Detected with No Cross-Reaction with Other *cor* Genes from *Citrus*.** Because of the high sequence homology of *Crcor15* cDNA from Fortune mandarin with that of *cor19* (69.3% identity) and *cor11* (67.9% identity) genes from the close relative *P. trifoliata*, we studied whether the *Crcor15* cDNA probe may cross-hybridize with the Fortune *cor19* and *cor11* orthologues. Since the predicted size of *Crcor15* mRNA (800 nt) is between that of *cor19* (900 nt) and *cor11* (700 nt), a northern blot analysis on a high-resolution polyacrylamide gel under denaturing conditions was required to discriminate among different *Cor* mRNAs on the basis of their different molecular size. **Figure 2A** shows the part of the gel between 1000 and 600 nt and how a unique hybridization mRNA band was detected in flavedo of freshly harvested fruit, which was similar in size (about 800 nt) to the in vitro synthesized mRNA from *Crcor15* cDNA. This indicates that our probe specifically monitored the levels of genuine *CrCor15* mRNA and not that of *cor19* or *cor11*.

**Estimation of the *Crcor15* mRNA Abundance.** The high abundance of *Crcor15* mRNA in the flavedo of Fortune fruits was well exemplified by the strong hybridization signal obtained when northern blots were exposed to the autoradiography films without intensifying screens. To estimate the relative abundance of *Crcor15* mRNA, a northern analysis was performed with poly$(A)^+$ RNA from flavedo of freshly harvested fruits and leaves of Fortune and increasing concentrations of the in vitro synthesized *Crcor15* mRNA (0.001−1% of the total mRNAs). The results confirmed that the relative abundance of *Crcor15* mRNA in the flavedo of freshly harvested Fortune fruit was higher than 1%, while that in leaves was estimated between 0.01 and 0.1% (**Figure 2B**).

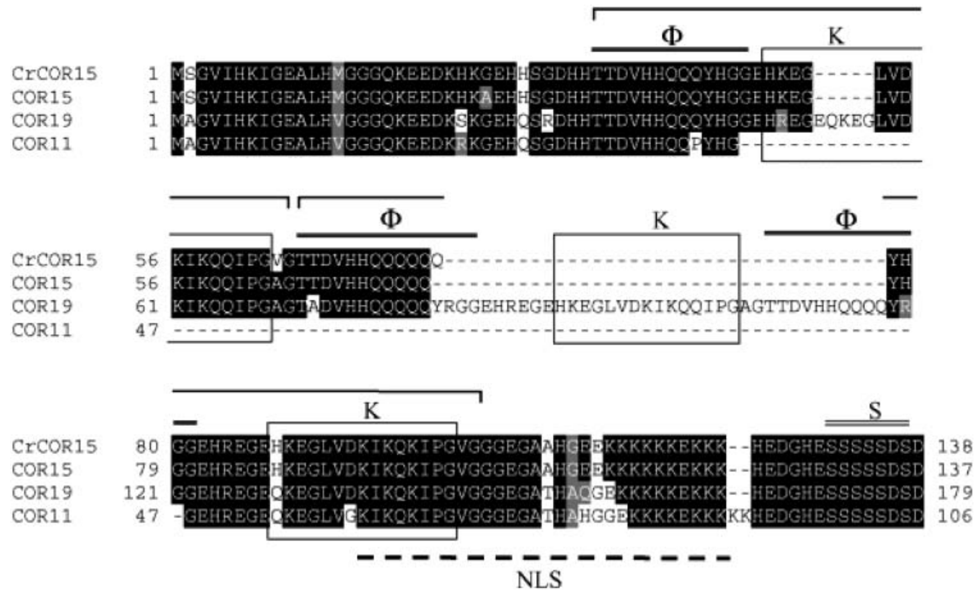


**Figure 1.** Alignment of the deduced amino acid sequences of the CrCOR15 (AY327515) from Fortune mandarin (*Citrus clementine* × *Citrus reticulata*), COR15 (AY032975) from grapefruit (*Citrus paradisi*), and COR19 (L39004) and COR11 (L39005) from *Poncirus trifoliata*. Identical amino acids and conservative substitutions are shown on a black or gray background, respectively. Tandem repeats are marked with a single black line, the highly rich polar domains (Φ) are marked with a bold gray bar, the putative K-segments are boxed, the Ser track is marked with a double bar, and the putative bipartite motif, which resembles a nuclear localization signal (NSL), is marked with a dashed line.

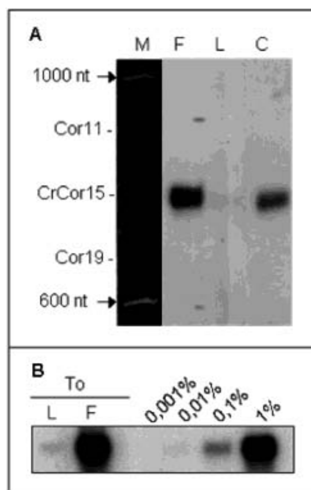


**Figure 2.** Specificity of the Crcor15 probe and estimation of its relative mRNA abundance in flavedo and leaves of Fortune mandarin. (**A**) Northern blot analysis of mRNA (600–1000 nucleotides) by high-resolution PAGE under denaturing conditions; the *Crcor15* probe was used to discriminate among different *Cor* mRNAs from Citrus. Eight micrograms of total denatured RNA from the flavedo of freshly harvested fruits (F) and from leaves (L) were separated on a 5% polyacrylamide–urea gel, blotted, and hybridized with the *Crcor15* probe. Lane M corresponds to the ethidium bromide staining of the RNA molecular weight marker. In vitro synthesized *Crcor15* transcript (0.001 $\mu$g) was used as control (C). The estimated sizes of *cor19*, *Crcor15*, and *cor11* are indicated. (**B**) Denatured poly-(A)+ RNA (0.4 $\mu$g) from flavedo of freshly harvested fruits (F) and from leaves (L) of Fortune, and increasing concentrations of the in vitro synthesized transcript (0.001–1% of the mRNAs total), were separated on 1.2% agarose–formaldehyde gels, blotted, and hybridized with the *Crcor15* probe.

**Analysis of *Crcor15* Gene Expression in the Flavedo of Citrus Fruits.** The pattern of *Crcor15* gene expression in the flavedo of the chilling-sensitive Fortune fruit in response to chilling (2 °C) and nonchilling (12 °C) temperatures was investigated. Accumulation of *Crcor15* mRNA was very high in the flavedo of freshly harvested fruit and remained constant throughout the storage period at both temperatures (**Figure 3A**). Under these storage conditions, chilling symptoms developed after 7 days at 2 °C, and after 28 days of storage the chilling index was 2.4. A similar pattern of mRNA accumulation was also obtained for the flavedo of the chilling-tolerant Hernandine fruits stored at 2 °C, which did not develop chilling injury (*7*).

To further study the potential involvement of *Crcor15* in the acquisition of fruit tolerance to low temperature, we studied the effect of a heat-conditioning treatment (37 °C for 3 days), which enhances fruit chilling tolerance, on the accumulation of *Crcor15* mRNA in fruit of both the chilling-sensitive Fortune and the chilling-tolerant Hernandine. *Crcor15* mRNA levels were rapidly depleted in both mandarins after heat treatment. However, upon transference to 2 °C the level of the transcript began to accumulate faster in heat-treated Hernandine fruits (7 days) than in the chilling-sensitive Fortune (14 days). The effect of heat conditioning was observed even after prolonged cold storage, as the levels of the transcript in both cultivars were always lower in heat-treated than in nontreated fruits (**Figure 3B**).

Since *Crcor15* gene was highly and constitutively expressed in the flavedo of mature fruits, we studied accumulation of the mRNA during maturation of Fortune fruits, from mature-green fruit harvested in October to full mature-orange fruit of March. The results of **Figure 3C** show a high and similar accumulation of *Crcor15* mRNA in the flavedo of fruit at the seven ripening stages analyzed, indicating that the maturation process did not affect *Crcor15* gene expression.

**Stress-Induced Expression of *Crcor15* in Fortune Leaves.** To examine whether stress conditions may induce the expression of *Crcor15* in vegetative tissues, accumulation of *Crcor15* mRNA was studied in leaves from Fortune seedlings exposed to 2 °C and also in detached water-stressed leaves. In contrast with the flavedo, *CrCor15* was barely detected in leaves under normal growth conditions. Fortune leaves accumulated *Crcor15* transcript by 2 days of exposure to 2 °C and increased thereafter for up to 14 days (**Figure 4A**). In detached leaves, accumulation

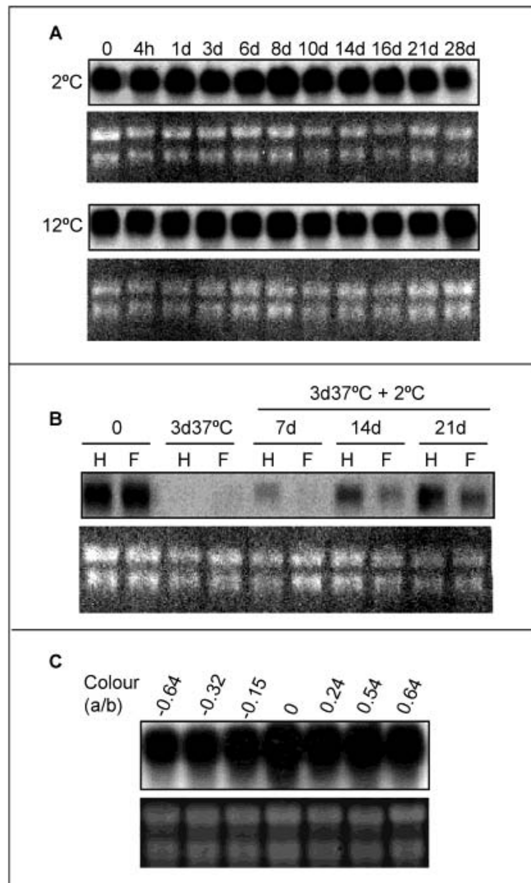


**Figure 3.** Accumulation of *Crcor15* mRNA in the flavedo of (**A**) the chilling-sensitive Fortune mandarin fruits stored at 2 and 12 °C; (**B**) fruits of the chilling-resistant Hernandine (H) mandarin and from the chilling-sensitive Fortune (F) heat-conditioned for 3 days at 37 °C and then stored at 2 °C for up to 21 days; and (**C**) fruits of Fortune mandarin harvested at different maturation stages. Color of the flavedo is indicated as *a/b* Hunter ratio, where negative values represent green fruits, 0 is the color break point, and positive values are colored fruits. Each *a/b* value is the mean of 10 fruits. Each lane contained 4 $\mu$g of total RNA, which were fractioned, blotted, and hybridized with the *Crcor15* probe. The equivalence of RNA loading of the lanes was demonstrated by ethidium bromide staining.

of *Crcor15* mRNA was detected as soon as 1 h after dehydration at 20 °C, and thereafter it increased in parallel with the progression of water loss (**Figure 4B**).

**Protective Activity of Recombinant CrCOR15 against Dehydration and Freezing Inactivation.** Dehydrin proteins have been shown to confer protection against enzyme denaturation induced by different stress conditions. To determine if CrCOR15 protein from Fortune may also have an enzyme protective activity, purified His-tagged CrCOR15 protein was produced by pQE expression system in *E. coli*. After purification and dialysis of the recombinant protein, a single band of around 22 kDa, slightly higher than the predicted size of CrCOR15, was identified by SDS−PAGE (**Figure 5A**). MDH and LDH enzymatic activities were used to test the effect of recombinant CrCOR15. These enzymes were selected because their activities are known to be significantly affected by dehydration (MDH) and freeze−thawing (LDH). To compare the potential enzyme-stabilizing activity of CrCOR15 with that of other proteins known as cryoprotectants, BSA (a well-known additive for the stability of enzyme preparations) and RNase A (a protein closer in its low molecular mass to CrCOR15 and very hydrophilic and stable) were included in parallel assays.

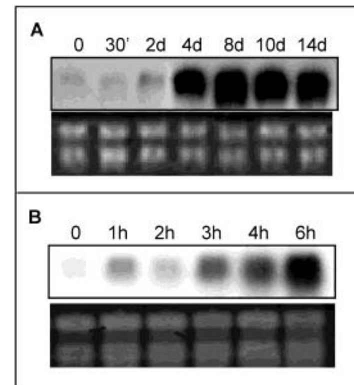


**Figure 4.** Accumulation of *Crcor15* mRNA in cold (**A**) and water-stressed (**B**) leaves of Fortune mandarins. Two year-old seedlings were exposed to 2 °C for 8 h at night/14 °C for 16 h light and individual leaves were taken at the time indicated. Detached Fortune leaves were dehydrated at 20 °C and 80–85% RH for up to 6 h. Each lane contained 8 $\mu$g of total RNA, which was fractioned, blotted, and hybridized with the *Crcor15* probe. The equivalence of RNA loading of the lanes was demonstrated by ethidium bromide staining.

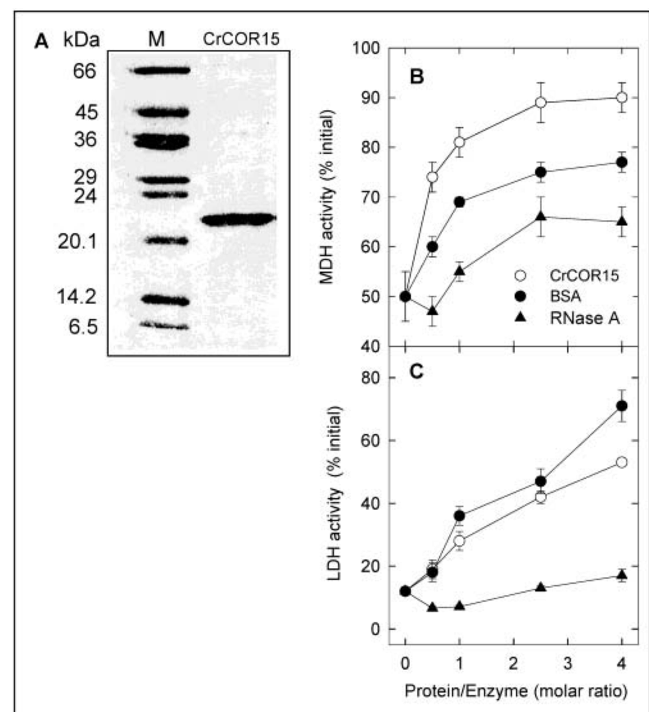


**Figure 5.** Expression of recombinant CrCOR15 protein and dehydration and cryoprotection assays. (**A**) Purified CrCOR15 protein was analyzed by SDS–PAGE (12% polyacrylamide) and stainined by CBB. Sizes of molecular mass markers are indicated on the left. MDH (**B**) and LDH activity (**C**) in aliquots containing 250 nM enzyme (monomer) and increasing concentrations of CrCOR15 protein, BSA, and RNase A, subjected to dehydration and freeze–thawing inactivation, respectively. Enzyme activity was determined in three replicate samples, in at least two independent experiments. Results are expressed as percentage of initial MDH and LDH activity and are the mean ± standard error of six replicates.

Our dehydration protocol resulted in a 50% reduction of MDH activity (**Figure 5B**) and provided the basis for testing the protective activity of recombinant CrCOR15. Recombinat CrCOR15, at protein:enzyme molar ratios from 0.5 to 4, conferred an important protection of MDH activity under controlled desiccation. At molar ratios above 2.5, almost 90%

of the initial activity was recovered in the presence of CrCOR15. This protective activity provided by CcCOR15 was higher on a molar basis than that achieved by BSA or RNase (75% and 66% of the initial MDH activity, respectively) (**Figure 5B**).

The cryoprotective activity of recombinant CrCOR15, BSA, and RNase was then assessed by determining whether they could protect LDH against freeze inactivation. Without the addition of a cryoprotectant, two freeze–thaw cycles resulted in more that 80% reduction of LDH activity (**Figure 5C**). Addition of CrCOR15 or BSA, in protein:enzyme molar ratios from 0.5 to 4, significantly protected LDH activity. Furthermore, CrCOR15 was shown to be as efficient as BSA in protecting LDH at molar ratios up to 2.5, recovering with both close to 40% of the initial LDH activity. At a molar ratio of 4, BSA maintained around 70% of initial LDH activity, while with CrCOR15 it was about 55%. Therefore, at high concentrations BSA was more effective than CrCOR15 in protecting LDH activity against freeze inactivation. RNase A, in contrast, did not provide a significant cryoprotective effect on LDH activity (**Figure 5C**).

## DISCUSSION

Dehydrins are a family of highly hydrophilic proteins that accumulate at the onset of seed desiccation and in vegetative tissues in response to environmental stresses with a dehydration component, such as drought, low temperature, and salinity (*9*, *10*, *26*). In *Citrus*, different members of a closely related dehydrin gene family have been isolated and all of them have common structural features that are differentiated from other plant dehydrins (*20*–*22*). The *Crcor15* cDNA isolated from the flavedo of the hybrid Fortune mandarin shows high sequence identity with the *cor15* dehydrin from grapefruit (*22*). Both proteins have two tandem repeats of an unusual K-segment, similar to that of gymnosperms, and a S-segment at the C-terminus that is not always present in other dehydrins (**Figure 1**) (*10*). Therefore, according to the classification established by Close (*27*), the Fortune and grapefruit dehydrins belong to the $K_2S$ dehydrin type, whereas the COR19 of *Poncirus trifoliata* and *Citrus unshiu* are $K_3S$ dehydrins and the COR11 from *P. trifoliata* is a KS type. Despite the high sequence homology between all the *Citrus* dehydrins that only differ in the number of tandem repeats, our results showed that the *Crcor15* probe did not cross-hybridize with orthologues *cor11* and *cor19* (**Figure 2A**).

One of the striking characteristics of *Crcor15* was the extremely high abundance of the transcript and its constitutive expression pattern in the flavedo of Fortune during maturation and after storage at two different temperatures (2 and 12 °C) (**Figure 3**). In the flavedo of mature grapefruits, however, homologous *cor15* mRNA declined after cold storage (*22*). Whether the unusually high accumulation of the *Crcor15* transcript in mandarin fruits represents a species-specific behavior remains to be determined, but similar results were also found in fruits of different *Citrus* species (*Citrus sinensis* L.) (*28*). In other plant species it has been shown that abundance of dehydrin mRNAs and proteins under inducing conditions generally ranged from 0.1% to 5% of the total (*10*), but to our knowledge this is the first report showing such a high accumulation of a dehydrin mRNA in a fruit tissue. Experiments designed to estimate the relative abundance of *Crcor 15* mRNA revealed that it was higher than 1% of total mRNA in the flavedo of mature fruits, whereas the steady-state level in nonstressed leaves was 10–100 times lower (**Figure 2B**). This unusual accumulation of *Crcor15* mRNA in the peel of citrus fruits was observed throughout the whole maturation process (**Figure 3C**). In citrus fruits, maturation lasts for several months and negative values of water potential are usually found in flavedo tissue (*29*–*31*). Moreover, it has been shown that the water flow movement to and within the peel in citrus fruits is very limited, in contrast to the leaves, where their elevated transpiration rate imposes a high water sink (*32*). Therefore, it is conceivable that accumulation of *Crcor15* in the flavedo may be part of the adaptive mechanism developed against dehydration under conditions that favored transpiration and water loss from the outer cell layers of the fruit. This possible explanation would be in agreement with the assumption that accumulation of dehydrins in unstressed plants would reflect the cellular regulation of the osmotic potential to maintain the water balance (*33*). Colmenero-Flores et al. (*34*) have shown accumulation of the PvLEA-18 mRNA and the corresponding protein in the epidermis and vascular cylinders of well-irrigated bean seedlings, correlating with more negative water potential of these tissues. In some woody perennial crops, it has been shown that dehydrins accumulate during chilly winters (*35*, *36*). However, accumulation of *Crcor15* mRNA in the flavedo of Fortune mandarin was already detected in green fruits, growing before the major decline in field temperature (*37*), which suggests that other factors rather than chilling may be responsible for the accumulation of *Crcor15* transcript.

Accumulation of *Crcor15* mRNA in the flavedo of Fortune fruits remained high and constant during storage, irrespective of the temperature regime and of the appearance of chilling injury symptoms (**Figure 3A**). Furthermore, a comparison of the changes in transcript level did not reveal significant differences between the chilling-tolerant (Hernandine) and the chilling-sensitive (Fortune) mandarin cultivars. These results suggest that the expression of *Crcor15* appears not to be related to the chilling tolerance in mandarin fruits. In addition, the heat-conditioning treatment that enhances cold tolerance in Fortune fruits strongly suppressed accumulation of *Crcor15* transcript (**Figure 3C**). Depletion of mRNA levels was not a specific effect on *Crcor15* since a similar response has been also observed in the accumulation of different cold-regulated mRNAs, such as PAL (*7*). After exposure to 2 °C, accumulation of the transcript increased in heat-conditioned fruits of both the chilling-tolerant and the chilling-sensitive mandarin, although it was faster in the former. However, *Crcor15* transcript remained lower in conditioned than in nonconditioned fruits. From these data it is difficult to assign a cold-protective role for *Crcor15* in the flavedo of mandarin fruits. In grapefruit, a hot water and brushing treatment, which enhanced chilling tolerance, avoided the decline of homologous *cor15* mRNA occurring after prolonged storage at 2 °C, suggesting a role for *cor15* in the acquisition of chilling tolerance (*22*).

In contrast to the constitutive accumulation of *Crcor15* mRNA in the flavedo of Fortune mandarin, *Crcor15* transcripts showed a clear and rapid induction by both cold and water stress in leaves (**Figure 4**). Interestingly, cold stress did not produce visual lesions in Fortune leaves but originated rolling, a symptom associated with dehydration. Therefore, we cannot discriminate whether *Crcor15* induction by low temperature may be related to the water deficit component of this stress or reflects a cold-specific induction. Other dehydrin genes isolated in *Citrus*, such as *cor11* and *cor19,* were also cold-induced but repressed (*20*) or weakly stimulated (*15*) by water stress.

In vitro functional analyses have demonstrated antifreeze activity of different plant dehydrins (*13*, *14*), including the $K_3S$

CuCOR19 from citrus (*15*). In this study, we demonstrate that CrCOR15 had both dehydration and freezing protection activity (**Figure 5B,C**). Assays for dehydration and freeze−thawing enzyme inactivation were done with MDH and LDH, respectively, because of their sensitivity to different stresses. A comparison of the protection conferred by CrCOR15 revealed that it was substantially higher than that of BSA (a well-known potent protein stabilizer) (*38*) or RNase A (a stable hydrophilic protein) against dehydration but similar to that of BSA against freezing. Using the same in vitro enzymatic assays, Reyes et al. (*39*) have shown that different dehydrins from *Craterostigma plantagineum* and *A. thaliana* conferred higher protection against controlled desiccation than other nondehydrin LEA proteins. Our results with CrCOR15 corroborate the assigned functional role for dehydrins in the protection against dehydration and indicate that the $K_2S$ dehydrin CrCOR15 may also have a dual function as cryoprotectant. Hara et al. (*40*) demonstrated that the dehydrin CuCOR19, in addition to its cryoprotective activity, also has a potent antioxidant activity, scavenging the free radicals generated under cold stress. CrCOR15 may also function not only as a water-binding protein but also as a stabilizing or cohesive molecule, protecting the integrity of proteins and cellular membranes.

In conclusion, we have identified a $K_2S$ member of the dehydrin gene family in Fortune mandarin that displays in vitro high protective activity against dehydration and moderate protective activity against freezing. *Crcor15* mRNA accumulates at very high levels in the flavedo throughout fruit maturation and during storage at 2 and 12 °C of both a cold-sensitive and a cold-resistant mandarin fruit. Moreover, a heat-conditioning treatment that enhances chilling tolerance suppressed *Crcor15* gene expression, suggesting that this dehydrin is not a main factor associated with the acquisition of chilling tolerance in mandarin fruit. *Crcor15* mRNA was induced by water stress and low temperature in leaves, indicating that in this tissue it may contribute to the protection against these stress conditions.

## ABBREVIATIONS USED

ABA, abscisic acid; BSA, bovine serum albumin; Cor, cold-responsive genes; EDTA, ethylenediaminetetraacetic acid; LDH, lactate dehydrogenase; LEA, late embryogenesis abundant; MDH, malate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

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