Plant Molecular Biology **25**: 693–704, 1994.

Two related low-temperature-inducible genes of *Arabidopsis* encode proteins showing high homology to 14-3-3 proteins, a family of putative kinase regulators

José Antonio Jarillo, Juan Capel, Antonio Leyva, José Miguel Martínez-Zapater and Julio Salinas*
*Departamento de Protección Vegetal, Centro de Investigación y Tecnología, INIA, Ctra. de la Coruña Km. 7, Madrid 28040, Spain (*author for correspondence)*

Received 20 December 1993; accepted in revised form 11 April 1994

Key words: Arabidopsis, cold acclimation, cold-inducible genes, freezing tolerance, 14-3-3 proteins, protein kinase regulators

Abstract

We have isolated two *R*are *C*old-*I*nducible (*RCI1* and *RCI2*) cDNAs by screening a cDNA library prepared from cold-acclimated etiolated seedlings of *Arabidopsis thaliana* with a subtracted probe. RNA-blot hybridizations revealed that the expression of both *RCI1* and *RCI2* genes is induced by low temperature independently of the plant organ or the developmental stage considered. However, *RCI1* mRNA accumulates faster and at higher levels than the *RCI2* one indicating that these genes have differential responsiveness to cold stress. Additionally, when plants are returned to room temperature, *RCI1* mRNA decreases faster than *RCI2*. In contrast to most of the cold-inducible plant genes characterized, the expression of *RCI1* and *RCI2* is not induced by ABA or water stress. The nucleotide sequences of *RCI1* and *RCI2* cDNAs predict two acidic polypeptides of 255 and 251 amino acids with molecular weights of 29 and 28 kDa respectively. The alignment of these polypeptides indicates that they have 181 identical amino acids suggesting that the corresponding genes have a common origin. Sequence comparisons reveal no similarities between the RCI proteins and any other cold-regulated plant protein so far described. Instead, they demonstrate that the RCI proteins are highly homologous to a family of proteins, known as 14-3-3 proteins, which are thought to be involved in the regulation of multifunctional protein kinases.

Introduction

Plants must adjust their physiology to changes in environmental conditions in order to prevent damage and ensure survival. One of the most variable environmental factors to which plants are exposed is temperature. Changes in temperature affect many metabolic processes in the plant cell, and may also modify its structural components [30]. When exposed to low temperatures,

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X74140 (*RCI1*) and X74141 (*RCI2*).

plants can show very different responses. The majority of tropical and subtropical species are chilling-sensitive in that they are injured or killed by exposure to low non-freezing temperatures [41]. In contrast, plants from temperate regions can cold-acclimate and survive by adapting their physiology to low temperatures [41]. Many of these species also increase their freezing tolerance during the period of cold acclimation [41, 51].

The process of cold acclimation has been increasingly studied with the aim of understanding the molecular basis of freezing tolerance [20, 23, 24, 51]. Biochemical analyses have shown that during this process a variety of changes are produced in the plant cells including alterations in lipid, protein and carbohydrate composition [41, 51, 54]. Many of these changes have been described as the result of changes in gene expression [see 24, 55 for recent reviews], and several authors have isolated and characterized genes whose expression is induced by cold although the function of the corresponding proteins, and their putative role in the development of freezing tolerance, remain to be illustrated [see 4, 15, 22, 29, 42, 48, 59, 60 for some recent examples].

Some of the cold-inducible genes encode proteins that share similarity with the RAB/LEA/DHN (response to ABA/late embryogenesis-abundant/dehydration-induced) class of polypeptides [18, 22, 29, 40, 60]. In fact, ABA levels have been shown to increase in certain plants after exposure to both low temperature and drought [6, 58], and most of the cold-inducible genes characterized so far are also induced by water deficit and ABA [22, 25, 38, 39, 40, 45, 48]. This is in agreement with the observation that both drought and exogenous ABA treatments can increase the freezing tolerance of some temperate species, mimicking the effect of low temperature exposure [7, 8]. However, the expression of some *Arabidopsis* cold-regulated genes, such as *LTI78*, *COR6.6*, and *COR47*, is induced by low temperature even in ABA-deficient and -insensitive mutants [17, 47], and some cold-regulated genes do not seem to be inducible by external application of ABA [21, 29, 59, 60]. Taken together, all these results suggest that both ABA-dependent and ABA-independent signal transduction pathways exist between the reception of the cold signal and the resulting gene expression.

Here we report the isolation and characterization of two cDNAs, called *RCI1* and *RCI2* (for *R*are *C*old-*I*nducible cDNAs), corresponding to genes which expression is induced by low temperature but not by ABA or water stress. These genes belong to a small gene family, and encode proteins showing strong similarity with a family of proteins, named 14-3-3 proteins, which were initially characterized in mammals and have been involved in the regulation of multifunctional protein kinases [2]. We show that the expression of the two *RCI* genes is differentially regulated by low temperature, and that this expression is not regulated in a developmental stage- or tissue-specific way. The potential roles of these genes in the development of freezing tolerance through their influence in regulating phosphorylation mechanisms involved in cold signal transduction pathways are discussed.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh., ecotype Columbia, was originally obtained from Chris Somerville (MSU-DOE, USA). Four-day-old etiolated seedlings and 3- to 4-week-old plants were used for the experiments. Plant growth conditions and temperature, ABA, and water stress treatments were as described previously [35]. For etiolated seedlings, seeds were sown, under sterile conditions, in Petri dishes containing mineral nutrient solution [26] solidified with 0.8% agar, and germinated 4 days in the dark. Cold treatment of etiolated seedlings was carried out at 4 °C, in the dark, for different lengths of time. Plants were grown at 22 °C, under constant illumination (100 μmol m^{-2} s^{-1}), in pots containing a mixture of perlite, vermiculite and sphagnum (1:1:1), and irrigated with mineral nutrient solution (see above). Low-temperature treatments of 3- to 4-week-old plants were performed at 4 °C under constant

illumination (50 μmol m^{-2} s^{-1}) for different periods of time. For ABA treatments, plants were sprayed with 100 μM ABA every 6 h and the leaves were collected 24 h after the first treatment. Control treatments were given by spraying water instead of ABA. Water stress was induced by dessicating cut leaves till they had lost 50% of their initial fresh weight.

RNA and DNA extraction

Plant material was frozen in liquid nitrogen, ground using a mortar and pestle, and stored at − 80 °C until use. Total RNA and genomic DNA were isolated as described by Logeman *et al.* [43] and Dellaporta *et al.* [10] respectively. Poly(A)$^+$ RNA was selected by oligo(dT) cellulose (type 7, Pharmacia) affinity chromatography accordingly to manufacturer's protocol. Plasmid DNA was prepared following standard protocols [52].

cDNA library construction and screening

Poly(A)$^+$ RNA isolated from three day cold-acclimated etiolated seedlings of *Arabidopsis* ecotype Columbia, was used to construct a cDNA library in the plasmid pcDNA II using the Librarian Cloning System (Invitrogene, USA). Aproximately 5×10^4 recombinant colonies from the library were screened in duplicate with a subtracted probe. This probe was obtained by subtracting single-stranded cDNA from cold-acclimated etiolated seedlings with photobiotinylated mRNA from unacclimated etiolated seedlings according to the Subtractor System (Invitrogene, USA). Labelling of the subtracted probe was performed by random primer extension with [α-^{32}P]dCTP [16] and the hybridizations were performed as described [52]. To obtain a full-length *RCI1* cDNA clone, a synthetic oligonucleotide (530-AGACCAAGCCTTATCGGGTGAGTA-553) corresponding to the 5′ region of the partial clone was labelled with [α-^{32}P]dCTP using terminal transferase (Promega, USA), as described by Ausubel *et al.* [3], and employed to screen the cDNA library [52]. The *RCI2* cDNA clone was isolated in the same screening.

RNA- and DNA-blot hybridizations

For RNA-blot hybridizations, 1 μg of poly(A)$^+$ RNA was denatured in formamide and formaldehyde, and separated by electrophoresis on 1% formaldehyde gels. For DNA-blot hybridizations, 3 μg of genomic DNA were digested with *Eco* RI, *Bam* HI and *Hin*d III, and separated on 1% agarose gels. Transfer of DNA and RNA to nitrocellulose filters (Schleicher & Schuell) was performed as recommended by the manufacturer. In all cases, the probes used were the 565 and 550 bp DNA fragments corresponding to the 3′ regions of the *RCI1* and *RCI2* clones respectively. They were always labelled by random primer extension [16] with [α-^{32}P]dCTP. Hybridizations were carried out following standard protocols [52].

In order to control the integrity and the amount of poly(A)$^+$ RNA loaded in each lane of the RNA blots, filters were hybridized with a [α-^{32}P]-labelled 20-mer oligo-dT prepared using terminal transferase [3]. The signal hybridizing with the oligo-dT probe in each lane was quantified with respect to the signal found in the control lanes, which were considered as the unit, by using a laser scanning densitometer (Molecular Dynamics, England). After hybridization with specific probes, the intensity of the bands was also quantified by densitometry and corrected for the loading differences detected with the oligo-dT probe.

Sequencing

DNA sequencing was carried out by the dideoxy method [53] using the Sequenase version 2.0 DNA sequencing kit (USB, USA). The nucleotide sequences were determined in both orientations by using synthetic oligonucleotides as primers. The sequence databases provided by EMBL were searched for sequence similarities using the FASTA program [49] from the

Genetics Computer Group's sequence analysis software package, version 7.1 [11]. Multiple sequence alignments and dendrograms were constructed by using the simultaneous alignment and phylogeny CLUSTAL program [27] from the PC/GENE software package (Intelligenetics).

Results

Isolation of RCI1 *and* RCI2 *cDNA clones*

A cDNA library, prepared with poly(A)$^+$ RNA from *Arabidopsis* etiolated seedlings acclimated at 4 °C for three days, was screened with a subtracted cDNA probe (see Materials and methods). Several hybridizing cDNA clones were identified and sequenced. When compared to the EMBL database, the sequence of one clone showed a strong similarity with a class of genes encoding proteins involved in the regulation of multifunctional protein kinases. Because the transcript corresponding to this clone could only be detected when using poly(A)$^+$ RNA, we called it *R*are *C*old-*I*nducible 1 (*RCI1*).

The *RCI1* clone was 565 bp long and hybridized with a cold-inducible 1.2 kb transcript, which was barely detectable in non-acclimated seedlings. As estimated by densitometric analysis, the accumulation of *RCI1* transcript in etiolated seedlings acclimated three days at 4 °C was 6–7-fold the basal levels (Fig. 1). In order to obtain an *RCI1* full-length clone, a synthetic oligonucleotide corresponding to the 5′ end of the *RCI1* partial clone (see experimental procedures) was used as a probe to rescreen the cold-acclimated cDNA library. Twelve clones were identified that hybridized to the probe, and on the basis of their restriction maps, they were pooled in two groups. One group corresponded to *RCI1* clones, whereas the other one represented a new cDNA class that we called *RCI2*.

RNA-blot hybridization with a 550 bp DNA fragment corresponding to the 3′ region of the *RCI2* clone allowed the identification of a single transcript of approximately 1.1 kb. This transcript could be preferentially detected in poly(A)$^+$ RNA

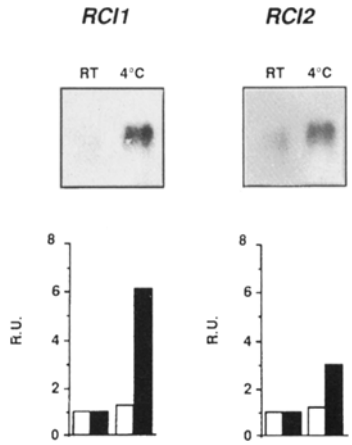

Fig. 1. Accumulation of *RCI1* and *RCI2* mRNAs in cold-acclimated seedlings of *Arabidopsis*. RNA-blot hybridizations of 1 μg poly(A)$^+$ RNA extracted from 7-day-old control etiolated seedlings which had not been acclimated (RT) and from 4-day-old etiolated seedlings which had been acclimated 3 days at 4 °C. The probes used were 565 and 550 bp fragments corresponding to the 3′ regions of the *RCI1* and *RCI2* cDNAs respectively. Histograms represent the relative quantification of the hybridization signals as obtained by densitometric analyses (see Material and methods). White bars represent the relative amount of poly(A)$^+$ RNA loaded in each lane with respect to control lanes. Black bars represent the quantification of the signals obtained after hybridization with the specific probes and correction for the loading differences detected with the oligo-dT probe. R.U., relative units.

prepared from etiolated seedlings acclimated at 4 °C for three days, indicating that, similarly to the *RCI1* gene, *RCI2* was also inducible by low temperature (Fig. 1). A densitometric analysis of the hybridizing bands revealed that the cold-induced expression of *RCI2* gene was about two times lower than that of *RCI1* (Fig. 1).

Nucleotide sequences of RCI1 *and* RCI2 *cDNAs and their predicted amino acid sequence*

The nucleotide sequence of the longest *RCI1* clone contained a long open reading frame (ORF) of 765 nucleotides that would encode a protein of 255 amino acids with an ATG at nucleotide 30 and the poly(A)$^+$ tail starting 192 nucleotides downstream from the predicted stop codon (Fig. 2). The calculated molecular weight of the

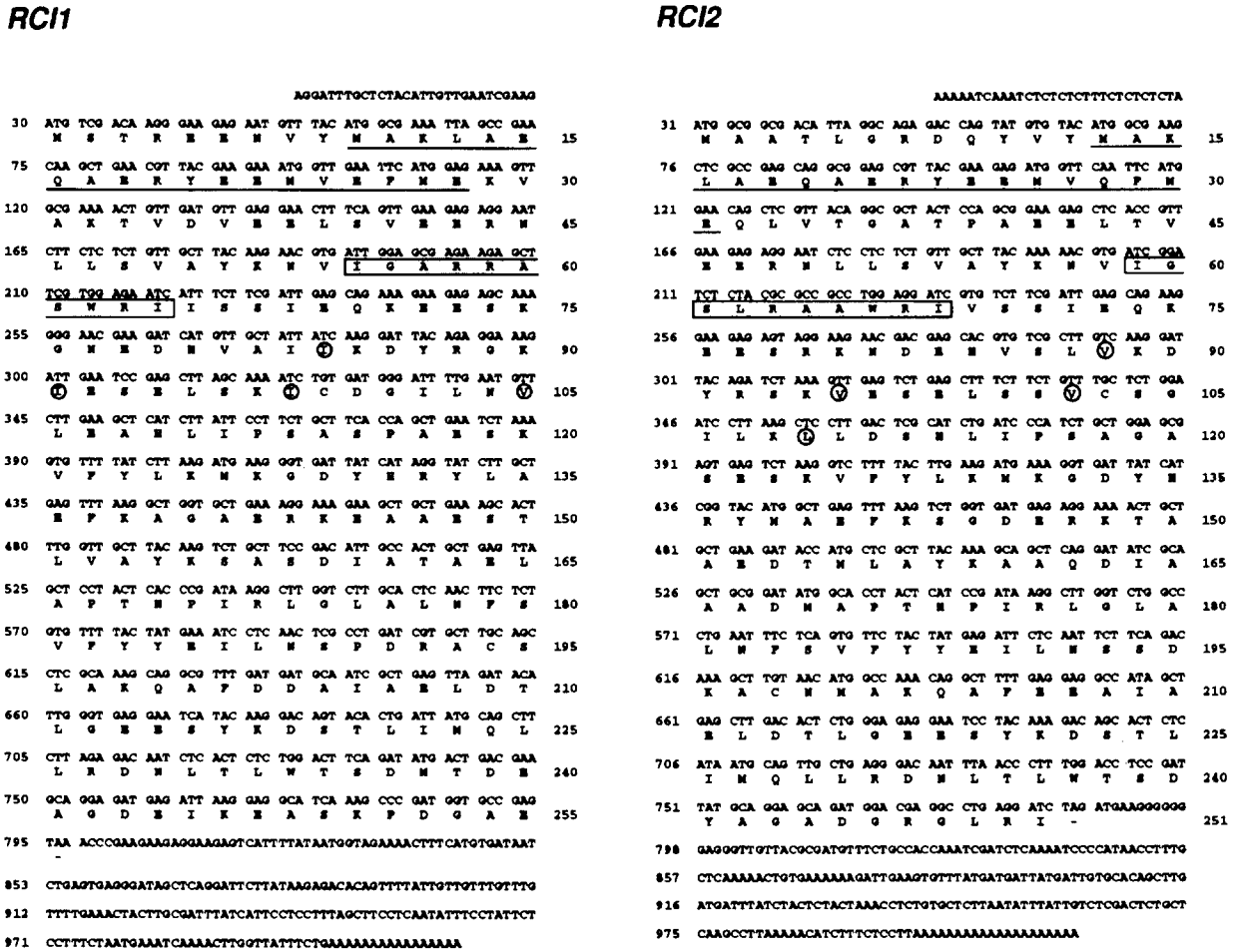

Fig. 2. Nucleotide and deduced amino acid sequences of *RCI1* and *RCI2* cDNAs. Underlined amino acids indicate a putative amphipatic α-helix. Boxed amino acids correspond to potential protein kinase A and C phosphorylation sites as well as to the 'pseudosubstrate' domain for protein kinase C. Circles indicate amino acids residues that are potentially involved in protein-protein interactions.

deduced protein, which we named RCI14A for Rare Cold-Inducible 14-3-3, is 29 kDa. It shows a strong negative charge at both the amino and carboxyl termini and has a calculated isoelectric point of 4.5. Furthermore, the N-terminal region can be modeled as an amphipatic α-helix with distinct negatively charged and hydrophobic faces. Highly acidic regions are a feature common to various eukaryotic transcriptional activators, including GAL4 [19] and VP16 [9]. The predicted protein also presents a sequence containing four isoleucine-valine heptad repeats resembling a leucine zipper. This sequence, however, is not preceded by a basic domain as is characteristic in transcription factors of the bZip family [57]. In addition, RCI14A reveals potential recognition sites for protein kinases A and C in its N-terminal region [36], as well as a 'pseudosubstrate' domain for protein kinase C, which has been proposed to be involved in protein kinase C inhibition [1] (Fig. 2).

The nucleotide sequence of the longest *RCI2* clone (Fig. 2) revealed that *RCI2* differs from *RCI1* mainly in the 5′- and 3′-untranslated regions, the similarity of both clones along the coding region being much higher (72%) (not shown).

These data suggest that the *RCI1* and *RCI2* cDNAs correspond to closely related genes. The *RCI2* cDNA contained an ORF of 753 nucleotides that would encode a protein of 251 amino acids. This ORF starts at the first methionine codon at nucleotide 31, and is followed by a 3′-untranslated region of 237 bases. The predicted molecular weight for the 251 amino acid polypeptide, that we named RCI14B, is 28 kDa with a calculated isoelectric point of 4.7, which are very similar to the values calculated for the RCI14A deduced protein (see above). As in the case of the RCI14A protein, an acidic α-helix forming region, potential recognition sites for protein kinases A and C, a kinase C 'pseudosubstrate' domain, as well as a sequence resembling a leucine zipper are also present in RCI14B (Fig. 2).

The two deduced proteins, RCI14A and RCI14B, are very similar in length and their alignment shows 181 identical and 35 similar amino acids residues (Fig. 3). A computer search of sequence data banks revealed no similarities between the RCI proteins and other plant protein so far described as cold regulated. Instead, a high degree of homology was found between the deduced RCI proteins and a class of proteins, initially identified in mammals, described as kinase-dependent activators of tryptophan and tyrosine hydroxylases (14-3-3 proteins) [31] (Fig. 3). The presence of these proteins has been later shown in yeast and in a wide variety of animal and plant species [5, 12, 28, 32, 37, 44]. Figure 3 also reveals a strong homology among all the plant 14-3-3 proteins so far characterized.

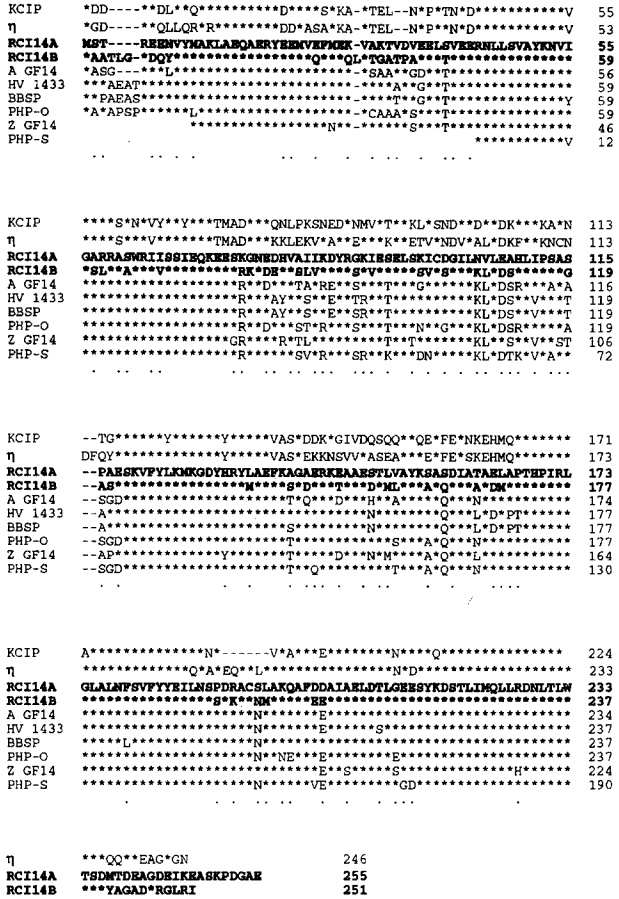

Fig. 3. Amino acid sequence alignment between RCI14A, RCI14B and other 14-3-3/KCIP proteins from various species. The amino acid sequences deduced from *Arabidopsis RCI1* and *RCI2* cDNA sequences shown in Fig. 2 (RCI14A and RCI14B) (bold) were aligned with those deduced from cDNA clones encoding bovine 14-3-3 η chain [31], *Arabidopsis* AGF14 protein [44], barley HV1433 protein [5], rice BBSP protein [37], *Oenothera* PHP-O protein [28], maize ZGF14 protein [12] and spinach PHP-S protein [28]. In addition, the alignment also includes the peptide sequence derived from the ovine kinase C inhibitor protein (KCIP) [1]. Amino acid residues identical to the *Arabidopsis* RCI14A sequence at a given position are indicated by an asterisk. Homologous amino acids are marked by points.

Genomic organization of RCI *genes*

The genomic organization of the *RCI* genes was determined by DNA-blot hybridization of genomic DNA from *Arabidopsis* ecotype Columbia digested with *Eco* RI, *Bam* HI and *Hin*d III. When the 565 bp DNA fragment corresponding to the 3′ region of the *RCI1* clone was used as a probe, it hybridized strongly with a single restriction fragment in each digestion (Fig. 4). However, the presence of 5 to 6 additional hybridizing bands in each digestion suggests that the *RCI1* gene could belong to a small gene family.

DNA-blot hybridization using the 550 bp DNA fragment corresponding to the 3′ region of the

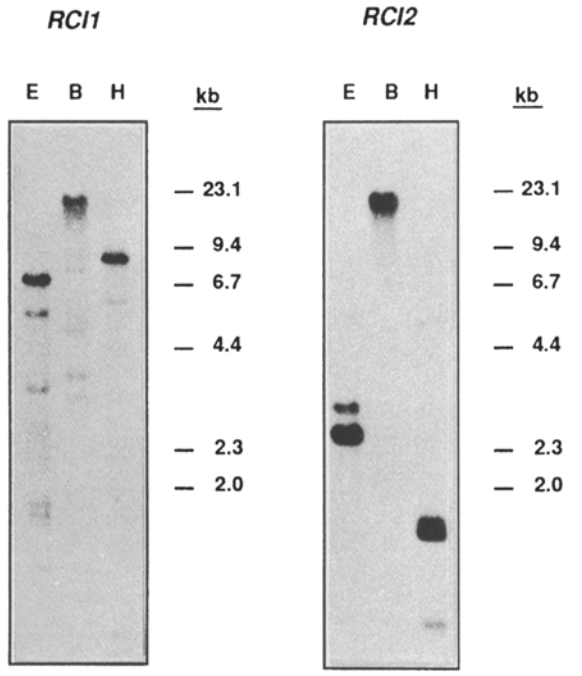

Fig. 4. Genomic organization of *RCI1* and *RCI2*. DNA-blot hybridizations of *Arabidopsis* genomic DNA (3 μg) digested with *Eco* RI (E), *Bam* HI (B) and *Hind* III (H). The probes used were the 3′ fragments of *RCI1* and *RCI2* cDNAs described in Fig. 1. The position of molecular size markers is indicated at right.

RCI2 clone revealed a different pattern of restriction fragments than that revealed by *RCI1* (Fig. 4). Under lower-stringency conditions, additional fragments hybridized with the *RCI2* cDNA probe. Several of these fragments had similar molecular size as some of the restriction fragments detected by the *RCI1* probe (not shown), confirming that both *RCI1* and *RCI2* genes are members of the same gene family.

The expression of RCI1 *and* RCI2 *genes is induced by low temperature in a developmental stage- and organ-independent way*

In order to determine whether the expression of *RCI1* and *RCI2* genes was also induced by low temperature in adult plants, we analyzed their expression in different organs of 4-week-old-*Arabidopsis* plants exposed to 4 °C for three days. Poly(A)$^+$ RNA obtained from leaves, stems and flowers of these plants and of non-acclimated control ones were hybridized with the 3′ cDNA fragments used as probes in previous experiments. In acclimated plants, a transcript homologous to the *RCI1* probe accumulated in all the organs analysed (Fig. 5) in similar amounts as in acclimated etiolated seedlings (Fig. 1). The same result was observed when using the *RCI2* probe (Fig. 5) although, as found in etiolated seedlings (Fig. 1), the accumulation of *RCI2* mRNA was, in all cases, two times lower than that of *RCI1* mRNA (see histograms of Fig. 5). These results

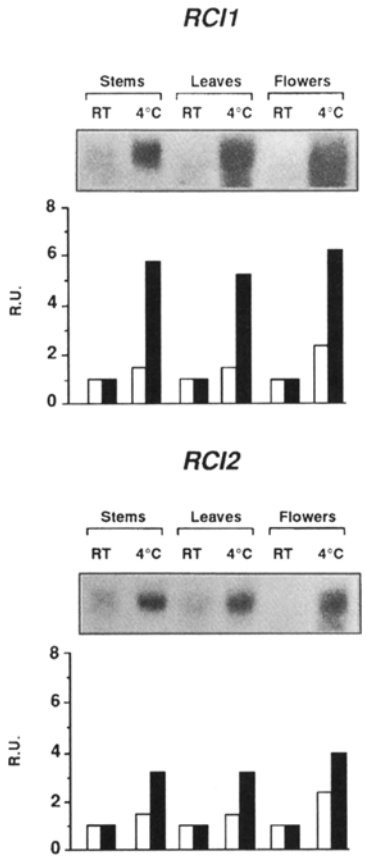

Fig. 5. Expression of *RCI1* and *RCI2* in different organs of *Arabidopsis* in response to low temperature. RNA-blot hybridizations of 1 μg of poly(A)$^+$ RNA extracted from stems, leaves, and flowers of 3–4-week-old plants which had not been acclimated (RT), or acclimated for 3 days at 4 °C (4 °C). The probes used were the 3′ fragments of *RCI1* and *RCI2* cDNAs described in Fig. 1. Histograms represent the relative quantification of the hybridization signals as described in Fig. 1.

indicate that the expression of both *RCI1* and *RCI2* genes is induced by low temperature in a developmental stage- and organ-independent way.

The expression of RCI1 *and* RCI2 *genes is differentially regulated by low temperature, but is not induced by ABA or water stress*

To determine the kinetics of accumulation of *RCI1* and *RCI2* transcripts in response to low temperature, the *RCI1* and *RCI2* cDNA probes mentioned above were used in RNA-blot hybridizations of poly(A)$^+$ RNA from leaves of *Arabidopsis* plants exposed to 4 °C during different times. A first set of experiments showed that an increase of the *RCI1* mRNA level was already detectable after 24 h of low temperature exposure, reaching the maximal level of accumulation between 3 and 7 days (Fig. 6). The *RCI2* mRNA did not accumulate to a detectable level until the third day of cold treatment (Fig. 6). At the time of maximal induction, the *RCI1* mRNA had accumulated up to 6–8 times the basal levels while the *RCI2* mRNA had increased three times over its correspondent control (see histograms of Fig. 6). Additional RNA-blot analyses demonstrated that the transcripts of both *RCI1* and *RCI2* genes remained at elevated levels for as long as the plants were kept in the cold (up to 15 days) (Fig. 6). When plants were returned to room temperature (22 °C) after 7 days of cold acclimation, there was a rapid decline in the levels of *RCI1* transcript after 24 hours (Fig. 6). However, after one day of deacclimation, the levels of *RCI2* transcript were still high compared to the control unacclimated levels, and only began to decrease after the third day of deacclimation (Fig. 6). Taken together, these results indicate that the expression of both *RCI1* and *RCI2* genes is differentially regulated in response to low temperature.

Since most of the cold inducible genes characterized so far are also induced by ABA and water stress, it was of interest to determine whether the expression of the *RCI* genes was responsive to these treatments. This was studied by RNA-blot

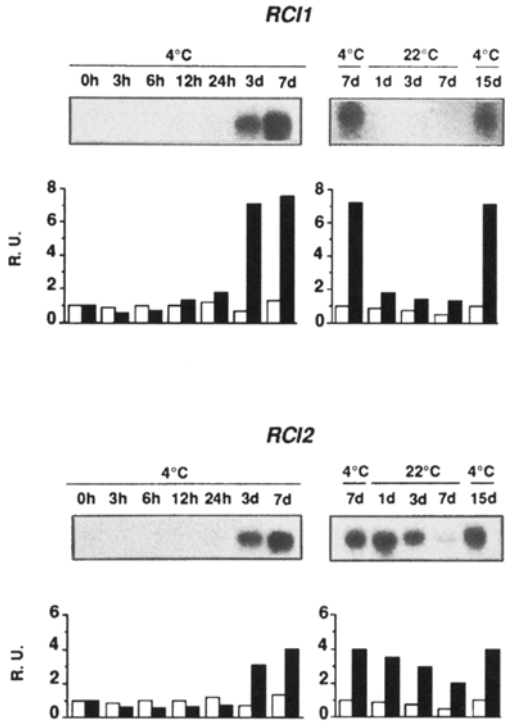

Fig. 6. Expression of *RCI1* and *RCI2* during cold acclimation and deacclimation. RNA-blot hybridizations of 1 μg of poly(A)$^+$ RNA extracted from leaves of 3–4-week-old *Arabidopsis* plants which had been acclimated at 4 °C, or deacclimated at 22 °C, for the indicated time. The plants used in the deacclimation experiments had been cold-acclimated for 7 days. The probes used were the 3′ fragments of *RCI1* and *RCI2* cDNAs described in Fig. 1. Histograms represent the relative quantification of the hybridization signals as described in Fig. 1.

hybridization analyses of poly(A)$^+$ RNA isolated from leaves sprayed with a solution containing ABA (100 μM), or from dehydrated cut leaves that had lost 50% of their initial fresh weight. The probes used were as in previous experiments. The results indicated that the transcript levels of *RCI1* and *RCI2* genes did not increase upon application of ABA or water stress (Fig. 7). That these results were an accurate reflection of the responsiveness of both genes to ABA and water stress, and not a consequence of possible inherent limitations of the experimental protocols, was confirmed by monitoring, in the same membranes, the transcript levels for the *COR47* gene, which have been shown to be induced by ABA and water stress [25] (Fig. 7).

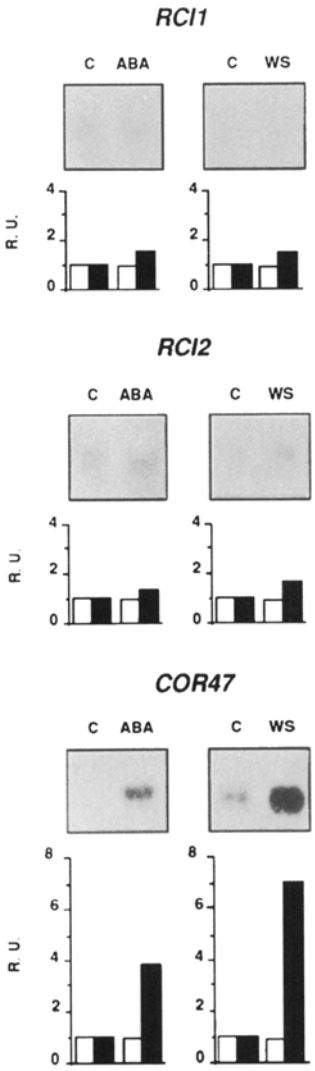

Fig. 7. Expression of *RCI1* and *RCI2* in response to ABA and water stress. RNA-blot hybridizations of 1 μg of poly(A)$^+$ RNA extracted from leaves of 3–4-week-old *Arabidopsis* plants which had been sprayed with 100 μM ABA (ABA) or cut and dehydrated till loosing 50% of their initial fresh weight (WS). Control leaves were sprayed with water for ABA experiments, while untreated leaves were used for water stress experiments. The probes used to detect *RCI1* and *RCI2* transcripts were the ones described in Fig. 1. To detect the *COR47* transcript, the probe used was a cDNA clone homologous to that described by Gilmour *et al.* [18]. Histograms represent the relative quantification of the hybridization signals as described in Fig. 1.

Discussion

One approach that can provide information on the molecular mechanisms responsible for the development of freezing tolerance in plants consists in isolating and characterizing genes specifically expressed during cold acclimation. With this aim, we used a subtracted probe to isolate cDNA clones corresponding to genes expressed in etiolated *Arabidopsis* seedlings after three days of exposure to 4 °C. We reasoned that by choosing etiolated seedlings as the RNA source, and by employing a subtracted probe enriched in cold-induced mRNAs, we would eliminate other environmental conditions that have an effect on low-temperature responsiveness [20, 35, Leyva *et al.*, in preparation), and increase the probability of isolating cDNAs corresponding to rare low-abundant cold-inducible mRNAs difficult to detect by differential screening.

In this paper, we present the isolation and characterization of two of these cDNAs, *RCI1* and *RCI2*, both of which correspond to genes that are inducible by low temperature. Our results demonstrate that the cold-inducible expression of *RCI1* and *RCI2* genes is not developmental stage-specific, since they are induced in etiolated seedlings as well as in adult plants. Moreover, this expression is not organ-specific because *RCI1* and *RCI2* mRNAs accumulate, to similar amounts, in leaves, stems and flowers. Although both genes encode very similar protein products, their expression seem to be differentially regulated by low temperature since, during cold acclimation, the *RCI1* mRNA accumulates to a higher level than the *RCI2* mRNA. Furthermore, when plants are returned to control temperature the two *RCI* transcripts show different rates of decline. The *RCI1* mRNA begins to decrease after 24 h of deacclimation, while the *RCI2* mRNA was still detectable after 7 days of deacclimation. The differential expression manifested by *RCI1* and *RCI2* in response to low temperature might reflect differences in their transcriptional regulation and/or in the stability of their mRNAs during and after the stress. Differential expression patterns have also been recently described for two other pairs of related genes (*LTI65* and *LTI78*, and *KIN1* and *KIN2*) that are cold-inducible in *Arabidopsis* [39, 48], and may indicate that different genes belonging to the same

gene family can develop different roles in cold acclimation and/or freezing tolerance through differences in their expression patterns.

When compared to cold-regulated genes described so far [see 4, 15, 22, 29, 42, 48, 59, 60 and references therein], *RCI1* and *RCI2* are more slowly induced, reach lower final levels and need more time to decay after deacclimation. Furthermore, the expression of these genes is not induced by ABA or water stress, two treatments that have been shown to increase plant freezing tolerance [7, 8]. These results support the idea proposed by Gilmour and Thomashow [17] and Nordin *et al.* [47] that both ABA-dependent and ABA-independent signal transduction pathways exist between the signal produced by low temperature and the resulting gene expression.

RCI14A and RCI14B are highly homologous to the 14-3-3 proteins. The high level of sequence conservation existing among these proteins suggests an important role for them in a broad range of biological systems. A computer-generated dendrogram comparing the plant 14-3-3 proteins to representatives of the mammalian and yeast 14-3-3 isoforms (Fig. 8) reveals that *Arabidopsis* proteins are more related to the other plant known sequences than to the mammalian isoforms. However, the ε mammalian isoform is closer to the yeast and plant 14-3-3 proteins than to the other mammalian isoforms, suggesting that, as proposed by Aitken *et al.* [2], some members of the 14-3-3 proteins family evolved and diverged before the separation of plants and animals.

Several functions have been proposed for the 14-3-3 proteins, based on their distribution in mammalian tissues and their *in vitro* activity [2]. Currently, the general thinking is that the 14-3-3 proteins may function as regulators in signal transduction/phosphorylation mechanisms [2]. The rise in *RCI1* and *RCI2* transcript levels observed in *Arabidopsis* in response to low-temperature treatment suggests a role for RCI14A and RCI14B proteins in the ABA-independent signal transduction pathway of cold response. The RCI proteins might, for example, modulate the phosphorylation/dephosphorylation of other proteins involved in the response to low temperature

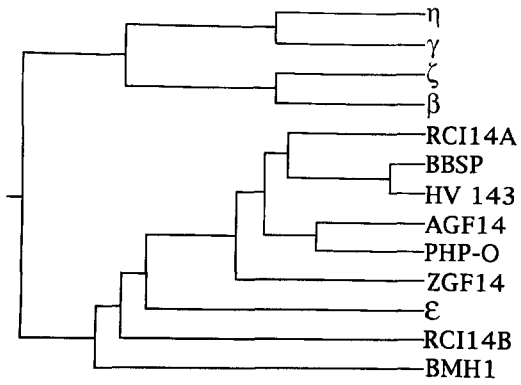

Fig. 8. Sequence relationships between different plant and mammalian 14-3-3 proteins. The amino acid deduced sequences of different plant and mammalian 14-3-3 proteins were aligned by using the simultaneous alignment and phylogeny CLUSTAL program. The sources of the 14-3-3 sequences are as follows: bovine η [31], bovine γ and β [33], bovine ζ [34], sheep ε [50], *Arabidopsis* RCI14A and RCI14B (this work), *Arabidopsis* AGF14 [44], rice BBSP [37], barley HV1433 [5], *Oenothera* PHP-O [28], maize ZGF14 [12] and yeast BMH1 [56]. The published partial sequences of 14-3-3 homologues are excluded from this alignment.

[13], acting at the level of protein kinase regulation. On this way, the involvement of protein kinases in the response of cell cultures to low temperature has been demonstrated [46]. Changes in protein phosphorylation/dephosphorylation have also been shown to be involved in many other signal transduction pathways, like, for example, the response to fungal elicitors [14]. The fact that the expression of *HV1433* gene from barley is induced following inoculation with fungal pathogen *Erysiphe graminis* [5] suggests that plant 14-3-3 proteins might act as regulators of phosphorylation processes occurring in response to different kinds of stress. If different members of the family are differentially involved in specific signal transduction pathways remains to be known. On the other hand, maize [12] and *Arabidopsis* GF14 [44] have been found to be associated to the G-box binding complex. Although the significance of this association is still unclear, it will be interesting to test whether RCI14A and RCI14B proteins can also associate to the G-box binding complex. Detailed physiological and biochemical characterization of the purified RCI proteins, together with the production of trans-

genic plants expressing sense and antisense *RCI* mRNAs will help to elucidate the function of these proteins during cold acclimation.

Acknowledgements

This work was supported by the Spanish Ministry of Agriculture Research Project INIA 9062 and E.E.C. PTP project BIOT CT93–0400.

References

1. Aitken A, Ellis CA, Harris A, Sellers LA, Toker A: Kinase and neurotransmitters. Nature 344: 594 (1990).
2. Aitken A, Collinge DB, van Heusden BPH, Isobe T, Roseboom PH, Rosenfeld G, Soll J: 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. Trends Biochem Sci 17: 498–501 (1992).
3. Ausubel FM, Brent R, Kingston RE, Moore DD, Seideman JG, Smith JA, Struhl K: Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Interscience, New York (1987).
4. Binh LT, Oono K: Molecular cloning and characterization of genes related to chilling tolerance in rice. Plant Physiol 99: 1146–1150 (1992).
5. Brandt J, Thordal-Christensen H, Vad K, Gregersen PL, Collinge DB: A pathogen-induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. Plant J 2: 815–820 (1992).
6. Chen HH, Li PH, Brenner ML: Involvement of abscisic acid in potato cold acclimation. Plant Physiol 71: 362–365 (1983).
7. Chen THH, Gusta LV: Abscisic acid induced freezing resistance in cultured plant cells. Plant Physiol 73: 71–75 (1983).
8. Cloutier Y, Siminovitch D: Correlation between cold- and drought-induced frost hardiness in winter wheat and rye varieties. Plant Physiol 69: 256–258 (1982).
9. Cress WD, Triezenberg JJ: Critical structural elements of the VP16 transcriptional activation domain. Science 251: 87–90 (1991).
10. Dellaporta SL, Wood J, Hicks JB: A plant DNA minipreparation: version II. Plant Mol Biol Rep 1: 19–21 (1983).
11. Devereux J, Haerberli P, Smithies O: A comprehensive set of sequence-analysis programs for the VAX. Nucl Acids Res 12: 387–395 (1984).
12. de Vetten NC, Lu G, Ferl RJ: A maize protein associated with the G-box binding complex has homology to brain regulatory proteins. Plant Cell 4: 1295–1307 (1992).
13. Dhindsa RS, Monroy A, Wolfraim LA, Dong G: Signal transduction and gene expression during cold acclimation of alfalfa. In: Li PH, Christersson L (eds) Advances in Plant Cold Hardiness, pp. 57–71. CRC Press, Boca Raton, FL (1993).
14. Dietrich A, Mayer JE, Hahlbrock K: Fungal elicitor triggers rapid transient, and specific protein phosphoprylation in parsley cell suspension cultures. J Biol Chem 265: 6360–6368 (1990).
15. Dunn MA, Hughes MA, Zhang L, Pearce RS, Quigley AS, Jack PL: Nucleotide sequence and molecular analysis of the low temperature induced cereal gene *BLT4*. Mol Gen Genet 229: 389–394 (1991).
16. Feinberg AP, Vogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13 (1983).
17. Gilmour SJ, Thomashow MF: Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol 17: 1233–1240 (1991).
18. Gilmour SJ, Artus NN, Thomashow MF: cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol Biol 18: 13–21 (1992).
19. Giniger E, Ptashne M: Transcription in yeast activated by a putative amphipatic α-helix linked to a DNA binding unit. Nature 330: 670–672 (1987).
20. Graham D, Patterson BD: Response of plants to low, nonfreezing temperatures: proteins, metabolism and acclimation. Annu Rev Plant Physiol 33: 347–372 (1990).
21. Grossi M, Cativelli L, Terzi V, Stanca AM: Modification of gene expression induced by ABA in relation to drought and cold stress in barley shoots. Plant Physiol Biochem 30: 97–103 (1992).
22. Guo W, Ward RW, Thomashow MF: Characterization of a cold-regulated wheat gene related to *Arabidopsis cor47*. Plant Physiol 100: 915–922 (1992).
23. Guy CL, Niemi KJ, Brambl R: Altered gene expression during cold acclimation of spinach. Proc Natl Acad Sci USA 82: 3673–3677 (1985).
24. Guy CL: Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol 41: 187–223 (1990).
25. Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF: Molecular cloning and expression of *cor* (cold regulated) genes in *Arabidopsis thaliana*. Plant Physiol 93: 1246–1252 (1990).
26. Haughn G, Somerville C: Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet 204: 430–434 (1986).
27. Higgins DG, Sharp PM: CLUSTAL: a package for performing multiple sequence alignments on microcomputer. Gene 73: 237–244 (1988).
28. Hirsch S, Aitken A, Bertsch U, Soll J: A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor. FEBS Lett 296: 222–224 (1992).
29. Houde M, Danyluk J, Laliberté JF, Rassart E, Dhindsa RS, Sarhan F: Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol 99: 1381–1387 (1992).
30. Hughes MA, Dunn MA: The effect of temperature on

plant growth and development. Biotechnol Genet Eng Rev 8: 161–187 (1990).
31. Ichimura T, Isobe T, Okuyama T, Takahashi N, Araki K, Kuwano R, Takahashi Y: Molecular cloning of a cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. Proc Natl Acad Sci USA 85: 7084–7088 (1988).
32. Ichimura T, Sugano H, Kuwano R, Sunaya T, Okuyama T, Isobe T: Widespread distribution of the 14-3-3 protein in vertebrate brains and bovine tissues: correlation with the distributions of calcium-dependent protein kinases. J Neurochem 56: 1449–1451 (1991).
33. Isobe T, Ichimura T, Sunaya T, Okuyama T, Takahashi N, Kuwano R, Takahashi Y: Distinct forms of the protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. J Mol Biol 217: 125–132 (1991).
34. Isobe T, Hiyane Y, Ichimura T, Okuyama T, Takahashi N, Nakajo S, Nakayama K: Activation of protein kinase C by the 14-3-3 protein homologous with exo1 protein that stimulate calcium-dependent exocytosis. FEBS Lett 308: 121–124 (1992).
35. Jarillo JA, Leyva A, Salinas J, Martínez-Zapater JM: Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling tolerant plant. Plant Physiol 101: 833–837 (1993).
36. Kennelly PJ, Krebs EG: Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J Biol Chem 266: 15555–15558 (1991).
37. Kiduo S, Umeda M, Kato A, Uchimiya H: Isolation and characterization of a rice cDNA similar to the bovine brain-specific 14-3-3 protein gene. Plant Mol Biol 21: 191–194 (1993).
38. Kurkela S, Franck M: Cloning and characterization of a cold- and ABA-induced *Arabidopsis* gene. Plant Mol Biol 15: 137–144 (1990).
39. Kurkela S, Borg-Franck M: Structure and expression of *Kin2*, one of two cold- and ABA-induced genes of *Arabidopsis thaliana*. Plant Mol Biol 19: 689–692 (1992).
40. Lang V, Palva ET: The expression of a rab-related gene, *rab 18*, is induced by abscisic acid during the cold acclimation process of *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 20: 951–962 (1992).
41. Levitt J: Responses of plants to environmental stresses: chilling, freezing and high temperatures stresses. In: Kozlowsky TT (ed) Physiological Ecology: A Series of Monographs, Texts and Treatises, vol 1, 2nd ed., pp. 23–64. Academic Press, New York (1980).
42. Lin C, Thomashow MF: DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the COR 15 polypeptide. Plant Physiol 99: 519–525 (1992).
43. Logeman J, Schell J, Willmitzer L: Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16–20 (1987).
44. Lu G, DeLisle AJ, de Vetten NC, Ferl RJ: Brain proteins in plants: an *Arabidopsis* homolog to neurotransmitter pathway activators is part of a DNA binding complex. Proc Natl Acad Sci USA 89: 11490–11494 (1992).
45. Mohapatra SS, Poole RJ, Dhindsa RS: Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa. Plant Physiol 87: 468–473 (1988).
46. Monroy AF, Sarhan F, Dhindsa RS: Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. Plant Physiol 102: 1227–1235 (1993).
47. Nordin K, Heino P, Palva ET: Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 16: 1061–1071 (1991).
48. Nordin K, Vahala T, Palva ET: Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 21: 641–653 (1993).
49. Pearson WR, Lipman DJ: Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 2444–2448 (1988).
50. Roseboom PH, Weller JL, Namboodiri MAA, Toker A, Aitken A, Klein DC: 14-3-3 proteins: isolation, cloning and distribution of the ε isoform. FASEB J 6: 1516 (1992).
51. Sakai A, Larcher W: Frost Survival of Plants. Responses and Adaptations to Freezing Stress. Springer-Verlag, Berlin (1987).
52. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
53. Sanger F, Nicklen S, Coulsen AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467 (1977).
54. Steponkus PL, Lynch DV: Freeze/thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. J Bioenerg Biomembr 21: 21–41 (1989).
55. Thomashow MF: Molecular genetics of cold acclimation in higher plants. Adv Genet 28: 99–131 (1990).
56. van Heusden GPH, Wenzel TJ, Lagendijk EL, Steensma HY, van der Berg JA: Characterization of the yeast *BMH1* gene encoding a putative protein homologous to mammalian protein kinase II activators and protein kinase C inhibitors. FEBS Lett 302: 145–150 (1992).
57. Vinson CR, Sigler PB, McKnight SL: Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science 246: 911–916 (1989).
58. Walton DC: Biochemistry and physiology of abscisic acid. Annu Rev Plant Physiol 31: 453–489 (1980).
59. Weretilnyk E, Orr W, White TC, Iu B, Singh J: Characterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. Plant Physiol 101: 171–177 (1993).
60. Wolfraim LA, Langis R, Tyson H, Dhindsa RS: cDNA sequence, expression and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicago sativa*) cells. Plant Physiol 101: 1275–1282 (1993).