Plant Physiol. (1997) 115: 569–576

Two Homologous Low-Temperature-Inducible Genes from Arabidopsis Encode Highly Hydrophobic Proteins[1]

Juan Capel[2], José A. Jarillo[3], Julio Salinas*, and José M. Martínez-Zapater

Departamento de Biología Molecular y Virología Vegetal, Centro de Investigación y Technologia, Instituto Nacional de Investigaciones Agrarias, Carretera de la Coruña, Km. 7, 28040 Madrid, Spain

We have characterized two related cDNAs (*RCI2A* and *RCI2B*) corresponding to genes from *Arabidopsis thaliana*, the expression of which is transiently induced by low, nonfreezing temperatures. *RCI2A* and *RCI2B* encode small (54 amino acids), highly hydrophobic proteins that bear two potential transmembrane domains. They show similarity to proteins encoded by genes from barley (*Hordeum vulgare* L.) and wheatgrass (*Lophophyrum elongatum*) that are regulated by different stress conditions. Their high level of sequence homology (78%) and their genomic location in a single restriction fragment suggest that both genes originated as a result of a tandem duplication. However, their regulatory sequences have diverged enough to confer on them different expression patterns. Like most of the cold-inducible plant genes characterized, the expression of *RCI2A* and *RCI2B* is also promoted by abscisic acid (ABA) and dehydration but is not a general response to stress conditions, since it is not induced by salt stress or by anaerobiosis. Furthermore, low temperatures are able to induce *RCI2A* and *RCI2B* expression in ABA-deficient and -insensitive genetic backgrounds, indicating that both ABA-dependent and -independent pathways regulate the low-temperature responsiveness of these two genes.

Many plant species from temperate regions are able to increase their freezing tolerance after being exposed to low, nonfreezing temperatures. This adaptive process, known as cold acclimation (Levitt, 1980; Sakai and Larcher, 1987), involves a number of biochemical and physiological changes, including increased levels of soluble sugars, proteins, amino acids, and organic acids, as well as the modification of membrane lipid composition (Thomashow, 1994). Many of these changes have been shown to be regulated through changes in gene expression, and a number of genes, the expression of which is induced by low temperatures, have been isolated and characterized from a wide range of both dicot and monocot species (for review, see Thomashow, 1994, and Hughes and Dunn, 1996). The precise role that such changes in gene expression play in the cold-acclimation process and the biological function for the majority of the low-temperature-responsive genes remain to be determined.

Although the expression of some cold-inducible genes seems to be specifically regulated by low temperature, in most cases it is also regulated by water stress and ABA (Hughes and Dunn, 1996). This is in agreement with the observation that both drought and exogenous ABA treatments can mimic the effects of low temperatures and increase the freezing tolerance of several temperate species (Cloutier and Siminovitch, 1982; Chen and Gusta, 1983; Mäntylä et al., 1995). Moreover, it has been shown that the endogenous levels of ABA increase in response to low temperature and water stress (Chen et al., 1983; Lang et al., 1994). These results, together with the fact that Arabidopsis ABA-deficient (*aba*) mutants are impaired in cold acclimation (Thomashow, 1994), indicate that ABA is involved in the response. However, the expression of several cold-responsive genes is induced by low temperatures in *aba* and ABA-insensitive (*abi*) mutants, demonstrating that they do not require ABA for this induction (Shinozaki et al., 1996). Taken together, these observations demonstrate that at least two separate regulatory pathways, one ABA dependent and another ABA independent, function in gene expression during cold stress. Additionally, other cold-regulated genes are also induced by salt stress (Kurkela and Borg-Franck, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994), pathogen infection (Molina and Garcia-Olmedo, 1993), hypoxia (Jarillo et al., 1993), and light stress (Leyva et al., 1995).

Most of the low-temperature-inducible genes have been isolated by differentially screening cDNA libraries from cold-acclimated plants. Since this technique identifies cDNAs corresponding to transcripts that are present at elevated levels in the mRNA populations, most of the isolated genes are induced at high levels by low temperature. In an attempt to isolate genes induced at medium-low levels during cold acclimation that could have a role in regulation or signal transduction, we screened a cDNA library from cold-acclimated seedlings of Arabidopsis with a subtracted probe enriched in cold-induced transcripts. In a previous report (Jarillo et al., 1994) we described the isolation and molecular characterization of two Rare Cold Inducible cDNAs, *RCI1A* and *RCI1B* (formerly named *RCI1* and *RCI2*, respectively), corresponding to genes encoding

[1] This work was supported by INIA grant no. SC94-124 and European Union grant no. BIOT CT93-0400. J.A.J. was supported by a fellowship from the INIA.

[2] Present address: Departamento de Genética, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad de Almería, La Cañada s/n, 04120 Almeria, Spain.

[3] Present address: Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104.

* Corresponding author; e-mail salinas@inia.es; fax 34–1–357–3107.

Abbreviation: ORF, open reading frame.

14-3-3 proteins, which are thought to be involved in the regulation of multifunctional protein kinase activity (Aitken et al., 1992).

In this work we present the isolation and characterization of *RCI2A* and *RCI2B*, two cDNAs corresponding to a new, small family of Arabidopsis genes that are induced in response to low temperatures. Both genes encode small, highly hydrophobic proteins containing two potential transmembrane domains and showing a high degree of homology. We show here that the expression of *RCI2A* and *RCI2B* is transiently regulated during the cold-acclimation process independently from the developmental stage of the plant. However, this expression is differentially regulated in different organs of Arabidopsis. Furthermore, RCI2A and RCI2B expression is also up-regulated by ABA and water stress, and by low temperature in *aba1* and *abi1* mutants of Arabidopsis, indicating that both ABA-dependent and -independent pathways regulate the low-temperature responsiveness of *RCI2A* and *RCI2B* genes. Based on these results, the potential roles of *RCI2A* and *RCI2B* in cold acclimation and freezing tolerance are discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (L.) Heyhn., ecotype Columbia (Col), was purchased from Lehle Seeds (Tucson, AZ). The ecotype Landsberg *erecta* (*Ler*) and the *aba1* and *abi1* mutants were kindly provided by Maarten Koornneef (Wageningen Agricultural University, The Netherlands). Four-day-old etiolated seedlings, 4-week-old plants, and 8-week-old plants were used for the experiments. For etiolated seedlings, seeds were sown under sterile conditions in Petri dishes containing mineral nutrient solution (Haughn and Somerville, 1986) solidified with 0.8% agar, and germinated for 4 d in the dark at room temperature. Cold treatment of etiolated seedlings was carried out at 4°C in the dark for different periods of time. Plants were grown at 22°C under long-day photoperiods (16 h of cool-white fluorescent light, photon flux of 70 μmol m^{-2} s^{-1}) in pots containing a mixture of perlite, vermiculite, and sphagnum (1:1:1), and irrigated with water and mineral nutrient solution (Haughn and Somerville, 1986) once a week. Low-temperature treatments of plants were performed at 4°C under the same light and photoperiodic conditions.

All treatments were performed on 4-d-old etiolated seedlings. For ABA treatments, seedlings were sprayed with 100 μM ABA and harvested 3 h later. The ABA stock solution was prepared in DMSO, and control treatments were given by spraying with water containing the same final concentration of the ABA solvent. Water stress was induced by transferring the seedlings to new Petri dishes without agar medium and allowing them to lose 50% of their fresh weight. Salt stress was performed by transferring the seedlings to Petri dishes containing the agar medium plus 250 mM NaCl and maintaining the seedlings in this medium for 24 h. Anaerobiosis was produced by placing the Petri dishes containing the seedlings on an N_2 atmosphere for 24 h. After the treatments, the seedlings were immediately frozen in liquid N_2 and stored at −80°C until their use. All treatments and manipulations with etiolated seedlings were performed under a green safelight (Safe Light no. 3, Kodak).

Molecular Biology Methods

Genomic DNA extractions were carried out following the method of Dellaporta et al. (1983). The construction and screening of the cDNA library has been previously described (Jarillo et al., 1994). Restriction digestions, cloning, and DNA-blot hybridizations were performed following standard protocols (Sambrook et al., 1989). To obtain full-length cDNA clones from the partial clones originally isolated, we used the *Ampli*FINDER RACE kit (Clontech, Palo Alto, CA). DNA sequencing was carried out by the dideoxy method (Sanger et al., 1977) using the Sequenase 2.0 kit (United States Biochemical) in the presence of [α-^{35}S]dATP. The nucleotide sequences were determined in both orientations. The sequence databases were searched for sequence similarities using the BLAST program (Altschul et al., 1990). Comparison of the nucleotide and amino acid sequences were performed with the software package PC/Gene 6.5.

Total RNA was isolated from etiolated seedlings and different plant organs following the method of Nagy et al. (1988). Poly(A)$^+$ RNA was purified from total RNA using the Quick-Prep mRNA purification kit (Pharmacia). RNA-blot hybridizations were performed following standard protocols (Sambrook et al., 1989). For DNA- and RNA-blot hybridizations, DNA probes were radioactively labeled with [α-^{32}P]dCTP using the Megaprime kit (Amersham). The *RCI2A*-specific probe that we used was a cDNA fragment containing 10 nucleotides of the ORF and the complete 3′ noncoding region. For *RCI2B*, we used the 3′ noncoding region of the transcript as a specific probe. For probes, we also used a 2.5-kb fragment from the Arabidopsis *ADH* gene (Chang and Meyerowitz, 1986) and the *RD29A* coding region (Yamaguchi-Shinozaki and Shinozaki, 1993).

To control the integrity and the amount of poly(A)$^+$ RNA loaded in each lane of the RNA blots, filters were hybridized with an [α-^{32}P]-labeled 20-mer oligo-dT that was prepared using terminal transferase (Sambrook et al., 1989). The hybridization signals were quantified by using a Howteck Scanmaster 3+ scanner and Bioimage 3.3 software from Millipore. Poly(A)$^+$ RNA samples from each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

RESULTS

Isolation and Molecular Characterization of *RCI2A* and *RCI2B*, Two cDNAs Corresponding to Rare Cold-Inducible Genes

RCI2A was isolated by screening a cDNA library prepared from cold-acclimated etiolated seedlings of Arabidopsis, with a subtracted cDNA probe enriched in cold-induced transcripts. RNA-blot experiments with poly(A)$^+$

RNA extracted from cold-acclimated and nonacclimated etiolated seedlings indicated that *RCI2A* specifically hybridized with a cold-induced transcript of approximately 500 nucleotides (data not shown). Sequence analyses showed that the *RCI2A* clone only contained 265 bp of the 3′ region, as indicated by the presence of a poly(A)$^+$ tail. Using the *Ampli*FINDER RACE kit we identified a longer clone that contained an ORF overlapping the one of the 265-bp partial clone, as well as a 5′ noncoding region, and that corresponded to a full-length *RCI2A* cDNA.

Comparison of the *RCI2A* full-length sequence to the expressed sequence tags present in the dbEST database uncovered the existence of one highly homologous expressed sequence tag (31D10). RNA-blot experiments using a 3′ noncoding, region-specific probe of 31D10 allowed the identification of a single transcript that had approximately 500 bp and was preferentially detected in poly(A)$^+$ RNA isolated from cold-acclimated etiolated seedlings (data not shown). These results indicated that 31D10 corresponded to another gene we called *RCI2B*, the mRNA of which also accumulated in response to low temperature.

The nucleotide sequence of the *RCI2A* cDNA contained an ORF of 162 nucleotides that would encode a protein of 54 amino acids with an ATG codon at nucleotide 26, and the poly(A)$^+$ tail starting 234 nucleotides downstream from the stop codon (Fig. 1). The estimated molecular mass of the predicted polypeptide, RCI2A, is 5.9 kD with a pI of 6.2. The nucleotide sequence of the *RCI2B* cDNA differed from *RCI2A* mainly in the 5′ and 3′ untranslated regions, the similarity of both cDNAs along the coding region being much higher (84%). *RCI2B* cDNA also contains an ORF of 162 nucleotides, which is preceded by a 5′ untranslated fragment of 53 nucleotides and followed by a 3′ untranslated region of 165 nucleotides (Fig. 1). The RCI2B protein would also have 54 amino acids, which is equivalent to a molecular mass of 6.0 kD, but a pI of 8.2.

RCI2A and RCI2B proteins are the same length, and their sequence alignment revealed that 78% of their amino acids are identical and 96% are similar (Fig. 2A). These proteins have a compositional bias for Leu (17%) and Ile (17%), which account for more than 30% of the residues and do not contain Gln, Asn, Asp, or His residues. Hydropathy analyses (Kyte and Doolittle, 1982) indicated that they are highly hydrophobic (Fig. 2B), and functional-domains analyses (Klein et al., 1985) suggested that RCI2A and RCI2B contain two potential transmembrane domains covering most of their length (Fig. 2A). In addition, comparison of RCI2A and RCI2B with the SBASE, a database of protein domains (Pongor et al., 1992), uncovered N-terminal peptides with consensus features found in signal peptides of different transmembrane proteins (Fig. 2A).

A computer search of sequence databanks revealed that RCI2A and RCI2B are highly similar to two proteins, BLT101 from barley (*Hordeum vulgare* L.) and ESI3 from wheatgrass (*Lophopyrum elongatum* L.), which are encoded by genes that are inducible by different abiotic stresses (Goddard et al., 1993; Gulick et al., 1994). BLT101 and ESI3 share identical sequences, although their comparison with RCI2A showed that 68% of their amino acids are identical and 93% are similar (Fig. 2A). The comparison of BLT101 and ESI3 with RCI2B revealed that 61% of their amino acids are identical and 98% are similar (Fig. 2A).

RCI2A

```
                    AGAGTAAAGAAGATAAAAAACACAATTTGA   30

AGCTTTTATAATATTTTCTCAGAAACTTTCAAAGAGCTTAGAAAA   75

ATGAGTACAGCTACTTTCGTTGATATTATTATCGCCATCCTCTTG  120
 M  S  T  A  T  F  V  D  I  I  I  A  I  L  L   15

CCTCCACTCGGTGTCTTTCTCAGATTTGGTTGCGGGGTTGAGTTT  165
 P  P  L  G  V  F  L  R  F  G  C  G  V  E  F   30

TGGATATGTTTGGTTTTGACGCTACTTGGGTATATTCCTGGGATC  210
 W  I  C  L  V  L  T  L  L  G  Y  I  P  G  I   45

ATATACGCCATTTATGTCCTCACCAAATGATTTACCATCTATCAT  255
 I  Y  A  I  Y  V  L  T  K                      54

CATCTCCTTGAACAGCTGTTCCGTCGTGTTCTCCTATCTTTGTGA  300

CTGATTCAGCGTTTCCTTTTCTTTCATCAGAGTTTTTATGTTTCA  345

AGTAATTTAATTAATCATCACTGTTGTGTTTGCATTGTTATATAA  390

ATGTTGTGTTGATATAAAAGAAGAGAGCGTTGGTTTGTACTTTGT  435

GTGAAGATTTTTTAAAAATATAGTTGGTTTATTACAATA        474
```

RCI2B

```
                                    CCACGCGT    8

CCGCAGCATAATAAATATTTTCTTTGAAATTTGAAGAGCTTGAAA   53

ATGAGTACAGCCACTTTCGTAGAGATTATTCTTGCTATCATCTTG   98
 M  S  T  A  T  F  V  E  I  I  L  A  I  I  L   15

CCTCCTCTCGGCGTCTTTCTCAAATTTGGTTGCAAGGTTGAGTTT  143
 P  P  L  G  V  F  L  K  F  G  C  K  V  E  F   30

TGGATATGTTTGATTTTGACGCTGTTTGGTTATCTTCCCGGAATC  188
 W  I  C  L  I  L  T  L  F  G  Y  L  P  G  I   45

CTTTACGCTCTTTATATCATCACCAAGTGATTTTTCCTATCTCCT  133
 L  Y  A  L  Y  I  I  T  K                      54

CTGTTCTTTCCTCTTGCTCCTCGAAGAACAGCTGTTTCGTCGTGC  178

TCTTCTCCCTTTGGGACTGATTCATCATTTATTTTATGTTATGAG  223

CAATTGTATTCTCATTGTTGTATTTGCATTATTGTATACTTTCCT  268

GCTGACATCAAG                                   380
```

Figure 1. Nucleotide sequences of *RCI2A* and *RCI2B* cDNAs. Nucleotides in bold correspond to the putative initiation and termination codons.

The genomic organization of *RCI2A* and *RCI2B* was analyzed by DNA-blot hybridizations. Genomic DNA from Arabidopsis ecotype Col was purified and digested with *Eco*RI, *Xba*I, and *Hin*dIII. The probes used in these experiments were DNA fragments corresponding to the 3′ untranslated regions of *RCI2A* and *RCI2B* (see "Materials and Methods"). The results obtained revealed that each probe hybridized strongly with a single restriction fragment in each digestion, demonstrating that they are able to specifically recognize the corresponding gene without producing cross-hybridization (Fig. 3). The fact that specific probes recognizing *RCI2A* and *RCI2B* genes hybridized with a 5-kb common *Eco*RI restriction fragment strongly suggested that both genes were localized in such a fragment. The cloning and characterization of the fragment allowed us to confirm that this is the case (data not shown).

A

```
RCI2A    MSTATFVDIIIAILLPPLGVFLRFGCG  27
RCI2B    MSTATFVEIILAIILPPLGVFLKFGCK  27
BLT101   MGSATVLEVILAIILPPVGVFLRYKLG  27
ESI3     MGSATVLEVILAIILPPVGVFLRYKLG  27
         *..** ...*.**.***.****..

RCI2A    VEFWICLVLTLLGYIPGIIYAIYVLTK  54
RCI2B    VEFWICLILTLFGYLPGILYALYIITK  54
BLT101   VEFWICLLLTILGYIPGIIYAVYVLVV  54
ESI3     VEFWICLLLTILGYIPGIIYAVYVLVV  54
         *******.**..**.***.**.*...
```

B

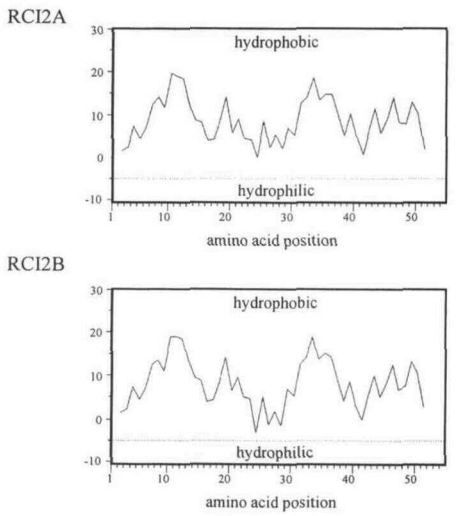

Figure 2. Deduced amino acid sequences of RCI2A and RCI2B proteins. A, Sequence alignment between the RCI2A and RCI2B proteins of Arabidopsis and the related proteins BLT101 from barley (Goddard et al., 1993) and ESI3 from *L. elongatum* (Gulik et al., 1994). Underlined amino acids indicate putative transmembrane domains. Amino acid residues identical to the Arabidopsis RCI2A protein sequence are indicated by asterisks. Similar amino acids are marked by points. B, Hydropathic plots of RCI2A and RCI2B proteins, as determined by the method of Kyte and Doolittle (1982), using a window of five amino acid residues.

Regulation of *RCI2* Gene Expression by Low Temperature at Different Stages of Development and in Different Organs of Arabidopsis

To determine whether the expression of *RCI2* genes is induced by low temperature at different stages of development, poly(A)$^+$ RNA was prepared from 4-day-old etiolated seedlings and leaves from 4-week-old plants of Arabidopsis exposed to 4°C during different periods of time. *RCI2A* and *RCI2B* transcripts were identified by RNA-blot hybridizations with the specific probes described above.

In etiolated seedlings both *RCI2A* and *RCI2B* mRNAs accumulated with very similar kinetics in response to low temperatures (Fig. 4A). An increase of *RCI2* transcript levels was already detectable after 12 h of low-temperature exposure, reaching the maximal levels of accumulation after 24 h. This increase was transient, since transcript levels started to decrease after 1 d of 4°C treatment. At the time of maximal induction (24 h), *RCI2A* and *RCI2B* mRNAs accumulated up to 10 times the basal levels (Fig. 4A). Transcripts corresponding to *RCI2A* and *RCI2B* also accumulated with a similar pattern in leaves of Arabidopsis exposed to low temperature, and this pattern paralleled that shown by etiolated seedlings (Fig. 4B). Taken together, these results indicate that in Arabidopsis low temperature induces a transient accumulation of both *RCI2A* and *RCI2B* transcripts independently of the developmental stage of the plant.

To analyze the accumulation of *RCI2A* and *RCI2B* mRNAs in different organs of Arabidopsis in response to low temperature, poly(A)$^+$ RNA was extracted from roots, stems, flowers, and siliques of 8-week-old plants that had been exposed to 4°C for 1 d. As controls, poly(A)$^+$ RNAs from the same organs of 8-week-old plants grown at 20°C were also isolated. RNA-blot hybridizations were carried out with the specific probes used in previous experiments. The results obtained showed that *RCI2A* transcripts accumulated in response to low-temperature treatment in all of the organs that were analyzed (Fig. 5). However, maximal accumulation levels were different for each organ, with the highest levels in stems and the lowest in roots. The *RCI2B* mRNA accumulated in stems, flowers, and siliques of plants exposed to 4°C, but not in roots, where *RCI2B* expression was constitutive (Fig. 5). The maximal level of accumulation was in stems and the minimal in siliques. Thus, the expression of *RCI2A* and *RCI2B* genes is differentially regulated by low temperature in different organs of Arabidopsis.

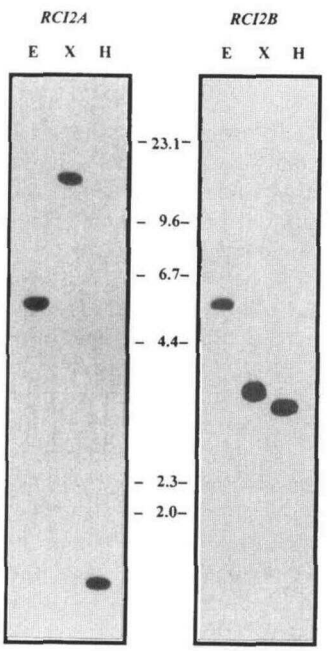

Figure 3. Genomic organization of *RCI2A* and *RCI2B* genes. DNA-blot hybridizations of Arabidopsis genomic DNA (4 μg) digested with *Eco*RI (E), *Xba*I (X), and *Hin*dIII (H). The specific probes used were the 3′ fragments of *RCI2A* and *RCI2B* cDNAs described in "Materials and Methods." The position of molecular size markers is indicated in the center of the figure.

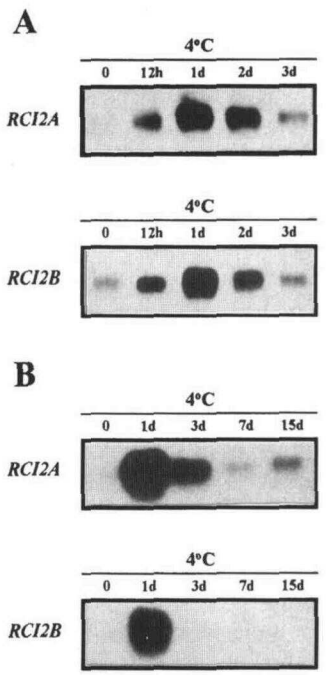

Figure 4. Low-temperature-induced accumulation of *RCI2A* and *RCI2B* transcripts in different developmental stages of Arabidopsis. RNA-blot hybridizations were performed with poly(A)$^+$ RNA (1 μg per lane) isolated from seedlings or leaves that had been exposed to 4°C for the indicated time. The specific probes used were the 3′ fragments of *RCI2A* and *RCI2B* cDNAs described in "Materials and Methods." A, Accumulation of *RCI2A* and *RCI2B* mRNAs in 4-d-old etiolated seedlings. B, Accumulation of *RCI2A* and *RCI2B* mRNAs in leaves from 4-week-old plants.

The Expression of *RCI2* Genes Is Regulated by ABA and Dehydration

Most cold-regulated genes are also responsive to other related treatments such as ABA, water stress, salt stress, or anaerobiosis (Hughes and Dunn, 1996). To determine whether the accumulation of *RCI2* mRNAs was specifically regulated by low temperature or was also responsive to other signals, 4-d-old etiolated seedlings were sprayed with 100 μM ABA, treated with 250 mM NaCl, exposed to anaerobiosis for 24 h, or dehydrated until losing 50% of their initial fresh weight. Poly(A)$^+$ RNAs isolated from these seedlings, from seedlings sprayed with the solvent used to solubilize ABA, and from 5-d-old untreated control seedlings were used to perform RNA-blot hybridizations with *RCI2A*- and *RCI2B*-specific probes. Figure 6 shows that *RCI2* transcripts accumulated in response to ABA and dehydration, attaining levels similar to those obtained in response to low temperature (see above, Fig. 4). In contrast, *RCI2* transcripts did not accumulate in response to salt stress or anaerobiosis (Fig. 6).

As positive controls for the treatments, hybridizations with an *RD29A* probe, which recognizes two homologous Arabidopsis genes inducible by water and salt stress (Yamaguchi-Shinozaki and Shinozaki, 1993), and an *ADH* probe, which recognizes a gene from Arabidopsis that is induced by anaerobiosis (Chang and Meyerowitz, 1986),

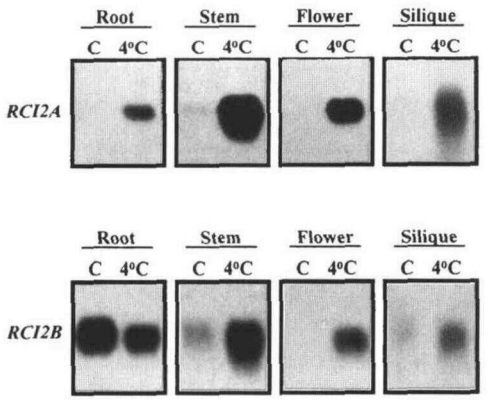

Figure 5. Accumulation of *RCI2A* and *RCI2B* transcripts in different organs of Arabidopsis in response to low temperatures. RNA-blot hybridizations were performed with poly(A)$^+$ RNA (1 μg per lane) isolated from roots, stems, flowers, and siliques of 8-week-old plants that had been grown at 22°C (C) or exposed to 4°C for 24 h (4°C). The specific probes used were the 3′ fragments of *RCI2A* and *RCI2B* cDNAs described in "Materials and Methods."

were also performed (Fig. 6). These results show that the accumulation of *RCI2* transcripts is also promoted by ABA and water-stress treatments but is not a general response to stress conditions, since it is not induced by other treatments such as salt stress or anaerobiosis.

An important question raised by the finding that *RCI2* transcripts accumulated in response to exogenous applica-

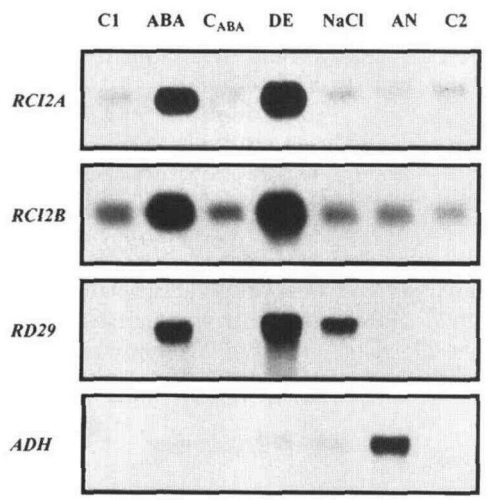

Figure 6. Effect of different stresses and treatments on the expression of *RCI2A* and *RCI2B*. RNA-blot hybridizations were performed with poly(A)$^+$ RNA (1 μg per lane) isolated from 4-d-old etiolated seedlings that had been exposed to different stresses and treatments. The probes used were the 3′ fragments of *RCI2A* and *RCI2B* cDNAs, and the fragments of the *RD29A* and *ADH* genes described in "Materials and Methods." C1, Control 4-d-old etiolated seedlings; ABA, 4-d-old seedlings harvested 3 h after being sprayed with 100 μM ABA; C$_{ABA}$, 4-d-old seedlings harvested 3 h after being sprayed with the ABA solvent; DE, 4-d-old seedlings dehydrated until losing 50% of their fresh weight; NaCl, 4-d-old seedlings exposed to a solution of 250 mM NaCl for 24 h; AN, 4-d-old seedlings exposed to anaerobiosis for 24 h; and C2, control 5-d-old etiolated seedlings.

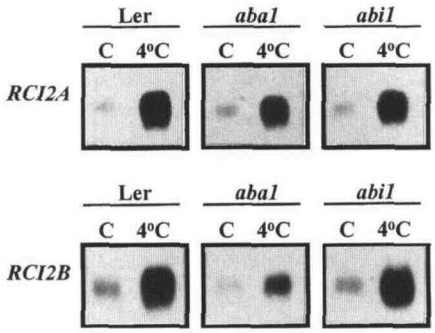

Figure 7. Accumulation of *RCI2A* and *RCI2B* transcripts in *aba1* and *abi1* mutants in response to low temperature. RNA-blot hybridizations were performed with poly(A)$^+$ RNA (1 μg per lane) isolated from 4-d-old etiolated seedlings of *aba1* and *abi1* mutants grown at 22°C and then exposed to 4°C for 24 h (4°C) or maintained at 22°C for 1 additional d (C). Ler corresponds to the wild-type genetic background of the mutants. The specific probes used were the 3′ fragments of *RCI2A* and *RCI2B* cDNAs described in "Materials and Methods."

tion of ABA was whether the cold-regulated expression of the corresponding genes is mediated by ABA. As a first step to answering this question, we examined the *RCI2* mRNA levels in the *aba1* and *abi1* mutants of Arabidopsis after low-temperature exposure. Poly(A)$^+$ RNAs were isolated from 4-d-old etiolated seedlings of L*er* ecotype and *aba1* and *abi1* mutants that had been exposed to 4°C for 1 additional d, as well as from 5-d-old L*er*, *aba1*, and *abi1* control etiolated seedlings. RNA-blot hybridizations with the specific *RCI2* probes showed that the accumulation of both *RCI2A* and *RCI2B* transcripts in cold-treated L*er* and *abi1* seedlings increased by 10-fold (Fig. 7), which was very similar to that observed in etiolated seedlings of the Col ecotype (Fig. 4). In *aba1* seedlings the levels of *RCI2A* mRNA after low-temperature exposure were only 70% of those detected in the L*er* and *abi1* mutants (Fig. 7). The levels of *RCI2B* mRNA in both control and cold-treated *aba1* seedlings were one-half of those found in the L*er* or *abi1* mutant (Fig. 7). These observations suggest that ABA is partially required for the accumulation of *RCI2* transcripts in seedlings subjected to low temperature in a pathway that does not require ABI1.

DISCUSSION

The process of cold acclimation is associated with intricate physiological and biochemical changes that are mediated through changes in the levels of mRNAs and proteins. The complexity of this process is exemplified by the number of different cold-responsive genes that have been isolated from various plant species (Thomashow, 1994; Hughes and Dunn, 1996). For most of these genes the transcript levels increase dramatically in response to low temperatures and remain high for as long as the plants are exposed to them. However, these genes are not representative of all low-temperature-responsive genes, since the techniques employed to isolate them do not always identify genes expressed at medium-low levels or genes transiently expressed during cold acclimation. We have used a subtracted probe to identify two additional Arabidopsis cDNA clones, *RCI2A* and *RCI2B*, corresponding to transcripts induced at medium levels in cold-acclimated plants.

Sequence analyses and DNA-blot hybridizations indicate that *RCI2A* and *RCI2B* are very homologous genes constituting a small gene family contained in a 5-kb *Eco*RI fragment. Their high degree of homology suggests that they probably arose by duplication of the same ancestral gene. The same origin has been proposed for four pairs of Arabidopsis low-temperature-responsive genes, in which the homologous genes are arranged in tandem in the genome; they are *kin1* and *cor6.6/kin2* (Gilmour et al., 1992; Kurkela and Borg-Franck, 1992), *cor15a* and *cor15b* (Wilhelm and Thomashow, 1993), *lti78/rd29A/cor78* and *lti65/rd29B* (Nordin et al., 1991; Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993), and *lti45/lti29* and *cor47* (Welin et al., 1994). It is interesting that in each case both homologous genes have diverged in their regulation by low temperature, suggesting they have different roles in cold acclimation or freezing tolerance. In this way, Smith (1990) suggested that phenotypic plasticity of plants in response to different environmental factors might be due to the differential expression of different members of gene families.

The analyses of expression patterns showed that the cold-inducible expression of both *RCI2A* and *RCI2B* during Arabidopsis development is very similar and not stage-specific, since they are induced in etiolated seedlings as well as in adult plants. However, the expression of *RCI2* genes is differentially regulated by low temperature in different organs of the plant. In contrast to the stable accumulation kinetics showed by the transcripts corresponding to most cold-regulated genes characterized in Arabidopsis (Thomashow, 1994; Hughes and Dunn, 1996), the accumulation of *RCI2* transcripts by low temperature is transient, reaching the maximal levels after 24 h of exposure. This accumulation pattern suggests that *RCI2* genes should be involved in responses that are transiently produced when plants are exposed to low temperatures. The expression of *RCI2A* and *RCI2B* is not regulated by anaerobiosis, which allows us to discard the possibility that the accumulation of *RCI2* mRNAs by cold treatment is a consequence of the anaerobic metabolism transiently produced by low temperatures (Jarillo et al., 1993). Furthermore, transcripts corresponding to *RCI2A* and *RCI2B* do not accumulate in response to salt stress, which differentiates them from other cold-responsive genes such as *kin2* (Kurkela and Borg-Franck, 1992), *lti78/rd29A/cor48*, or *lti65/rd29B* (Yamaguchi-Shinozaki and Shinozaki, 1994). However, the two *RCI2* genes, like most of the cold-inducible genes (Thomashow, 1994; Hughes and Dunn, 1996), are induced by drought, a treatment that has been shown to increase plant freezing tolerance (Cloutier and Siminovitch, 1982; Chen and Gusta, 1983; Mäntylä et al., 1995). Since both low temperature and drought can be considered to be dehydration stresses (Steponkus, 1984), it is therefore reasonable to imagine that they can induce common gene products.

We have also found that the levels of *RCI2A* and *RCI2B* transcripts increase in response to ABA, another treatment

that enhances freezing tolerance (Chen and Gusta, 1983) and induces the expression of the majority of cold-inducible genes (Hughes and Dunn, 1996). Endogenous levels of ABA have been shown to transiently increase in response to low temperature and water stress (Lang et al., 1994) with kinetics that parallel those of the cold-induced *RCI2* mRNA accumulation. This suggests that ABA could be responsible, at least in part, for the accumulation of *RCI2* mRNAs in response to low temperature and drought. The accumulation of *RCI2* mRNAs in the *abi1* mutant is identical to that occurring in L*er*, indicating that ABI1 is not required for mediating the low-temperature response. Accumulation of *RCI2A* and *RCI2B* mRNAs in the *aba1* mutant is reduced with respect to the wild type. Moreover, this reduction is higher in *RCI2B* than in *RCI2A* transcripts, indicating that the ABA-independent pathway is the most important for the accumulation of *RCI2A* mRNA in response to low temperature. In *RCI2B* mRNA it seems that both the ABA-dependent and -independent pathways are required to achieve full cold-induced accumulation. In conclusion, *RCI2* genes are induced by low temperature in *abi1* and *aba1* mutants, indicating that, as described for other cold-responsive genes (Nordin et al., 1991; Gilmour and Thomashow, 1991), the accumulation of *RCI2* mRNAs by low temperature is regulated by at least two different signal transduction pathways, one ABA dependent and the other ABA independent.

A database search revealed that RCI2A and RCI2B are highly similar to BLT101 and ESI3 proteins from barley and *L. elongatum*, respectively. These proteins have identical sequences and are encoded by genes regulated by different treatments. *ESI3* expression is induced by osmotic stress, salt stress, and ABA treatment, but its response to low temperature has not been described (Gulik et al., 1994). On the other hand, *BLT101* mRNA accumulates in response to low temperature but not in response to drought or ABA. However, *BLT101* does not respond transiently to low temperature as *RCI2* genes do, but respond all the time that low temperature is maintained (Goddard et al., 1993).

RCI2 genes are two of the few cold-induced genes identified in plants that encode hydrophobic proteins. They contain two potential membrane-spanning domains, as predicted by the method of Klein et al. (1985), and they could be integral membrane proteins. The first transmembrane domain, the amino terminal one, contains three consecutive polar amino acids in its 3′ region, whereas the second contains seven scattered polar amino acids. A similar distribution of polar amino acids is frequently found in transmembrane domains that interact with other membrane proteins (Lewin, 1994). Although we do not know the kind of membrane to which RCI2 proteins are directed, the absence of signals for organelle targeting suggests that their final destination could be the plasma membrane, which is considered to be a primary site of injury during freezing (Lyons, 1973). In this way they could play a role in maintaining membrane function and/or integrity in water-stressed situations provoked by low temperatures, freezing, or other environmental conditions that reduce water availability. Alternatively, they could interact with other membrane proteins to maintain the hydric equilibrium of the cells. Phenotypic analyses of transgenic plants overexpressing *RCI2* mRNAs in both sense and antisense orientations would help to elucidate the role of these genes during cold acclimation and the function of the corresponding proteins in the development of freezing tolerance.

ACKNOWLEDGMENTS

We wish to thank Dr. M. Koorneef for providing us with seeds of ABA-related mutants and Dr. M.J. Jorquera for her technical assistance.

Received April 16, 1997; accepted June 30, 1997.

LITERATURE CITED

Aitken A, Collinge DB, van Heusden BPH, Isobe T, Roseboom PH, Rosenfeld G, Soll J (1992) 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. Trends Biochem Sci **17:** 498–501

Altschul F, Gish F, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Chang C, Meyerowitz EM (1986) Molecular cloning and DNA sequence of *Arabidopsis thaliana* alcohol dehydrogenase gene. Proc Natl Acad Sci USA **83:** 1408–1412

Chen HH, Li PH, Brenner ML (1983) Involvement of abscisic acid in potato cold acclimation. Plant Physiol **71:** 362–365

Chen THH, Gusta LV (1983) Abscisic acid induced freezing resistance in cultured plant cells. Plant Physiol **73:** 71–75

Cloutier Y, Siminovitch D (1982) Correlation between cold- and drought-induced frost hardiness in winter wheat and rye varieties. Plant Physiol **69:** 256–258

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Gilmour SJ, Artus NN, Thomashow MF (1992) cDNA sequence analysis and expression of two cold regulated genes of *Arabidopsis thaliana*. Plant Mol Biol **18:** 13–21

Gilmour SJ, Thomashow MF (1991) Cold acclimation and cold-regulated gene expression in ABA mutants of *Arabidopsis thaliana*. Plant Mol Biol **17:** 1233–1240

Goddard NJ, Dunn MA, Zhang AJ, White PL, Jack PL, Hughes MA (1993) Molecular analysis and spatial expression pattern of a low-temperature-specific barley gene, *blt 101*. Plant Mol Biol **23:** 871–879

Gulick PJ, Shen W, An H (1994) *ESI3*, a stress-induced gene from *Lophopyrum elongatum*. Plant Physiol **104:** 799–800

Haughn G, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet **204:** 430–434

Horvath DP, McLarney BK, Thomashow MF (1993) Regulation of *Arabidopsis thaliana* L. (Heyn) *cor78* in response to low temperature. Plant Physiol **103:** 1047–1053

Hughes MA, Dunn MA (1996) The molecular biology of plant acclimation to low temperature. J Exp Bot **47:** 291–305

Jarillo JA, Capel J, Leyva A, Salinas J, Martínez-Zapater JM (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. Plant Mol Biol **25:** 693–704

Jarillo JA, Leyva A, Salinas J, Martínez-Zapater JM (1993) Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidopsis thaliana*, a chilling-tolerant plant. Plant Physiol **101:** 833–837

Klein P, Kanehisa M, DeLisi C (1985) The detection and classification of membrane-spanning proteins. Biochim Biophys Acta **815:** 468–476

Kurkela S, Borg-Franck M (1992) Structure and expression of *Kin2*, one of two cold- and ABA-induced genes of *Arabidopsis thaliana*. Plant Mol Biol **19:** 689–692

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 108–132

Lang V, Mäntylä E, Welin B, Sundberg B, Palva ET (1994) Alterations in water status, endogenous abscisic acid content, and expression of *rab18* gene during the development of freezing tolerance in *Arabidopsis thaliana.* Plant Physiol **104:** 1341–1349

Levitt J (1980) Responses of Plants to Environmental Stresses: Chilling, Freezing and High Temperature Stresses. Academic Press, New York

Lewin B (1994) Genes V. Oxford University Press, New York

Leyva A, Jarillo JA, Salinas J, Martinez-Zapater JM (1995) Low temperature induces the accumulation of *phenylalanine ammonia-lyase* and *chalcone synthase* mRNAs of *Arabidopsis thaliana* in a light dependent manner. Plant Physiol **108:** 39–46

Lyons JM (1973) Chilling injury in plants. Annu Rev Plant Physiol **24:** 445–466

Mäntylä E, Lang V, Palva T (1995) Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in *Arabidopsis thaliana.* Plant Physiol **107:** 141–148

Molina A, Garcia-Olmedo F (1993) Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. Plant J **4:** 983–991

Nagy F, Kay SA, Chua N-H (1988) Analysis of gene expression in transgenic plants. *In* SB Gelvin, RA Schilperoort, eds, Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–29

Nordin K, Heino P, Palva ET (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol **187:** 169–183

Pongor S, Skerl V, Cserzo M, Hatsagi Z, Simon G, Bevilacqua V (1992) The SBASE domain library release 2.08: a collection of annotated protein sequence segments. Nucleic Acids Res **21:** 311–315

Sakai A, Larcher W (1987) Frost survival of plants. *In* Responses and Adaptation to Freezing Stress. Springer-Verlag, New York

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulsen AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opinion Biotechnol **7:** 161–167

Smith H (1990) Signal perception, differential expression within multigene families and the molecular basis of phenotypic plasticity. Plant Cell Environ **13:** 585–594

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Thomashow MF (1994) *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. *In* EM Meyerowitz, CR Somerville, eds, *Arabidopsis.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 807–834

Welin BV, Olson A, Nylander M, Palva ET (1994) Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in *Arabidopsis thaliana.* Plant Mol Biol **26:** 131–144

Wilhelm K, Thomashow MF (1993) *Arabidopsis thaliana cor 15b,* an apparent homologue of *cor 15a,* is strongly responsive to cold and ABA, but not drought. Plant Mol Biol **23:** 1073–1077

Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of the expression of a desiccation-responsive *rd29* gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet **236:** 331–340

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature or high-salt stress. Plant Cell **6:** 251–264