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An unusual Group 2 LEA gene family in citrus responsive to low temperature

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Abstract

Six cDNAs representing unique cold-induced sequences have been cloned from the hardy citrus relative *Poncirus trifoliata*. Among these, pBCORc115 and pBCORc119 were found to belong to the same gene family. Sequencing data indicated that pBCORc115 and pBCORc119 each contained an open reading frame, coding for a 19.8 kDa protein (COR19) and a smaller 11.4 kDa protein (COR11) respectively. Inspection of the deduced amino acid sequences revealed three large repeats in COR19, but only one was present in the COR11. Two elements: a Q-clustered tract and a K-rich motif were identified in each repeat. The K-rich motifs were similar to those of cotton D-11 and Group 2 LEA proteins. A Serine-cluster, a common feature in many Group 2 LEA-like proteins, was also found in these proteins, but it was in an unusual position at the carboxy-terminus. A bipartite motif of basic residues, similar to known nuclear targeting sequences, was also present in COR19 and COR11, suggesting that members of this protein family may have a nuclear targeting function. The expression of COR19 mRNA in response to cold acclimation, drought, flooding, and salinization was examined. COR19 expression in leaf tissue was induced in response to cold acclimation, but repressed during drought and flooding stress.

Introduction

Many plants exhibit an increase in freezing tolerance when exposed to low nonfreezing temperatures [39]. This process, termed cold acclimation, involves a series of physiological and metabolic changes, including alterations of protein synthesis and gene expression [2, 25, 26, 48, 52]. An important characteristic of these changes is the accumulation of specific proteins or mRNAs following exposure to inductive temperature [18, 21, 29, 36].

Freezing tolerance in plants is considered to be a quantitative genetic trait, influenced by many genes. The accumulation of specific gene products during cold acclimation raises the important question of whether the gene products induced during cold acclimation play a key role in acquired tolerance to freezing stress. Genes preferentially expressed during cold acclimation have

been cloned and characterized from several plants, including alfalfa [41, 42], *Arabidopsis* [20, 31, 35], barley [10, 16], spinach [34, 45], and wheat [32]. Some genes associated with cold acclimation encode proteins related to late embryogenesis-abundant proteins (LEAs), or proteins responsive to water stress (dehydrins) and/or abscisic acid (RABs). These families of genes have several features in common such as high hydrophilicity, boiling solubility and presence of repeating motifs [20, 22, 40, 44, 45].

Although a number of cold stress-responsive genes have common sequence features, this information has yet to reveal a basis for understanding their functional role in freezing tolerance mechanisms [42], because the bases of cold hardiness remain mostly unknown. However, in one case, an alanine-rich protein, Kinl, from *Arabidopsis* may be functionally homologous to certain alanine-rich fish antifreeze proteins [35, 46]. If Kin1 has antifreeze properties, then its presence inside cells could help to inhibit ice crystal growth and protect cells from ice seeding [23]. Other studies have implicated certain enzymes, like glutathione reductase in red spruce [30], and specific plasma membrane proteins in winter wheat [55] with the development of freezing tolerance. To better understand the mechanisms of cold acclimation, it will be necessary to characterize the full ensemble of genes that are induced during cold acclimation.

Considerable effort has been directed at understanding the molecular mechanisms of cold acclimation and freezing tolerance in herbaceous plant species. In contrast, very few studies have delved into the molecular responses of fruit trees and woody perennials to low temperature. Cold tolerance in these species is particularly important because of their perennial nature and because tolerance mechanisms may be more complex, involving dormancy and deciduousness or other developmentally regulated traits. Members of the subtropical genus *Citrus* and its relatives are known to acclimate to cold when exposed to inductive temperature [53, 54]. Studies by Guy *et al.* [28] and Durham *et al.* [18] have shown that several proteins in *Citrus* were accumulated while others declined when exposed to low temperature. To date, none of the cold-induced proteins or their genes from this genus have been characterized. We report here on the cloning and characterization of several cDNAs for cold-responsive genes, including two members related to cotton D-11 and Group 2 LEA-like proteins, from the cold-hardy citrus relative *Poncirus trifoliata*.

Materials and methods

Plant materials

Two interfertile species, *Poncirus trifoliata* (L.) Raf. and *Citrus grandis* (L.) Osb., used in this study were previously described [8, 18, 19]. *P. trifoliata* is a cold-hardy, deciduous *Citrus* relative, while *C. grandis* is cold-sensitive having little freezing tolerance. Eight-week old seedlings were used for extraction of mRNA for construction of cDNA libraries. One-year old seedlings were used for environmental stress expression studies and clonally propagated plants were used for genome organization studies.

Extraction of mRNA and construction of cDNA libraries

Messenger RNA was extracted from plants subjected to seven days of cold acclimation (CA) and control plants kept under nonacclimating conditions. Prior to RNA isolation the CA plants were treated as follows. Eight-week old seedlings were maintained for one week at 4 °C, under 12 h photoperiod, and an irradiance of 470 μmol m^{-2} s^{-1}. Control seedlings were maintained under the same conditions except the temperature was 25 °C. Leaf samples were collected and stored at −80 °C. Total RNA was extracted from leaf tissue using phenol/chloroform extraction and a LiCl precipitation procedure [3]. Poly(A)$^+$ RNA was purified using an oligo-dT-cellulose column as described by Sambrook *et al.* [50]. Poly(A)$^+$ RNA extracted from CA seedlings of *P. trifoliata* was used to construct a cDNA library. The cDNA

synthesis and cloning were conducted using the Uni-Zap unidirectional lambda cloning kit (Stratagene, San Diego, CA) following the manufacturer's protocols [45].

Differential screening of the cDNA library

The cDNA library was differentially screened using ^{32}P-labelled single-stranded total cDNA synthesized from CA and control poly(A)$^+$ RNA as described by Sambrook *et al.* [50]. Plaques showing positive signals to the CA probe but not to the control probe were collected and subjected to a second round screening using the same procedure. Phage collected from the second round screening were further purified using dot-blot hybridization analysis [7]. Clones confirmed in the dot-blot hybridization analysis were subsequently rescued by *in vivo* excision from lambda and maintained as a Bluescript plasmid. A cross-hybridization analysis was performed to test for the presence of overlapping or homologous sequences in the different clones. Differential expression was examined with selected clones by hybridization with RNA blots containing CA and control RNA from *P. trifoliata* and *C. grandis*.

DNA sequencing analysis

cDNAs were sequenced using an automated sequencing technique [51]. The complete sequences of both strands were obtained using synthetic primers. The Blast program [1] was used to conduct computer searches of the GenBank databases.

Genomic DNA blot hybridization analysis

Genomic DNA was extracted from young leaf tissue of *P. trifoliata* and *C. grandis* and their F_1 hybrid [19] taken from plants that were cultivated in a greenhouse. DNA digested with *Eco* RI, *Pst* I or *Bam* HI (BRL Life Tech) was separated on 1% agarose gel in TAE and blotted onto nylon membrane [3]. Clones, pBCORc115, pBCORc410, pBCORc510, pBCORc720 and pBCORc102, were used to probe DNA blots as previously described [19].

Environmental stress treatments

P. trifoliata and *C. grandis* were subjected to the following stress treatments.

- *Cold acclimation.* Plants were maintained at 4 °C and irrigated as needed. Control plants were kept at 25 °C.
- *Flooding.* The pots of one-year old seedlings were immersed in water until the soil was completely covered. The immersion water was changed every other day. Control plants were maintained with normal irrigation.
- *Drought.* Water was withheld from plants maintained in a controlled environment at 25 °C. Controls were watered daily to keep the rooting medium at field capacity.
- *Salinization.* Plants were irrigated with 50 mM NaCl in 100 mg/l Peter's solution once every two days in place of normal irrigation. Excess saline irrigation solution was added to the pots and allowed to drain from the soil to prevent salt accumulation during the progression of the experiment.

Leaf tissue was collected at various intervals up to 30 days after the beginning of the stress treatment and stored at −80 °C. Total RNA was isolated from the leaf samples as described above and used for RNA-blot analysis using standard procedures [50]. Clone pBCORc115 was used to probe all the blots. Another clone homologous to pBCORc115, pBCORc119, was used to probe blots containing CA mRNA to verify mRNA size. The final washing for all RNA blots was at 65 °C in 0.2 × SSC + 0.2% SDS [8].

Results

Differential screening of a *P. trifoliata* cDNA library resulted in the initial isolation of nine clones. Cross-hybridization experiments demonstrated

that five clones shared homologous sequences, while the rest contained unique sequences. A partial cDNA clone from the cross-hybridizing group, pBCORc112 (480 bp), was used to rescreen the cDNA library, and 13 clones were obtained by screening 50 000 plaques. From these, two clones, pBCORc115 and pBCORc119, contained the largest inserts and were selected for further characterization. Table 1 lists the clones that were examined in this study. RNA blot hybridization analysis using these cDNAs as probes (Fig. 1) affirmed the differential expression between CA and control seedlings of *P. trifoliata*. This analysis also indicated, in some cases, a differential expression pattern between the two species. An abundant mRNA was revealed by pBCORc115 in both *P. trifoliata* and *C. grandis*. However, its size was slightly different between the two species. The *P. trifoliata* mRNA was ca. 950 nt, i.e. ca. 60 nt larger than that of *C. grandis*. The mRNA for pBCORc410 was the same size in both species (about 1500 nt), but its abundance in *P. trifoliata* was greater than that of *C. grandis*. pBCOR720 also revealed similar-sized mRNAs (about 900 nt) in both species. The hybridization signal with this probe was relatively weak for both species, suggesting a lower abundance. However, the relative levels in cold-acclimated and unacclimated leaves appeared to be similar for the two species. After hybridization with pBCORc102 and pBCORc510, no detectable signal for *C. grandis* was observed. The mRNA size was roughly 720 nt for pBCORc102 and 600 nt for pBCORc510.

Table 1. Cold acclimation-responsive clones from the cDNA library of *Poncirus trifoliata* and the size of the corresponding mRNA.

Name	cDNA size (bp)	mRNA size (nt)	Accession number
pBCORc115[1]	850	950	L39004
pBCORc119[1]	678	700	L39005
pBCORc720	220	900	–[2]
pBCORc410	477	1500	L39006
pBCORc102	395	720	L39003
pBCORc510	332	600	L39007

[1] Another four clones, pBCORc120 (0.42 kb), pBCORc112 (0.43 kb), pBCORc730 (0.48 kb), and pBCORc530 (0.47 kb), share sequence homology with these clones. [2] This clone was not sequenced.

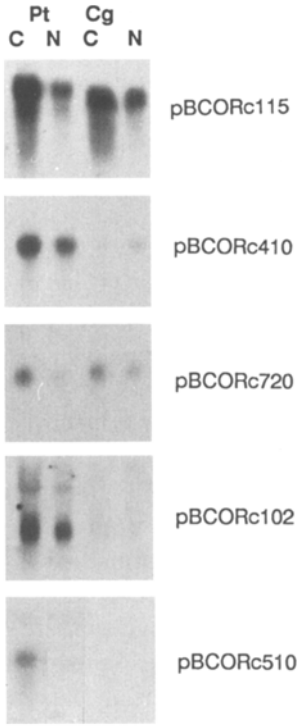

Fig. 1. RNA-blot analysis of mRNA from cold-acclimated and unacclimated *P. trifoliata* and *C. grandis* hybridized with five cDNA clones isolated from a *P. trifoliata* cDNA library. The cDNA inserts were labelled with ^{32}P-dCTP using the Random Primer Labeling Kit (BRL). Membranes hybridized with labelled probes were washed in a solution containing 0.2× SSC and 0.1% SDS at 65 °C at the last washing step and exposed to X-ray film for two days. Total RNA (15 μg) was loaded in each lane. The first two lanes were samples from cold-acclimated (C) and unacclimated (N) *P. trifoliata* (Pt). The next two lanes were loaded with samples from acclimated (C) and unacclimated (N) *C. grandis* (Cg).

cDNA sequence analysis

Sequence analyses of pBCORc115 and its closely homologous clone pBCORc119 revealed two distinctive open reading frames. pBCORc115 was 850 bp not including the poly(A) tail, while pBCORc119 was 678 bp in length. The open reading frame in pBCORc115 started at nucleotide 13

and terminated with a stop codon at nucleotide 552. This open reading frame encoded a 179 residue protein with a molecular weight of 19.8 kD, which was designated as COR19 (Fig. 2). The deduced protein was found to be rich in Gly (G, 16%), Gln (Q, 12%), Lys (K, 12%), Glu (E, 12%) and His (H, 11%). It had a calculated pI of 6.9. Three repeats ranging from 36 to 38 residues were observed. The difference in length of the repeats appeared to be related to the number of Q residues which varied from 3 to 5 among the repeats. Each repeat contained a Q-clustered tract and a K-rich motif. The Q-clustered tract was preceded by two H residues and followed by Y R or Y H. A dot homology plot of COR19 versus itself verified the presence of a number of repeating elements within the predicted coding region (Fig. 3A).

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CCGTTGCTGTCGATGGCGGGAGTTATTCACAAGATCGGAGAAGCCCTTCA   50
            m  a  g  v  i  h  k  i  g  e  a  l  h     13
CGTGGGAGGAGGGCAAAAGGAGGAGGACAAGAGCAAGGGAGAGCACCAGA  100
  v  g  g  g  q  k  e  e  d  k  s  k  g  e  h  q      29
GTAGGGACCACCACACTACTGATGTTCACCATCAGCAGCAGTATCACGGT  150
s  r  d  h  h  t  t  d  v  h  h  q  q  q  y  h  g     46
GGGGAACACAGGGAGGGCGAACAGAAGGAGGGCTTGGTGGACAAGATCAA  200
g  e  h  r  e  g  e  q  k  e  q  l  v  d  k  i  k     63
GCAGCAGATCCCCGGTGCCGGTACTGCTGATGTTCACCATCAGCAGCAGC  250
q  q  i  p  g  a  g  t  a  d  v  h  h  q  q  q        79
AGCAGTATCGAGGCGGGGAACACAGGGAGGGCGAGCACAAGGAGGGCTTG  300
q  q  y  r  g  g  e  h  r  e  g  e  h  k  e  q  l     96
GTGGACAAGATCAAGCAGCAGATCCCTGGTGCCGGCACTACTGATGTTCA  350
v  d  k  i  k  q  q  i  p  g  a  g  t  t  d  v  h     113
CCATCAGCAGCAGCAGTATCGCGGAGGGGAACACAGGGAGGGCGAACAGA  400
h  q  q  q  q  y  r  g  g  e  h  r  e  g  e  q        129
AGGAGGGCTTGGTGGACAAGATCAAGCAGAAGATACCCGGTGTCGGCGGC  450
k  e  q  l  v  d  k  i  k  q  k  i  p  g  v  g  g     146
GGCGAGGGCGCCACCCACGCCCAAGGTGAAAAGAAGAAAAAGAAGGAGAA  500
g  e  g  a  t  h  a  q  g  e  k  k  k  k  k  e  k     163
GAAGAAGCACGAGGACGGACACGAGAGCAGCAGCAGCAGTGACAGCGATT  550
k  k  h  e  d  g  h  e  s  s  s  s  s  d  s  d        179
AAATTAATAAACTTTAATGAGAGATGTGTGACGCATCATCATCAGCATAA  600
.
TCATCCATCATCCTAGATTTATTCTATAATAAAAATTTGGTGTGTGTATG  650
TGGTCACAGTATGAGAGCTAGCTGCTTTATTTTTGGGGGTGTCTCCTCAA  700
CGGTGGTGTTGCGTTTAAATTATTGCCTCTGAGAAACTCTGTTTTTTAATT  750
TACCCAGTCGGTGTGTAATTTGCTTTTTTTGTCAACGTAAATTTCGATTA  800
TCAATCATATGATGATAAGATTAGTTATTAAAAAAAAAAAAAAAAAAAAA  850
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Fig. 2. The nucleotide sequence of pBCORc115 and deduced amino acid sequence of the coding region which encodes a 19.8 kDa protein (COR19). The shadowed areas indicate repeat sequences; single underlines indicate glutamine (Q) clusters; double underlines indicate the lysine (K)-rich motifs; the serine cluster is in bold.

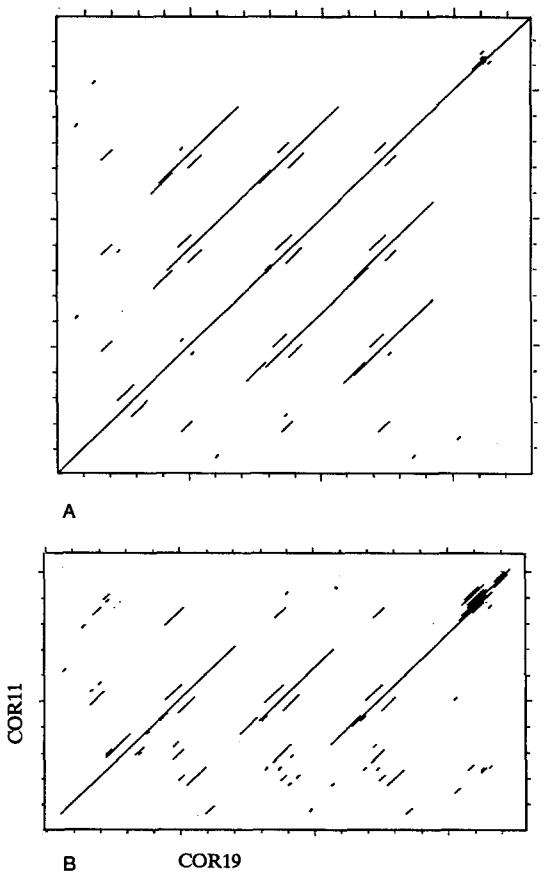

Fig. 3. Dot homology plot analysis of COR19 versus itself (A) and versus COR11 (B). The analysis was conducted in a window of 11 amino acids and the stringency was 9 matches of 11 for self comparison, and 8 matches of 11 for comparison between COR19 and COR11. Synonym amino acids (functionally similar to each other) were considered to be identical: D = E, K = R, N = Q, T = S, P = Y = W and A = V = I = L = M.

The open reading frame in pBCORc119 started at nucleotide 56 and ended at nucleotide 376. It coded for a 106 residue protein with a molecular weight of 11.4 kDa, and was designated as COR11. An inframe stop codon was present prior to the first ATG, suggesting that pBCORc119 was a near full-length clone and represented a distinct sequence from that of pBCORc115. The deduced amino acid sequence and its comparison with COR19 is shown in Fig. 4. Two large repeats present in COR19 were absent in COR11.

Sequence comparison using the 'Bestfit' command of GCG showed no significant similarity

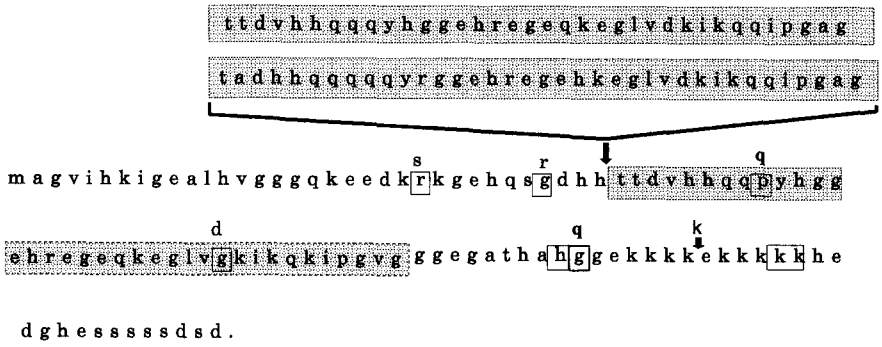

Fig. 4. Comparison of the deduced amino acid sequence of COR11 and COR19. The boxes mark the amino acids different or missing from COR19. The shadowed boxes mark the repeat sequence. Arrows point to positions where amino acids exist in COR19 but not in COR11.

between pBCORc115 and pBCORc119 and the other cDNAs. A computer search of the Genbank database revealed no significant homology to other known sequences in the database for any of the cDNAs except for COR19 and COR11. CORs 11 and 19 possessed short regions within the repeating sequences coding for the Lys-rich motif which were characteristic of Group 2 LEAs and dehydrin proteins. Also, a 95 base sequence (469–554 bp) overlapped an alfalfa cDNA, cas15b, and its homologous member [42] with a 79% identity. This region matched a 30 amino acid residue portion of the C-terminus of cas15b with 76.6% identity. Additionally, a sequence coding for a K-cluster region near the C-terminus was similar to sequences coding for the bipartite motif of basic residues in nuclear localization signal (NLS) sequences reported from tobacco [33], *Xenopus* [49], *Agrobacterium* [12] and yeast [38].

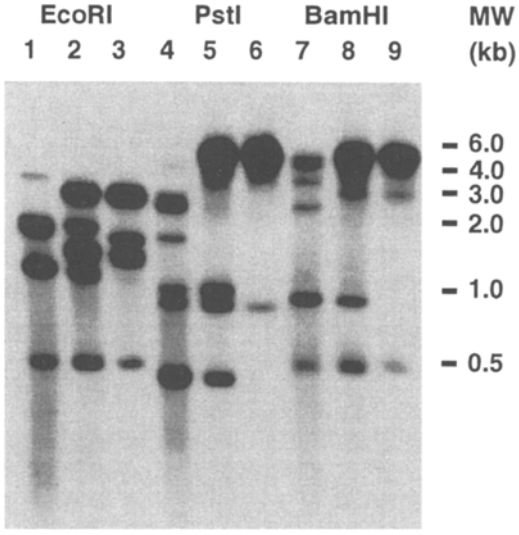

Fig. 5. DNA blot analysis of genomic DNA hybridized with pBCORc115. Genomic DNA from *C. grandis* (lanes 1, 4 and 7), *P. trifoliata* (lanes 3, 6 and 9) and their F_1 (lanes 2, 5 and 8) were digested with *Eco* RI (lanes 1–3), *Pst* I (lanes 4–6) and *Bam* HI (lanes 7–9). Each lane was loaded with 4 μg genomic DNA. The hybridized nylon membrane was washed in 0.2 × SSC and 0.1% SDS at 65 °C at the last washing step and subsequently exposed to X-ray film for four days.

DNA blot hybridization analysis

DNA blot hybridization of the genomic DNA from *P. trifoliata*, *C. grandis* and their F_1 with pBCORc115 revealed multiple bands with different restriction endonuclease treatments (Fig. 5). Several bands were detected in *Eco* RI-, *Bam* H1- and *Pst* I-digested DNA. The banding patterns from *P. trifoliata* and the F_1 digested with *Pst* I and *Bam* HI suggested the possibility of parental specific DNA methylation patterns at several restriction sites. From this and other studies, the loci causing these methylation patterns have been genetically analyzed and mapped in the *Citrus* genome [9]. A four-gene model for the banding patterns revealed in *Eco* RI-restricted DNA was inferred from analysis of segregation in a backcross [*C. grandis* × (*C. grandis* × *P. trifoliata*)]

population and 3 of the 4 gene loci were placed on a genetic linkage map [8]. Two gene loci, pBCORc115b and pBCORc115c, were assigned to linkage group IV while pBCORc115a was placed on linkage group VIII. The other inferred locus was found to be monomorphic and could not be positioned on the linkage map.

DNA hybridization analyses conducted with all the other clones listed in Table 1 can be briefly summarized as follows (data not shown). Hybridization with pBCOR410 revealed one band in *Eco* RI-digested DNA and two bands in *Pst* I-digested DNA. An upper band (about 3 kb) was present in *P. trifoliata* and the F_1 and a lower band (about 2 kb) existed in both species and their hybrid. In *Bam* HI-digested samples, an upper band existed in *P. trifoliata*, a lower band was present in *C. grandis*, and the F_1 possessed both bands. The pBCOR510 probe showed one band in *P. trifoliata* and the F_1, but no detectable signal in *C. grandis* after restriction with all three enzymes. The size of the band was 0.6 kb for *Eco* RI, 1.3 kb with *Pst* I and 2.5 kb with *Bam* HI digestion. The pBCORc102 probe gave a detectable signal consisting of three bands (0.6 kb, 1.0 kb and 1.2 kb) after *Eco* RI digestion. The 0.6 kb band existed in parentals and F_1, while the 1.0 kb and 1.2 kb were present respectively in *P. trifoliata* and *C. grandis*. Both bands were present in the F_1. Three bands (0.9 kb, 3.0 kb and 3.5 kb) were present following *Pst* I digestion and revealed the same pattern as observed with *Eco* RI digestion. The DNA banding patterns generated by the three restriction enzymes suggested that pBCOR102 may represent at least two loci while the patterns for pBCOR410 and pBCOR510 may represent single genes. Hybridization with the pBCORc720 probe gave very poor results. The two hybridizing bands revealed for the parental and progeny DNAs following *Eco* RI digestion were too weak to accurately determine the number of loci present.

Responses to different stresses

The steady-state mRNA levels of pBCORc115 during cold, flooding, drought and salinization stress were analyzed in *P. trifoliata* and *C. grandis*. RNA blot analysis of RNA levels during cold acclimation is shown in Fig. 6. At 5 °C, the mRNA for pBCORc115 in *P. trifoliata* was main-

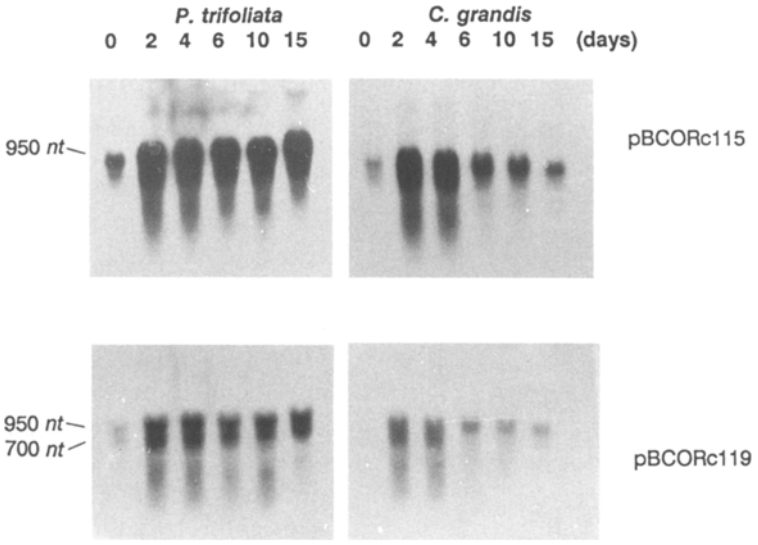

Fig. 6. RNA blot analysis of *P. trifoliata* and *C. grandis* during cold acclimation. For each species, total RNA was isolated at 0 (control), 2, 4, 6, 10 and 15 days after initiation of cold acclimation. Each lane was loaded with 20 μg total RNA. Blots were sequentially probed with pBCORc115 and pBCORc119.

tained at a high level (roughly 10-fold) over the control. In contrast, expression of pBCORc115 in *C. grandis* was highest at 2 and 4 days of acclimation and then gradually declined until 15 days when the mRNA level was only about 3-fold greater than the control. The blot probed with pBCORc119 cDNA gave two bands with almost the same intensity. One band was ca. 950 nt in size while the other was about 700 nt (Fig. 6). The 950 nt band was concluded to be that of the pBCORc115 mRNA and the 700 nt RNA to be the corresponding pBCORc119 mRNA.

Salinization of *P. trifoliata* with 50 mM NaCl did not elicit a change in the pBCORc115 steady-state mRNA level except at 30 days when the level was reduced compared to the control (Fig. 7). The same treatment of *C. grandis* appeared to repress the expression of pBCORc115 after 15 days. No signal was observed in the 10 day samples from either species, and was attributed to technical problems during RNA isolation. In contrast to what was observed during cold acclimation or salinization, the mRNA levels in the leaves of drought and flooded plants were dramatically reduced (Fig. 7). Drought-treated *P. trifoliata* showed very low mRNA levels as the plants became progressively more water-stressed. Surprisingly, the control, mildly water-stressed (6 days), and rewatered plants contained detectable levels of the mRNA. Similarly, droughted *C. grandis* had low steady-state levels in comparison to the control. Equally dramatic was the repression of expression observed in the flooding experiment. Samples from the two species over a period of 2 to 15 days of flooding exhibited no detectable hybridization signal.

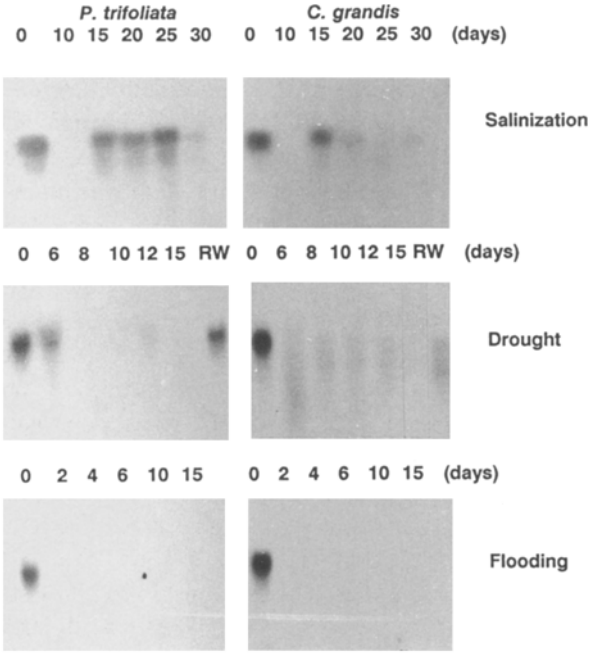

Fig. 7. RNA blot analysis of *P. trifoliata* and *C. grandis* mRNA hybridized with pBCORc115 during salinization, drought, and flooding. Following the initiation of salinization samples were collected at 0 (control), 10, 15, 20, 25 and 30 days. For drought stress, samples were collected at 0 (control), 6, 8, 10, 12 and 15 days after the last watering. Flooding samples were collected at 0 (control), 2, 4, 6, 10 and 15 days after the initiation of flooding. Each lane was loaded with 15 μg of total RNA.

Discussion

It has been previously shown that the trifoliate orange, *P. trifoliata*, is considerably more cold hardy than the pummelo, *C. grandis*. Both the cold hardy *P. trifoliata* and relatively cold-sensitive *C. grandis* can respond to cold acclimation by increasing their cold tolerance, but the cold-tolerance level induced in *P. trifoliata* is much greater than that induced in *C. grandis* [18, 28]. The mechanism responsible for the difference between these two species is unknown. In earlier studies, low-temperature exposure of *P. trifoliata* led to the appearance of novel polypeptides and their presence was correlated to the induced freezing tolerance. Here, the differential expression of six genes between CA and control seedlings were compared. Three cDNAs, pBCORc410, pBCORc102 and pBCORc510, showed cold induction specific for *P. trifoliata* in that their mRNA levels were found to increase during cold acclimation, while in *C. grandis* no detectable mRNA levels were revealed in either CA or control seedlings. With pBCORc410 and pBCORc102, the signal specificities cannot be attributed to sequence divergence between the two species because DNA blot analyses indicated that

the two clones can readily hybridize to the genomic DNAs from both species at high stringency. Therefore we consider that the hybridization signal difference between these two species reflects a authentic difference in the mRNA levels for these two genes. In contrast, the signal difference revealed with pBCORc510 may be due to sequence differentiation or complete absence of this sequence in *C. grandis* since DNA blot hybridization with this probe showed a signal both in *P. trifoliata* and in the F_1 but not in *C. grandis*. The specificity revealed with these clones correlates with the higher level of induced cold tolerance in *P. trifoliata*. Although pBCORc115 and pBCORc119 mRNA levels were actively induced in both species, the possibility their expression may also be correlated with the higher level of induced cold tolerance in *P. trifoliata* cannot be ruled out. In view of the high abundance and stability of the mRNA levels, pBCORc115 and its family may be connected with induced cold hardiness in both *P. trifoliata* and *C. grandis*.

A very high-molecular-mass polypeptide (160 kDa) was identified in *Citrus* in earlier studies. This protein was found to be cold inducible in *P. trifoliata* and sweet orange, but not in *C. grandis* [18, 28]. Among the cDNAs cloned in this study, none have the capacity to encode this large protein since the largest size of the mRNAs hybridizing with these cDNA probes is about 1500 nt and therefore could encode a protein no larger than about 50 kD.

Sequencing data showed that of a group of 7 pBCORc112 cross-hybridizing clones, including pBCORc115 and pBCORc119, 2 clones had exactly the same sequence and the others exhibited a 90–98% identity. Among these, two pairs pBCORc112/pBCORc115 and pBCORc730/pBCORc119 had higher homology (about 98% identity) when compared within each pair. This implied that each pair may belong to allelic members of the same gene locus while the clones outside the pairs may originate from different loci. Should this hold true, then the cDNAs characterized in this study may originate from each of the four gene members of this gene family [8]. However, it will be necessary to more fully characterize all these gene members, including allelic members, in order to comprehend the mechanisms of their genetics, regulation, and evolution and role in cold tolerance related functions.

The deduced open reading frames of COR19 and COR11 may be full-length. This conclusion is supported by two features: an inframe stop codon exists in the sequence before the start codon in COR11 and the two cDNAs are close in length the corresponding mRNA size.

Examination of the deduced amino acid sequence for COR19 revealed four notable structural features. First, three repeats consisting of 36–38 residues were found, accounting for more than 50% of the total residues present in the protein. However, two repeats in this protein are completely absent in COR11, and thus may provide a important clue for understanding the evolutionary pathway of this gene family. The phenomenon of repetitions in amino acid sequence has been reported in many other cold-inducible proteins, among them several Group 2 LEA proteins [17, 20, 42, 45]. Three 21-mer Lys-rich repeats were reported in a cold-induced protein, COR47, from *Arabidopsis* [20] and four small repeats of ten residues were identified in the cold-responsive cas15b from alfalfa [42]. In a more extreme case, 11 repeats each with 22 residues were found in CAP85 from spinach [45]. The variable number repeats in these cold-induced proteins may confer specific functions due to the unique structural features of each protein. Dure *et al.* [17] have suggested that multiple and contiguous repeats might allow for the formation of unique secondary and tertiary structures necessary for protein function during stress. The second structural feature of CORs 11 and 19 is that a Q-cluster tract and a K-rich motif are involved in each repeat. The Q-cluster tract is usually preceded by two H residues and followed by Y, H or Y, R residues. It is interesting that the two flanking residues are always basic amino acids. The number of Q residues in this tract varies from repeat to repeat. This repeated tract has not been previously described in other cold-induced proteins. It is possible that this element may have a function specific to this protein. In contrast to this

element, the K-rich motif is well known in many proteins especially those that are cold- or ABA-inducible, dehydrin-like and LEA-related proteins [4, 6, 17, 20, 37, 45]. A comparison of the K-rich motif of COR19 with those of LEA-related proteins is shown in Fig. 8. Among the proteins shown in Fig. 8, the K-rich motif of the COR19 family is most homologous to the motifs reported for COR47 from *Arabidopsis* [20] and that for CAP85 from spinach [45]. It has been suggested that the conservation of this region may provide insight as to the function and evolution of these proteins [45]. The third feature is the sequence tract KIKQKIPGVG ....KKKKKEKKK that is highly homologous to the bipartite motif of nuclear localization sequences such as nucleoplasmin and N1 in *Xenopus* [49], VirE2 from *Agrobacterium* [12], TGAia in tobacco [33] and NSR1 in yeast [38]. The spacer region between the two basic residue termini of the NLS is known to be variable in length without affecting its nuclear-targeting function. However, according to Robbins *et al.* [49], some basic residues such as

Poncirus trifoliata	COR19	keglvd-kikqkipg-vg
		keglvd-kikqqipg-ag
	COR11	keglvg-kikqkipg-vg
Rice	RAB21	kkgike-kikeklpggnk
Arabidopsis	RAB18	kkgitq-kikeklpg-hh
Arabidopsis	COR47	kkglve-kikeklpg-hh
		kkgile-kikeklpg-yh
Wheat	COR39	kkgvmenkikdklpgg-h
Spinach	CAP85	kggvld-kikdklpg-qh
Barley	B-18	kkgike-kikeklpgg-h
Cotton	D-11	kkglke-rlkekipg-nk
Maize	M-3	kkgike-kikeklpgg-h

Fig. 8. Comparison of Lys-rich motif between COR19 and its homologous member and selected Group 2 LEA-like proteins: RAB21 from rice [43], RAB18 (37) and COR47 [20] from *Arabidopsis*, COR39 from wheat [24], CAP85 from spinach [45], B-18 from barley [13], D-11 from cotton [4] and M-3 from maize [13].

K and R are absolutely necessary for the nuclear targeting function. A similar NLS has been reported for the cold-inducible protein CAS15b in alfalfa [42]. Involvement of a putative bipartite motif necessary for nuclear targeting in COR19 and its homologous member may suggest that this protein family may be nuclear-targeted. A Ser-cluster tract was also found in COR19 and COR11. This tract represents a common feature for many of the Group 2 LEAs [20]. However, its location in the COR19 family is different from that previously described [13, 17, 20]. In COR19 and COR11, the Ser cluster is located at the C-terminus, while in other Group 2 LEAs, it is located on the N-terminal side of the Lys-rich repeat. This implies that COR19 may have a structure quite distinctive from the other members of the family. However, a hydropathy plot analysis showed no obvious difference in hydropathy of this protein from that of other Group 2 LEA proteins. COR19 and COR11 are highly hydrophilic and have a short hydrophobic leading sequence of about 10 amino acids. This feature is common to this family. Since COR19 and COR11 possess features related to Group 2 LEAs and to nuclear targeted proteins, it raises the question as to whether these proteins may have a function in the nucleus. Further studies addressing this question will be critical to understanding their function in woody perennial cold stress responses.

Many studies have demonstrated that a wide variety of proteins induced by cold acclimation are also induced by water stress and/or ABA [11, 14, 27, 34, 44, 45]. Water stress and/or ABA application can induce plants to develop cold tolerance under nonacclimating conditions [11, 15, 27]. Studies focussing on regulatory mechanisms for cold, water stress, and ABA-inducible genes [5, 31, 40] have provided some initial insights as to the mechanism controlling gene expression in response to osmotic stresses. These studies have suggested that water and cold stress and ABA-regulated gene expression may be mediated by similar *cis*-acting elements or by independent but closely linked *cis*-acting elements. In the case of the *cor15a* gene from *Arabidopsis*, the location of

the *cis*-acting elements was confined to a −305 to +78 region [5]. In order to determine whether accumulation of COR19 mRNA in *Citrus* is also regulated by stresses other than low temperature, the RNA levels in response to salinization and water stress, including drought and flooding were examined by RNA blot hybridization. The results demonstrated that the COR19 mRNA expression was specifically promoted during cold acclimation, showed no marked response to salt stress, and displayed a strong repression in response to flooding and drought stress. A similar phenomenon has been reported for the cold regulated gene encoding a 200 kDa protein in wheat [47].

This study has characterized the cDNAs for COR19 and COR11. The evidence provided here implicates this protein family as having a role in citrus cold tolerance. However, to understand the function of this gene family in cold stress responses more work will be required including purification and the biochemical and physical characterization of the encoded proteins.

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