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# Characterization of a pea gene responsive to low temperature

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**Abstract.** A cold-induced gene, *peaci11.8*, was isolated by differential screening of a subtracted cDNA library from cold-acclimated etiolated seedlings of pea (*Pisum sativum* L.). The expression of *peaci11.8* gene was investigated. The accumulation of the transcripts began within 6 h of cold treatment and peaked between 4 and 10 days of treatment. The expression of this gene was rapidly down-regulated after the acclimated seedlings were transferred to ambient temperature. This indicated the correlation of the *peaci11.8* gene expression and cold treatment. Further studies showed that the expression was also highly stimulated by water stress. The amino acid sequence deduced from the nucleotide sequence of *peaci11.8* shared significant homology with several plant cold-induced genes. In addition, the sequence analysis revealed the presence of two common features of many late embryogenesis abundant (LEA) proteins, the lysine-rich motif and the serine cluster. The availibility of *peaci11.8* will facilitate the structure-function studies of the cold-inducible gene product.

**Keywords:** Cold acclimation; Gene expression; *Pisum sativum*.

**Abbreviations: LEA,** late embryogenesis abundant; **NTS,** nuclear-targeting signal; **ORF,** open reading frame; **SSC,** 150 mM NaCl/15 mM sodium citrate.

## Introduction

Temperature is one of the major environmental factors affecting nutrient distribution, growth, and development of plants. Cold-sensitive plants, especially those of tropical or subtropical origins, are readily injured or killed at low, nonfreezing temperatures (Levitt, 1980). In contrast, plants of temperate areas are more tolerant to freezing when previously exposed to low positive temperatures, a process known as cold acclimation. Comparative analyses have revealed that various biochemical and physiological changes occur during cold acclimation, including alterations in lipid and carbohydrate compositions as well as protein contents and enzyme activities (Guy, 1990; Thomashow, 1990).

It has been well-demonstrated that changes in gene expression and protein synthesis are associated with induction of freezing tolerance during cold acclimation (Gilmour et al., 1988; Guy et al., 1985). Thus far, cold-acclimation-specific transcripts have been identified in various plant species including *Arabidopsis thaliana* (Kurkela and Franck, 1990), *Brassica napus* (Orr et al., 1992), *Hordeum vulgare* (Cativelli and Bartels, 1990), *Medicago sativa* (Wolfraim and Dhindsa, 1993), *Oryza sativa* (Aguan et al., 1991), *Spinacia oleracea* (Neven et al., 1993), and *Triticum aestivum* (Chauvin et al., 1993). Although functions of these proteins and the molecular mechanisms regulating expression of these genes remain largely unknown, they are thought to be closely related to cold acclimation. Recently, we found a 19-kDa thylakoid membrane-associating protein increased remarkably when pea (*Pisum sativum*) plants were exposed to low temperature (Ma et al., 1990). To obtain a better insight into the mechanisms of cellular acclimation, efforts were made to isolate and characterize cDNA clones corresponding to cold-induced transcripts from pea. In this report, we describe the cloning and characterization of a novel gene of pea that responds to low temperature.

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## Materials and Methods

### *Plant Material and Treatments*

Seeds of pea (*Pisum sativum* L. cv. Taichung No. 9) were rinsed with tap water and germinated in rolls of moist paper towel at ambient temperature (28°C) in a dark growth chamber. Two-day-old seedlings were either harvested without cotyledons or used for cold (4°C) treatment. For cold acclimation, seedlings were rinsed with ice-cold water, then transferred to cold in dark and harvested at prescribed time intervals. For deacclimation, seedlings acclimated for 4 days were transferred back to ambient temperature and harvested at a specified time. For other stresses, seedlings were harvested 1 day after being transferred to 20% PEG 6,000 or 100 mM ABA at ambient temperature, respectively.

### *RNA Isolation*

Total and poly(A)$^+$ RNA were extracted according to the method of Zurfluh and Guilfoyle (1982), except that the poly(A)$^+$ RNA was precipitated in the presence of 0.3 M ammonium acetate and two volumes of ethanol.

### *Construction and Screening of a cDNA Library*

A subtracted cDNA library, constructed by using the Subtractor II kit (Invitrogen, NV Leek, Netherlands) according to the manufacturer's instructions, was used for differential screening. Poly(A)$^+$ RNA from etiolated pea seedlings cold-acclimated for 3 days was used to construct a subtracted cDNA library. Single-stranded cDNA was synthesized and hybridized to an excess of photobiotinylated poly(A)$^+$ RNA from nonacclimated seedlings (grown at 28°C). The resulting RNA/cDNA hybrids were complexed with streptavidin and removed from the hybridization mixture by phenol/chloroform extraction.

The subtracted cDNA was tailed with dGTP by terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and used for synthesis of second strand cDNA primed with oligo(dC). The double-stranded cDNA was ligated to *Eco*RI linkers, cloned into the *Eco*RI site of the λgt10 vector (Promega, Madison, WI, USA) using *Escherichia coli* as the host cells.

Differential screening was carried out as follows: Duplicate plaque lifts of the library were prepared using Hybond-C extra filters (Amersham). Filters were then hybridized with [$^{32}$P]dCTP-labeled single-stranded cDNA generated from poly(A)$^+$ RNA of nonacclimated plants and plants acclimated for 3 days, separately. Recombinant λgt10 clones that hybridized preferentially to the probe prepared from acclimated plants were isolated, and the cDNA inserts were then subcloned into pGEM-11Zf(+) (Promega) for further analysis.

### *Northern Hybridization*

Total RNA was heat-denatured in the presence of 0.3× electrophoresis buffer (1× electrophoresis buffer equals 200 mM 3-[N-morpholino]propanesulfonic acid/50 mM sodium acetate, pH 6.0/10 mM EDTA), 0.2% formaldehyde, and 15% formamide at 65°C for 10 min and subjected to electrophoresis through a 1% agarose gel containing 1× electrophoresis buffer and 1.2% formaldehyde. RNA samples were transferred onto Hybond-C extra membranes, hybridized with $^{32}$P-labeled probes, washed, and then subjected to autoradiography by the standard procedures (Sambrook et al., 1989). To prepare the probe used for Northern analyses, *Eco*RI fragment of cDNA was gel-purified and labeled with [$^{32}$P]-dCTP by using Prime-a-Gene system (Promega).

### *DNA Sequencing and Analysis*

DNA sequence was determined by the dideoxy method of Sanger et al. (1977). Sequence analysis and alignment were performed with DNASTAR software (DNASTAR, Madison, WI, USA) or the BESTFIT program.

### *Isolation and Southern Analysis of Genomic DNA*

Two grams fresh weight of frozen pea seedlings were ground to a powder in the presence of liquid nitrogen and dispersed in 8 ml of extraction buffer (50 mM Tris-HCl [pH 8.0]/50 mM NaCl/20 mM EDTA/100 μg/ml proteinase K). After adding N-lauroyl sarcosine to a final concentration of 1% (w/v), the homogenate was incubated for 1 to 2 h at 55°C. The mixture was then extracted with an equal volume of water-saturated phenol, and twice more with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Total DNA was precipitated with a 0.7 volume of isopropanol. The recovered pellet was washed with 70% ethanol and resuspended in water. RNA was removed by adding ribonuclease to a final concentration of 10 μg/ml and incubating at 37°C for 1 h. Proteins were removed by adding 50 mM NaCl and 100 μg/ml proteinase K and incubating for another 2 h at 50°C. The solution was extracted with phenol:chloroform:isoamyl alcohol, then precipitated with ethanol. The pellet was resuspended in water. DNA samples were digested with appropriate restriction endonucleases prior to agarose gel electrophoresis. Southern blot was carried out and analyzed according to standard procedures (Sambrook et al., 1989).

## Results

### *Isolation and Expression of the peaci11.8 Gene*

cDNAs corresponding to cold-inducible mRNAs were isolated by differential screening and confirmed by northern blot analysis. One clone, *peaci11.8*, was isolated from $1.4 \times 10^5$ plaques of the subtracted cDNA library. By using randomly primed insert of *peaci11.8* as a probe, northern analysis including nonacclimated and cold-acclimated samples identified a single transcript of approximately 750 nucleotides in the RNA from acclimated seedlings. Figure 1 shows steady-state expression of *peaci11.8* mRNA during cold acclimation and deacclimation. No hybridization in RNA prepared from nonacclimated seedlings was detectable (0 acclimated). The mRNA appeared during the first 6 h of cold treatment and peaked between 4 and 10 days. Subsequently, the accumulation of the transcripts gradually decreased and remained steady for at least

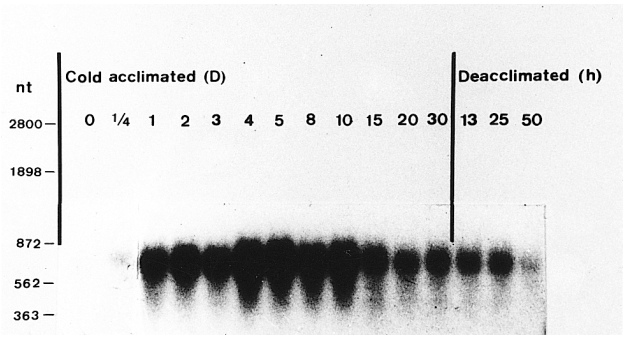


**Figure 1.** Cold acclimation induced the expression of *peaci11.8* gene in pea. Forty micrograms of total RNA prepared from seedlings grown at ambient temperature (28°C) and seedlings treated as indicated was subjected to northern blot analysis. Deacclimation was executed after the seedlings were cold-acclimated for 4 days. Molecular standards are indicated in nucleotides at the left.

30 days. When 4-day-acclimated seedlings were transferred back to ambient temperature, the level of transcripts declined but remained detectable for at least 50 h.

Induction of *peaci11.8* responding to various stresses was also investigated. Figure 2 reveals that in addition to cold, the expression of *peaci11.8* was also induced by water stress and exogenous ABA. When the seedlings were treated with 20% PEG 6,000, the accumulation of *peaci11.8* transcript was identical to that of cold treatment. The gene expression was induced by exogenous ABA, but to a lower level when compared with cold treatment. About one-tenth of the transcripts induced by cold were detected when seedlings were incubated with 100 µM ABA at ambient temperature.

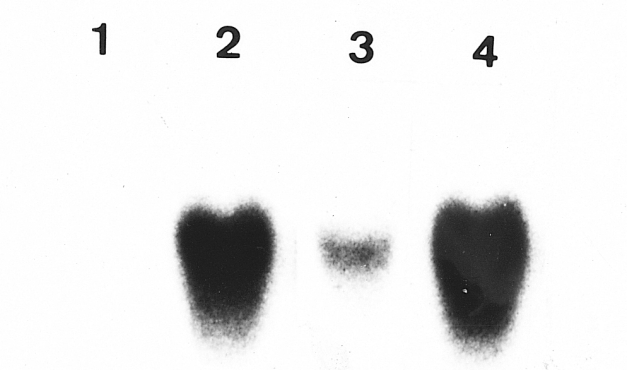


**Figure 2.** Effect of various stress treatments on the accumulation of the *peaci11.8* mRNA. Forty micrograms of total RNA was subjected to northern blot analyses. Lane 1, seedlings grown at ambient temperature; Lane 2, seedlings cold-acclimated for 1 day; Lanes 3 and 4, seedlings treated with 100 µM ABA or 20% PEG 6000, respectively, at ambient temperature for 1 day.

## *Sequence Analysis of the peaci11.8 cDNA*

The DNA sequence was determined to be 607 bp, not including the poly(A) tail. Sequence analyses of *peaci11.8*

A

```
peaci11.8                                  aattaattccggaattaattccggaattatagaaa   35

peaci11.8  atggcaggaatcattaacaagattggtgatgctcttcatataggaggagacaagaaagaa   95
PEACI11.8  M  A  G  I  I  N  K  I  G  D  A  L  H  I  G  G  D  K  K  E     20

peaci11.8  ggagaacaacacataggagagggccatggacatggacatggacatgaacatggacatggt  155
PEACI11.8  G  E  Q  H  I  G  E  G  H  G  H  G  H  G  H  E  H  G  H  G     40

peaci11.8  gctgagtacaaaggagaagaacatggttttggccatggagagcacaagccaggtcaatac  215
PEACI11.8  A  E  Y  K  G  E  E  H  G  F  G  H  G  E  H  K  P  G  Q  Y     60

peaci11.8  caaggggcgcaacacaaggaaggaattgttgacaaggtcaagcacaagatccatggtgaa  275
PEACI11.8  Q  G  A  Q  H  K  E  G  I  V  D  K  V  K  H  K  I  H  G  E     80

peaci11.8  ggtggagcaggtgaaaagaaaaagaaggagaggaagaaacgtgaagatggtcatgaacat  335
PEACI11.8  G  G  A  G  E  K  K  K  K  E  R  K  K  R  E  D  G  H  E  H    100

peaci11.8  ggccatgatagcagcagtagtgatagtgattagatcttaattcacatgcttcataatctt  395
PEACI11.8  G  H  D  S  S  S  S  D  S  D  *                              110

peaci11.8  gaaaggtgtatggaaccatatgtgacgactatgttctatgtttttatttcttgttatgat  455

peaci11.8  tatatctatgtatgtgagtgagaatgtagttgctaattcgagttgttgtacaaataatttt  515

peaci11.8  gcggctagattggtttgtgaaacccatgatgctttgttaatgtttggttgtattattgttg  575

peaci11.8  tcaagaaaattataaggatttctattgttagtaaaaaaaaaaaaaaaaaa             625
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B

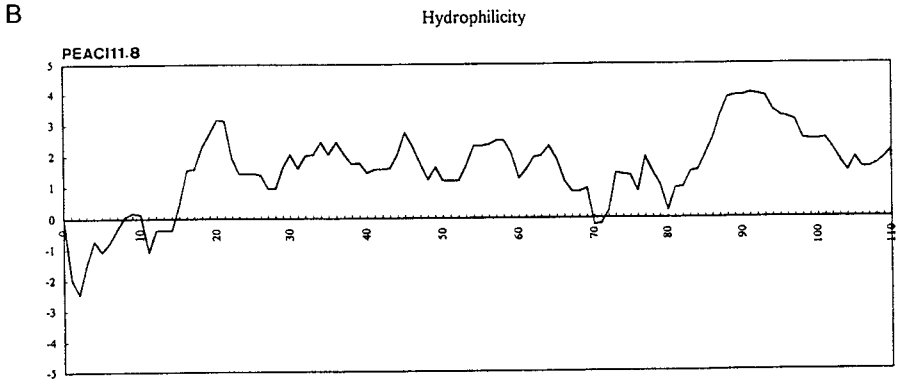


**Figure 3.** A, Nucleotides and deduced amino acid sequences of cloned *peaci11.8* cDNA. The nucleotides and amino acids are numbered at the ends of lines. The direct repeats of the dodenucleotide are dashed underlined. The glycine-histidine-rich clusters are shown in bold. The NTS-like sequence is singly underlined, and the serine cluster is doubly underlined. The stop codon is indicated by asterisks, and the *cis* elements in the polyadenylation signal are shown in bold italics. B, Hydropathy profile of PEACI11.8 protein generated according to the procedure of Kyte and Doolittle (1982) using a 6-amino acid averaging window. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide sequence Databases under the accession number U24398.

revealed a putative open reading frame (ORF). The nucleotide sequence and the deduced amino acid sequence are shown in Figure 3A. The 5' untranslated region contained two direct repeats of 5'-AATTCCGGAATT-3'. Polyadenylation signals, including a near-upstream element 5'-AATTATAA-3', located 17 bp upstream from the poly(A) tail, and two copies of far-upstream element 5'-TTGTA-3', were identified. The ORF, starting at nucleotide 36 and ending with the stop codon at nucleotide 368, encoded a 110-residue polypeptide with predicted molecular mass of 11.8 kDa and isoelectric point of 6.9. This deduced polypeptide, designated PEACI11.8, was rich in glycine (22.7%) and contained a high percentage of residues such as histidine (15.4%), lysine (13.6%), and glutamate (11.8%). These four kinds of residues accounted for 63.6% of the polypeptide. On the other hand, cysteine, threonine, and tryptophan were absent from the polypeptide. Possible motif survey of the PEACI11.8 sequence revealed two glycine-histidine-rich clusters in the middle, a serine cluster at the C-terminal end, and a nuclear targeting signal (NTS)-like sequence (Cai et al., 1995; Monroy et al., 1993) at the C-terminal region. The putative NTS sequence contained a bipartite motif, which started at amino acid 72, KVKHK——KKKKERKKR, with nine spacer residues. The hydrophathy profile suggested it was hydrophilic (Figure 3B).

Search of the sequence data base indicated that PEACI11.8 showed significant homology with CAS15B (69.1%) from *M. sativa* (Monroy et al., 1993), WSI724 (42.7%) from *O. sativa* (Takahashi et al., 1994), and COR19 (44.1%) and COR11 (45.2%) from *Poncirus trifoliata* (Cai et al., 1995), all of which were responsive to low temperature induction. The alignment of the derived amino acid sequences of these genes is shown in Figure 4. The regions of highest homology were located at both termini. Among these, the N-terminal 20 residues of these five polypeptides were almost identical. Additionally, they all possessed NTS-like sequence and serine clusters at the C-terminal ends.

```
PEACI11.8  MAGIINKIGDALHIGGDKKE-----------------------------------GEQH  24
CAS15B     MAGIMNKIGDALHGGGDKKEGEH-KGEQHGHVGGEHHGEYKGEQHGFVGGHAGDHKGEQH  59
COR19      MAGVIHKIGEALHVGGGQKEEDKSKGEHQSRDHHTTDVHHQQQYHGGEHRE-----GEQK  55
COR11      MAGVIHKIGEALHVGGGQKEEDKRKGEHQSGDHHTTDVHHQQPYHGGEHRE-----GEQK  55
WSI724     MAGIIHKIEEKLHMGG---------GEHHEDEHKKE--------------------GEHH  31
           MAG.:.KI:.:LH GG  ::    .::.    .           ..          GE:.

PEACI11.8  IGEGHGHGHGHEHGH-GAE-------YKGEEH--------------------GFGHGEHK  56
CAS15B     -GFVGGHGGD----------------YKGEQH--------------------GFGHGDHK  82
COR19      EGLVDKIKQQIPGAGTADVHHQQQQQQYRGGEHREGEHKEGLVDKIKQQIPGAGTTDVHHQ 115
COR11      ------------------------------------------------------------  55
WSI724     -------------------------KKDGEH----------------------------  37
            .                        ... .:                   .     .

PEACI11.8  PGQYQGAQH-----KEGIVDKVKHKIHGEGGA--------GEKKKK-ERKK--R-EDGHE  99
CAS15B     EG-YHGEEH-----KEGFADKIKDKIHGEGAD--------GEKKKKEKKK--H-GEGHE 125
COR19      QQQYRGGEHREGEQKEGLVDKIKQKIPGVGGGEGATHAQ-GEKKKKEKKK--H-EDGHE 171
COR11      ---------------EGLVGKIKQKIPGVGGGEGATHAHGGE-KKKKEKKKKH-EDGHE  98
WSI724     --------------KEGVVEKIKDKITGDHGDGGE-HKEKKDKKKKKEKK---HGEEGHH  79
              . .  .     :EG :.K:K KI G ::  .  .   :::KKK:E:K:  : :.GH:

PEACI11.8  H-GHDSSSSDSD                                                110
CAS15B     H-GHDSSSSDSD                                                136
COR19      ----SSSSSDSD                                                179
COR11      ----SSSSSDSD                                                106
WSI724     HDGHSSSSSDSD                                                 91
           . ...SSSSDSD
```

**Figure 4.** Comparison of the deduced amino acid sequences of PEACI11.8, CAS15B (accession number: L12461), COR19 (L39004), COR11 (L39005), and WSI724 (D26538). The amino acids are numbered at the ends of lines. Dashes present insertions in the deduced polypeptide sequences to increase sequence alignment. Identical amino acids are indicated below the sequences. Marks below are for amino acids identical in three (.) and four (:) proteins compared, respectively. Other symbols are indicated as in Figure 3.

### *Genomic Characterization of peaci11.8 Gene*

Southern blot analysis of *P. sativum* genomic DNA provided insight into the organization of the gene. Genomic DNA was purified, digested with restriction endonucleases and probed with *peaci11.8*. The hybridization pattern is shown in Figure 5; only a single band was observed in the genomic DNA digested with the restriction enzymes used. From these results and the fact that the *peaci11.8* transcript was polyadenylated, it was suggested that *peaci11.8* exists as a single nuclear gene.

**Figure 5.** Southern blot analysis of *P. sativum* genomic DNA. Lanes B, E, H, S, and X, repesent genomic DNA digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, and *Xba*I, respectively. Twenty micrograms of DNA was used in each lane. Molecular standards are indicated in nucleotides at the left.

## Discussion

A cDNA clone, *peaci11.8*, corresponding to a cold-induced transcript was isolated from *P. sativum* by differential screening of a subtracted cDNA library. The results of northern analyses clearly showed a relationship between expression of the *peaci11.8* gene and cold treatment. Accumulation of the corresponding transcript was observed within the first 6 h of cold treatment and remained at a high level for 10 days. When seedlings were returned back to ambient temperature after 4 days of cold treatment, the amount of the mRNA diminished rapidly. These observations indicated that pea responds to cold treatment quickly and the modulation of the *peaci11.8* gene is stringently regulated at low temperature. The turnover of *peaci11.8* mRNA was slow during deacclimation. The level of this mRNA remained detectable after deacclimation for up to 50 h.

In addition to cold, *peaci11.8* was responsive to water stress and exogenous ABA. It has been reported (Amundson et al., 1993) that cold tolerance could be contributed to by moderate water stress. Their results suggested that water stress may also cause the cold tolerance response in pea. Although treatment with exogenous ABA induced freezing tolerance and the expression of cold-regulated genes in *Arabidopsis thaliana* (Chandler and Robertson, 1994), only a low level of *peaci11.8* transcript was induced in pea seedlings treated with ABA at ambient temperature when compared with cold treatment. Therefore, the expression of the *peaci11.8* gene in pea seems to be regulated in a different manner. This observation is consistent with the hypothesis that independent but converging signal pathways may regulate the expression of genes in response to low temperatures and exogenous ABA treatment (Nordin et al., 1991).

Polyadenylation signals of plant genes are composed of several *cis* elements: far-upstream element, near-upstream element, the actual polyadenylation site and/or downstream element (Hunt, 1994). Two copies of 5'-TTGTA-3', occurring in the far-upstream element in the pea *rbcS*-E9 gene (Mogen et al., 1992), were found between 40 and 107 upstream from the polyadenylation site in *peaci11.8* cDNA. An AATAAA-like motif, 5'-AATTATAA-3', in the near-upstream element in *peaci11.8* gene was proposed.

According to DNA sequence analysis, this gene could potentially encode a polypeptide, PEACI11.8. This ORF appeared to encode a small, hydrophilic protein with abundant glycine, histidine, lysine, and glutamate residues. Examination of the deduced amino acid sequence revealed three structural features. First, the arrangement of the basic amino acids in the putative bipartite motif near the carboxy terminus of PEACI11.8 was highly homologous with those of NTS sequences (Cai et al., 1995; Monroy et al., 1993). The spacer between the two basic regions of the NTS is known to be variable in length and sequence.

| | | |
|---|---|---|
| *P. sativum* | PEACI11.8 | KegivdKvKhKihg |
| *A. thaliana* | COR47 | KKglveKiKeKlpg |
| | | KKgileKiKeKlpg |
| *Gossypium hirsutum* | D-11 | KKglKerlKeKipg |
| *H. vulgare* | B-17 | KKglKdKiKeKlpg |
| | | KKgimdKiKeKlpg |
| *Lycopersicon esculentum* | LE4 | KKglKeKimeKmpg |
| *O. sativa* | RAB21 | KKgikeKiKeKlpg |
| *Poncirus trifoliata* | COR19 | KegivdKiVqKipg |
| | | KeglvdKiKqqipg |
| *S. oleracea* | CAP85 | KggvldKiKdKlpg |

**Figure 6.** Comparison of lysine-rich motif between PEACI11.8 and a selected group 2 LEA-relative proteins: COR47 from *A. thaliana* (Gilmour et al., 1992), D-11 from *Gossypium hirsutum* (Baker et al., 1988), B-17 from *H. vulgare* (Close et al., 1989), LE4 from *Lycopersicon esculentum* (Kahn et al., 1993), RAB21 from *O. sativa* (Mundy and Chua, 1988), COR19 and COR11 from *Poncirus trifoliata* (Cai et al., 1995) and CAP85 from *S. oleracea* (Neven et al., 1993).

It is highly likely that the protein product is transported to nuclei. The second feature was the glycine-histidine-rich sequence tracts. Such a structure has not yet been observed in other cold-induced proteins. The third feature was the serine cluster located at the C-terminus. This is a common feature of the five homologous cold-induced proteins represented in Figure 3 and is common in many LEA-like proteins, but in a different position. The glycine-histidine cluster was demonstrated in one of the *fsh* proteins (Haynes et al., 1989) and the *pipsqueak* protein (Weber et al., 1995) of *Drosophila*; the serine cluster motif was also found in the *fsh* protein. Both *Drosophila* proteins are critical in regulating the expression of other genes during development. Whether or not these characters in PEACI11.8 play the same regulatory function as *pipsqueak* and *fsh* proteins requires further investigation. Moreover, a lysine-rich motif, well known in group 2 LEA proteins, was found. A comparison of the lysine-rich motifs between *peaci11.8* and selected LEA-relative proteins is shown in Figure 6. These sequence characterizations together with the expression pattern of *peaci11.8* and the hydrophilicity of PEACI11.8 suggest that it could be one of the LEA proteins.

Although it remains unclear how the expression of *peaci11.8* contributes to the development of freezing tolerance during the cold acclimation of pea, it is possible for its product to play an important function in plant metabolism during cold acclimation. The availability of *peaci11.8* cDNA clone should facilitate further investigation into mechanisms of cold adaptation and modulation of gene expression of plants at low temperatures.

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