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A Rapeseed Cold-Inducible Transcript Encodes a Phospho*enol*pyruvate Carboxykinase[1]

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We have isolated a clone corresponding to a new cold-regulated gene from a cDNA library made from rapeseed (*Brassica napus* cv Samouraï) cold-acclimated etiolated seedlings. Sequence analysis and homology searches showed that this clone encodes a protein highly homologous to the ATP-dependent phospho*enol*pyruvate carboxykinase (PEPCK; EC 4.1.1.49) from *Saccharomyces cerevisiae*, *Trypanosoma*, *Rhizobium* sp., and *Escherichia coli*; we refer to the *B. napus* clone as BnPEPCK. A potential ATP-binding site existing in all PEPCK proteins was also found in BnPEPCK. Although there was a basal expression of BnPEPCK in seedlings grown at control, room temperature, the steady-state level of the transcripts increased at 4°C and decreased to normal levels when the seedlings were returned to control temperature (22°C). Using antibodies made against a recombinant histidine-BnPEPCK fusion protein, we demonstrated that BnPEPCK protein level is correlated with the accumulation of the *BnPEPCK* transcript.

Several biochemical and physiological changes have been demonstrated during cold acclimation (for reviews, see Guy, 1990; Thomashow, 1990; Palva, 1993). These changes include a general increase in soluble proteins, an increase in free sugar concentration, changes in some specific amino acids, an increase in the level of unsaturation of membrane fatty acids, and changes in nonenzymatic proteins, isoenzyme composition, and enzyme conformation. Most of these changes are the result of modification of gene expression. Although many cold-inducible genes have been described, in most cases their functions are not known. The mechanisms underlying their induction are also poorly understood, some of them being ABA dependent, whereas the induction of others is mediated by another, as-yet-unidentified signal.

Investigations of *Arabidopsis thaliana*, alfalfa, rice, and barley revealed a series of genes that are presumed to be involved in cold acclimation and to play a role in antifreeze mechanisms, although their exact function is not known. They fall into several classes and encode proteins such as KIN1 and KIN2, RAB 18 and dehydrin-like proteins, Ala-rich proteins, and COR15 and BN115 chloroplast proteins (Gilmour et al., 1992; Lin and Thomashow, 1992; Kurkela and Borg-Franck, 1992; Lang and Palva 1992; Weretilnyk et al., 1993). Several of these genes or their homologs have also been shown to be induced in various other stress conditions, and the question arises as to the specificity of their induction by cold treatment. For instance, *Rab18* and *kin1* genes are normally expressed during silique and seed development at normal temperature (Delseny et al., 1993; Parcy et al., 1994).

Another approach consists of looking for several enzyme genes that are likely to be regulated by cold. Christie et al. (1991) showed the increase of alcohol dehydrogenase-1 mRNA and protein activity in maize and rice.

Similar results have been reported in *A. thaliana* (Jarillo et al., 1993). Fatty acid ω-3 desaturase is another example of a cold-regulated gene (Gibson et al., 1994). Furthermore, it was demonstrated in winter wheat that the Suc synthase polypeptide and mRNA levels increase with low-temperature treatment (Crespi et al., 1991). Finally, several genes encoding putative regulatory proteins have been described, although their place and role in the various transduction pathways involved in the response to cold treatment are far from clear (Aguan et al., 1993; Sáez-Vásquez et al., 1993; Jarillo et al., 1994).

We previously showed changes in protein patterns and the populations of translatable mRNA in cold-acclimated *Brassica napus* seedlings, including repression of the small subunit of Rubisco (Meza-Basso et al., 1986). Subsequently, we demonstrated that L-Phe ammonia-lyase was one of the cold-induced polypeptides (Parra et al., 1990). More recently we have isolated and characterized several new cDNA clones corresponding to transcripts that accumulate following a transfer of etiolated seedlings to 4°C, two of which correspond to a protein homologous to the human tumor breast basic protein (Sáez-Vásquez et al., 1993). Now, we report the isolation and characterization of another clone encoding a cold-induced protein, identified as the PEPCK (EC 4.1.1.3.2). This is a key enzyme in gluconeogenesis of animal cells and may also contribute to increased sugar in plant cells during cold acclimation.

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Abbreviations: EST, expressed sequence tag; His-BnPEPCK, fusion protein of His and *B. napus* PEPCK; PEPCK, PEP carboxykinase; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Plant Material

Rapeseed (*Brassica napus* L. cv Samouraï) seeds were germinated at room temperature (22°C) in the dark on wet filter paper. After 72 h of germination, seed coats were removed and the seedlings divided into two batches. One of them was incubated for a further 48 h in a cold room (4°C); the other was incubated at room temperature (22°C) as a control. In both cases, seedlings were grown in the dark.

RNA and DNA Extraction

Total RNA and DNA were prepared by grinding plant tissue in liquid nitrogen and extracting as previously described (Sáez-Vásquez et al., 1993). Total RNA was extracted from cold-treated (4°C) and control seedlings (22°C). Genomic DNA was prepared from young leaves of 8-d-old seedlings.

Isolation of cDNA Clones

Construction of a cDNA library in the λ-ZAPII phage using poly(A)$^+$ RNA isolated from cold-acclimated etiolated seedlings of *B. napus*, cv Samouraï, was previously described (Sáez-Vásquez et al., 1993). Differential screening of this library was carried out on approximately 10^6 plaques using [^{32}P]dCTP-labeled first-strand cDNA probes prepared with poly(A)$^+$ RNA isolated from cold-acclimated or unacclimated seedlings. Hybridizations were carried out at 65°C in 5× SSC (0.75 M NaCl, 0.075 M Na_3 citrate), 5× Denhardt's solution (0.1% [w/v] BSA, 0.1% [w/v] Ficoll, 0.1% [w/v] PVP), 0.5% (w/v) SDS, and denatured calf thymus DNA at 25 μg/mL. Nitrocellulose membranes (BA85, Schleicher & Schuell) were washed twice for 10 min in 2× SSC, 0.1% (w/v) SDS at room temperature and once for 15 min in 1× SSC, 0.1% (w/v) SDS at 65°C. Phage corresponding to positive clones following differential screening were plaque purified three times for characterization and sequence analysis.

Northern and Southern Blot Analysis

Total RNA (15 or 20 μg) was fractionated on 1% formaldehyde agarose gels (Maniatis et al., 1982), stained with ethidium bromide, and transferred to nitrocellulose (BA85, Schleicher & Schuell). The filters were air dried and baked for 2 h at 80°C under vacuum. Northern blot hybridizations were carried out overnight at 42°C in 50% formamide, 5× SSC, 2× Denhardt's solution, 0.1% SDS, 100 μg/mL calf thymus DNA, and 100 μg/mL poly(U). We used the isolated 980-bp pBnC10 cDNA or the 270-bp fragment corresponding to the 3′ noncoding region of the pBnC10 cDNA (nucleotides 1241–1509) as probes. The filters were washed twice for 30 min in 0.2× SSC, 0.1% SDS at room temperature (980-bp probe) or 30 min in 2× SSC, 0.1% SDS at 65°C (270-bp probe) and autoradiographed at −80°C. After hybridization the filters were hybridized with the radish 18S rRNA probe, pRG3 (Grellet et al., 1989), as an internal standard.

Total *B. napus* DNA (5 μg) was digested with *Eco*RI, *Hin*dIII, *Sac*I, or *Xba*I, and the resulting fragments were fractionated on 0.8% agarose gels. The digested DNA fragments were transferred to Hybond-N membranes (Amersham) and hybridized according to the manufacturer's instructions. After hybridization, the filters were washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature and once in 1× SSC, 0.1% (w/v) SDS at 65°C.

[^{32}P]dCTP-labeled probes (pBnC10, 980 bp, and pRG3) were prepared from gel-purified cDNA fragments using the Megaprime DNA labeling system (Amersham). To prepare a specific pBnC10 cDNA probe, the noncoding region corresponding to nucleotides 1241 to 1509 of the cDNA clone pBnC10 (pBnC10, 270 bp) was amplified and [^{32}P]dCTP labeled by polymerase chain reaction using the primers 5′-GAGAGAACACTATGATGT-3′ and 5′-TCCTCTTAAATTTTTCTT-3′. Filters were exposed for autoradiography at −80°C using x-ray films (General Electric) and Hyperscreen intensifying screens (Amersham).

DNA Sequencing and Analysis

DNA sequences were determined by the dideoxy terminator method on double-stranded DNA templates using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 373A automated DNA-sequencing apparatus (Applied Biosystems). Sequence analysis and computation were carried out using the FASTA and BLAST service (Pearson, 1990). The sequences were aligned using the CLUSTAL V program (Higgins, 1994).

Expression of BnPEPCK Protein in *Escherichia coli*

The pBnPEPCK cDNA was mutated using PCR to introduce an *Nde*I restriction site at the 5′ end and an *Xho*I restriction site just after the stop codon. The two oligonucleotides 5′-GCATATGTCAGCTAGAGCTTACCCA-3′ and 5′-CCTCGAGTTAAAAGATAGGACCAGC-3′, containing the *Nde*I and *Xho*I sites (underlined), respectively, were purchased from Bioprobe (Montreuil-sous-bois, France). After amplification, the purified insert was digested with *Nde*I and *Xho*I and ligated into *Nde*I- and *Xho*I-digested plasmid pET16b (Novagen, Madison, WI) to create a His-BnPEPCK fusion protein. The construction was analyzed by sequencing to be sure that the fusion occurred in the correct reading frame. The recombinant plasmid was transformed into *E. coli* BL21(DE3) and the fusion protein induced with isopropylthio-β-galactoside (1 mM final concentration) for 3 h at 37°C. The induced bacterial cells containing the expressed fusion protein were collected by centrifugation, and the bacterial pellet was suspended in 10 mM Tris-HCl buffer, pH 8.0. After one freeze-thaw step, the cells were disrupted by sonication, and the extract was centrifuged at 10,000*g* for 15 min. The supernatant was collected, and the fusion protein was pu-

rified by preparative SDS-PAGE (12.5%) and electroelution using an electroeluter model 422 (Bio-Rad) according to manufacturer's instructions.

Antiserum against His-BnPEPCK Fusion Protein and Western Blots

Polyclonal antibodies against His-BnPEPCK were produced in rabbit by injecting 100 μg of purified protein followed by three injections, 14, 28, and 56 d later, of the same amount of protein. These antibodies were custom-made by Eurogentec (Seraing, Belgium).

Total protein from unacclimated and cold-acclimated (48 h) seedlings was solubilized in extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% [w/v] Suc, 1% β-mercaptoethanol, and 50 μg/mL PMSF). The homogenate was centrifuged at 10,000*g* for 30 min to obtain the soluble fraction. Proteins were separated on a 12.5% SDS gel and transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad) in 125 mM Tris, pH 8.3, 192 mM Gly, 20% methanol. The membranes were blocked with 3% gelatin in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) and probed overnight with a 1:5000 dilution of the His-BnPEPCK antibodies (in TBS supplemented with 0.1% Tween 20 and 1% milk powder). The immunoblots were incubated for 2 h at 37°C with anti-rabbit IgG conjugated to peroxidase. For staining, 0.5 mg/mL horseradish peroxidase Color Development Reagent (Bio-Rad) and 0.05% (v/v) H_2O_2 were used in TBS buffer. Coomassie blue staining was used to verify that the same amount of protein was loaded on gels.

RESULTS

Isolation and Expression Analysis of a cDNA for a Cold-Inducible mRNA

A cold-acclimated *B. napus* cDNA library was differentially screened with single-strand cDNA probes prepared from poly(A)$^+$ RNA from cold-acclimated and control plants. A λ phage cDNA clone that showed much stronger hybridization with the "cold-acclimated" probe than with the control was isolated and recovered as a pBluescript KS(+) clone by in vivo excision, resulting in the plasmid pBnC10.

Northern blot experiments, using the isolated pBnC10 cDNA fragment as a probe and total RNA from cold-treated and nontreated seedlings, indicated that pBnC10 hybridized with a single class of mRNA of about 2.4 kb that is present at a low level in control seedlings but is expressed at much higher levels in cold-acclimated seedlings (Fig. 1). The kinetics of accumulation of *BnC10* transcripts was followed during of the cold acclimation period. With this clone an increase in mRNA level was detected within 8 h after the transfer of the seedlings to low temperature (4°C). However, a significant signal was detected in the control (not shown), and further work demonstrated that there were several genes. Therefore, the experiment was repeated using the 3′ noncoding region of pBnC10 cDNA as a more specific probe (270-bp probe). The *BnC10* transcripts increased within 4 h after the transfer of the seedlings to low temperature (4°C) and decreased to control levels after 6 h of deacclimation (22°C) (Fig. 2A). In parallel, the blots were hybridized with a radish 18S rRNA probe, pRG3, to ensure that similar amounts of RNA were loaded on the gels (Fig. 2B). By scanning the corresponding autoradiograms we estimated that the specific transcript increased approximately 5-fold.

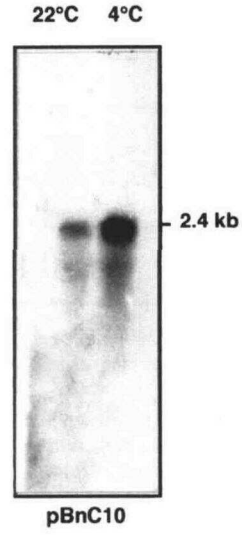

Figure 1. Northern blot analysis of BnPEPCK mRNA. Total RNA (20 μg) isolated from cold-treated (4°C) and nontreated (22°C) *B. napus* etiolated seedlings was fractionated on a 1% formaldehyde agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ^{32}P-labeled pBnC10 cDNA (980 bp) insert. The transcript size is given in kb.

Further Screening and Characterization of BnC10 Sequence

Sequence analysis of the pBnC10 clone showed that it contained a 980-bp insert. Because the first amino acid was not a Met and because the corresponding mRNA is 2.4 kb long, this open reading frame is not complete. In an attempt to find full-length cDNAs, the cold-acclimated cDNA library was screened again using the pBnC10 insert as probe. Approximately 10^6 plaque-forming units from the cDNA library were screened, and six cDNA clones hybridizing to the pBnC10 probe were isolated. The nucleic acid sequences of these clones showed that they were derived from the same gene. The longest cDNA insert was 1508 bp. The cDNA sequence includes neither the 5′ untranslated sequences nor the Met codon that initiates translation; however, the 3′ end is likely to be complete because it ends with a 32-bp poly(A) tail. The partial open reading frame is 1236 bp and encodes a deduced polypeptide of 412 amino acids with a molecular mass of about 45 kD (Fig. 3). Further attempts to isolated a full-length cDNA from this library or to characterize the 5′ end sequence by reverse transcription-polymerase chain reaction were not successful.

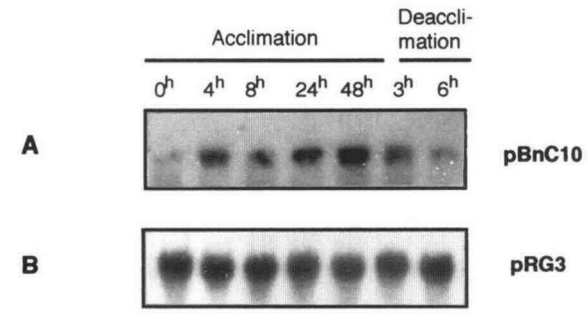

Figure 2. A, Northern blot analysis of the accumulation of BnPEPCK mRNAs during acclimation and deacclimation. Acclimation was at 4°C and deacclimation at 22°C. RNA was extracted at the indicated time after the beginning of the experiments, and 15 μg were loaded in each well, electrophoresed, and transferred to a nitrocellulose membrane and hybridized with a ^{32}P-labeled gene-specific probe (270-bp 3′ end fragment). B, As a control the same filter was hybridized with a radish rRNA clone probe (pRG3).

A data base search showed that pBnC10 encodes a protein that is homologous to the ATP-dependent PEPCK protein. The deduced PEPCK protein of *B. napus* showed considerable similarity to sequences of ATP-dependent PEPCK from *Saccharomyces cerevisiae* (57% identity; Stucka et al., 1988), *Trypanosoma cruzei* (53% identity; Linss et al., 1993), *Trypanosoma brucei* (51% identity; Parsons and Smith, 1989), *Rhizobium* (51% identity; Osteras et al., 1991), and *E. coli* (47% identity; Medina et al., 1990) (Fig. 4). Furthermore, it is 51% identical with a partial sequence downstream from the *ompR* and *envZ* genes from *Salmonella typhimurium* (Liljestrom et al., 1988). No homology was found with the animal GTP-dependent PEPCK.

In addition to this significant average homology, several regions are almost perfectly conserved. A consensus ATP-binding site (GXXGXGKT) was located in BnPEPCK at position 110 (GLSGTGKT) (Higgins et al., 1988). This region is highly conserved in all five PEPCK proteins described in Figure 4. Similarly, the calcium-binding site found in *E. coli* PEPCK sequence (DXTXDXXDXSXXE) was partially conserved in *B. napus* (position 180) as well as in the sequences from yeast, *Rhizobium*, and *Trypanosoma*. The reactive Cys^{364} identified in yeast (Alvear et al., 1992) is also conserved in *B. napus* (Cys^{212}). Accordingly, the pBnC10 clone was renamed pBnPEPCK. Additionally, four partial cDNA sequences (ESTs) for *A. thaliana* that are homologous to the *BnPEPCK* gene were reported from the French systematic cDNA-sequencing program (Höfte et al., 1993). The deduced amino acid sequences of ATTS3142 (154 amino acids), ATTS2525 (108 amino acids), and ATTS 1817 (55 amino acids) are, respectively, 94, 98, and 91% identical with the amino-terminal sequence of BnPEPCK; ATTS0572 (33 amino acids) showed 94% identity with the corresponding carboxyl extremity sequence of the deduced amino acid sequence of BnPEPCK. A maize peptide sequence also derived from an EST (ZMT12738) shows 75% identity with PEPCK of *B. napus* and *A. thaliana*. This high amino acid sequence conservation is remarkable given the phylogenetic distance between Cruciferae and cereals.

Genomic Southern Blot Analysis

To investigate the genomic organization of *BnPEPCK* gene(s) we performed a Southern blot hybridization analysis using genomic DNA digested with *Eco*RI, *Hin*dIII, *Sac*I, or *Xba*I, four enzymes that do not cut within the *BnPEPCK* insert. Hybridization and washing in moderately stringent conditions revealed several fragments hybridizing to the pBnPEPCK insert probe, indicating the presence of several closely related homologous genes (Fig. 5). Although this could have resulted from the tetraploid origin of *B. napus*, which contains both the *Brassica campestris* and *Brassica oleracea* genomes, this is not the case. An additional Southern blot analysis using the two parental genomes of *B. napus* showed that each genome contains at least two related genes (data not shown).

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1     TCA GCT AGA GCT TAC CAC TCT CTC TTC ATG CAC AAC ATG TGT ATC CGA CCA
1      S   A   R   A   Y   H   S   L   F   M   H   N   M   C   I   R   P

52    ACT CCA GAG GAG CTT GAG AGC TTC GGT ACT CCG GAC TTC ACT ATA TAC AAC
18     T   P   E   E   L   E   S   F   G   T   P   D   F   T   I   Y   N

103   GCT GGG CAT TTA AGT GTA AAC CGT TAC ACT CAC TAC ATG ACT TCC TCC ACT
35     A   G   H   L   S   V   N   R   Y   T   H   Y   M   T   S   S   T

154   AGC GTA GAC CTG AAT CTC AGT AGG AGG GAG ATG GTT ATA CTG GGA ACT CAG
52     S   V   D   L   N   L   S   R   R   E   M   V   I   L   G   T   Q

205   TAT GCT GGT GAG ATG AAG AAG GGT CTC TTC AGT GTG ATG CAC TAC CTT ATG
69     Y   A   G   E   M   K   K   G   L   F   S   V   M   H   Y   L   M

256   CCT AAG CGT CGG ATC CTC TCC CTT CAC TCT GGT TGC AAT ATG GGC AAA GAA
86     P   K   R   R   I   L   S   L   H   S   G   C   N   M   G   K   E

307   GGA GAT GTT GCT CTC TTC TTT GGA CTC TCA GGG ACT GGG AAG ACA ACG CTG
103    G   D   V   A   L   F   F   G   L   S   G   T   G   K   T   T   L

358   TCT ACT GAT CAC AAC AGG TAC CTG ATT GGA GAT GAT GAG CAT TGT TGG ACT
120    S   T   D   H   N   R   Y   L   I   G   D   D   E   H   C   W   T

409   GAG ACT GGT GTC TCG AAC ATT GAG GGT GGA TGC TAT GCC AAG TGC GTT GAT
137    E   T   G   V   S   N   I   E   G   G   C   Y   A   K   C   V   D

460   CTT TCG AGG GAG AAG GAG CCT GAT ATC TGG AAC GCT ATC AAG TTT GGA ACA
154    L   S   R   E   K   E   P   D   I   W   N   A   I   K   F   G   T

511   GTT TGG GAA AAT GTT GTG TTT GAT GAG CAC ACC AGA GAA GTG GAT TAT TCT
171    V   W   E   N   V   V   F   D   E   H   T   R   E   V   D   Y   S

562   GAT AAA TCC GTT ACA GAG AAC ACA CGT GCT GCT TAC CCT ATT GAG TTC ATT
188    D   K   S   V   T   E   N   T   R   A   A   Y   P   I   E   F   I

613   CCA AAC GCG AAA ATA CCT TGC ATT GGT CCA CAC CCC AAG AAT GTG ATT CTT
205    P   N   A   K   I   P   C   I   G   P   H   P   K   N   V   I   L

664   CTG GCA TGT GAT GCC TTT GGT GTT CTC CCA CCT GTG AGC AAG CTG AAC CTG
222    L   A   C   D   A   F   G   V   L   P   P   V   S   K   L   N   L

715   GTG CAA ACC ATG TAC CAC TTC ATT AGT GGT TAC ACT GCT CTG GTT GCT GGG
239    V   Q   T   M   Y   H   F   I   S   G   Y   T   A   L   V   A   G

766   ACA GAG GAC GGT ATC AAG GAG CCA ACA GCT ACA TTC TCA GCA TGC TTT GGT
256    T   E   D   G   I   K   E   P   T   A   T   F   S   A   C   F   G

817   GCA GCT TTC ATA ATG CTG CAT CCT ACC AAG TAC GCA GGT ATG TTA GCC GAG
273    A   A   F   I   M   L   H   P   T   K   Y   A   A   M   L   A   E

868   AAG ATG AAG ACG CAA GGT GCT ACT GGA TGG CTC GTT AAC ACT GGC TGG TCT
290    K   M   K   T   Q   G   A   T   G   W   L   V   N   T   G   W   S

919   GGT GGC AGC TAT GGA GTT GGA AAC AGA ATC AAG CTG GCG TAT ACT AGG AAG
307    G   G   S   Y   G   V   G   N   R   I   K   L   A   Y   T   R   K

970   ATC ATT GAT GCA ATC CAT TCA GGT ACC TTG TTG AAG GCG AAC TAC AAG AAA
324    I   I   D   A   I   H   S   G   T   L   L   K   A   N   Y   K   K

1021  ACC GAA ATA TTT GGG TTT GAA ATC CCA ACT GAG ATC GAA GGG ATA CCA TCA
341    T   E   I   F   G   F   E   I   P   T   E   I   E   G   I   P   S

1072  GAG ATC TTG GAT CCG ATC AAC TCG TGG TCT GAT AAG AAA GCA CAC AAG GAG
358    E   I   L   D   P   I   N   S   W   S   D   K   K   A   H   K   E

1123  ACT CTG TTG AAG CTG GGA GGT TTG TTC AAG AAG AAC TTT GAG ACG TTC GCT
375    T   L   L   K   L   G   G   L   F   K   K   N   F   E   T   F   A

1174  AAC CAC AAG ATT GGT GTG GAT GGT AAG CTC ACG GAG GAG ATT CTC GCT GCT
392    N   H   K   I   G   V   D   G   K   L   T   E   E   I   L   A   A

1225  GGT CCT ATC TTT TAA GAGAGAACACTATGATGTTGTCGAAACAAACAAGAAGAAGGATATTA
409    G   P   I   F   *
1287  TCAAGAATAAAATGTGACTTGTGTGTTGTATCTCTTTGTCCGAGTTATTTTATATGAAGTGGTTACT
1354  TTGCGAGGCAAAAGAACTTCCAAATATGGTGGATATGAAATATGACCTTATGTTATTTGATTTTCAT
1421  ATATTGTCGTCATTTATTTTTTCCTTAGTCTCTTACGAGAGATATTCTTCTGTTTTATTTTCCAATG
1488  AACAAGAAAAATTTAAGAGGA(A)32
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Figure 3. Nucleotide sequence and deduced partial amino acid sequence of *BnPEPCK*. The DNA sequence is numbered from the first nucleotide of the sequenced fragment. The ATP-binding site is underlined, and the consensus sequence within it is shown in boldface. Residues of a potential calcium-binding site are in boldface. The stop codon is indicated by an asterisk (*).

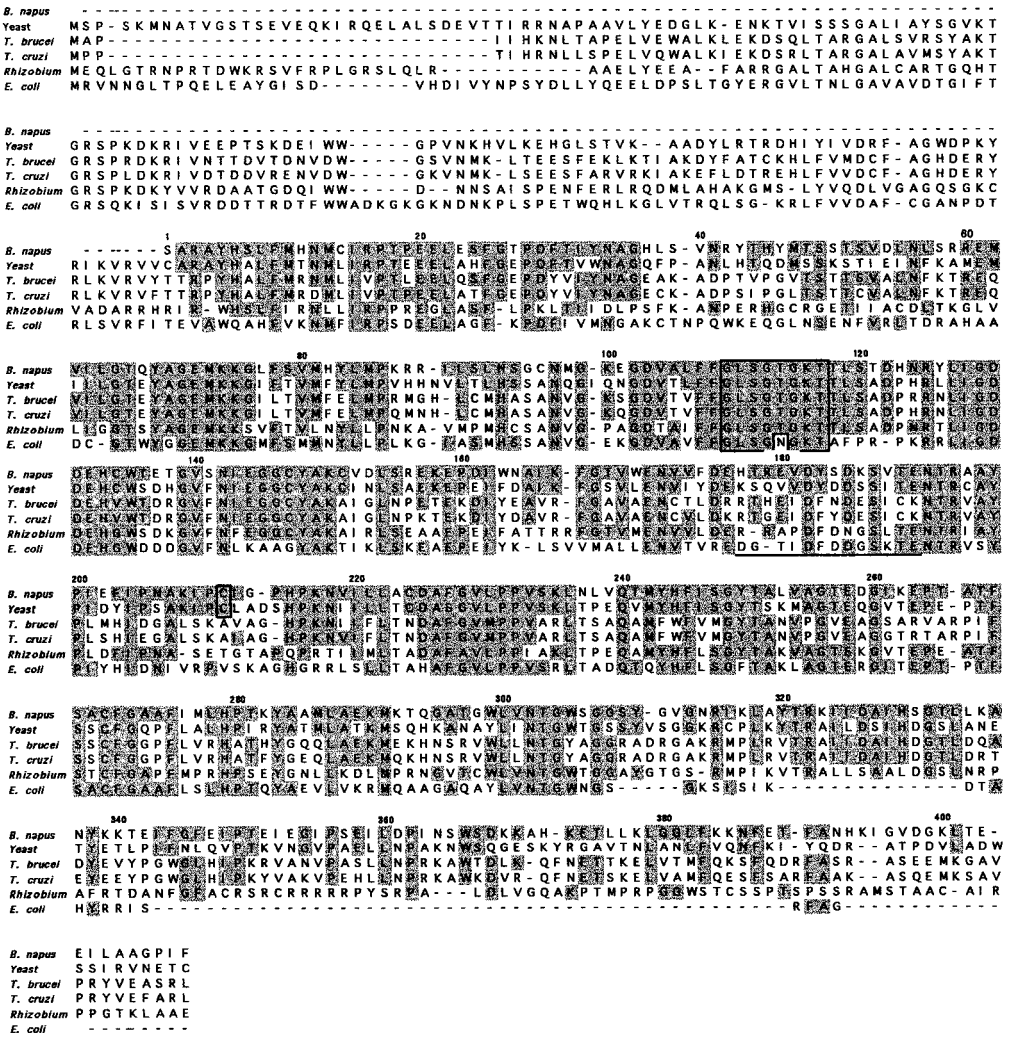

Figure 4. Comparison of the amino acid sequence of BnPEPCK of *B. napus* with PEPCK proteins from yeast (accession No. X13096), *T. brucei* (accession No. P13735), *T. cruzi* (accession No. M91163), *Rhizobium* sp. (accession No. X63291), and *E. coli* (accession No. M59823). Sequences were aligned with the program CLUSTAL V. Identities between the amino acid sequence of the *B. napus* PEPCK and the others PEPCK sequences are shaded. The ATP-binding site and the active Cys residue are boxed. The putative calcium-binding site is underlined.

Expression of a His-BnPEPCK Fusion Protein and Immunodetection of BnPEPCK during Cold Treatment

To determine whether the levels of BnPEPCK proteins also varied during cold acclimation, we prepared antibodies from a recombinant fusion protein. The pBnPEPCK cDNA was subcloned in the vector pET16b to produce a His-BnPEPCK fusion protein. Expression of pET/His-BnPEPCK produced a protein of approximately 45 kD. This recombinant protein was purified by electroelution and the antibodies were produced in rabbit. Attempts to purify the His-BnPEPCK fusion protein by affinity chromatography using His-Bind metal chelation resin (Novagen) were unsuccessful.

Equal amounts of soluble proteins extracted from unacclimated (22°C) and acclimated (4°C) seedlings were separated on 12.5% SDS-PAGE gels and transferred to nitrocellulose for western blot analysis. Preimmune serum showed no cross-reaction with any protein of the plants extracts. The results of Figure 6 show that the antibodies against His-BnPEPCK reacted with two polypeptides that were more abundant in cold-acclimated than in unacclimated seedlings. When the seedlings were returned to control temperature, the protein concentration did not significantly change within the next 24 h (not shown). The higher molecular mass band corresponds to a polypeptide of apparent molecular mass of 67 kD. The lower molecular mass band might correspond to a degradation product, since its intensity increased with storage of the protein extract. Alternatively, it could correspond to an isoform.

DISCUSSION

In this paper we have described a cDNA clone corresponding to a new cold-inducible gene in etiolated rape-

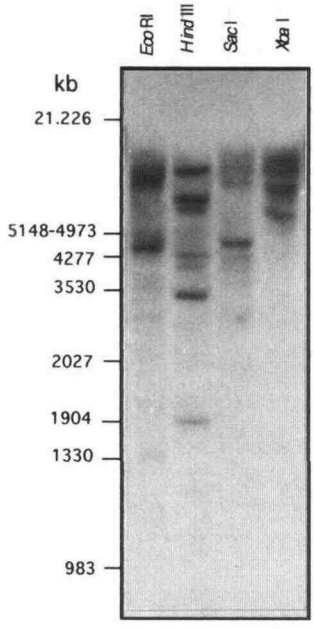

Figure 5. Southern blot analysis of the *BnPEPCK* gene. Total genomic DNA (5 μg) extracted from young leaves was digested with either *Eco*RI, *Hin*dIII, *Sac*I, or *Xba*I and hybridized with the pBnPEPCK probe. The filter was washed under medium stringency conditions. Standard molecular sizes are given in kb.

seed seedlings. Although this cDNA is not full length, enough sequence (1508 bp) was available to identify it, by homology search, as the ATP-dependent PEPCK. The deduced polypeptide shows, on average, 57% identity with the yeast PEPCK protein, and several domains including an ATP-binding domain, a calcium-binding site, and the catalytic site are very well conserved. In addition, several other blocks of amino acids (e.g. 71–76, 127–135, 227–233) are perfectly conserved. When this work was almost completed the sequence of the ATP-dependent PEPCK cDNA from cucumber was reported (Kim and Smith, 1994). It is 2401 bp long and encodes a protein of 74 kD. Sequence comparison indicates 91% homology between the common part of the two sequences. The size of the *B. napus* mRNA (2.4 kb) and the estimated size of the protein (67 kD) are comparable in both species.

Our results demonstrate that the steady-state level of the corresponding mRNA increases throughout a 48-h period of cold acclimation (4°C) and that the protein concentration also increases. When the seedlings were returned to the control temperature, the mRNA concentration returned to its basal level within 6 h. However, the protein concentration remained stable for at least 24 h. The situation is indeed more complex because Southern blots suggest that, in contrast with cucumber, there are several genes in *B. napus* as well as in other diploid *Brassica*. EST analysis also suggests the occurrence of two genes in Arabidopsis. Northern blots carried out with a coding sequence probe indicate that there is some constitutive expression. Therefore, we evaluated the increase in mRNA using a probe for the 3′ untranslated region of the mRNA and came to the conclusion that several genes are probably expressed and that at least one of them is differentially regulated by the cold treatment. Whether the increase in steady-state level of mRNA is due to a transcriptional activation or to stabilization of mRNA is not clear, but preliminary run-on experiments strongly suggest that there is a transcriptional regulation.

It is interesting that this new gene encodes a protein involved in sugar metabolism. PEPCK is a key enzyme in the gluconeogenic pathway by which pyruvate is converted back into Glc. The role of this enzyme has been emphasized in photosynthesis of C_4 plants and more recently in senescing cotyledons of cucumber, a C_3 species (Kim and Smith, 1994). In *B. napus* cold-acclimated seedlings, the increase of PEPCK may contribute to the accumulation of Glc, although there are many other ways for Glc to accumulate at low temperature. The accumulation of Glc, Suc, and Fru during cold acclimation has been reported in a number of species including *A. thaliana* (Ristic and Ashworth, 1993), Puma rye (Koster and Lynch, 1992), ryegrass (Bredemeijer and Esselink, 1994), and spinach (Guy et al., 1992). Therefore, the accumulation of Glc or Suc synthesized from the phosphorylated form of Glc might function as a cryoprotectant in plants exhibiting cold hardiness. In agreement with our results, the up-regulation of aldose reductase and phosphoglucomutase in bromegrass (Lee and Chen, 1993) and Suc synthase in wheat (Crespi et al., 1991) indicates that there is a requirement for genes involved in sugar metabolism during cold acclimation. Whether these changes are biologically significant for the acclimation process or are simply a consequence of adaptation remains to be determined. A possible approach to answering this question is to map the corresponding gene on the chromosomes and establish a linkage between the gene and a favorable phenotype such as cold tolerance. In this respect, it is interesting to note that our clone pBnPEPCK (renamed PEP/4) has been recently mapped on linkage group 3 of *Brassica rapa* (Teutonico and Osborn, 1994) with two other cold-inducible genes, *Cor15a* and

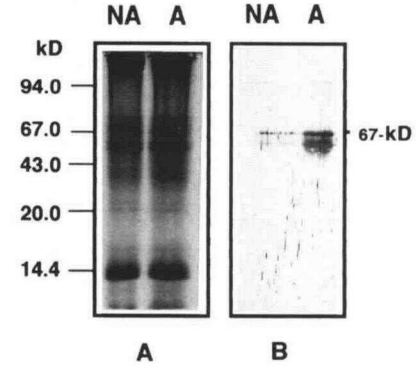

Figure 6. Western blot analysis. Total soluble protein isolated from nonacclimated (NA) and acclimated (A) seedlings (48 h of acclimation) was separated by SDS-PAGE on an 12.5% gel and visualized by Coomassie blue staining (A) or transferred to nitrocellulose (B). The membrane was treated with the His-BnPEPCK antiserum and the bound antibody was revealed as described in "Materials and Methods."

Cor78b. Further analysis of quantitative trait locus should provide additional information concerning the biological significance of this gene for cold tolerance.

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