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Characterization of Three Related Low-Temperature-Regulated cDNAs from Winter *Brassica napus*[1]

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A cDNA clone, pBN115, encoding a low-temperature-regulated transcript in winter *Brassica napus* has been isolated. Northern blot analyses show that levels of transcripts hybridizing to pBN115 increase within 24 h of exposure of *B. napus* to low temperature, peak at 3 d, and then remain at an elevated level for the duration of the cold treatment (up to 10 weeks). Transferring plants from 2°C to room temperature results in the loss of detectable transcripts hybridizing to pBN115 within 1 d. The transcript was not detected in RNA isolated from roots of cold-acclimated *B. napus*. Results of in vivo labeling of nascent RNA in leaf discs of *B. napus* with thiouridine suggest that regulation of expression may be transcriptional, at least at the onset of cold temperature. Although drought stress leads to a slight increase in transcript level at room temperature, neither a brief exposure to elevated temperatures nor exogenous application of abscisic acid resulted in the appearance of the transcript represented by pBN115. Furthermore, transcripts hybridizing to pBN115 were present at the same levels whether the plants were acclimated in the light or dark. Hybridization experiments show that pBN115 hybridizes strongly to cold-regulated transcripts in *Arabidopsis thaliana*, *Descurania sophia*, and spring *B. napus*, all of which are cruciferous plants capable of cold acclimation. No hybridizing transcript could be detected in cold-acclimated *Spinacea oleracea*, winter *Secale cereale*, or cold-grown *Nicotiana tabacum*. DNA sequence analysis of pBN115 reveals a single open reading frame that potentially encodes a protein of 14.8 kD. This size closely approximates that of a polypeptide produced by in vitro transcription/translation experiments. Two additional cDNA clones, pBN19 and pBN26, with divergent 5′- and 3′-untranslated regions, were also isolated and found to encode similar, but not identical, polypeptides.

Low temperature is a major trigger for the acquisition of freezing tolerance in plants capable of cold acclimation (Levitt, 1980). Although it has been shown that biochemical, morphological, and physiological changes occur in plant cells during cold acclimation (Sakai and Larcher, 1987; Johnson-Flanagan and Singh, 1988; Singh and Laroche, 1988; Guy, 1990), direct evidence was obtained only recently to show that low temperature regulated the accumulation of specific mRNAs during cold acclimation (Thomashow, 1990). The appearance of novel transcripts during cold acclimation has been observed in alfalfa (Mohapatra et al., 1989), wheat (Lin et al., 1990; Houde et al., 1991), barley (Cattivelli and Bartels, 1990; Dunn et al., 1990), *Arabidopsis* (Hajela et al., 1990; Kurkela and Franck, 1990; Nordin et al., 1991; Gilmour et al., 1992), and *Brassica* (Orr et al., 1992). Furthermore, DNA sequences corresponding to these cold-specific or cold-regulated transcripts have also been isolated and characterized by differential screening of cDNA libraries constructed from these species. These mRNAs appear rapidly upon exposure of the plant to low temperatures, and deduced amino acid sequences of the products of some of these cold-regulated genes have been determined.

A number of cold-induced transcripts have been shown to hybridize to transcripts of *rab* (responsive to ABA) genes (Hahn and Walbot, 1989) or to encode polypeptides containing amino acid sequence motifs (Guo et al., 1991; Gilmour et al., 1992; Houde et al., 1992) found in *rab* proteins (Skriver and Mundy, 1990), suggesting that they may play a role in the toleration of the cellular desiccation stress that accompanies extracellular freezing. Other cold-induced transcripts encode polypeptides containing stretches rich in alanine and glycine (Kurkela and Franck, 1990; Gilmour et al., 1992; Orr et al., 1992), suggesting that they may play an antinucleating role, although their functions have not been elucidated. The product of one cold-regulated gene has been reported to show cryoprotective properties in vitro (Lin and Thomashow, 1992). The isolation and characterization of cold-regulated transcripts will lead to the identification not only of gene products and mechanisms of cellular frost tolerance, but also of the molecular regulatory elements responsible for gene expression in response to cold temperature and dehydrative stresses. Using differential screening of cDNA libraries prepared from poly(A)$^+$ RNA isolated from leaves of cold-acclimated winter *Brassica napus* cv Jet neuf, we have isolated cDNAs corresponding to four distinct low-temperature-regulated transcripts. In this paper, we report the expression characteristics and nucleotide sequences of the most abundant set of these mRNAs, BN115, BN19, and BN26.

MATERIALS AND METHODS

Plant Material and Stress Conditions

Winter *Brassica napus* L. cv Jet neuf plants were grown in controlled-environment growth chambers at 20°C under a 16-h day at 250 μE m^{-2} s^{-1} light intensity day and an 8-h, 15°C night. For the cold treatment, seedlings were grown under the above conditions for 3 weeks and then transferred to a growth chamber set at 2°C (250 μE m^{-2} s^{-1} and 16-h day) and left at this temperature for varying lengths of time. Heat stress was carried out by exposure of 3-week-old plants at either 37 or 42°C for 2 h. Plants were allowed to recover from heat stress at 20°C for 2 to 9 h before the leaves were

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harvested. Drought stress was imposed by withholding water from plants grown at 20°C until they showed visible signs of wilting (approximately 3 d). The wilted leaves were able to regain turgor upon rewatering, and the relative water content of the wilted leaves was between 60 and 73%.

Spring *B. napus* L. cv Topaz, *Arabidopsis thaliana* L. Heynh ecotype Columbia, *Descurania sophia*, and *Nicotiana tabacum* cv Delgold were grown in controlled-environment growth chambers as described above for winter *B. napus* cv Jet neuf and cold treated at 2°C for 1 week. *Secale cereale* cv Puma was grown under previously described conditions (Johnson-Flanagan and Singh, 1986). *Spinacia oleracea* cv Longstanding Bloomsdale was grown at 325 μE^{-2} s^{-1} at 24°C, 8-h day and 20°C, 16-h night and transferred to a chamber set at 2°C with an 8-h photoperiod for 7 d of cold treatment.

ABA Application and Determination

Plants grown at 24°C were treated with a foliar spray containing 100 μM ABA in 0.02% (v/v) Tween 20, pH 5.5 (Hajela et al., 1990). Leaf samples were collected 2, 5, 24, and 48 h after spraying. Additionally, 1-cm diameter leaf discs were excised from plants grown at 24°C and vacuum infiltrated (Daie and Wyse, 1983) with the ABA solution used for foliar spraying. Infiltrated discs were left in contact with the ABA solution at room temperature for 6 and 18 h. For determination of ABA uptake, leaf discs infiltrated with ABA were washed extensively with the infiltration media minus ABA, blotted to remove excess liquid, weighed, and lyophilized in the dark. ABA was measured by HPLC (Dumbroff et al., 1983).

RNA Extraction

Total RNA was isolated from leaf and root tissues following a phenol/chloroform extraction procedure (Sambrook et al., 1989). Poly(A)$^+$ RNA was isolated from the total RNA samples by binding to Hybond-mAP paper (Amersham) according to the manufacturer's instructions.

cDNA Library Preparation and Screening

Two cDNA libraries were constructed. The first cDNA library was prepared using poly(A)$^+$ RNA isolated from leaves of *B. napus* grown at 2°C for 10 weeks. The cDNA library construction was completed by Clontech Custom Library Services (Clontech Laboratories Inc., Palo Alto, CA) using 25 μg of poly(A)$^+$ RNA. The library was constructed using the vector λgt10 with oligo(dT) as primer and *Eco*RI adapters. This cDNA library was amplified before screening. A second cDNA library was prepared subsequent to that constructed by Clontech. For the second library, poly(A)$^+$ RNA was isolated from winter *B. napus* cv Jet neuf grown at 2°C for 4 d. This cDNA library was prepared in λgt10 with *Eco*RI adaptors using 10 μg of poly(A)$^+$ RNA (Gubler and Hoffman, 1983). This library was not amplified before screening.

Differential screening was carried out using the cDNA library generated by Clontech Custom Library Services. Duplicate plaque lifts of the library were prepared using Nytran filters (0.45 μm; Schleicher & Schuell) and UV cross-linking following recommendations of the manufacturer. Filters were then hybridized with [^{32}P]dATP-labeled (New England Nuclear) single-stranded DNA prepared from poly(A)$^+$ RNA isolated from nonacclimated winter *B. napus* cv Jet neuf (grown at 24°C) and from plants acclimated at 2°C for 1 week. Recombinant λgt10 clones that hybridized specifically to the single-stranded cDNA probe generated from poly(A)$^+$ RNA from acclimated plants were isolated and purified. All differential clones from this library had either one or both *Eco*RI-cloning sites lost. However, in one clone, a 0.4-kb insert could be excised from the vector by *Eco*RI/*Hin*dIII restriction enzyme digestion, and this insert was subcloned into the plasmid pUC119. This 0.4-kb subcloned fragment was subsequently excised, purified by agarose gel electrophoresis, and labeled by randomly primed incorporation of digoxigenin-labeled dUTP with a commercial kit (Boehringer Mannheim). This labeled probe was then used to screen the second, unamplified λgt10 library following the protocols for hybridization and immunological detection suggested by the manufacturer. Fifteen positive clones were detected in the second screen from among 10,000 recombinant clones. The same clones were also detected by differential screening of duplicate plaque lifts made from the same plates. All phage isolates were purified, and cDNA inserts were subcloned into pUC119 for further analyses.

Restriction analyses of the cDNA inserts indicated that they could be divided into three groups: those containing an internal *Hin*dIII and an *Xba*I site, those with only a *Hin*dIII site, and those with neither. A single plasmid subclone (containing the longest insert) from each subgroup was selected. The clones with both *Hin*DIII and *Xba*I sites, with the single *Hin*dIII site, and with neither were designated pBN115, pBN19, and pBN26, respectively. These clones were subsequently used for further studies.

DNA Sequence Analysis

Single-stranded plasmid DNA was prepared (Sambrook et al., 1989) and used to generate deletion subclones (Dale and Arrow, 1987). Single-stranded plasmid clones and deletion subclones were sequenced by the Sanger dideoxynucleotide chain-termination method using either the Klenow DNA Polymerase (Sambrook et al., 1989) or the T7 DNA Polymerase Sequencing Kit (Pharmacia) or Sequenase version 2.0 (United States Biochemical) and following the manufacturers' instructions.

RNA Fractionation and Hybridization Analyses

For RNA hybridization, 5 or 10 μg of total RNA was denatured, subjected to electrophoresis in formaldehyde/1.5% agarose gels, and then transferred to Nytran membrane. Filters were hybridized with the ^{32}P-labeled insert of pBN115 prepared using the Random Primers DNA Labeling System (Gibco/BRL). The size of the transcript hybridizing to the radiolabeled probe was estimated by comparison to the relative mobilities of RNA mol wt standards (Boehringer Mannheim).

Thiouridine Labeling and Purification of Thiouridine-Labeled RNA

Discs (1.5 cm diameter) were excised from leaves of *B. napus* plants grown at 24 and 2°C. One gram each of leaf discs was vacuum infiltrated in 50 mL of distilled water containing 0.02% Tween 20 and 1 mM 4-thiouridine (Sigma). The discs were then maintained in the light at either 24 or 2°C for 18 h. Rate of RNA synthesis during the incubation period was estimated by inclusion of 10 μCi of [^{3}H]uridine (New England Nuclear) in the incubation medium. After the discs were incubated, total RNA was isolated from treated tissue and then applied to phenyl mercury columns (Bio-Rad). Separation of newly synthesized RNA was carried as described previously (Woodford et al., 1988). RNA synthesized before incubation will pass through this affinity matrix, whereas nascent, thiouridine-labeled RNA will be adsorbed. Adsorbed RNA was selectively recovered by elution with 2-mercaptoethanol. Both the nonderivatized and thiouridine-labeled fractions of RNA were purified and analyzed by northern blot hybridization.

In Vitro Transcription and Translation

The cDNA insert of pBN115 was subcloned into pGEM4 (Promega), and the in vitro transcription was directed by SP6 RNA polymerase using the Promega Riboprobe System II kit according to the manufacturer's instructions. The capped RNA was used to direct in vitro translation using a rabbit reticulocyte lysate (Promega) and [^{35}S]methionine (New England Nuclear). The ^{35}S-labeled polypeptides were separated by SDS-PAGE and located by fluorography.

RESULTS

A cDNA library was constructed from poly(A)$^+$ RNA isolated from leaves of winter *B. napus* cv Jet neuf grown at low temperature. A clone, pBN115, was isolated from the library by differential screening. Use of randomly primed insert of pBN115 in a northern blot hybridization of total RNA isolated from leaves of nonacclimated and cold-acclimated *B. napus* identified a single transcript of approximately 0.8 kb that could only be detected in the RNA prepared from cold-acclimated tissue (Fig. 1). No signal could be detected in the lane containing RNA isolated from nonacclimated material despite prolonged autoradiographic exposure of the filter to x-ray film or application of 1 μg of poly(A)$^+$ RNA per lane (Fig. 1, lanes 1–3). The mRNA corresponding to the insert of pBN115 is present in abundance in leaves but absent in roots and mature seeds (data not shown). The transcript appears to reach a maximum after 3 d in the cold (Fig. 1, lane 10, based on densitometer analysis, which showed the intensity of labeling of lane 10 to be 1.5-fold that of lane 8). After 10 weeks in the cold, the transcript was still present but at a level about 50% of that after the first week of exposure of the plants to 2°C (Fig. 1, lanes 4–6). Leaf discs excised from plants grown at 20°C and infiltrated in 2°C water for 18 h gave a hybridization signal as strong as leaves from plants acclimated at 2°C for 1 week (Fig. 2, lane 3 versus lane 7).

Exposure of the plants to either 37 or 42°C for 2 h did not result in the appearance of a hybridization signal (Fig. 2, lanes 1 and 2). When cold-grown plants were transferred to room temperature, the mRNA hybridizing to pBN115 could no longer be detected in total leaf RNA after 22 h (Fig. 2, lane 4). ABA was applied at 20°C either by foliar spray or by infiltration of leaf discs. RNA from leaf samples collected 18 h after the foliar application of ABA did not show the appearance of the transcript hybridizing to pBN115 (Fig. 2, lane 5). Samples collected as early as 2 and 6 h following ABA application also did not show a hybridizing signal (data not shown). Similarly, leaf discs infiltrated in ABA at 20°C for 18 h did not show an accumulation of pBN115 transcript (lane 6), in contrast to leaf discs infiltrated at 2°C in the same media minus ABA (lane 7). ABA analysis of infiltrated leaf discs showed an 8-fold uptake of ABA above endogenous levels (data not shown). Drought stress of *B. napus* plants at room temperature resulted in the appearance of a very weak signal hybridizing to pBN115 (lane 8).

Leaf discs of winter *B. napus* Jet neuf grown at 24°C were vacuum infiltrated with a solution containing [^{3}H]uridine and 4-thiouridine. Incorporation of [^{3}H]uridine into RNA was measured for both temperatures to assess the level of RNA synthesis at the two temperatures. The estimated rate of [^{3}H]uridine incorporation into poly(A)$^+$ RNA is approximately 7.5 times higher at 20°C than at 2°C (4.7×10^5 versus 6.2×10^4 cpm/g fresh weight leaf discs). This newly synthesized RNA was purified by phenyl mercury affinity chromatography and analyzed by northern blot hybridization. Figure 3 shows that at 20°C no transcript corresponding to pBN115 could be detected in the fraction of total RNA applied to the column or in the affinity-purified fraction of RNA (lanes 1–3). At 2°C, however, a strong hybridizing signal to pBN115

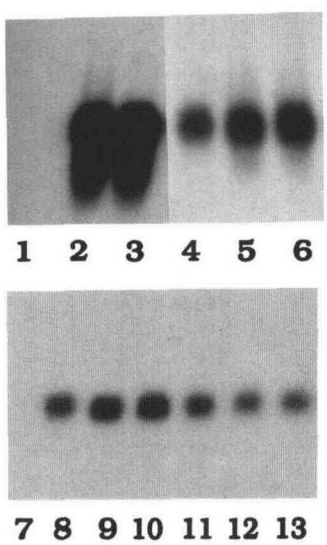


Figure 1. Hybridization of BN115 to a northern blot of RNA isolated from leaves of *B. napus* cv Jet neuf grown at 2 and 20°C. Lane 1, 1 μg of poly(A)$^+$ mRNA from plants grown at 20°C. Lanes 2 and 3, 1 μg of poly(A)$^+$ mRNA from plants grown at 2°C. Lanes 4 to 6, 10 μg of total RNA from plants grown at 2°C for 10, 2, and 1 week, respectively. Lane 7, 5 μg of total RNA from plants grown at 20°C. Lanes 8 to 13, 5 μg of total RNA from plants grown at 2°C for 2, 3, 4, 5, 6, and 7 d, respectively.

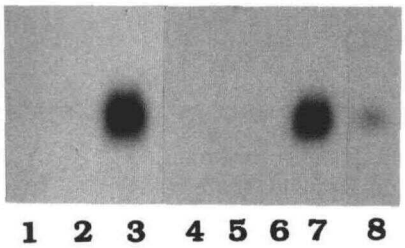


Figure 2. Hybridization of BN115 to a northern blot of RNA isolated from leaves of heat-shocked and ABA- and drought-treated *B. napus* cv Jet neuf plants. Lanes 1 and 2, Total RNA from plants exposed to 37 and 42°C for 2 h, respectively. Lane 3, Total RNA from plants exposed to 2°C for 1 week. Lane 4, Total RNA from plants exposed to 2°C for 1 week and returned to 20°C for 22 h. Lane 5, Total RNA from plants exposed to 20°C for 18 h after foliar application of ABA. Lanes 6 and 7, Total RNA from leaf discs infiltrated for 18 h at 20°C in the presence of ABA and at 2°C in the absence of ABA, respectively. Lane 8, Total RNA from plants subjected to drought.

is observed in spite of the overall lower levels of incorporation of uridine into RNA (Fig. 3, lanes 4 and 5). More specifically, this signal is found only in the RNA fraction that binds to the affinity matrix and, hence, in the nascent fraction of RNA (Fig. 3, lane 8). The proportion of derivatized RNA that did not bind to the column is very low, as indicated by the weak signal in lane 7.

Northern hybridization using total RNA isolated from other cruciferous plants including spring *B. napus*, *D. sophia*, and *A. thaliana* shows that a single cross-hybridizing RNA species can be detected in all of these plants but only, as is true for winter *B. napus*, in samples of RNA from cold-acclimated plants (Fig. 4). Leaves from all three species are capable of developing frost tolerance after cold acclimation (Gilmour et al., 1988; Laroche et al., 1992). Neither the cold-

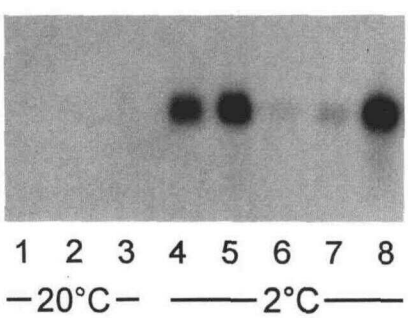


Figure 3. Hybridization of BN115 to a northern blot of newly synthesized RNA from leaf discs exposed to 20 and 2°C. Lane 1, Total RNA (10 μg) from leaf disc exposed to 20°C. Lane 2, Total RNA that did not bind to the affinity column (10 μg) from a leaf disc exposed to 20°C. Lane 3, Total RNA that was bound to the affinity column (10 μg) from a leaf disc exposed to 20°C. Lanes 4 and 5, Total RNA, 5 and 10 μg, respectively, from a leaf disc exposed to 2°C. Lanes 6 and 7, Total RNA that did not bind to the affinity column, 5 and 10 μg, respectively, from a leaf disc exposed to 2°C. Lane 8, Total RNA that was bound to the affinity column (10 μg) from a leaf disc exposed to 2°C.

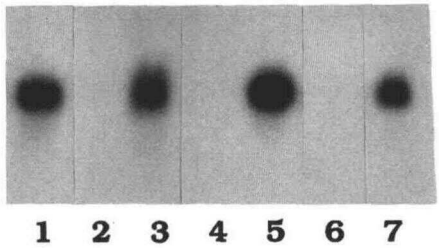


Figure 4. Hybridization of BN115 to a northern blot of total RNA from leaves of different members of the *Cruciferae* grown at 2 and 20°C. Total RNA (10 μg) was used for each lane. Lane 1, Winter *B. napus* cv Jet neuf grown at 2°C for 8 weeks. Lanes 2 and 3, Spring *B. napus* cv Topas grown at 20°C and at 2°C/8 weeks, respectively. Lanes 4 and 5, *D. sophia* grown at 20°C and at 2°C/4 weeks, respectively. Lanes 6 and 7, *A. thaliana* (Columbia) grown at 20°C and at 2°C/4 weeks, respectively.

acclimated dicot spinach nor cold-acclimated monocot rye appeared to possess a transcript sufficiently homologous for hybridization, even when low-stringency washing conditions were used. The same is true for northern analysis of RNA from cold-grown tobacco, in which no cross-hybridizing mRNA species could be detected (data not shown).

Because the cold-induced transcripts hybridizing to pBN115 were found in leaf but not in root or seed RNA, the effect of light on the cold accumulation of BN115 transcripts was investigated. Plants were kept in the dark at 20°C for 24 h and then transferred to 2°C in the dark, and the transcript levels were analyzed after 3 d in the dark (Fig. 5, lane 2). These were compared with plants grown at 2°C for 3 d under a normal photoperiod (compare lanes 2 and 4). Similar levels of transcript hybridizing to BN115 were observed under dark and normal photoperiod conditions during cold exposure. Changing plants from the dark to a normal photoperiod condition or vice versa during cold acclimation did not have an effect on transcript accumulation (lanes 3 and 5), at least in the short term (after 3 d). The transcript could still be detected, although at a lower level, in plants kept at 2°C in the dark for 25 d (lane 6).

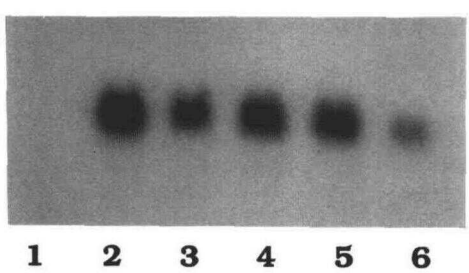
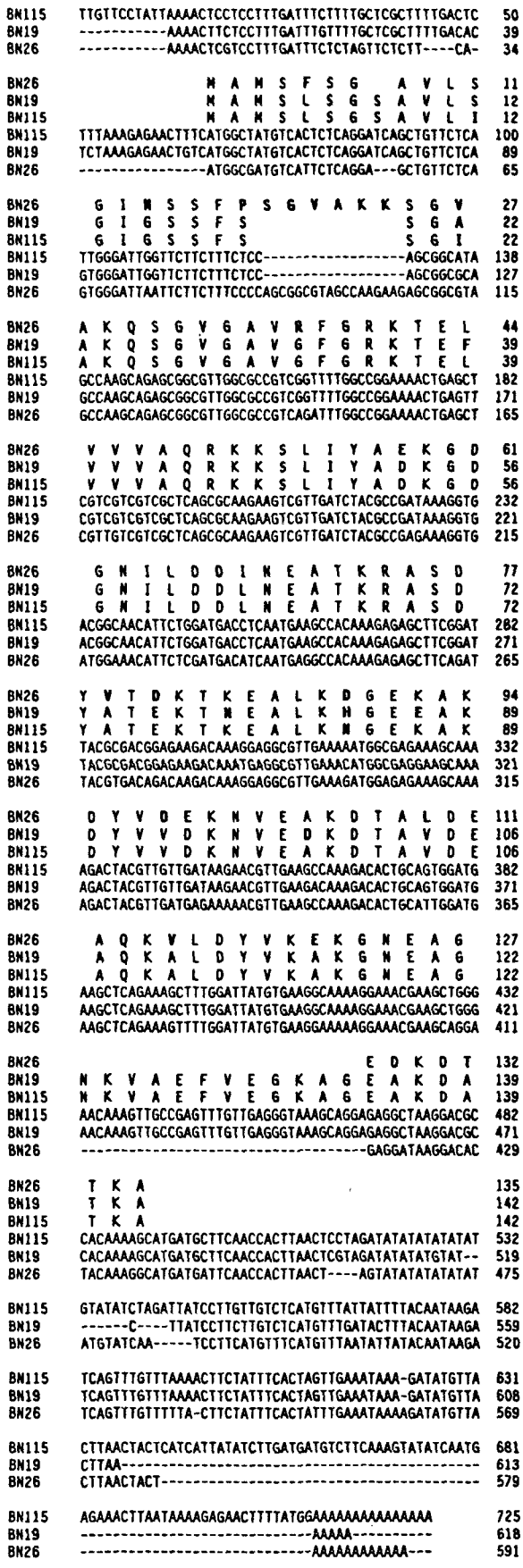


Figure 5. Hybridization of BN115 to northern blots of total RNA from leaves of *B. napus* cv Jet neuf plants exposed to 2°C in the light and dark. All lanes contained 5 μg of RNA. Plants were kept in the dark for 24 h at 20°C (lane 1), then transferred to the dark at 2°C for 3 d (lane 2), and finally returned to light at 2°C for 3 d (lane 3). Plants that were acclimated for 3 d at 2°C (lane 4) were transferred to the dark at 2°C for 3 d (lane 5), and the same plants were maintained for 25 d in the dark at 2°C (lane 6).



The nucleotide sequence of pBN115 is presented in Figure 6. Analysis of the deduced amino acid sequence indicates that the transcript directs the translation of a polypeptide of 142 amino acid residues with a predicted molecular mass of 14.8 kD, which is consistent with the mass of approximately 14 kD for a polypeptide obtained by translation of an in vitro transcript of the cDNA (Fig. 7). The deduced polypeptide is rich in glycine/alanine (26%) and lysine (14%) but does not contain any cysteine, histidine, proline, or tryptophan.

Hybridization of restricted *B. napus* cv Jet neuf DNA with pBN115 suggests that the gene encoding pBN115 may be a member of a small gene family (Fig. 8). In fact, two other cDNA clones (pBN19 and pBN26) that cross-hybridized with the insert from pBN115 were isolated (see "Materials and Methods"). Nucleotide and deduced amino acid sequence analyses indicated that, for at least pBN26, considerable divergence from pBN115 both in the 3′- and 5′-untranslated and coding regions exists (Fig. 6). Most notably, BN26 has five extra amino acids near the N terminus and 12 fewer amino acids near the C terminus, resulting in a total of 135 instead of 142 residues as compared with BN115 and BN19.

DISCUSSION

A cDNA clone (pBN115) corresponding to a cold-induced transcript was isolated from winter *B. napus* cv Jet neuf by differential screening. pBN115 is almost full length because it is missing only 11 bp from the putative cap site identified by sequence analysis of the corresponding genomic clone (White et al., 1992). Both nucleotide and deduced amino acid sequence analyses of BN115 indicated that it is similar to a cold-induced transcript (COR15) isolated from *A. thaliana* (Lin and Thomashow, 1992). Unlike COR15, however, BN115 was not inducible by application of ABA at room temperature. No transcript hybridizing to BN115 was observed in RNA isolated from leaves of room-temperature-grown *B. napus* cv Jet neuf after exogenous application of ABA by both foliar spray and infiltration of leaf discs. In contrast, two other cold-induced clones, pBN28 and pBN59, isolated from the same cDNA library by differential screening, showed increased accumulation in response to ABA application under similar experimental conditions (Orr et al., 1992a, 1992b). These results support the observation that BN115 is not inducible by ABA at room temperature, at least not under the conditions of ABA application used in this study.

Labeling of nascent mRNA by derivatization with thiouridine followed by northern blot analyses has been used to show rapid regulation of mRNA synthesis (Stetler and Thorner, 1984; Cramer et al., 1985). Although this technique selectively enriches the population of de novo synthesized mRNA, pBN115 transcripts were detected only in the low- and not the room-temperature nascent populations. Although this cannot entirely preclude the role of low temperature in pBN115 transcript stability, it suggests that the

Figure 6. Comparisons of nucleotide and deduced amino acid sequences of BN115, BN19, and BN26. Divergences in amino acid sequences among the three cDNAs are highlighted in bold.

accumulation of the transcript(s) hybridizing to pBN115 is regulated mainly at the transcriptional level, at least in the early stages of exposure to low temperature. Maintenance of elevated levels of the transcript corresponding to pBN115 for up to 10 weeks could be accomplished by continued transcription and/or enhanced stabilization of transcripts at 2°C and may be consistent with the requirement for long periods of cold acclimation and vernalization required by winter *B. napus* to develop freezing tolerance and the ability to flower, respectively (Laroche et al., 1992). Our observation that pBN115 hybridized to transcripts observed in cold-acclimated spring *B. napus* and *Arabidopsis* suggests that the gene encoding BN115 is unlikely to be involved only in vernalization, because spring *Brassica* and *Arabidopsis* do not require a period of low-temperature growth to flower. A strong hybridization signal to pBN115 was also observed for RNA isolated from leaves of *D. sophia* (flixweed) acclimated at 2°C. Flixweed cold acclimates more quickly and is capable of developing a higher degree of freezing tolerance than any other crucifer tested (Laroche et al., 1992).

Two other cDNA clones, pBN19 and pBN26, were isolated by screening an unamplified library with pBN115. On the basis of the total number of the three clones obtained, the abundance of the transcript(s) after 4 d of cold acclimation was estimated to be in the order of 0.1 to 0.15%. Divergence among the three clones, especially between pBN115 and pBN26, may either result from pBN115 being a member of a small gene family or reflect the origin of *B. napus*, which contains both the *B. campestris* and *B. oleracea* genomes. Experiments to resolve this are in progress.

pBN115 hybridized to mRNA isolated from cold-grown leaves of three members of the cruciferous family that are capable of developing frost tolerance. Nonetheless, the function of the gene product of BN115 in relation to cellular frost tolerance is not known. The nucleic acid and deduced amino acid sequences of pBN115 show similarities to a cold-regulated gene, COR15, from *Arabidopsis* (Lin and Thomashow, 1992). COR15 was reported to possess in vitro cryoprotective properties in addition to being targeted to the chloroplast stroma, with the first 50 amino acid residues serving as a transit peptide that is cleaved during or after import (Lin and Thomashow, 1992). Analyses of the deduced amino acid sequences for the full-length polypeptides encoded by BN115, BN19, and BN26 resulted in calculated isoelectric points of 9, 5, and 7, respectively. However, the theoretical isoelectric points without the putative transit peptides were very similar (4.6, 4.3, and 4.2). It will be interesting to determine whether the three gene products are similarly localized and whether the processed polypeptides have overlapping functions in cold adaptation.

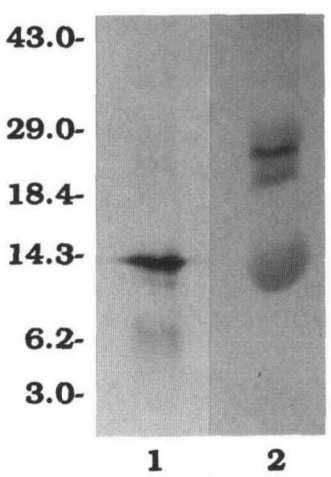


Figure 7. Fluorograph of translation products of the in vitro transcript of BN115. Numbers on the left refer to molecular mass in kD. Lanes 1 and 2 represent the in vitro translation products in the presence and absence of the capped transcript in a rabbit reticulocyte lysate system, respectively. Equal amounts of incorporated radioactivity (5×10^4 cpm) were loaded onto each lane.

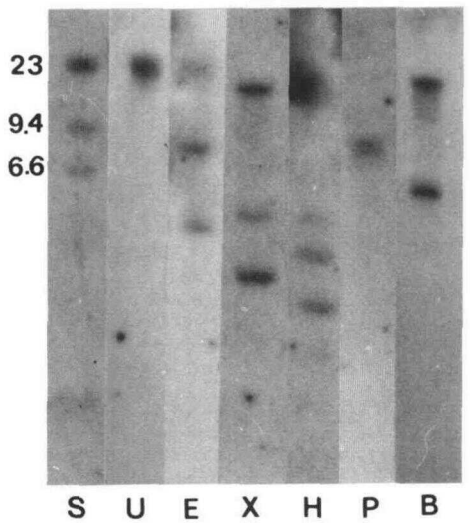


Figure 8. Hybridization of BN115 to a Southern blot of restriction enzyme-digested genomic DNA of winter *B. napus* cv Jet neuf. Lanes S, U, E, X, H, P, and B represent genomic DNA restricted with *Sst*I, undigested, and digested with *Eco*RI, *Xba*I, *Hin*dIII, *Pst*I, and *Bam*HI, respectively. DNA (10 μg) was used in each lane, and numbers on the left represent molecular mass markers in kb.

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The cDNA sequence of BN115 and the restriction map of the genomic clone of BN115 was reported in poster form at the Third International Congress of the International Society for Plant Molecular Biology, October 6–11, 1991, Tucson, AZ.

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LITERATURE CITED

Cattivelli L, Bartels D (1990) Molecular cloning and characterization of cold regulated genes in barley. Plant Physiol **93:** 1504–1510

Cramer CL, Ryder TB, Bell JN, Lamb CJ (1985) Rapid switching of plant gene expression induced by fungal elicitor. Science **227:** 1240–1243

Daie J, Wyse R (1983) ABA uptake in source and sink tissue of sugar beet. Plant Physiol **72:** 430–433

Dale RMK, Arrow A (1987) A rapid single-stranded cloning, sequencing, insertion and deletion strategy. Methods Enzymol **155:** 204–214

Dumbroff EB, Walker MA, Dumbroff PA (1983) Choice of methods for the determination of ABA in plant tissue. J Chromatogr **256:** 439–446

Dunn MA, Hughes MA, Pearce RS, Jack PL (1990) Molecular characterization of a barley gene induced by cold treatment. J Exp Bot **41:** 1405–1413

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

Gilmour SJ, Hajela RK, Thomashow MF (1988) Cold acclimation in *A. thaliana*. Plant Physiol **97:** 745–750

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene **26:** 263–269

Guo WW, Ward R, Thomashow MF (1991) Cold regulated genes of wheat and *Arabidopsis*. Presented at the Third International Congress, International Society of Plant Molecular Biology, Tucson, Arizona, Program and Abstracts: 1624

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu Rev Plant Physiol Plant Mol Biol **41:** 787–796

Hahn M, Walbot V (1989) Effects of cold-treatment on protein synthesis and mRNA levels in rice leaves. Plant Physiol **91:** 930–938

Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold regulated) genes in *Arabidopsis thaliana*. Plant Physiol **93:** 1246–1252

Houde M, Danyluk J, Laliberte J-F, Dhindsa RS, Sarhan F (1991) Cloning and characterization of four cDNAs specifically induced by low temperature in wheat (abstract No. 1063). Plant Physiol **96:** S-161

Houde M, Danyluk J, Laliberte J-F, Rassart E, Dhindsa RS, Sarhan F (1992) Cloning, characterization and expression of a cDNA encoding a 50 kD protein specifically induced by cold acclimation in wheat. Plant Physiol **99:** 1381–1387

Johnson-Flanagan AM, Singh J (1986) Membrane deletion during plasmolysis in hardened and nonhardened cells. Plant Cell Environ **9:** 299–305

Johnson-Flanagan AM, Singh J (1988) Protein synthesis and freezing tolerance in plant cells. CRC Crit Rev Plant Sci **7:** 279–302

Kurkela S, Franck M (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. Plant Mol Biol **15:** 137–144

Laroche A, Geng X-M, Singh J (1992) Differentiation of freezing tolerance and vernalization responses in *Cruciferae* exposed to low temperatures. Plant Cell Environ **15:** 439–445

Levitt J (1980) Responses of Plants to Environmental Stress. Chilling, Freezing and High Temperatures, Ed 2. Academic Press, New York

Lin C, Guo WW, Everson E, Thomashow MF (1990) Cold acclimation in *Arabidopsis* and wheat. Plant Physiol **94:** 1078–1083

Lin C, Thomashow MF (1992) DNA sequence analysis of a cDNA for cold regulated *Arabidopsis* gene *cor15* and characterization of the COR15 polypeptide. Plant Physiol **99:** 519–525; **100:** 546–547

Mohapatra SS, Wolfraim L, Poole RJ, Dhindsa RS (1989) Molecular cloning and relationship of freezing tolerance of cold acclimation specific genes of alfalfa. Plant Physiol **89:** 375–380

Nordin K, Heino P, Palva ET (1991) Separate signal pathways regulate the expression of a low temperature-induced gene in *Arabidopsis thaliana*. Plant Mol Biol **16:** 1061–1071

Orr W, Iu B, White TC, Robert LS, Singh J (1992a) Complementary sequence of a low temperature induced *B. napus* gene with homology to the *A. thaliana kin1* gene. Plant Physiol **98:**1532–1534

Orr W, White TC, Singh J (1992b) Expression of the gene encoding the 70 kDa subunit of the *B. napus* vacuolar ATPase is enhanced by cold (abstract No. 740). Plant Physiol **99:** S-124

Sakai A, Larcher W (1987) Frost Survival of Plants. Responses and Adaptations to Freezing Stress. Springer-Verlag, Berlin

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A laboratory manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Singh J, Laroche A (1988) Freezing tolerance in plants: a biochemical overview. Biochem Cell Biol **66:** 650–657

Skriver K, Mundy J (1990) Gene response to ABA and osmotic stress. Plant Cell **2:** 503–512

Stetler GL, Thorner J (1984) Molecular cloning of hormone responsive genes from the yeast *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA **81:** 1144–1148

Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. Adv Genet **28:** 99–131

White TC, Simmonds D, Singh J (1992) Identification of a *Brassica napus* cold-inducible promoter (abstract No. 467). Plant Physiol **99:** S-78

Woodford TA, Schlegel R, Pardee AB (1988) Selective isolation of newly synthesized mammalian mRNA after *in vivo* labeling with 4-thiouridine or 6-thioguanosine. Anal Biochem **171:** 166–172