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# Expression of a Low-Temperature-Induced Protein in *Brassica napus*[1]

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**BN28 is a low-temperature-induced, boiling-soluble protein in *Brassica napus*. We used antibodies raised against a recombinant BN28 to examine the expression of this protein in cold-acclimating plants and to investigate its relationship to plant freezing tolerance. Changes in the steady-state levels of BN28 protein appear to lag several days behind those of the mRNA. BN28 is first detected on immunoblots after approximately 8 d of exposure to low temperature, and thereafter levels remain stable while plants are maintained at 4°C. Radiolabeling studies indicate that BN28 is synthesized at a relatively low rate. A decline in protein levels is observed soon after returning plants to control temperatures, and little or no protein can be detected after 7 d of deacclimation. The disappearance of the protein precedes a loss in freezing tolerance, suggesting that BN28 is not involved in maintaining plasma membrane integrity. Expression of BN28 is observed primarily in leaves and appears to be low-temperature specific. Quantitative analysis indicated that BN28 accumulates to approximately 82.7 pmol $mg^{-1}$ total protein in cold-acclimated leaves. This concentration is similar to that reported for two group 2 late-embryogenesis-abundant-like proteins.**

Many species of plants are capable of adapting to low temperature and freezing conditions. Generally, this adaptation requires a prior period of acclimation at low, nonfreezing temperatures, during which time a number of morphological, physiological, and molecular changes occur (Singh and Laroche, 1988; Guy, 1990; Alberdi and Cocuera, 1991). In recent years considerable effort has been directed toward the study of changes in gene expression that occur as a result of cold acclimation. Low-temperature-induced genes have now been described in a number of different plant systems, including wheat (Guo et al., 1992), spinach (Neven et al., 1993), alfalfa (Laberge et al., 1993; Monroy et al., 1993), barley (Cattivelli and Bartels, 1990; Dunn et al., 1994), Arabidopsis (Kurkela and Franck, 1990; Gilmour et al., 1992; Kurkela and Borg-Franck, 1992; Lin and Thomashow, 1992a; Horvath et al., 1993), and *Brassica* (Orr et al., 1992; Weretilnyk et al., 1993).

The regulation of these genes appears to be complex and dependent on the particular gene being studied. In some cases, expression is found to be low-temperature specific (Chauvin et al., 1993; Ouellet et al., 1993; Sáez-Vásquez et al., 1993; Weretilnyk et al., 1993; Jarillo et al., 1994), whereas many other genes are also induced after dehydration and salt stress and in response to ABA (Kurkela and Franck 1990; Kurkela and Borg-Franck, 1992; Wilhelm and Thomashow, 1993; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). In Arabidopsis, evidence suggests that separate signal transduction pathways may exist for the regulation of single genes by low temperature, dehydration, and ABA (Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994). Three other pairs of Arabidopsis genes, *kin1/kin2* (Kurkela and Borg-Franck 1992), *cor15a/cor15b* (Wilhelm and Thomashow, 1993), and *lti78* (*rd29a*)/*lti65* (*rd29b*) (Nordin et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994), that belong to small, highly homologous gene families exhibit differential expression in response to cold, ABA, dehydration, and salt stress.

The function of most low-temperature-induced proteins is unknown, although alignment of predicted amino acid sequences has revealed regions within some of these proteins that are homologous to those induced by dehydration, such as the dehydrin and Lea families (Gilmour et al., 1992; Guo et al., 1992; Neven et al., 1993). The expression of these families of proteins under conditions of dehydration and the fact that they all appear to be very hydrophilic suggest that they may function in helping the plant to cope with desiccation stress (Dure et al., 1989). Other low-temperature-induced proteins have been reported to have cryoprotective (Volger and Heber, 1975; Hincha et al., 1990; Lin and Thomashow, 1992b) or antifreeze (Hon et al., 1994) properties in vitro, and one, the KIN1 protein from Arabidopsis, has been shown to have some degree of sequence and structural homology with a winter flounder type I antifreeze protein (Kurkela and Franck, 1990).

To better understand the role of proteins in the acquisition of freezing tolerance, it is necessary to examine their expression not only at the transcriptional level, but also at the level of the protein itself. Steady-state levels of proteins can be more directly correlated with the physiological aspects of cold acclimation and freezing tolerance than can those of transcripts. Antibodies raised against low-temperature-induced proteins allow detection of structural simi-

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Abbreviations: cor, cold-regulated; IPTG, isopropyl β-D-thiogalactopyranoside; Lea, late embryogenesis abundant; $TL_{50}$, temperature of 50% electrolyte leakage.

larities between proteins that may be important for their function and, further, are useful for tissue and intracellular localization. Quantitative analysis of protein levels represents another means of comparing between different stress-induced proteins and also provides valuable information for the design and evaluation of in vitro assays of function.

In the present study, polyclonal antibodies to a low-temperature-induced protein from *Brassica napus* (Orr et al., 1992), BN28, were used to analyze the expression of this protein under various stress conditions and in different tissues within the plant. Steady-state levels of BN28 during cold acclimation and deacclimation were correlated with plant freezing tolerance measured through electrolyte leakage tests. We have also determined the concentration of BN28 in cold-acclimating leaves.

## MATERIALS AND METHODS

### Plant Material

*Brassica napus* cv Cascade (a winter-type canola) plants were grown under controlled conditions with a 16-h photoperiod at temperatures of 22 and 14°C (day/night) to the four-leaf stage. All samples were taken from the fourth-emerged leaf of control or treated plants.

Plants that were to be cold acclimated were transferred to 4°C under the same photoperiod and harvested as required. For deacclimation, plants that had been cold acclimated for 42 d were returned to the control temperatures and photoperiod regime.

### Evaluation of Freezing Tolerance

Freezing tolerance was evaluated for control, cold-acclimated, and deacclimated leaves using an electrolyte leakage assay. Leaves were harvested and washed with distilled water. One-centimeter-diameter discs of tissue were punched from leaves with a cork borer and placed on moistened filter paper in small Petri dishes (one or two discs/dish). The Petri dishes were placed in a programmable freezer and the temperature was lowered to 0°C, at which point the first set of samples was removed and transferred to 4°C. The temperature was then lowered to −2.5°C, and ice nucleation was initiated by touching a small chip of dry ice to the edge of the moistened filter paper. Samples were maintained at −2.5°C overnight. The temperature was then lowered at a constant rate of 2.5°C $h^{-1}$ and samples were collected at 2.5°C intervals between −2.5 and −17.5°C. All samples were allowed to thaw at 4°C for at least 12 h prior to determining electrolyte leakage.

Electrolyte leakage was determined by placing samples with the accompanying filter paper in tubes containing 10 mL of glass-distilled water, shaking overnight at room temperature, and measuring conductivity with a Radiometer CDM 83 conductivity meter (Bach-Simpson Ltd., London, Ontario, Canada). Total electrolytes were determined after boiling for 3 min, cooling to room temperature for 1 h with shaking, adjusting the volume to 10 mL and remeasuring the conductivity. Electrolyte leakage was expressed as a percentage of the total, and $TL_{50}$ was used as an indicator of lethal damage (Sukumaran and Weiser, 1972).

### ABA and Environmental Stress Treatments

ABA-treated plants were watered daily with a 100-μM solution of ABA (mixed isomers, Sigma) for 14 d. Plants subjected to salt stress were watered daily with a 450-mM solution of sodium chloride, and samples were collected after 14 d. For drought treatment, water was withheld from plants, and samples were collected after wilting was observed. Relative water content (*RWC*) was calculated as described by Guo et al. (1992)

$$RWC = \frac{FW - DW}{TW - DW} \times 100\%$$

where *FW* is fresh weight, *DW* is dry weight, and *TW* is turgid weight. *TW* was measured after floating leaves on glass-distilled water until no increase in weight was observed, usually 6 to 12 h. All nontemperature treatments were carried out on plants at the four-leaf stage under control temperature and photoperiod regimes.

### RNA Extraction and Northern Analysis

Leaf tissue was ground to a fine powder with mortar and pestle while frozen in liquid nitrogen. All subsequent operations were performed on ice, and centrifugations were carried out at 4°C unless otherwise indicated. RNA extraction buffer (4 M guanidinium thiocyanate, 2% [w/v] sarcosyl, 50 mm Tris, pH 8.0, 10 mM EDTA, 1% β-mercaptoethanol) (5 mL $g^{-1}$ fresh weight) was added and grinding continued until a thawed slurry was formed. The slurry was transferred to a centrifuge tube and 0.2 volumes of 10% (w/v) SDS, 0.1 volume of 2 M sodium acetate (pH 4.0), 1.0 volume of phenol, and 0.2 volume of chloroform: isoamyl alcohol (24:1) were added. The mixture was vortexed vigorously and incubated at room temperature for 15 min with agitation before centrifuging at 7500*g* for 20 min. Isopropanol (0.8 volume) was added to the aqueous phase, and RNA precipitated on ice for at least 1 h and then was centrifuged at 7500*g* for 20 min. The pellet was redissolved in diethyl pyrocarbonate-treated water, and RNA precipitated with an equal volume of 6 M lithium chloride at 4°C for at least 12 h. After centrifugation at 7500*g* for 20 min, the RNA pellet was rinsed with 2 M lithium chloride followed by 70% ethanol and then dissolved in diethyl pyrocarbonate-treated water. RNA concentration was determined spectrophotometrically from $A_{260}$.

Total RNA (20–40 μg) was separated on agarose-formaldehyde gels according to standard protocols (Sambrook et al., 1989). RNA was subsequently transferred to a Zeta-probe (Bio-Rad) membrane and hybridized with $^{32}P$-labeled probes that were radiolabeled using a multiprime labeling system, following the manufacturer's instructions (Amersham RPN-1601Z). Hybridizations and washes were carried out under high-stringency conditions. Hybridizations were performed in 50% formamide, 0.12 M $NaH_2PO_4$, pH 7.2, 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA at 42°C, and the final wash was in 0.1 × SSC, 0.1% SDS at 65°C.

### Expression and Purification of Recombinant BN28

The *bn28* cDNA (Orr et al., 1992) was subcloned into the pMAL-c (New England Biolabs) expression vector and transformed into *Escherichia coli*. This vector is under control of the *tac* promoter and upon induction with IPTG, expresses the cloned sequence fused to the C-terminal end of the maltose binding protein. The two proteins are separated by a factor Xa protease recognition site enabling recovery of the recombinant product after proteolytic cleavage of the fusion protein. *E. coli* overexpressing BN28 were grown to an $A_{600}$ of 0.5, induced with IPTG (final concentration 0.3 mM), and incubated for an additional 3 h at 37°C. The cells were pelleted, resuspended in 50 mM Tris, pH 8.0, and disrupted by sonication.

Soluble protein was recovered in the supernatant from disrupted cells after centrifugation at 10,000*g* for 20 min. This fraction was applied directly to a DEAE-cellulose column previously equilibrated in 50 mM Tris, pH 8.0. The column was thoroughly washed with the same buffer to remove all unbound protein and then drained completely by centrifugation at 1,000*g* for approximately 5 min. An equal volume of the same buffer containing factor Xa protease (New England Biolabs) at a concentration of approximately 0.2 units $mg^{-1}$ crude soluble protein was added back to the column, and the column was incubated at 4°C for 3 to 12 h. Under these conditions, factor Xa becomes bound to the column and rBN28 is subsequently eluted in pure form. Purity was confirmed by silver staining of samples that were separated on Tricine-SDS gels as described below.

### In Vivo Radiolabeling

Leaves were cut from plants at the petiole and the petiole was submerged in a solution of 200 mCi $mL^{-1}$ [$^{35}$S]Met (>1000 Ci $mmol^{-1}$, 10 mCi $mL^{-1}$, Amersham) and 1% (v/v) Tween-20 for 4 to 24 h. Soluble protein was extracted from radiolabeled leaves as described below.

*E. coli*-overexpressing rBN28 were grown and induced with IPTG as described above. One hour after induction, [$^{35}$S]Met (>1000 Ci $mmol^{-1}$, 10 mCi $mL^{-1}$, Amersham) was added to a final concentration of 200 $\mu$Ci $mL^{-1}$, and incubation was continued for another 2 h. Soluble protein was extracted as described above, and without further purification part of the sample was cleaved with factor Xa protease. Incorporation was determined by TCA precipitation of protein extracts.

### Preparation of Boiling-Soluble Proteins

Soluble protein extracts from either leaves or *E. coli* were placed in boiling water for 10 min and then allowed to cool on ice for approximately 15 min. Boiling-soluble proteins were recovered from the supernatant after centrifugation at 10,000*g* for 20 min.

### Protein Extraction and Electrophoresis

Leaves were frozen in liquid nitrogen and ground with a mortar and pestle. Protein extraction buffer was added, and grinding was continued until the tissue was thoroughly homogenized. For extraction of total proteins, 5 mL $g^{-1}$ fresh weight tissue of SDS buffer (50 mM Tris-HCl, pH 8.0, 2% [w/v] SDS, 1 mM PMSF, 10% [v/v] glycerol) were added, and soluble proteins were extracted in the same buffer without SDS. The extracts were centrifuged to remove insoluble debris. Protein concentrations were determined using the bicinchoninic acid method (Smith et al., 1985). Prior to electrophoresis, an equal volume of loading buffer (100 mM Tris-HCl, pH 8.0, 2% [w/v] SDS, 5% [v/v] $\beta$-mercaptoethanol, 10% [v/v] glycerol, 0.025% [w/v] bromphenol blue) was added to each sample and the samples were heated to 100°C for 2 min. Protein samples (30–60 $\mu$g) were electrophoresed on either 12 or 16.5% Tricine-SDS polyacrylamide gels (3% bis-acrylamide cross-linker) (Schägger and von Jagow, 1987). Radiolabeled proteins were detected by equilibrating gels in EN$^3$Hance (Amersham) and exposing dried gels to Fuji x-ray film (Innomed Christie Group Ltd., Edmonton, Alberta, Canada).

### Immunoblot Analysis and Quantitation

Purified rBN28 was conjugated to an equal mass of keyhole limpet hemocyanin via glutaraldehyde cross-linking, and polyclonal antibodies were raised in rabbits following standard protocols (Harlow and Lane, 1988). After electrophoresis on Tricine-SDS gels, the protein was electroblotted to a nitrocellulose membrane for 2 h at 300 mA in carbonate blot buffer (Dunn, 1986). Protein blots were reacted with anti-BN28 (1:200 dilution) and developed using an alkaline phosphatase-conjugated secondary antibody (1:3000 dilution, Sigma) and the 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt /*p*-nitroblue tetrazolium chloride reagent system.

For quantitative analysis, proteins blotted onto nitrocellulose membranes were reacted with anti-BN28 (1:2000 dilution) and developed using the enhanced chemiluminescence system (Amersham) with horseradish peroxidase-conjugated secondary antibody (1:3000 dilution) according to the manufacturer's directions. The concentration of BN28 was determined through laser densitometry of x-ray film that had been exposed to immunoblots that reacted with chemiluminescent substrate. Values from samples of cold-acclimated leaves were compared with a standard curve generated from immunoblots of known concentrations of purified rBN28. Immunoblots from leaf samples and standards were processed together to eliminate possible variability in reagents or handling. Each value represents the mean of at least three separate determinations.

## RESULTS

### Freezing Tolerance

Electrolyte leakage tests were used to determine the freezing tolerance of *B. napus* cv Cascade leaves (Fig. 1). Leaves from control plants exhibited very little freezing tolerance ($TL_{50}$ of approximately −2°C). The greatest increase in freezing tolerance was observed during the first 14 d of acclimation, when $TL_{50}$ values reached −11°C. There appeared to be little change between 14 and 28 d of

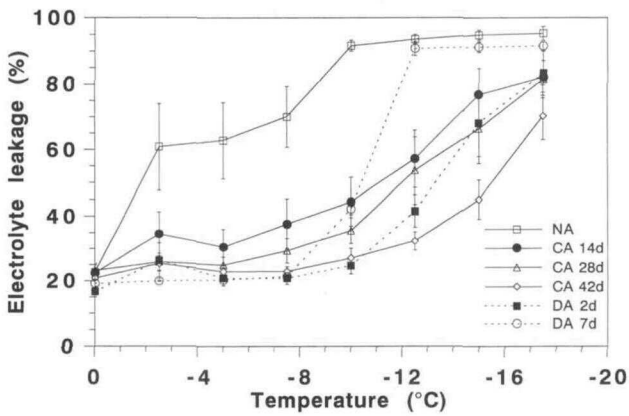



**Figure 1.** Electrolyte leakage test showing change in freezing tolerance of *B. napus* leaves associated with acclimation and deacclimation. Values for acclimated and deacclimated leaves represent the average ± SE of five and three independent measurements, respectively.

acclimation, but an additional increase in freezing tolerance was observed between 28 and 42 d of acclimation, with leaves attaining a $TL_{50}$ of −16°C. Leaves from plants acclimated for 42 d and then deacclimated for 2 or 7 d retained a high degree of freezing tolerance, with $TL_{50}$ values of −13 and −10°C, respectively.

### Gene Expression

The *bn28* gene was isolated previously through differential screening of a cDNA library derived from leaf tissue of acclimated *B. napus* cv Jet Neuf (Orr et al., 1992). A single transcript of approximately 0.5 kb was detected on northern blots that were hybridized with a radiolabeled *bn28* cDNA probe (Fig. 2). Analysis of the expression of *bn28* mRNA during acclimation and deacclimation revealed little or no message in nonacclimated leaves, but revealed induction within 1 d of exposure to low temperature. When plants acclimated for 42 d were returned to control temperatures, the mRNA disappeared almost completely within 12 h.

### Protein Expression

Expression of BN28 protein in acclimating and deacclimating plants was examined by probing immunoblots with a polyclonal antibody raised against a purified recombinant BN28 (rBN28) protein that had been overexpressed in *E. coli* (Fig. 3). These antibodies recognized a polypeptide that was present only in leaf samples from cold-acclimated plants. Although the molecular mass for this polypeptide appeared to be less than the approximate 6.6-kD value calculated from the predicted amino acid sequence, the relative mobility was the same as that for rBN28. There was no evidence of any significant cross-reactivity with any higher or lower molecular mass species.

Little or no BN28 was detected in SDS-soluble extracts from leaves of 1-d-acclimated plants. By 8 d of acclimation, sufficient amounts of BN28 had accumulated to enable consistent detection of the protein, and thereafter levels appeared to remain high. Previous attempts to detect BN28 in samples from leaves acclimated for 4 d gave inconsistent results; in some cases a band was detected, whereas in others it was not (results not shown). Upon deacclimation, levels of BN28 decreased. In some samples, a poorly resolved smear of cross-reacting material, possibly representing degradation products, was observed just below the main band after 1 to 2 d of deacclimation. Within 7 d of returning plants to control temperatures, BN28 became almost undetectable.

### Protein Synthesis

The appearance of *bn28* mRNA on northern blots before the protein could be detected on immunoblots suggested that BN28 accumulates at a relatively low rate. To investigate this question further, comparisons of newly synthesized proteins from control and acclimated plants were made. Since previous results (not shown) indicated that BN28 was boiling soluble, this property was used to enrich for BN28 by the removal of nonboiling-soluble proteins. Boiling-soluble proteins were prepared from samples containing equal quantities of acid-precipitable radioactivity and were separated on SDS gels. Measurement of radioactivity in samples before and after boiling indicated that the boiling-soluble fraction represented approximately 6% of the total newly synthesized soluble proteins in both control and acclimated leaves. A sample of boiling-soluble proteins from factor Xa-digested extracts of *E. coli* cells overexpressing rBN28 was included to determine the expected position of BN28 on these gels. Although sufficient quantities of BN28 had accumulated by 8 d of acclimation to enable detection on immunoblots (Fig. 3), no polypeptide of equivalent relative mobility could be detected among the newly synthesized leaf proteins after 1 or 8 d of acclimation (Fig. 4). By contrast, rBN28 was visible, as were two higher molecular mass, low-temperature-induced polypeptides that were present in samples from acclimated leaves. BN28 was also undetectable in samples from leaves acclimated

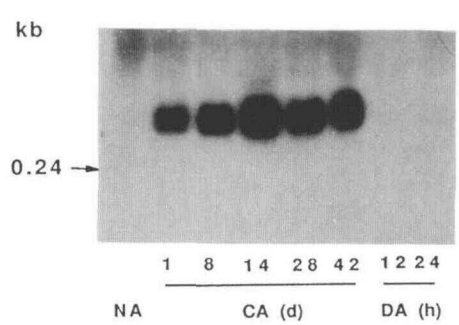
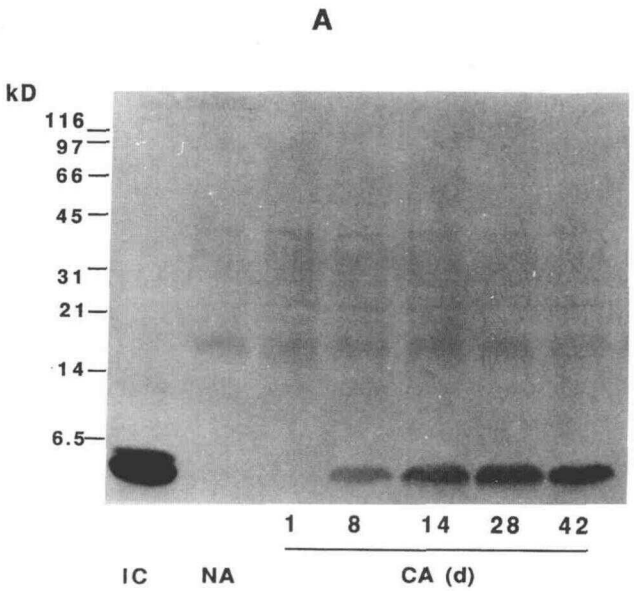



**Figure 2.** Northern blot analysis of *bn28* mRNA levels in acclimating and deacclimating *B. napus* leaves. Forty micrograms of total RNA from leaves were loaded onto each lane, and the blot was probed with a [$^{32}$P]dCTP-radiolabeled *bn28* cDNA. Leaves were collected from plants that were either nonacclimated (NA), cold acclimated (CA) for 1 to 42 d, or deacclimated (DA) for 12 or 24 h after 42 d of cold acclimation.



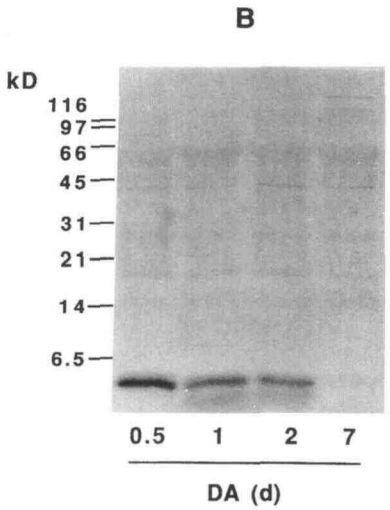




**Figure 3.** Immunoblot analysis of BN28 protein levels in acclimating and deacclimating *B. napus* leaves. Lane IC contains 5 μg of total soluble protein from induced *E. coli* cells overexpressing rBN28 that has been cleaved with factor Xa. The remaining lanes contain 60 μg of total (SDS-soluble) leaf protein. A, Nonacclimated (NA) and cold-acclimated (CA) for 1, 8, 14, 28, and 42 d. B, Deacclimated (DA) for 0.5, 1, 2, and 7 d. The relative mobilities of molecular mass standards are indicated on the left.

for 28 d or when using radiolabeling periods of 24 h (not shown).

### Response to ABA, Dehydration, and Salt

The specificity of BN28 induction was examined after exogenous application of ABA and after dehydration or salt stress (Fig. 5). None of these treatments resulted in the accumulation of detectable quantities of BN28. To determine if *bn28* mRNA was present but not translated under these conditions, northern blots from samples for each of

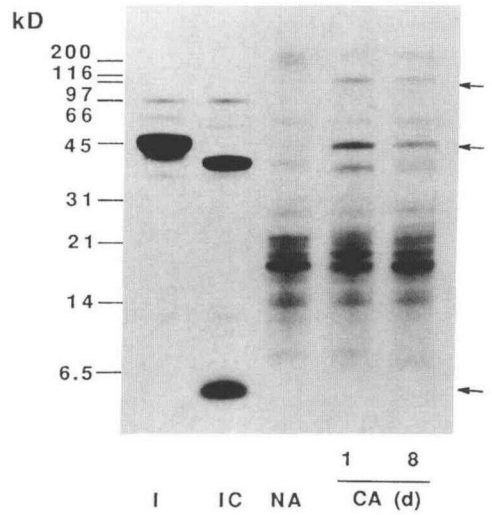



**Figure 4.** In vivo [$^{35}$S]Met-radiolabeled, boiling-soluble protein either from induced *E. coli* cells before (I) or after (IC) cleavage with factor Xa protease or from nonacclimated (NA) and cold-acclimated (CA) *B. napus* leaves. Each lane contains the boiling-soluble protein recovered from approximately 50,000 dpm of total soluble protein. Arrows indicate the expected position of BN28 and two higher molecular mass, low-temperature-induced proteins. The relative mobilities of molecular mass standards are indicated on the left.

the stress treatments were probed with radiolabeled *bn28* cDNA (Fig. 6A). In agreement with results from the immunoblots, *bn28* mRNA was detected only in response to low temperature. In the case of ABA treatment, similar results were also obtained when a leaf was cut at the petiole and placed in a solution of 100 μM ABA and 0.1% Tween 20 for 24 h or with a foliar application of the same solution (not shown). To verify plant uptake and responsiveness to ABA, northern blots were re-probed with *rab16a* (Mundy and Chua, 1988), an ABA-responsive gene from rice. Clear in-

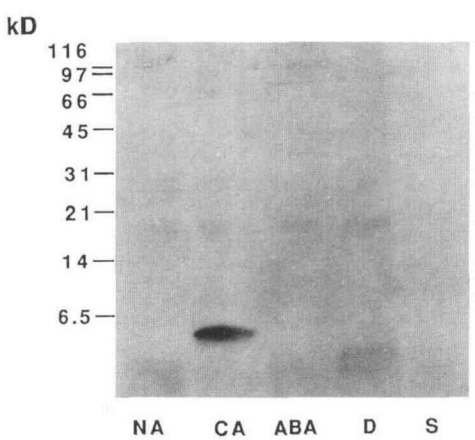



**Figure 5.** Immunoblot showing low-temperature specificity of BN28 expression. Each lane contains 60 μg of total SDS-soluble protein from leaves of nonacclimated (NA) and 14-d cold-acclimated (CA) plants or plants treated with 100 μM ABA for 14 d (ABA), drought to *RWC* of 36% (D) or 450 mM salt for 14 d (S). The relative mobilities of molecular mass standards are indicated on the left.

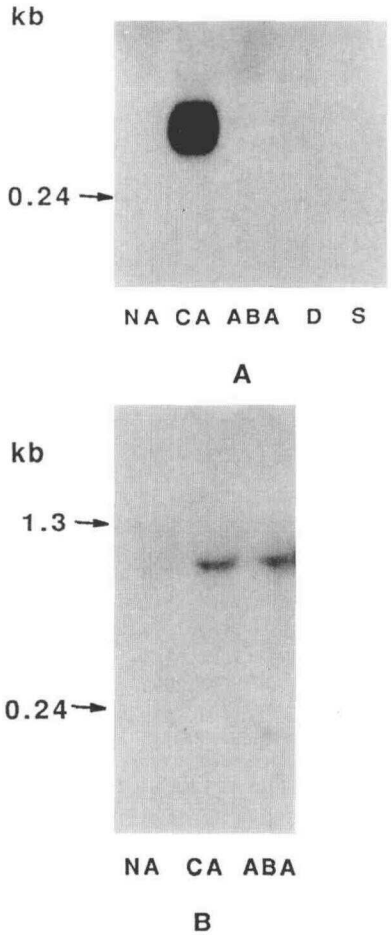



**Figure 6.** A, Northern blot showing low-temperature specificity of *bn28* mRNA expression. Lanes contain 40 μg of total RNA from leaves of nonacclimated (NA) and cold-acclimated (CA) plants or plants treated with ABA (A), drought to *RWC* of 43% (D), or salt (S) as described in Figure 5. B, Northern blot showing expression of *rab 16a* homologous mRNA in nonacclimated (NA), 14-d cold-acclimated (CA), and ABA-treated (ABA) leaves of *B. napus*.

duction of a homologous sequence in ABA-treated samples of *B. napus* was apparent (Fig. 6B).

### Tissue Specificity

The expression of BN28 was examined in samples collected from petioles, hypocotyls, and roots of plants acclimated for 42 d and from mature, dried seeds (Fig. 7). Although low levels of the protein could be detected in both petioles and hypocotyls, no BN28 was found in either roots or mature seeds. However, a protein(s) of approximately 33 kD present in mature seeds did cross-react with the BN28 antiserum. The identity of this protein(s) and the nature of its relationship with BN28 are unknown at this time.

### Quantitative Analysis

A quantitative analysis of BN28 accumulation was undertaken using a chemiluminescent immunodetection system. Immunoblots of samples from nonacclimated, cold-acclimated, and deacclimated leaves were reacted with a chemiluminescent substrate, and the resulting images on x-ray film were measured densitometrically. The quantity of BN28 in each sample was determined from a standard curve that was generated with known amounts of rBN28. Table I shows the concentrations of BN28 in samples taken over the 42-d period of acclimation. Although little difference was observed between different sampling times, the greatest amount of variability appeared to be associated with the 8-d-acclimated samples, corresponding to the initial period of BN28 accumulation. The protein reached maximum levels of approximately 0.542 μg $mg^{-1}$ total protein or 82.7 pmol $mg^{-1}$ total protein. BN28 could not be detected in samples from either nonacclimated or 7-d-deacclimated plants.

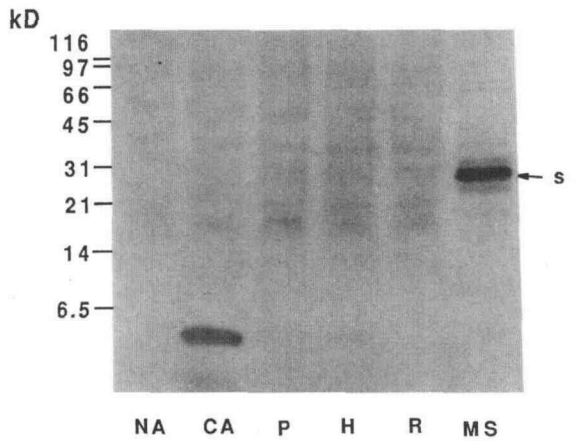



**Figure 7.** Immunoblot showing spatial expression of BN28. Each lane contains 60 μg of total SDS-soluble protein from leaves of nonacclimated plants (NA), leaves (CA), petioles (P), hypocotyls (H), or roots (R) of 42-d cold-acclimated plants, or mature, dry seed (MS). Arrow indicates the position of a higher molecular mass seed protein(s) cross-reacting with antibodies to BN28. The relative mobilities of molecular mass standards are indicated on the left.

## DISCUSSION

We have characterized the expression of *bn28*, a low-temperature-induced gene in *B. napus*. Antibodies raised

**Table I.** *Accumulation of BN28 during cold-acclimation (CA) in B. napus leaves*

The quantity of BN28 on immunoblots was determined through scanning densitometry and comparison with purified protein standards. Values are expressed as the ratio to total SDS-soluble protein. Each value represents the mean ± SE of at least three separate measurements.

| CA | μg BN28[a] | pmol BN28[a] |
|---|---|---|
| *d* | | |
| 8 | 0.414 ± 0.096 | 63.2 |
| 14 | 0.396 ± 0.040 | 60.5 |
| 28 | 0.542 ± 0.014 | 82.7 |
| 42 | 0.494 ± 0.026 | 75.4 |

[a] μg and pmol BN28 $mg^{-1}$ protein.

against a recombinant form of BN28 protein have been used to follow the kinetics of protein accumulation, to determine its concentration in cold-acclimating leaves, and to examine the correlation between its expression and plant freezing tolerance. As observed with many other low-temperature-induced genes (Cativelli and Bartels, 1990; Lin et al., 1990; Gilmour et al., 1992; Horvath et al., 1993; Monroy et al., 1993; Neven et al., 1993; Ouellet et al., 1993; Weretilnyk et al., 1993), little or no *bn28* mRNA was detected in leaves of control plants. Exposure of plants to low temperature resulted in a rapid increase in transcript levels that persisted throughout the period of cold acclimation and declined when plants were returned to control temperatures.

In contrast to the rapid increase in transcripts, BN28 protein appeared to accumulate relatively slowly. Visual inspection and quantitative analysis of immunoblots indicated that maximum levels of BN28 were not achieved until at least 8 d of cold acclimation. Between 14 and 42 d of acclimation, levels appeared to remain relatively constant and less variation was observed among different samples. The greater variation in BN28 levels that was observed among samples collected at 8 d probably indicates that maximum levels of the protein have not been reached in all of these samples. With deacclimation, a reduction in the levels of BN28 was observed, and little or no protein could be detected within 7 d of returning plants to control temperatures.

Few other studies have examined changes in the steady-state levels of low-temperature-induced proteins. The CAP85 (Guy et al., 1992a; Neven et al., 1993) and CAP160 (Guy et al., 1992a) proteins of spinach exhibit kinetics of accumulation and decline qualitatively similar to those of BN28. However, these proteins appear to reach peak levels more quickly during cold acclimation and to persist longer under deacclimating conditions. This difference in kinetics may reflect functional differences between the *Brassica* and spinach proteins. It has been suggested that a slow turnover of low-temperature-induced proteins under deacclimating conditions might enable the plant to retain freezing tolerance over short periods of warmer temperatures (Neven et al., 1993).

Radiolabeling studies failed to detect BN28 among newly synthesized polypeptides in either the earlier or later stages of acclimation. Three factors could contribute to these results: (a) insufficient number of Met residues; (b) synthesis as a higher molecular mass precursor protein; and (c) low relative levels of synthesis. Detection of radiolabeled rBN28 indicates that sufficient incorporation of Met occurred to enable detection provided that enough of the newly synthesized protein was present. It is also unlikely that BN28 is synthesized as an unprocessed, higher molecular mass precursor, since the amino acid sequence does not contain a putative signal peptide and increasing the radiolabeling period to 24 h did not result in the appearance of a lower molecular mass band. Furthermore, no difference in relative mobility was observed between rBN28 produced in *E. coli* and endogenous BN28 from leaves.

Although the incorporation of radiolabel by polypeptides of similar amino acid composition is proportional to their molecular mass, that is, a greater molar quantity of a small polypeptide is required to incorporate an equivalent amount of amino acids than a larger polypeptide, it seems unlikely that this alone accounts for the inability to detect BN28. Since BN28 is a boiling-soluble polypeptide, the use of this fraction for the protein-synthesis studies represents an enrichment of at least 20-fold, compensating for the reduced incorporation of lower molecular mass species. Thus, we believe that the relative level of BN28 synthesis is quite low. Together with results indicating that steady-state levels of the protein remain stable throughout the later period of cold acclimation, this implies low rates of both accumulation and turnover for BN28.

One of the primary sites of freezing injury is the plasma membrane (Steponkus, 1984). Leakage of electrolytes from cells after a freeze-thaw cycle is a readily visible and easily measured indicator of this type of injury and thus has often been used to follow the acquisition of freezing tolerance in plants. Early studies of changes in protein profiles with cold acclimation reported increases in the synthesis of certain polypeptides that paralleled the acquisition of freezing tolerance (Guy and Haskell, 1987; Mohapatra et al., 1987; Gilmour et al., 1988; Lång et al., 1989) and a decline in the synthesis of these polypeptides as freezing tolerance was lost (Guy and Haskell, 1987). These findings suggest that proteins may play a role in stabilizing the plasma membrane against either rupture or loss of its semipermeable characteristics. Since radiolabeling studies examine only whether or not a protein is actively synthesized and not its presence or absence, they may not provide an accurate representation of the correlation between low-temperature-induced proteins and freezing tolerance.

In the present study, where steady-state levels of protein were examined, although the initial accumulation of BN28 appeared to parallel somewhat the increase in freezing tolerance, its disappearance upon returning plants to control temperatures was not accompanied by a dramatic decline in freezing tolerance. In this respect BN28 differs from the spinach proteins CAP85/COR85 and CAP160, for which both the increase and decrease in protein levels appear to follow reasonably closely the gain and loss of freezing tolerance (Guy et al., 1992; Kazuoka and Oeda, 1992). Although these correlative studies cannot prove a role for proteins in the acquisition of freezing tolerance, our results would seem at least to preclude the involvement of BN28 in maintaining the functional integrity of the plasma membrane.

The absence of *bn28* expression in response to drought, salt, or application of ABA indicates that the regulation of this gene differs from that of a number of other low-temperature-induced genes (Hajela et al., 1990; Guo et al., 1992; Laberge et al., 1993; Neven et al., 1993), including the *cor6.6/kin* genes of Arabidopsis (Kurkela and Franck, 1990; Gilmour et al., 1992; Kurkela and Borg-Franck, 1992). These results are somewhat surprising in view of the fact that *Brassica* and Arabidopsis are closely related genera and the *bn28* and *cor6.6/kin* genes are highly homologous (Orr et

al., 1992). It has been shown that the Arabidopsis genes form a small gene family that are differentially expressed in response to different stresses (Kurkela and Borg-Franck, 1992). This also appears to be the case with another pair of homologous genes in Arabidopsis, *cor15a* and *cor15b* (Wilhelm and Thomashow, 1993; Baker et al., 1994). However, in these studies, the coding regions of each gene pair were found to be almost identical, and resolution of differential expression required the use of probes specific to the more variable 3′ untranslated region of the gene.

Since a full-length cDNA probe was used in the present study, we would expect to detect all members of a closely related gene family, and so our results suggest that *bn28* expression is in fact low-temperature specific. Interestingly, *bn115*, the *Brassica* gene homologous to *cor15* in Arabidopsis, is also not induced by ABA and is only weakly responsive to drought (Weretilnyk et al., 1993). Also, in wheat two related low-temperature-induced genes, *wcs120* (Houde et al., 1992) and *wcs200* (Ouellet et al., 1993), have been identified that appear to be low-temperature specific, despite the fact that they share considerable sequence homology with a number of ABA- and drought-induced genes. It is possible that genes or regulatory elements responsible for expression in response to ABA, drought, and salt have been lost in *Brassica*.

To date, a precise role has not been determined for any stress-induced proteins with the possible exception of a few enzymes for which participation in metabolic pathways were already known (Guy et al., 1992b). In an effort to gain insight into the function of low-temperature-induced proteins, attention has been directed to similarities in their expression, sequence, and biochemical properties. By virtue of its high hydrophilicity, BN28 belongs to the broad family of boiling-soluble proteins that, in addition to many low-temperature-induced proteins, includes those associated with cellular dehydration, either as a result of environmental stress (dehydrins) (Close et al., 1989) or during normal seed desiccation (Lea proteins) (Dure et al., 1989).

The cellular concentration of proteins represents another parameter by which different stress-induced proteins can be compared. In the present study, BN28 was found to accumulate to levels of 0.05% or 82.7 pmol $mg^{-1}$ of total protein and our unpublished results indicate that this concentration would at most double if the calculation were based on soluble protein alone. Although this value is substantially lower than that of the cotton LEA proteins D-7 at 1.77 nmol $mg^{-1}$ and D-113 at 2.21 nmol $mg^{-1}$ soluble protein (Roberts et al., 1993), it is reasonably similar to those of COR85 and a maize dehydrin, two group 2 LEA-like proteins. COR85 was found to constitute approximately 0.2% of the soluble protein in cold-acclimated spinach leaves (Kazuoka and Oeda, 1994), and the maize dehydrin constituted 0.24% of the soluble protein in seeds (Ceccardi et al., 1994). Based on the reported molecular masses of these proteins, these values correspond to 32 and 141 pmol $mg^{-1}$, respectively. It has been suggested that this concentration of dehydrins is compatible with a structural role, possibly in preventing protein denaturation and aggregation during dehydrative stress (Asghar et al., 1994). A similar role could be envisioned for BN28 under conditions in which the effects of both low temperature and freezing-induced dehydration may be acting to disrupt protein structure. Given the spatial specificity of BN28 expression, it is also possible that this protein is designed to meet the specific requirements of leaf tissues. Structural characterization and immunocytological localization studies may provide further insight into the possible role of BN28 in protecting plants against the deleterious effects of low temperature and freezing stress.

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