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Regulation of *BN115*, a Low-Temperature-Responsive Gene from Winter *Brassica napus*[1]

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The genomic clone for *BN115*, a low-temperature-responsive gene, was isolated from winter *Brassica napus* and its sequence was determined. A 1.2-kb fragment of the 5′ regulatory region (from bp −1107 to +100) was fused to the β-glucuronidase (GUS) reporter gene and *BN115*-promoted GUS expression was observed in green tissues of transgenic *B. napus* plants only after incubation at 2°C. No expression was observed after incubation at 22°C, either in the presence or the absence of ABA. Microprojectile bombardment of winter *B. napus* leaves with a *BN115* promoter/GUS construct yielded similar results and was used to analyze a series of deletions from the 5′ end of the promoter. Results obtained from transient expression studies showed that the low-temperature regulation of *BN115* expression involves a possible enhancer region between bp −1107 and −802 and a second positive regulatory region located between bp −302 and −274. Deletion analyses and results from replacement with a truncated cauliflower mosaic virus 35S promoter suggest that the minimal size required for any maintenance of low-temperature GUS expression is a −300-bp fragment. Within this fragment are two 8-bp elements with the sequence TGGCCGAC, which are identical to those present in the positive regulatory region of the promoter of the homologous *Arabidopsis cor15a* gene and to a 5-bp core sequence in the low-temperature- and dehydration-responsive elements identified in the promoter regions of several cold-responsive *Arabidopsis thaliana* genes.

In plants capable of cold acclimation, growth at low, nonfreezing temperatures leads to the acquisition of increased frost tolerance (Levitt, 1980) and, in many species, to the accumulation of specific mRNAs. Low-temperature-induced transcripts have been identified in several cold-acclimating species including spinach (*Spinacia oleracea*) (Neven et al., 1993), alfalfa (*Medicago sativa*) (Laberge et al., 1993; Wolfraim and Dhindsa, 1993; Wolfraim et al., 1993), barley (*Hordeum vulgare*) (Catevelli and Bartels, 1990; Hughes et al., 1992; Goddard et al., 1993), wheat (*Triticum aestivum*) (Houde et al., 1992; Chauvin et al., 1993), *Arabidopsis thaliana* (Kurkela and Franck, 1990; Nordin et al., 1991, 1993; Gilmour et al., 1992; Lång and Palva, 1992), and *Brassica napus* (Orr et al., 1992a, 1992b; Saez-Vasquez et al., 1993; Weretilnyk et al., 1993). For many of these transcripts, the corresponding clones have been isolated by differential screening of cDNA libraries and their sequences determined. Deduced amino acid sequences show that several of these low-temperature-induced transcripts (Gilmour et al., 1992; Houde et al., 1992; Lång and Palva, 1992; Neven et al., 1993) encode peptides that are similar to those that accumulate during dehydrative stress, i.e. late embryogenesis abundant proteins (Baker et al., 1988), dehydrins (Close et al., 1989; Bartels et al., 1990), and responsive-to-ABA proteins (Skriver and Mundy, 1990).

It is generally thought that such proteins may help plants tolerate the cellular desiccation that accompanies extracellular freezing. Indeed, both water stress (Siminovitch and Cloutier, 1982) and application of ABA (Chen and Gusta, 1983) at nonacclimating temperatures can induce increased freezing tolerance, and accumulation of proteins encoded by two spinach genes responsive to drought and low temperature was shown to be initiated by a change in the hydration state of the plant tissue (Guy et al., 1992). Other cold-induced transcripts encode peptides that are enriched in Ala or Gly (Kurkela and Franck, 1990; Gilmour et al., 1992; Orr et al., 1992a) or that are enriched in Ala, Lys, and Arg with homology to the human tumor gene *bbc1* (Saez-Vasquez et al., 1993). Still others have been shown to share homologies with LTP, a lipid transfer protein (Hughes et al., 1992), or with the translation elongation factor 1α (Dunn et al., 1993). In most cases, these low-temperature-induced transcripts are also induced, often to a lesser degree, by ABA (Hajela et al., 1990; Kurkela and Frank, 1991; Nordin et al., 1991; Lång and Palva, 1992; Neven et al., 1993). Endogenous ABA levels have been observed to increase transiently during the cold-acclimation process in potato (*Solanum tuberosum*) (Chen et al., 1983), spinach (Guy and Haskell, 1988), and *B. napus* (T.C. White and J. Singh, unpublished results).

We recently reported the isolation and characterization of cDNA clones of three related cold-induced transcripts, pBN115, pBN19, and pBN26 from winter *B. napus* cv Jet neuf (Weretilnyk et al., 1993). Transcripts hybridizing to pBN115 accounted for 0.15% of the mRNA in the leaves of cold-treated Jet neuf plants. *BN115* transcripts did not accumulate

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Abbreviations: ABRE, ABA-responsive element; CaMV, cauliflower mosaic virus; DRE, dehydration-responsive element; GUS, β-glucuronidase; LTRE, low-temperature-responsive element; LUX, luciferase; MS, Murashige and Skoog; MU, methyl umbelliferone.

in ABA-treated leaves or in the roots and mature seeds of cold-treated winter *B. napus* plants (Weretilnyk et al., 1993). The nucleotide and deduced amino acid sequences of pBN115 (and its relatives) are similar to those of *cor15a* and *cor15b*, two low-temperature-induced genes from *A. thaliana* (Lin and Thomashow, 1992; Wilhelm and Thomashow, 1993). Unlike *BN115*, probes prepared from the *cor15* cDNA clone hybridize to both low-temperature- and ABA-inducible transcripts (Hajela et al., 1990). The first 50 amino acids of *BN115* and *COR15* show similarities to chloroplast targeting sequences and it has been shown that a processed *COR15* peptide of approximately 9 kD is present in the stromal fraction of cold-acclimated *A. thaliana* chloroplasts (Lin and Thomashow, 1992).

The regulation of gene expression by low temperature is critical to understanding the process of cold acclimation in plants. Cold-induced genes have been shown to be regulated by separate ABA-independent and -dependent pathways on exposure to low temperatures (Gilmour and Thomashow, 1991; Nordin et al., 1991). The low-temperature regulation at the transcriptional level of one of these genes has been demonstrated in transgenic *Arabidopsis* (Horvath et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1993) and *cis* elements involved in this regulation have been identified (Yamaguchi-Shinozaki and Shinozaki, 1994).

To understand low-temperature regulation of gene expression in winter *B. napus*, the genomic clone of *BN115* was isolated and sequenced and the promoter region was studied by the transient expression of the GUS reporter gene from the *BN115* promoter after microprojectile bombardment of *B. napus* cv Jet neuf leaves. The development of a reliable transient expression system for the study of gene regulation in *B. napus* is useful because to date *Agrobacterium*-mediated procedures developed for *B. napus* transformation have been found to be relatively inefficient (Charest et al., 1988; Radke et al., 1988), making it difficult to generate homozygous transformants required for detailed promoter analysis. Transient expression systems using rice protoplasts have proven successful for the identification of the regulatory elements required for ABA-mediated expression of the wheat *Em* (Marcotte et al., 1989) and maize *rab28* (Pla et al., 1993) genes.

We have chosen to study the 5′ regulatory regions of *BN115* in particular because this gene is transcriptionally regulated, responds rapidly to low temperature (resulting in the appearance of *BN115* transcripts within 1 d of cold treatment), but does not respond to exogenous applications of ABA at 20°C (Weretilnyk et al., 1993). In addition, *BN115* transcripts are the most abundant of those induced by low temperature in the leaves of winter *B. napus* that we have identified so far. In this paper we demonstrate the ability of the regulatory region of *BN115* to direct gene expression at low temperature and identify regions containing potential *cis* elements involved in this regulation in *B. napus*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Winter *Brassica napus* L. cv Jet neuf and spring *Brassica napus* L. cv Westar were grown in controlled-environment growth chambers at 22°C under a 16-h, 250 μE m^{-2} s^{-1} light intensity day and an 8-h, 20°C night.

Library Construction and Isolation of the Genomic *BN115* Clone

Seeds of winter *B. napus* cv Jet neuf were surface sterilized with 3% sodium hypochlorite and germinated in the dark at room temperature on sterile, moistened filter paper in sterile, glass trays. DNA was isolated from 25 g of dark-grown cotyledons as described in unit 2.3 of *Current Protocols in Molecular Biology* (Ausubel et al., 1987) except that 2% SDS was used in place of 10% Sarkosyl and the DNA was extracted with 1 volume each of phenol saturated with extraction buffer and chloroform before the initial precipitation with ethanol. High mol wt DNA was partially digested with *Bam*HI and the fragments were fractionated by Suc gradient centrifugation (unit 5.3 of Ausubel et al., 1987). Fractions containing DNA fragments of 9 to 20 kb in length were pooled, purified (unit 5.3 of Ausubel et al., 1987), and ligated to the arms of the vector λ DASH (Stratagene, La Jolla, CA). The library was packaged using the Gigapack II Gold packaging extract (Stratagene) and transfected into the *McrA*$^-$, *McrB*$^-$ *Escherichia coli* host strain WA802 (Wood, 1966). The library was screened with a digoxigenin-dUTP-labeled probe prepared from the pBN115 cDNA clone (Weretilnyk et al., 1993) using the Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim) according to the manufacturer's protocol. Positive clones were visualized by immunological detection with anti-digoxigenin-dUTP/alkaline phosphatase conjugate using *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl-phosphate (*p*-toluidine salt) as substrates following the manufacturer's instructions. A genomic clone containing a 7.0-kb *Bam*HI fragment hybridizing to pBN115 was isolated and the fragment was subcloned into pUC119 (Yanisch-Perron et al., 1985). A 3.0-kb *Xba*I fragment containing the entire coding region and over 2 kb of the 5′ regulatory region was further subcloned into pUC119 in both orientations (pUC119-G28 and pUC119-G29) for sequencing.

Nested Deletions and DNA Sequencing

Nested deletions from both the 5′ and 3′ ends of the *Xba*I genomic subclone and from the 5′ end of the *BN115* promoter/GUS fusion were constructed using the Erase-a-Base kit (Promega, Madison, WI). Double-stranded sequencing of each deletion series was performed using the Sequenase Kit (United States Biochemical) or the T7 DNA Polymerase Sequencing Kit (Pharmacia). Sequences were aligned and analyzed using DNASIS sequence analysis software for IBM-compatible computers. Both strands of the genomic subclone were sequenced completely, but only one strand was sequenced to determine the location of the deletions in the *BN115* promoter/GUS fusion series.

Construction of *BN115* Promoter/GUS Fusions and pEPLux

A 2.1-kb fragment containing the GUS coding region and the nopaline synthase terminator was isolated from the bi-

nary vector pBI101.2 (Jefferson, 1987) by *Hin*dIII/*Eco*RI digestion and inserted into the polylinker of pUC119 (Yanisch-Perron et al., 1985) to generate pUC119-GUSter. A 1.2-kb *Sal*I/*Pvu*II fragment of the genomic *BN115* subclone was then ligated to pUC119-GUSter that had been digested with *Sal*I and *Sma*I. This generates the plasmid pUC119/115P-GUS (see Fig. 2) encoding an in-frame fusion protein of which the first eight amino acids are those encoded by *BN115*. The plasmid pEPLux, which expresses the firefly LUX gene under the control of an enhanced CaMV 35S promoter and serves as an internal standard for each bombardment, was constructed by replacing the 0.5-kb *Hin*dIII/*Sal*I CaMV 35S promoter fragment in pJD300 (Luehrsen et al., 1992) with a 0.8-kb fragment containing the CaMV 35S enhancer and promoter sequences excised from the plasmid pFF19G (Timmermans et al., 1990) by digestion with *Hin*dIII and *Sal*I. For *Agrobacterium tumefaciens*-mediated transformation of spring *B. napus* cv Westar, the 1.2-kb *Sal*I/*Pvu*II fragment containing the *BN115* promoter was inserted into the polylinker of the binary plasmid pBI101.2 (Jefferson, 1987), which had been digested with *Sal*I and *Sma*I, to generate pBI101.BN115P-GUS (see Fig. 2).

The region of the *BN115* promoter from −300 to −53 was amplified by the PCR (Saiki et al., 1988) from 1 ng of pUC119/115P-GUS using the primers 5′-GGGTAGATCTA-CTAACATG-3′ (upstream) and 5′-GGATTGCAGCTGGTC-TAG-3′ (downstream), generating *Bgl*II and *Pvu*II sites at −300 and −53, respectively. This fragment was inserted into the polylinker of pUC119 (Yanisch-Perron et al., 1985) and sequenced. An error-free subclone was isolated and the "−300" fragment was isolated by digestion with *Kpn*I and *Sph*I and the ends were blunted with T4 DNA polymerase. *Bgl*II linkers were then ligated onto the ends of the blunted fragment and inserted into the unique *Bgl*II site upstream of the truncated CaMV 35S promoter in the plasmid pEMBL-46GUS3C (Fang et al., 1989).

A. tumefaciens-Mediated Transformation of Spring *B. napus* cv Westar

Essentially, the method of Moloney et al. (1989) was used to transform *B. napus*. Cotyledons and petioles of spring *B. napus* cv Westar were co-cultured with the *A. tumefaciens* strain GV3101/pMP90 carrying the binary plasmid pBI101/BN115P-GUS (Fig. 2). After a 7-d recovery, positive primary transgenics were isolated by selection on 25 mg/L kanamycin and were rooted on media containing 0.5 mg/L each of α-naphthaleneacetic acid and IAA. Selfed seeds, collected from these primary transgenics, were germinated in Magenta boxes on MS agar (Murashige and Skoog, 1962) containing 100 mg/L kanamycin and reselected. Resistant progeny from these were selfed and the seeds obtained were again subjected to another round of germination and selection on 100 mg/L kanamycin to identify homozygous lines. Ten seeds from each independent transgenic line were germinated on MS agar (minus kanamycin) in Magenta boxes and placed at 22°C under a 16-h, 250 μE m^{-2} s^{-1} light intensity day. Seedlings (5 cm tall and containing the cotyledons and first two leaves) were either transferred to 2°C at the same light intensity, left at 22°C, or transferred to new medium containing 10^{-4} M ABA at 22°C for 72 h. After 3 d, the whole seedlings were assayed for GUS expression after incubation in 5-bromo-4-chloro-3-indoyl glucuronide for 2 h or overnight as described by Jefferson (1987). The seedlings were then bleached in a solution of ethanol:propionic acid (3:1, v/v) and rinsed with 70% ethanol.

Microprojectile Bombardment of Leaves

Young, expanding *B. napus* cv Jet neuf leaves (grown at 22°C as described above) were cut, sterilized first in 70% ethanol (30 s) and then in 1.4% calcium hypochlorite (plus a drop of Tween 20) for 4 min, rinsed three times with sterile, distilled water, and mounted on MS medium (Murashige and Skoog, 1962) containing 3% Suc and 0.6% phytagar in 35 × 10 mm Petri dishes (one leaf per dish). After an overnight incubation in a controlled-environment growth chamber set at 250 μE m^{-2} s^{-1} with a 16-h photoperiod (22°C light and 20°C dark), leaves were bombarded at 900 p.s.i. with 1.6-μm gold particles coated with a mixture of pEPLux plasmid and *BN115* promoter deletion/GUS fusion plasmid at a ratio of 1:4 (w/w) using the Biolistic PDS-1000/He system particle gun (Bio-Rad) as follows: 10 μg of DNA in 10 μL of 10 mM Tris, pH 8, 1 mM EDTA was combined (while vortexing) with 50 μL of suspended gold particles, 50 μL of 2.5 M $CaCl_2$, and 10 μL of 0.1 M spermidine; particles were allowed to settle for 10 min at room temperature, washed twice with 200 μL of ethanol, and resuspended in 30 μL of ethanol; 5 μL of resuspended DNA-coated gold particles were pipetted onto six flying discs that were used to bombard six individually mounted leaves.

Four of six leaves bombarded with a given deletion plasmid + pEPLux were transferred to semisolid MS medium in 60 × 15 mm Petri dishes and the other two were transferred to semisolid MS medium containing 100 μM (±)ABA. Two of the four leaves mounted on MS medium and the two leaves mounted on ABA-containing MS medium were incubated for 24 or 48 h in the same controlled-environment growth chamber as above. The remaining two leaves mounted on MS medium were incubated for 24 or 48 h in another chamber set at 2°C (constant) and approximately the same light intensity and a 16-h light period. Leaves continued to expand throughout the above manipulations. At least three sets of six leaves each were bombarded with each promoter deletion + pEPLux and no difference in the relative promoter activity was observed when incubating for either 24 or 48 h under any of the individual conditions.

Assay of GUS and LUX Activities in Extracts of Bombarded Leaves

Histochemical detection of GUS activity was performed as described by Jefferson (1987) using 5-bromo-4-chloro-3-indoyl glucuronide (Sigma) as substrate. To facilitate visualization of blue spots, the leaves were bleached in ethanol:propionic acid (3:1, v/v) and rinsed with 70% ethanol. For fluorimetric GUS and LUX assays, a single extract was prepared from one or two leaves from each treatment by homogenizing in 1.5 to 2.0 mL of extraction buffer (100 mM potassium phosphate, pH 7.8, 1 mM

Na_2EDTA, 10% glycerol, 7 mM β-mercaptoethanol) and clearing by centrifugation (15 min at 4°C in a microfuge). GUS activity in the supernatant was measured fluorimetrically using 4-methyl umbelliferyl β-D-glucuronide (Sigma) as substrate (Jefferson, 1987). LUX activity was measured as follows: 20 μL of extract was added to 200 μL of assay buffer (25 mM Tricine, pH 7.8, 15 mM $MgCl_2$, 5 mM ATP, 7 mM β-mercaptoethanol, 1 mg mL^{-1} BSA) in the sample cuvette and placed in the LKB 1250 Luminometer (LKB-Wallac, Turku, Finland) equilibrated to 25°C. The reaction was initiated by injection of 100 μL of 0.5 mM luciferin (Analytical Luminescence Laboratory, San Diego, CA) into the sample cuvette and the luminescence (in mV) was integrated over a 10-s interval. Protein concentrations were determined according to the method of Bradford (1976) using IgG as standard. For each extract (prepared from one or two bombarded leaves), the GUS activity (in nmol MU produced min^{-1} mg^{-1}) and LUX activity (in mV s^{-1} μg^{-1}) of control extract from a nonbombarded leaf were subtracted from the total observed GUS and LUX activities before calculating the ratio. The GUS/LUX activity ratios (in nmol MU produced min^{-1} mg^{-1}/mV s^{-1} μg^{-1}) represent the average (±SE) of at least three independent extracts each prepared from one or two leaves bombarded with one deletion construct + pEPLUX and incubated under one of the three incubation conditions.

Isolation of Total RNA and Northern Blot Analysis

Total RNA was isolated from leaf tissue using a phenol-chloroform extraction procedure (Sambrook et al., 1989). For northern blot analysis, 10 or 20 μg of total RNA was denatured, subjected to electrophoresis in a formaldehyde/1.5% agarose gel, and then transferred to Nytran (Schleicher & Schuell) membranes (Sambrook et al., 1989). Filters were hybridized with a ^{32}P-labeled single-stranded DNA probe prepared using the Random Primers DNA Labeling System (Gibco/BRL, Gaithersburg, MD) with the insert of pBN115 (Weretilnyk et al., 1993) or pBN28 (Orr et al., 1992a) as template and following the manufacturer's protocol.

Primer Extension Analysis

The transcriptional start site of *BN115* was determined by primer extension analysis (Sambrook et al., 1989) as follows: 20 μg of total RNA isolated from the leaves of room temperature or 4-d cold-treated winter *B. napus* was hybridized to a ^{32}P-labeled oligonucleotide DNA probe 5′-GAACAGCTGATCCTGAGAGTGACATAGCCATG-3′ (complementary to bp +75 to +106) and the DNA extended with avian myloblastosis virus reverse transcriptase (Pharmacia). Extension products were resolved on a 6% denaturing polyacrylamide gel alongside sequencing reactions performed using the same primer and compared to sequences produced from the 5′ end of the *BN115* gene.

RESULTS

Isolation of the *BN115* Genomic Clone

A genomic library of winter *B napus* L. cv Jet neuf was constructed in the vector λ DASH using total DNA that had been isolated from dark-grown cotyledons and partially digested with *Bam*HI. A positive clone was isolated by screening the library with a probe prepared from the cDNA clone pBN115 (Weretilnyk et al., 1993). A 3-kb *Xba*I fragment containing the entire coding region of *BN115*, a single 0.26-kb intron, and over 2 kb of the 5′ regulatory region was subcloned into the plasmid pUC119 (Yanisch-Perron et al., 1985) and over 2 kb of this clone was sequenced. The sequence of this genomic subclone of *BN115*, which is 100%

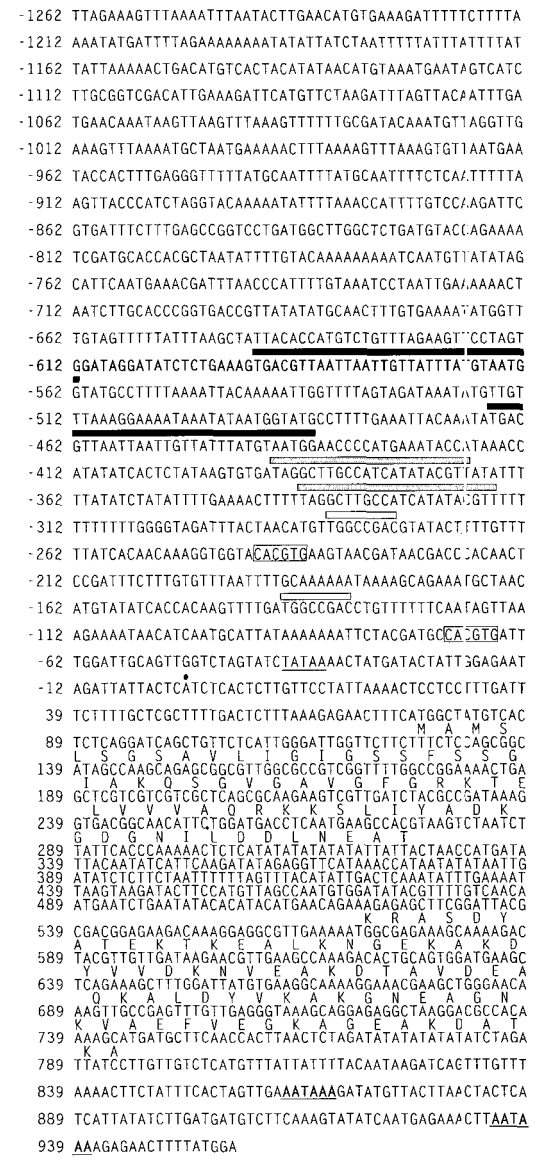

Figure 1. Genomic sequence of *BN115*. The location of the direct repeats of 31, 22, and 8 bp are indicated by solid, stippled, or unfilled bars, respectively, above the corresponding nucleotide sequences. The two G-boxes are boxed in and the putative TATA box and polyadenylation signals are underlined. The start of transcription, as determined by primer extension analysis, is designated with an asterisk. The deduced amino acid sequences for the protein coding regions are given below the corresponding nucleotide sequences.

identical in the protein coding regions to the cDNA clone pBN115 (Weretilnyk et al., 1993), is shown in Figure 1. The location of the transcription initiation site (+1) was determined by primer extension analysis (data not shown). The putative TATA box (TATAAA) is located from −39 to −35 and putative polyadenylation signals (AATAAA) are located at +862 to +867 and +935 to +940.

Analysis of the 5′ regulatory region showed that it contains two large direct repeats of 31 bp (−592 to −562 and −466 to −436) and 22 bp (−390 to −369 and −337 to −316) and several smaller direct repeats, including an 8-bp direct repeat (TGGCCGAC) located at bp −284 to −277 and −139 to −132. An identical 8-bp direct repeat is present within the similar region of the promoter of the homologous *A. thaliana cor15a* gene, a region found to be sufficient to direct the low-temperature-responsive expression of GUS in transgenic *Arabidopsis* (Baker et al., 1994). The last 5 bp are also found within repeated sequences (G/TA/GCCGACA/TT/A) observed in regulatory sequences of low-temperature-regulated genes *lti78* (*rd29A*) and *lti65* (*rd29B*) from *A. thaliana* (Nordin et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994).

Two G-boxes (Guiliano et al., 1988) are present in the *BN115* promoter (bp −242 to −237 and bp −71 to −66) and are staggered with the 8-bp repeat (Fig. 1). Although the core hexamer of these two G-boxes (CACGTG) is the same as those found in some ABREs (Marcotte et al., 1989), the flanking nucleotides are not. The observations that no ABA-inducible transcripts were found to hybridize to pBN115 (Weretilnyk et al., 1993) and that no ABA-induced GUS expression was observed in plants transformed with the *BN115* promoter (data below) suggest that the two G-boxes in the *BN115* promoter are probably not functioning as ABREs.

BN115-Promoted GUS Expression in Transgenic *B. napus* Plants

BN115 regulatory sequences from −1107 to +100 were fused in frame to the GUS reporter gene in the binary plasmid pBI101.2. The resulting plasmid, pBI101/BN115P-GUS (Fig. 2), was introduced into spring *B. napus* cv Westar via *A. tumefaciens*-mediated transformation (Moloney et al., 1989) and *BN115*-promoted GUS expression monitored in the transgenic seedlings. The spring cultivar was used as the host because transformation protocols for *B. napus* are found to be genotype (Charest et al., 1988; DeBlock et al., 1989) and/or laboratory dependent (Fry et al., 1987; Radke et al., 1988), with winter varieties being particularly difficult to transform (W. Keller, personal communication). It is expected that the spring cultivar will regulate the *BN115* promoter under low temperatures as in the winter type, since the spring cultivar does develop increased frost tolerance during cold acclimation (Laroche et al., 1992) and, more importantly, since it does accumulate transcripts hybridizing to *BN115* in response to low-temperature exposure (Weretilnyk et al., 1993).

Transgenic *B. napus* seedlings showed strong *BN115*-promoted GUS expression after incubation for 72 h at 2°C (Fig. 3a). Seedlings from seeds of five independent transformants were analyzed for low-temperature-induced GUS expression. All of these showed *BN115*-promoted GUS activity at low

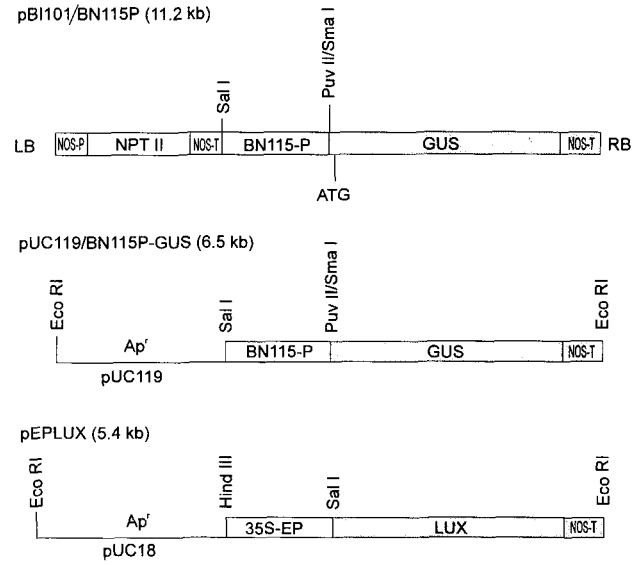

Figure 2. Plasmids used for microprojectile bombardments and transformations. The plasmids pBI101/BN115P-GUS, pUC119/BN115P-GUS, and pEPLux were constructed as described in "Materials and Methods." The relevant restriction sites used for the constructions are shown. BN115-P represents the promoter, and Ap^r represents the β-lactamase gene present in the plasmid vectors pUC119 and pUC18. NPTII represents the neomycin phosphotransferase gene that confers resistance to kanamycin in the transgenic plants. NosP and NosT represent the nopaline synthase promoter and 3′-termination sequences, respectively.

temperature. No GUS expression was detected in any of the transgenic seedlings incubated at 22°C, either in the presence or absence of ABA, even after an overnight incubation in the histochemical assay solution (Fig. 3a). Nontransformed control plants did not show GUS expression at either temperature (data not shown). Microscopic analysis of the leaves of cold-treated seedlings stained for GUS expression showed that *BN115*-promoted GUS expression occurs primarily in the mesophyll cells with little or no expression in the epidermis or trichomes (Fig. 3, b and c).

Assay of *BN115* Promoter Activity by Transient GUS Expression in Bombarded Winter *B. napus* L. cv Jet neuf Leaves

The 5′ regulatory region of *BN115* from bp −1107 to +100 was fused, in frame, to the GUS gene to form the plasmid pUC119/115P-GUS (Fig. 2), which was then delivered into Jet neuf leaves via microprojectile bombardment (Russell et al., 1992). Under the conditions used, the bombarded leaves suffered very little visible damage and continued to expand throughout the mounting, bombardment, and subsequent incubation procedures.

Histochemical staining for transient GUS activity in Jet neuf leaves bombarded with pUC119/BN115P-GUS resulted in approximately 10-fold more blue spots being observed in the leaves that had been incubated for 48 h at 2°C than in the leaves that had been incubated at 22°C on the same media plus or minus 100 μM ABA (Fig. 3, d–f).

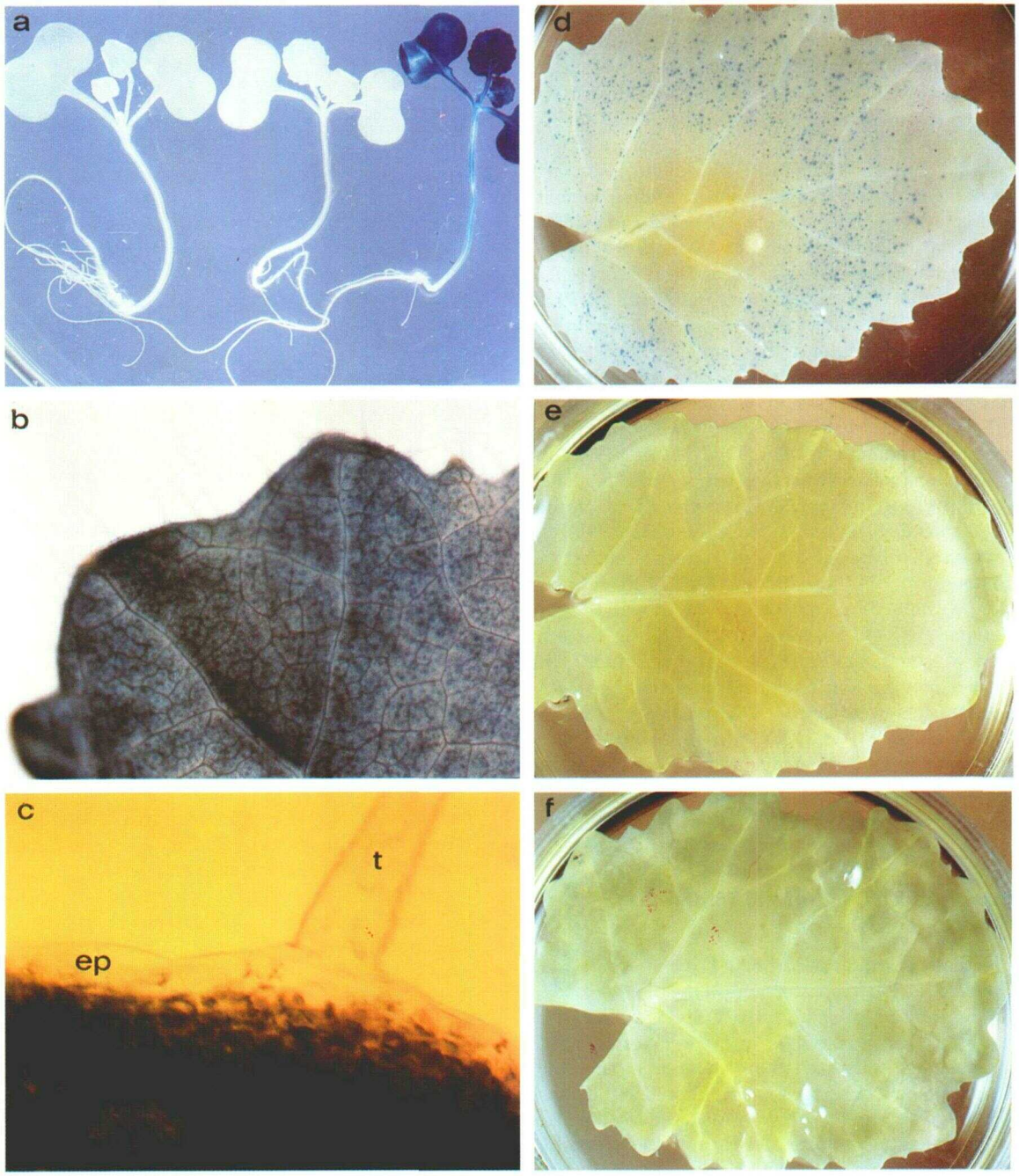

Figure 3. Histochemical GUS assay of transgenic *Brassica* seedlings and of bombarded *Brassica* leaves. a, Seedlings of *B. napus* cv Westar, transformed with pBI101/BN115P-GUS, were grown on sterile MS agar, incubated for 72 h at, left to right, 22°C, 22°C on MS agar containing 100 μM ABA, or 2°C, then stained for GUS overnight and cleared of Chl. b, 2°C incubation as in (a), higher magnification showing GUS activity in mesophyll cells after only 3 h of staining. c, 2°C as in (a), higher magnification showing the absence of GUS activity in epidermal cells (ep) and trichomes (t) even after staining in substrate overnight. d–f, Young, expanding winter *B. napus* cv Jet neuf leaves were bombarded with the plasmid pUC119/115P-GUS (*BN115* promoter/GUS) and incubated for 48 h on semisolid MS media at (d) 2°C; (e) 22°C; and (f) at 22°C on semisolid media containing 100 μM ABA.

Analysis of 5′ Deletions in the *BN115* Promoter

To identify regions containing the *cis*-acting elements involved in regulating the low-temperature expression of *BN115* in *B. napus* cv Jet neuf, a series of seven nested deletions from the 5′ end of the promoter region was constructed. Using fluorometric analyses of transient expression, the full-length *BN115* promoter/GUS construct (bp −1107 to +100) was found to be approximately 5-fold more active in leaves that had been incubated for 24 or 48 h at 2°C than in those incubated at 22°C on the same media with or without ABA (Fig. 4). Fusion of *BN115* sequences from bp −2200 to +100 (i.e. the whole cloned fragment) with the GUS reporter gene did not further increase the activity of the promoter in this transient expression system (data not shown).

Deletion of the 5′-distal 305 bp (from bp −1107 to −802) resulted in a 40% decrease in the activity of the *BN115* promoter at 2°C (Fig. 4) but had no effect on its activity at 22°C (plus or minus ABA). Thus, the cold inducibility of this promoter, as determined by fluorimetric GUS activity normalized to LUX activity, has been reduced from 5- to 3-fold, suggesting that this region may contain enhancer sequences. Deletion of the next 162 bp (to −640) decreased promoter activity at 2°C by an additional 25% (to about 35% of the full-length promoter activity). In addition, this deletion resulted in a 2-fold increase in the promoter activity at 22°C, but only in the absence of ABA (Fig. 4). Deletion of the next 204 bp (to −436) had little effect on the promoter activity at either 2 or 22°C (plus or minus ABA). Similarly, deletion of the region containing the 22-bp direct repeat (through bp −302) caused little, if any, decrease in activity at either temperature. However, subsequent deletion analysis showed that this 302-bp promoter fragment is the smallest that can still support GUS expression in the cold at a level equivalent to or greater than that at 22°C in bombarded Jet neuf leaves (Fig. 4).

Deletion to bp −274 caused a 2- to 3-fold decrease in the

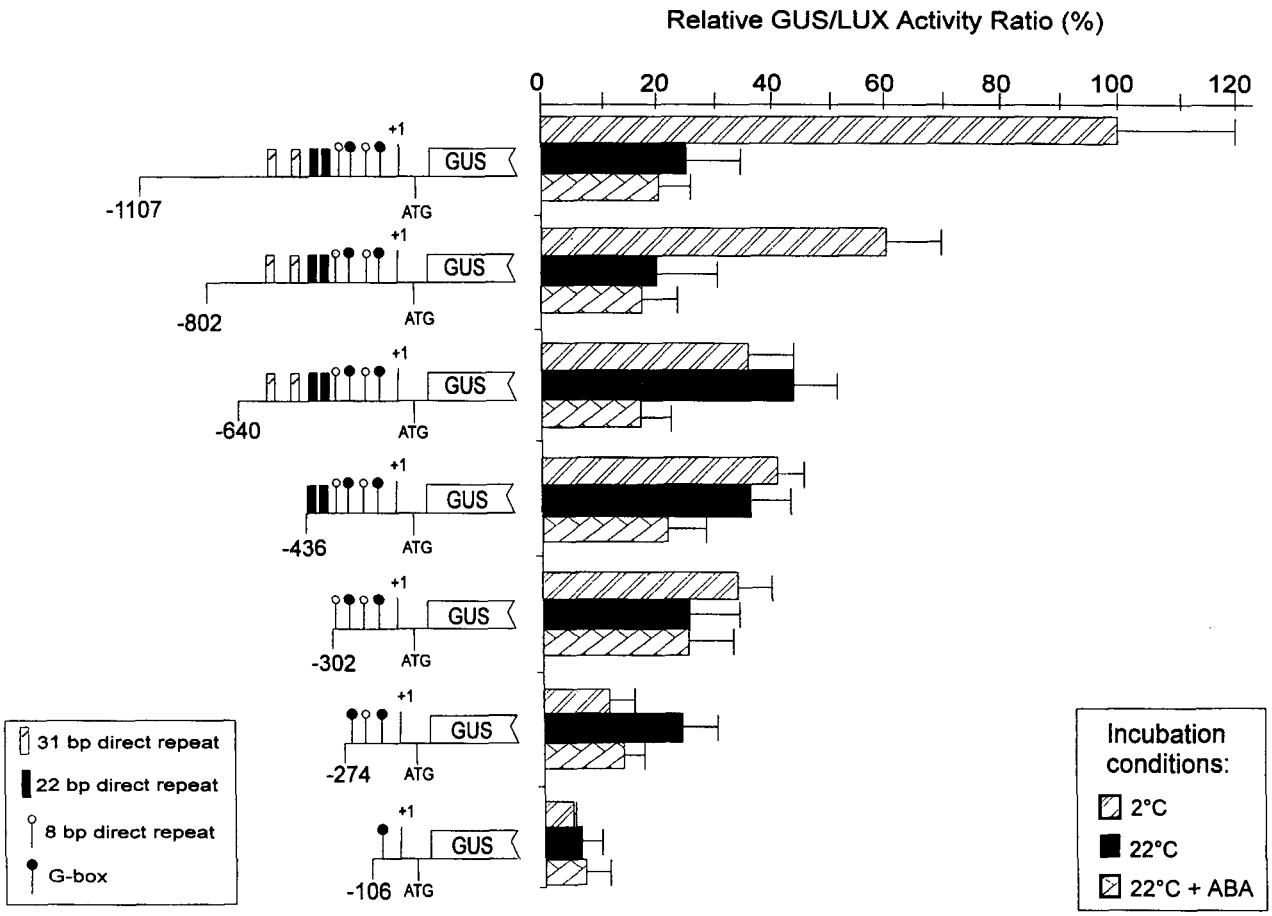

Figure 4. Effects of 5′ deletions on the transient expression of GUS by the *BN115* promoter. Linear maps of the inserts from the series of plasmids containing increasingly larger deletions from the 5′ end of the *BN115* promoter/GUS fusion in pUC119/115P-GUS are shown on the left. The stopping position of each deletion is shown along with remaining repeated elements. The histogram shows the relative GUS/LUX activity ratios measured in bombarded Jet neuf leaves after 24 to 48 h as described in "Materials and Methods." All activity ratios were normalized to that of the full-length promoter/GUS construct at 2°C set at 100%. Values shown represent the mean + SE net GUS/net LUX activity (in nmol MU produced min^{-1} mg^{-1}/mV s^{-1} μg^{-1}) determined for four to six independent experiments as described in "Materials and Methods."

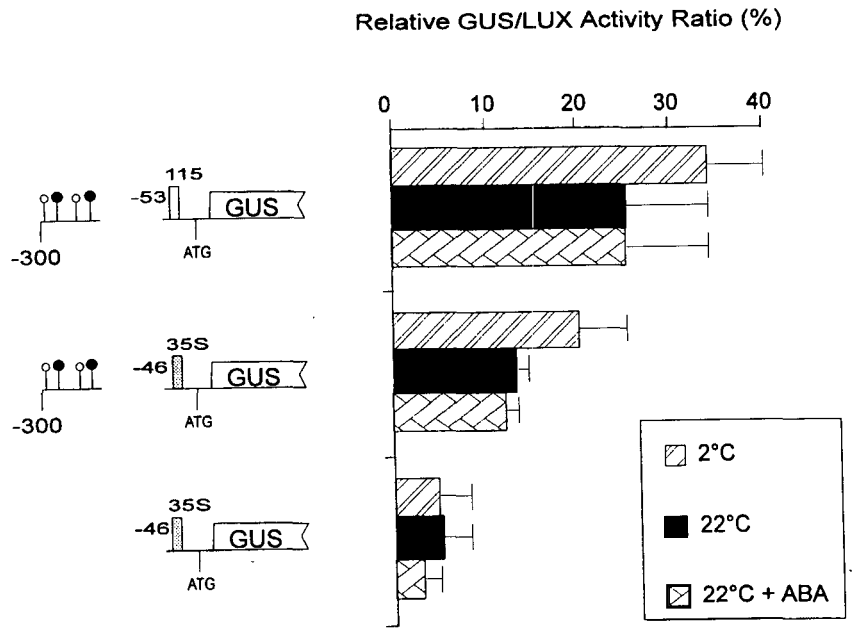

Figure 5. Test for the presence of cold-responsive elements in *BN115* promoter sequences from −300 to −53 by a gain-of-function experiment in bombarded *Brassica* leaves. DNA sequences from −300 to −53 of the *BN115* promoter were amplified by PCR and inserted upstream of the CaMV 35S promoter truncated to −46. pEMBL-46GUS3C and the −300/−46GUS fusion plasmids were introduced, along with the internal standard plasmid pEPLUX, by biolistic bombardment. The histogram shows the relative GUS/LUX activity ratios measured for each of these two constructs in bombarded Jet neuf leaves after 24 to 48 h of incubation at 2°C, 22°C, and 22°C plus ABA. All activity ratios were normalized to that of the full-length promoter/GUS construct at 2°C set at 100%. Values shown represent the average of the GUS/net LUX activity (in nmol MU produced min^{-1} mg^{-1}/mV s^{-1} μg^{-1}) determined for two independent experiments as described in "Materials and Methods." The activity of the −300 deletion under the three incubation conditions is shown for comparison. Filled circles represent the G-boxes; open circles represent the 8-bp motif TGGCCGAC. The open and shaded rectangles represent the truncated *BN115* and CaMV promoters, respectively.

activity of the promoter at 2°C from that of the previous deletion to −302 but did not affect the activity of the promoter at 22°C (no ABA) (Fig. 4). Thus, with the removal of only 28 bp, cold activity dropped to below that of room temperature and was virtually eliminated. Interestingly, this 28-bp fragment contained the first half of the 8-bp direct repeat (TGGCCGAC) that was present in the promoter of the *A. thaliana BN115* homolog *cor15* (Baker et al., 1994) and that contained a consensus sequence CCGAC that was present in the 9-bp (TACCGACAT) DRE identified in the upstream region of the *A. thaliana* cold-, salt-, and drought-induced gene *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994). A 71-bp fragment of this gene containing the downstream 9-bp DRE was shown to be able to direct the production of GUS transcripts in transgenic *A. thaliana* in response to cold treatment. In addition, the 9-bp DRE was not present in the promoter of the *rd29B* gene, which is not cold induced (Yamaguchi-Shinozaki and Shinozaki, 1994).

Subsequent deletion to bp −106, which removed the region containing the second 8-bp LTRE (TGGCCGAC), resulted in significant loss of promoter activity (to less than 10% of that of the full-length promoter at 2°C) under all conditions tested (Fig. 4). The remaining sequences, a single G-box and the putative TATA box, were therefore insufficient to maintain gene expression at a level equivalent to that of the full-length promoter at 22°C (20–25% of the full-length promoter at 2°C).

To verify that the region from bp −300 to −53 was able to direct low-temperature GUS expression, this region was amplified by the PCR (Saiki et al., 1988) and inserted upstream of a minimal CaMV 35S promoter in the plasmid pEMBL-46GUS3C (Fang et al., 1989). Transient GUS expression from the resulting plasmid, −300/−46GUS, was monitored in microprojectile-bombarded Jet neuf leaves as described for the deletion series. As shown in Figure 5, GUS expression from −300/−46GUS was 1.5-fold higher at 2°C than at 22°C. The −302 deletion of the *BN115* promoter/GUS fusion also showed a similar fold of cold induction although the absolute activities were higher with the *BN115*-deleted fragment (Fig. 5). In addition, GUS expression from −300/−46GUS at 2°C was about 5-fold higher than GUS expression from either pEMBL-46GUS3C or the −106 deletion (Fig. 4) at 2°C, suggesting that this fragment has the ability to direct low-temperature gene expression.

Effect of ABA on Transient GUS Expression from *BN115* Promoter Deletions in Winter *B. napus* Leaves

Within experimental error, no ABA-inducible GUS expression was detected in any of the extracts prepared from winter

B. napus cv Jet neuf leaves that had been bombarded with the *BN115* promoter/GUS fusion deletion plasmids (Fig. 4). Uptake of ABA by the leaves from the semisolid media was confirmed by the detection, on northern blots, of *BN28* transcripts, which have been shown to be induced by cold and, to a lesser degree, by ABA (Orr et al., 1992a) (Fig. 6a). However, no ABA-induced transcripts hybridizing to a *BN115* probe were detected in the RNA isolated from these same leaves (Fig. 6b). On the other hand, similar analysis of total RNA isolated from nonbombarded *A. thaliana* leaves that had been surface-sterilized and incubated on ABA-containing media for 6 h showed the accumulation of significant levels of *BN115*-hybridizing transcripts (Fig. 6c). These ABA-induced messages in *A. thaliana* most likely represent *cor15* transcripts, which have previously been shown to accumulate in ABA-treated leaves (Hajela et al., 1990). Thus, ABA uptake from the semisolid media is sufficient to induce the expression of *BN28* in winter *B. napus* leaves and *cor15* in *A. thaliana* leaves, but not *BN115* in winter *B. napus* leaves.

DISCUSSION

The 5′ regulatory region of the low-temperature-regulated gene *BN115* from winter *B. napus* L. cv Jet neuf was isolated, sequenced, and characterized. A 1.2-kb promoter fragment (bp −1107 to +100) was fused to the GUS reporter gene and found to direct the low-temperature- but not the ABA-regulated expression of GUS in transgenic *B. napus* seedlings. GUS expression in these transgenic seedlings was limited to green tissue. Within the leaves, GUS expression was highest in the mesophyll cells, with little or no expression in the epidermis or trichomes. These results are consistent with the product of the homologous gene from *A. thaliana*, *cor15*, being targeted to the chloroplast, where it accumulates as a 9-kD mature peptide (Lin and Thomashow, 1992) and with GUS expression observed in *A. thaliana* transformed with the *cor15a* promoter-GUS fusion (Baker et al., 1994). The observation that *BN115*-promoted GUS expression does not occur in the trichomes is in contrast, however, with recent results showing that the promoter of another cold-induced gene, *cor78* (*lti78*, *rd29A*), can direct GUS expression exclusively in the trichomes under conditions of moderate water stress (Horvath et al., 1993). Unlike *cor78* (*lti78*, *rd29A*), which is highly responsive to drought stress (Horvath et al., 1993; Nordin et al., 1993; Yamaguchi-Shinozaki and Shinozaki, 1994), *BN115* is only weakly drought responsive (Weretilnyk et al., 1993).

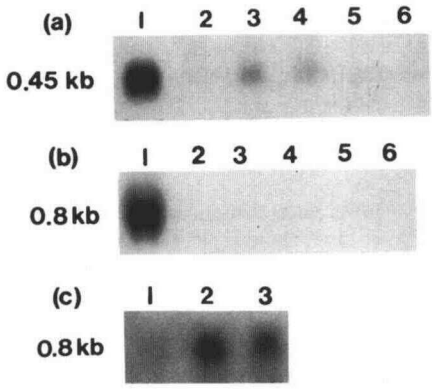

Figure 6. Northern blot analysis of leaves incubated on ABA-containing media. Hybridization of *BN28* (a) and *BN115* (b) probes to northern blots of total RNA isolated from *B. napus* cv Jet neuf leaves incubated for increasing lengths of time on semisolid MS media containing 100 μM ABA as described for bombardment experiments. Lanes 1, Leaves from whole plants exposed to 2°C for 4 d; lanes 2, leaves incubated for 24 h on semisolid MS media without ABA; lanes 3 to 6, leaves incubated on ABA-containing media for 6, 19, 24, and 48 h. All lanes contained 10 μg of RNA except lanes 3 to 6 for the *BN115* hybridization, which contain 20 μg of RNA. c, Hybridization of *BN115* probe to a northern blot of total RNA (10 μg/lane) isolated from *A. thaliana* leaves that had been surface-sterilized with 70% ethanol and incubated for 6 h on the same semisolid MS media as per bombardment conditions with or without 100 μM ABA. Lane 1, Leaves incubated at 22°C; lane 2, leaves incubated at 22°C plus ABA; lane 3, leaves incubated at 2°C.

Transient GUS expression from the *BN115* promoter was approximately 5-fold higher in bombarded leaves incubated at 2°C than at 22°C. No ABA-inducible GUS expression was detected at 22°C, within experimental error, in Jet neuf leaves bombarded with any of the deletion plasmids. The basis for the difference in ABA responsiveness of the homologous genes *BN115* and *cor15* from the two closely related species *B. napus* and *A. thaliana* is not clear at this time. It is worthwhile to note that in *A. thaliana*, *cor15* transcripts (Hajela et al., 1990) and endogenous ABA concentrations (Lång et al., 1994) both reach maximum levels within 1 d of low-temperature exposure. In contrast, *BN115* transcripts appear in winter *B. napus* leaves within 1 d of low-temperature exposure and reach maximum levels after 3 d (Weretilnyk et al., 1993), whereas ABA levels peak only after 7 d (data not shown). The absence of an ABA response suggests that the two G-boxes present in the *BN115* promoter do not appear to function as ABREs. Although the core hexamer, CACGTG, has been identified in the ABREs of several ABA-responsive genes, e.g. the maize *rab28* gene (Pla et al., 1993) and the wheat *Em1a* gene (Marcotte et al., 1989), it is also found within the positive regulatory regions of several other plant genes responding to a variety of stimuli (Williams et al., 1992). Furthermore, in contrast to *BN115*, the promoters of three low-temperature- and ABA-responsive genes from *A. thaliana*, a relative of *B. napus*, contain one or more of the imperfect G-box motifs TACGTG or GACGTG (Lång and Palva, 1992; Nordin et al., 1993; Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). It is possible that G-boxes present in *BN115*, like those found in many other non-ABA-responsive promoters (Williams et al., 1992), are common plant regulatory elements (Armstrong et al., 1992).

Alternatively, these G-boxes may be wound-responsive elements, responding to wound-induced increases in methyl jasmonate (Mason et al., 1993). Analysis of *BN115*-promoted GUS expression in green developing siliques from transgenic *B. napus* plants suggests that the *BN115* promoter in this organ is wound responsive in addition to being cold responsive (T.C. White and J. Singh, unpublished data). Comparison of the nucleotides flanking the upstream *BN115* G-box (at −237) with that found in the wound-responsive domain of the *versuspB* gene (Mason et al., 1993) shows that they are

similar but not identical. The potential wound response of this G-box may help to explain the relatively high GUS activity observed in the bombarded leaves incubated at 22°C compared to the almost absent GUS activity in the transgenic seedlings incubated at 22°C.

A second explanation for the higher 22°C GUS activity in the bombarded leaves over that observed at 22°C in the transgenic seedlings may result from the weak drought responsiveness of the *BN115* (Weretilnyk et al., 1993). The bombarded leaves, having been sterilized in hypochlorite and ethanol, may have experienced some osmotic stress that would weakly induce the *BN115* promoter. Since the GUS protein is relatively stable (Jefferson, 1987), active GUS protein may accumulate under conditions that do not allow for the accumulation of *BN115* transcripts (Fig. 5).

Transient expression of the GUS reporter gene in microprojectile-bombarded Jet neuf leaves from a series of plasmids with increasingly larger 5′ deletions in the *BN115* promoter was used to identify regions containing potential *cis* elements involved in the low-temperature regulation of *BN115* expression. The region between bp −1107 and −802 in the *BN115* promoter is an A/T-rich region that appears to contain an enhancer element for low-temperature expression. The next 162 bp (−802 to −640), in addition to possibly containing sequences that enhance *BN115* promoter activity at 2°C, may also contain possible negative regulatory elements that repress gene expression at 22°C. Deletion of this region resulted in an increase in *BN115*-promoted GUS expression in bombarded Jet neuf leaves at 22°C. Deletion of the regions of the *BN115* promoter containing the 31-bp direct repeat (−640 to −436) and the 22-bp direct repeat (−434 to −300) results in almost no change in promoter activity at both 2°C and 22°C. It has been reported that long direct repeats in the promoter of a *B. rapa* acyl carrier protein gene are nonessential for its expression (Scherer et al., 1992).

Deletion of the next 28 bp (to −274) markedly decreases transient expression at 2°C to a level below that at 22°C for the first time without affecting expression at 22°C in bombarded leaves (Fig. 4). Contained within this 28-bp fragment is the first half of an 8-bp direct repeat, TGGCCGAC, that may contain the basic low-temperature regulatory element or LTRE. This 8-bp motif, also found repeated in the promoter of *cor15a* (Baker et al., 1994), is similar to the repeated sequence G/TA/GCCGACA/TT/A-A/T present in the promoter regions of three low-temperature-induced genes from *A. thaliana*: *rab18* (Lång and Palva, 1992), *lti78*, and *lti65* (Nordin et al., 1993) (Fig. 7). Yamaguchi-Shinozaki and Shinozaki (1994) have identified a 9-bp core sequence, TACCGACAT, within a 20-bp repeated sequence in the *rd29A* (*lti78*) promoter as a DRE, which also appeared to be involved in the production of GUS transcripts in transgenic *A. thaliana* in response to cold treatment. In addition, the promoter of *rd29B*, which is not a cold-induced gene, does not have the 9-bp DRE (Yamaguchi-Shinozaki and Shinozaki, 1994). Interestingly, although the 9-bp DRE forms part of the larger 20-bp repeated element in the *rd29A* promoter of *Arabidopsis*, the 8-bp TGGCCGAC motif is separate from the large direct repeats in the *BN115* promoter in *B. napus* cv Jet neuf. Thus, the presence of identical or similar core motifs in low-temperature-responsive promoters isolated from two species, *A. thaliana* and *B. napus*, further implicates the involvement of the 8-bp repeated element, TGGCCGAC, in the low-temperature regulation of *BN115* gene expression.

rd29A/lti78(DRE-1/LTRE-2)	t c a T A C C G A C A T c a g
rd29A/lti78(DRE-2/LTRE-3)	t a a T A C C G A C A T g a g
lti65(LTRE)	t g g A C C G A C T a a a
lti78(LTRE-1)	t g g A C C G A C T a c t
lti78(LTRE-4)	c a a G C C G A C A - a a a
cor15a(LTRE-1)	g t T G G C C G A C a t a c a
BN115(LTRE-1)	g t T G G C C G A C g t a t a
cor15a(LTRE-2)	c a T G G C C G A C c t g c t
BN115(LTRE-2)	g a T G G C C G A C c t g t t

Figure 7. Alignment of the LTREs and DREs from the *Arabidopsis rd29A* (*lti78*), *lti65*, and *cor15a* genes with the TGGCCGAC direct repeat from the *BN115* promoter. The DRE and LTRE from *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994), putative LTRE from *lti78*, *lti65* (Nordin et al., 1993), and *cor15a* (Baker et al., 1994) genes, and the corresponding *BN115* sequences are shown. Uppercase letters indicate repeated or conserved nucleotides within each gene. For genes containing more than one LTRE, the number 1 designates the 5′-most element.

The observation of increased high-temperature GUS activity after deletion of parts of the *BN115* promoter sequence lends support to the possibility that low-temperature induction of *BN115* expression may result in part from repression of gene expression at higher temperatures. Repression of transcription was proposed to explain the observation that a protein factor from both control and drought-stressed *A. thaliana* can interact with the 9-bp DRE (TACCGACAT) from the *rd29A* gene (Yamaguchi-Shinozaki and Shinozaki, 1994). It will be interesting to determine whether a similar protein factor binds the TGGCCGAC motif from the *BN115* promoter and whether this factor is present in the nuclei from both control and cold-treated leaves.

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