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A novel protein from *Brassica napus* has a putative KID domain and responds to low temperature

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Summary

To identify factors that interact with histone deacetylase (HDAC) in *Brassica napus*, a yeast two-hybrid library was screened using the *Arabidopsis* HDA19 as bait. A novel protein, bnKCP1, containing a putative kinase-inducible domain (KID) was found to interact with HDA19. Southern blot analysis indicated that the *bnKCP1* gene belongs to a small gene family of at least three members. Northern blot analysis showed *bnKCP1* to be strongly expressed in stems, flowers, roots, and immature siliques, but not in leaf blades of seedlings. The accumulation of *bnKCP1* transcript in the leaf blades was induced significantly within 4 h of exposure of *B. napus* seedlings to cold stress, whereas treatment of leaf blades with inomycin, an ionophore of Ca^{2+}, caused a rapid (30 min) but transient induction of *bnKCP1* expression. In contrast to that observed in leaf blades, expression of *bnKCP1* in the stems was repressed upon cold treatment. *In vitro* and *in vivo* protein-binding assays showed that bnKCP1 interacts with HDA19 via the KID domain, and that S^{188} is critical for bnKCP1–HDA19 interaction. BnKCP1 also exerted modest transactivation of the *lac*Z reporter gene in yeast through its N-terminal region. These assays suggest that bnKCP1 may function as a transcription factor, which regulates gene expression through interaction with HDA19.

Keywords: *Brassica napus*, kinase-inducible domain, transcription factor, histone deacetylase, Ca^{2+}, cold stress tolerance.

Introduction

Regulation of eukaryotic gene expression is a highly coordinated, multifactorial process involving many components of the nuclear apparatus. In eukaryotic cells, gene expression requires that appropriate transcription factors gain access to regulatory regions located mainly at the 5′-upstream region of the gene. Accessibility to regulatory regions is determined by local chromatin structure, e.g. condensed 'closed' chromatin structure prevents transcription factors from reaching their target DNA-binding sites, whereas relaxed 'open' chromatin structure enhances transcription by allowing transcription factors to bind to target DNA sites (Ahringer, 2000). Chromatin structure is controlled, to a large extent, by the acetylation state of histones, which is governed by the actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs). In general, acetylation and deacetylation of histones are associated with activation and repression, respectively, of gene expression (Eberharter and Becker, 2002; Kuo and Allis, 1998; Sterner and Berger, 2000; Struhl, 1998). Therefore, recruitment of HAT and HDAC to specific promoters to control the acetylation state of histones in turn influences gene expression. Such recruitment of HAT and HDAC to specific chromosomal loci is generally achieved through interaction with transcription activators/co-activators and repressors/co-repressors, respectively (Peterson and Logie, 2000). Although significant progress has been made in characterizing chromatin re-modeling complexes and their recruiting factors in yeast and mammals (Hassan *et al.*, 2001), work in plants is still in its infancy.

In mammals, the cAMP-responsive element (CRE)-binding protein (CREB) binds to the CREB-binding protein (CBP) in response to extracellular stimuli that induce intracellular accumulation of secondary messengers Ca^{2+} and cAMP (Montminy, 1997). The KID domain is highly conserved in the CREB family of proteins including CREB, CREM, and ATF-1 (Montminy, 1997). Each protein in this family has a serine phosphorylation site ($RRPS^{133}$) within the KID domain that can be phosphorylated by protein kinase A

(PK-A). PK-A in turn is activated by outside stimuli that induce intracellular accumulation of Ca^{2+} and cAMP. CREB-binding activity is regulated through S^{133} phosphorylation, which is required for the interaction of the KID domain of CREB with the KIX domain of CBP (Montminy, 1997). Interestingly, CBP possesses intrinsic HAT activity (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996), suggesting that histone acetylation via the recruitment of CBP to target promoters by the transcription activator CREB may also contribute to the transcriptional activation of CRE-dependent genes.

In *Arabidopsis*, a *HAT* gene encoding an ortholog of the yeast GCN5 was found to bind *in vitro* to two proteins similar to the yeast HAT-adaptor proteins ADA2, ADA2a, and ADA2b (Stockinger *et al.*, 2001). Moreover, the transcription activator CBF1 was found to bind to both HAT and ADA2, indicating that these proteins might be recruited to specific cold-inducible genes by binding to CBF1 (Stockinger *et al.*, 2001). The finding that the *Arabidopsis ADA2* and *GCN5* genes share similarity with their counterparts in yeast and humans suggests that chromatin re-modeling complexes are conserved among evolutionarily distant organisms. However, chromatin in plants appears to be more diverse and complex as exemplified by the large number of genes encoding chromatin re-modeling factors. For example, the *Arabidopsis* genome encodes at least 12 HATs and 16 HDACs (Pandey *et al.*, 2002), and there is evidence which points to the presence of multiple *HAT* and *HDAC* genes in *Zea mays* (Dangl *et al.*, 2001; http://www.chromdb.org). Moreover, Bordoli *et al.* (2001) described four *Arabidopsis* orthologs, PCAT-1–4, of the human p300 and CBP proteins, and Pandey *et al.* (2002) reported that the *Arabidopsis* genome encodes five CBP-type HATs (HAC1, HAC2, HAC4, HAC5, and HAC12). CBP/p300 has intrinsic HAT activity and can act as a transcription co-activator by interacting with the CREB protein (Giles *et al.*, 1998). The amino acid sequence similarity between the PCAT-1–4 orthologs and CBP/p300 is confined to a region of about 600 amino acids at the C-terminus, which encompasses the HAT- and E1A-binding domains. However, PCAT-1–4 lack the bromo- and KIX-domains, which are present in the human p300/CBP proteins. However, Pandey *et al.* (2002) found that a weakly defined KIX-like domain exists in four of the five HATs. *Brassica napus*, a species related to *Arabidopsis*, is expected to encode homologs to the *Arabidopsis* genes encoding chromatin re-modeling factors because of the high coding sequence conservation and genomic collinearity between these two plant species (Cavell *et al.*, 1997; Parkin *et al.*, 2002; Paterson *et al.*, 2001; Ryder *et al.*, 2001; Vanoosthuyse *et al.*, 2001).

As part of our efforts to characterize nuclear factors that interact with HDAC, we report the cloning of bnKCP1 from *B. napus*. We demonstrate that bnKCP1 interacts with HDA19 in the yeast two-hybrid system and *in vitro*. bnKCP1 contains a putative KID domain and shares some functional similarities with the mammalian CREB family of proteins. To our knowledge, this is the only known protein with putative KID domain in higher plants, thereby adding credence to the suggestion that plants may have a gene regulatory system similar to that conveyed by the CREB-CBP/p300 system in mammals.

Results

Cloning of the B. napus *KCP protein*

To identify proteins that interact with HDAC in *B. napus*, the ORF of the *Arabidopsis RPD3*-type *HDA*, which is designated as *HDA19* (AtRPD3A) (Pandey *et al.*, 2002), was fused to the yeast GAL4 DNA-binding (GAL4 DB) domain and used as bait in a yeast two-hybrid screening of a *B. napus* cDNA library linked to the yeast GAL4 activation domain (GAL4 AD). Several positive clones were obtained on the basis of the induction of three reporter genes in yeast, *HIS*3, *URA*3, and *lacZ*. One of these clones (963 bp) encoded a 23.5-kDa protein that contained a putative kinase-inducible domain (KID), and was designated *B. napus* KID-containing protein (*bnKCP*). Alignment of deduced amino acid sequences indicated that bnKCP shared 82% amino acid identity with a putative KCP from *Arabidopsis* encoded by a gene of unknown function (AY088175, At5g24890). It also shared high similarity in a conserved region of approximately 60 amino acids (GKSKS domain) with two other predicted proteins from the *Arabidopsis* genome, atKCL1 (CAB45910, At4g31510) and atKCL2 (AAD23890, At2g24550) (Figure 1). However, atKCL1 and atKCL2 proteins lack the KID and the RRPS phosphorylation site (Figure 1).

To estimate the *bnKCP* gene copy number in *B. napus*, we carried out Southern blot analysis on total genomic DNA digested with restriction endonucleases using the entire open-reading frame of *bnKCP* as a probe under high stringency conditions (Figure 2). Digestion with *Eco*RI, *Hin*dIII, and *Eco*RV, none of which cleaves within *bnKCP*, resulted in the detection of multiple bands. Digestion with *Xba*I generated more bands due to the presence of an internal site for *Xba*I in the *bnKCP* gene. The results suggest that *bnKCP* belongs to a small gene family of at least three members in the *B. napus* genome. Therefore, the gene we have identified will be designated *bnKCP1*.

Structural features of the bnKCP1 protein

The open-reading frame of *bnKCP1* encodes a polypeptide of 215 amino acids with several functional motifs (Figure 3). Based on a search of protein localization sites using the PSORT program (Nakai and Kanehisa, 1992; http://

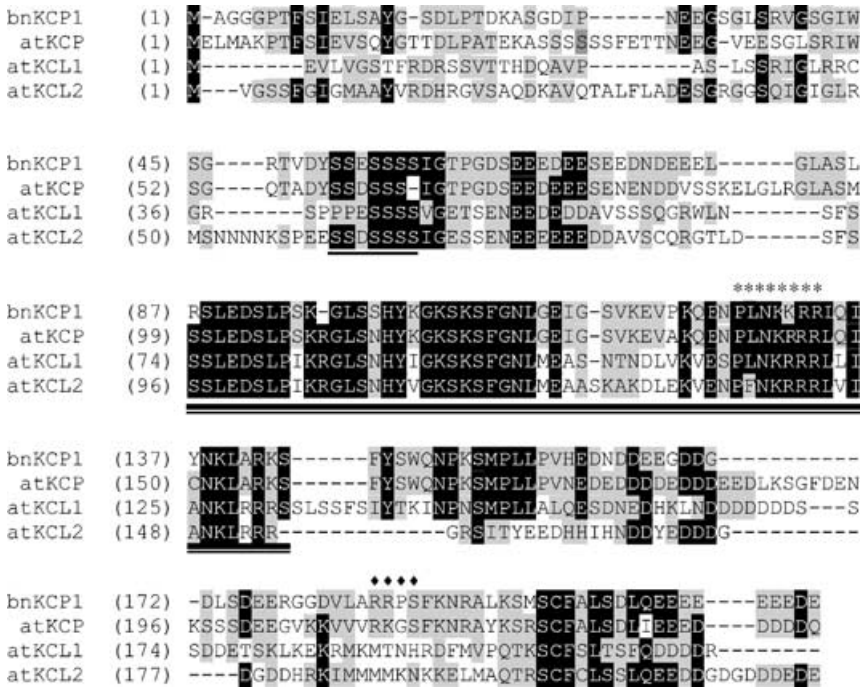

Figure 1. Alignment of the deduced amino acid sequences of bnKCP1, atKCP (At5g24890), atKCL1 (At4g31510), and atKCL2 (At2g24550) proteins.
Identical or highly conserved amino acids in the four proteins are shown as white letters on a black background, whereas amino acids with similarity are indicated as black letters on a gray background. Amino acids with no similarity are shown as black letters on a white background. The serine (S)-rich residues and the conserved region (GKSKS domain) among the four sequences are single and double underlined, respectively. The putative nuclear localization signal (NLS) and the phosphorylation site of protein kinase A are indicated by asterisks (*) and diamonds (◆), respectively.

psort. nibb.ac.jp), bnKCP1 appears to be a nuclear protein containing a putative pat7 nuclear localization signal (NLS), PLNKKRR (residues 127–133) within the charged motif GKSKS (residues 88–143), which is conserved in all four protein orthologs identified in *B. napus* and *Arabidopsis*

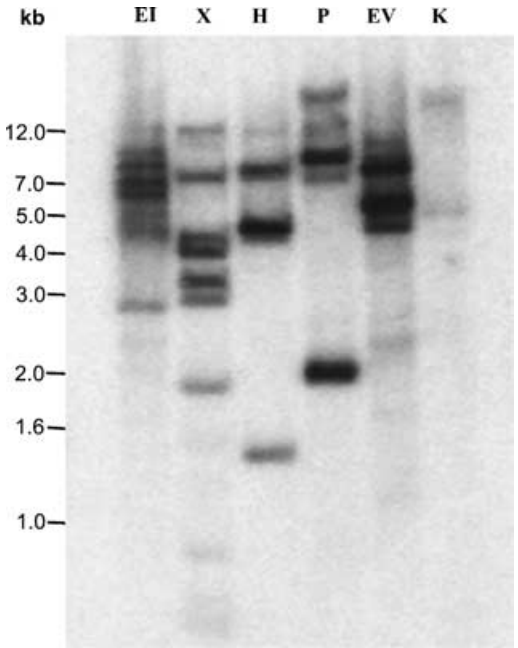

Figure 2. Southern blot analysis of *Brassica* genomic DNA.
Total genomic DNA (10 μg per lane) from *Brassica napus* cv. Westar was digested with restriction enzymes *Eco*RI (EI), *Xba*I (X), *Hin*dIII (H), *Pst*I (P), *Eco*RV (EV), and *Kpn*I (K). The entire ORF of *bnKCP1* was used as a probe.

(Figure 1). Three acidic motifs (I–III) and a serine (S)-rich region (residues 34–58) (Figure 3a) may function in transcription activation by bnKCP1 (Johnson *et al.*, 1993). Amino acid sequence analysis also revealed that bnKCP1 has a KID-like motif (residues 161–215) with alpha structure at its C-terminal region (Figure 3b). The KID is highly conserved amongst members of the mammalian CREB protein family and functions in transactivation and protein binding (see review Montminy, 1997). The KID in bnKCP1 has a higher degree of similarity to the one of CREB family member, ATF-1 (Figure 4), and contains a PK-A phosphorylation site ($RRPS^{188}$) that is also conserved in the CREB family of proteins (Figure 4). The secondary structure at the N-terminal region of bnKCP1 is rich in turns and coils (Figure 3b), suggesting that this region might bear a different function from that of the KID domain.

Interaction of bnKCP1 with HDA19

To confirm the interaction detected in the yeast two-hybrid system between the bnKCP1 protein and HDA19, GST pull-down affinity assays were performed using *in vitro* translated bnKCP1 labeled with [^{35}S]methionine. The bnKCP1 protein was tested for its ability to interact with recombinant GST–HDA19 fusion protein expressed in *Escherichia coli*. As shown in Figure 5(b), bnKCP1 bound to GST–HDA19 fusion protein, but not to GST alone.

To map the protein-binding domain of the bnKCP1 protein, we constructed two C-terminal truncated mutants of bnKCP1 lacking the KID domain, $\Delta bnKCP1^{1-160}$ and $\Delta bnKCP1^{1-80}$ as shown in Figure 5(a). These truncated

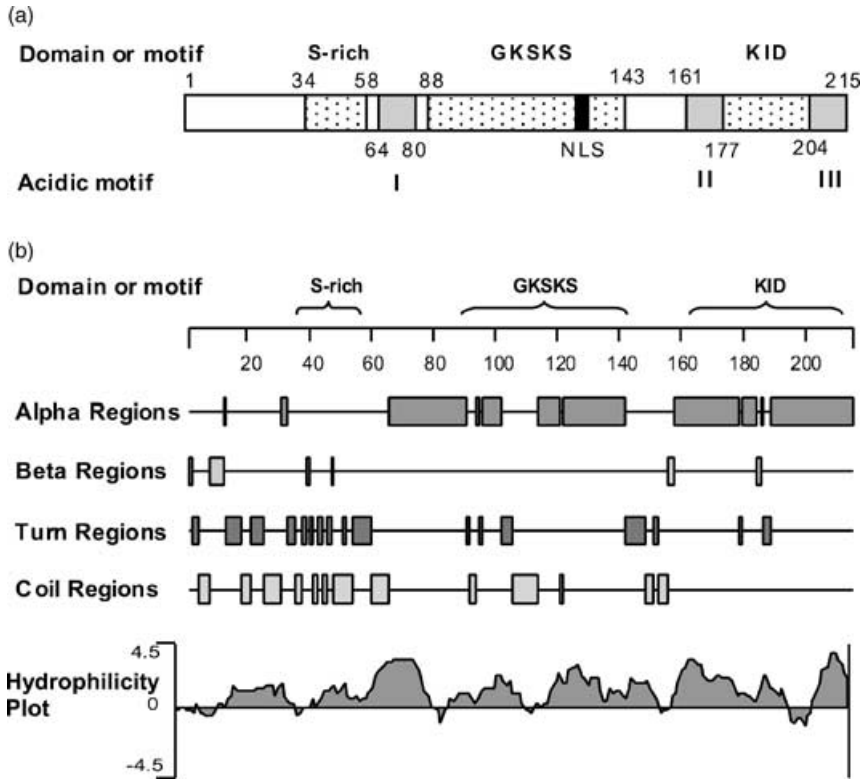

Figure 3. Structural features of bnKCP1.
(a) Schematic representation of entire bnKCP1 protein. Numbers above or under the boxes refer to positions of amino acid residues. S-rich (34–58), GKSKS (88–143), and KID (161–215) domains or motifs are shown in dotted boxes, the nuclear localization signal (NLS) in black box, and the three acidic motifs (I–III) in gray boxes.
(b) Secondary structure features and hydrophilicity of bnKCP1 analyzed using DNASTAR PROTEAN program.

mutants were assayed for *in vitro* interaction with the recombinant GST–HDA19 fusion protein. The two mutant proteins, ΔbnKCP1$^{1-160}$ and ΔbnKCP1$^{1-80}$, exhibited no interaction with GST–HDA19 (Figure 5b) indicating that the KID domain is essential for bnKCP1 binding to HDA19. The importance of the KID domain for protein binding was also determined *in vivo* using the yeast two-hybrid system. MaV203 yeast cells were co-transformed with pDBLeu–HDA19, and either pPC86–bnKCP1, pPC86–bnKCP1$^{1-160}$, pPC86–bnKCP1$^{1-80}$ or pPC86 alone (Figure 5). As shown in Figure 5(c), β-galactosidase activity was reduced by at least 50% when pDBLeu-expressing cells were transformed with plasmids expressing either ΔbnKCP1$^{1-160}$ or ΔbnKCP1$^{1-80}$, both of which lacked KID, as compared to the full-length bnKCP1. This finding demonstrates that KID is critical for bnKCP1 interaction with HDA19 *in vivo*.

The S^{188} residue in the KID domain is predicted to be subjected to phosphorylation by PK-A similar to S^{133} in CREB (Montminy, 1997). To investigate the importance of S^{188} for bnKCP1 interaction with HDA19, the S^{188} in bnKCP1 was mutated to G^{188} using site-directed mutagenesis to obtain bnKCP1G^{188} protein (Figure 6a). This mutated protein was then tested for binding to HDA19 *in vitro*. When

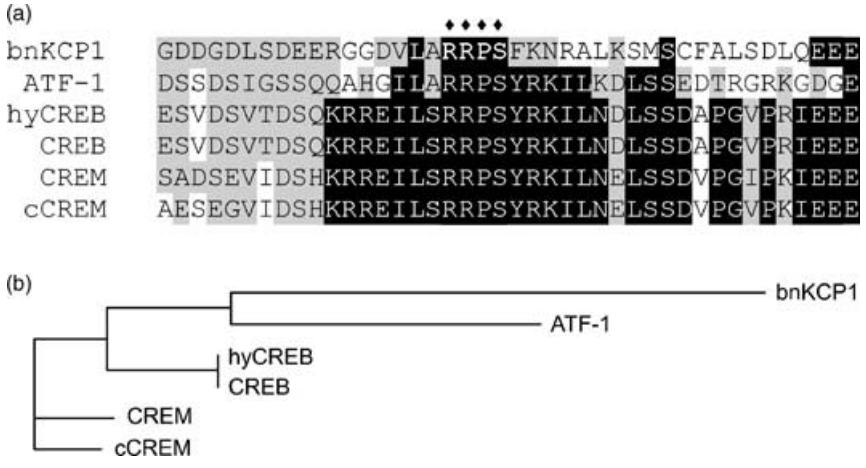

Figure 4. Sequence comparison of kinase-inducible domains (KIDs) in bnKCP1 and CREB family members.
(a) Alignment of the amino acid sequences of bnKCP1, hydra CREB (hyCREB), canfa CREM (cCREM), and mammalian ATF-1, CRBB, and CREM. Shadings are as indicated in Figure 1. Diamonds (◆) indicate the conserved phosphorylation site of protein kinase A.
(b) Phylogenetic tree of the KIDs sequences using the NTI Vector program.

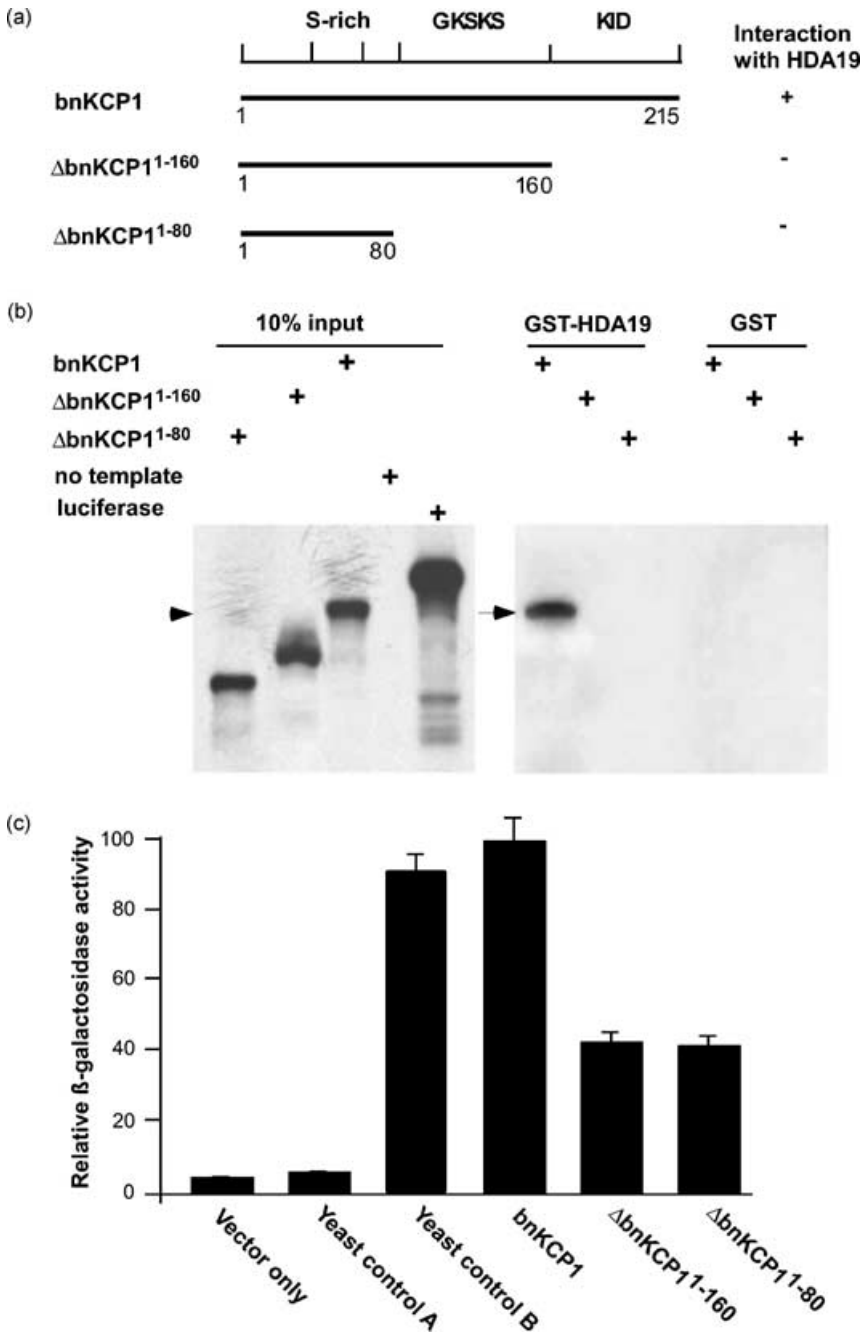

Figure 5. *In vitro* and *in vivo* interaction of wild-type and mutant bnKCP1 proteins with the GST–HDA19 fusion protein.
(a) Schematic representation of the bnKCP1 and its deletion mutants obtained by deletion of C-terminal regions of bnKCP1.
(b) Binding activities of bnKCP1 and its mutants with GST–HDA19 and GST alone (negative control), respectively. The wild-type bnKCP1, mutants ΔbnKCP1$^{1–160}$ and ΔbnKCP1$^{1–80}$, luciferase (positive control) and negative control (no template) were produced using *in vitro* transcription/translation reactions. The translation products were incubated with GST fusion proteins or GST and their binding activities were examined as described in Experimental procedures. Arrows point to bands representing the *in vitro*-translated bnKCP1 protein.
(c) Activation of *lac*Z reporter gene by bnKCP1 and its deletion mutants, ΔbnKCP1$^{1–160}$ and ΔbnKCP1$^{1–80}$, in yeast cells. MaV203 yeast cells carrying plasmid pDBLeu–HDA19 and the reporter gene were transfected with the plasmid pPC86–bnKCP1, pPC86–bnKCP1$^{1–160}$, pPC86–bnKCP1$^{1–80}$ or pPC86 vector only. Yeast strains A and B (GibcoL BRL, Life Technologies) were used as negative and positive controls, respectively. The β-galactosidase activity was assayed using chlorophenol red-β-D-galactopyranoside (CPRG) and was expressed as a percentage of activity conveyed by bnKCP1. Bars indicate the SE of three replicates.

compared to bnKCP1, the mutated protein, bnKCP1G^{188}, had significantly reduced binding to HDA19 (Figure 6b). This confirms that S^{188} is essential for optimal interaction between bnKCP1 and HDA19.

Expression pattern of the bnKCP1 *gene*

The expression pattern of the *bnKCP1* gene was analyzed by Northern blot analysis of total RNA extracted from various *B. napus* organs. As shown in Figure 7, *bnKCP1* transcripts accumulated in flowers, roots, stems, and immature siliques, but were undetectable in leaf blades of seedlings at room temperature.

To investigate the pattern of *bnKCP1* expression in response to environmental stress conditions, Northern blot analysis was conducted using total RNA isolated from leaf blades of four-leaf-stage *B. napus* seedlings that were subjected to low temperature (4°C), drought, high salt (NaCl), and ABA treatment. Two transcripts of similar sizes hybridized to *bnKCP1*, indicating the existence of two expressed homologs of *bnKCP1* mRNAs in *B. napus*, and both of the *bnKCP1* homologs accumulated in leaves in

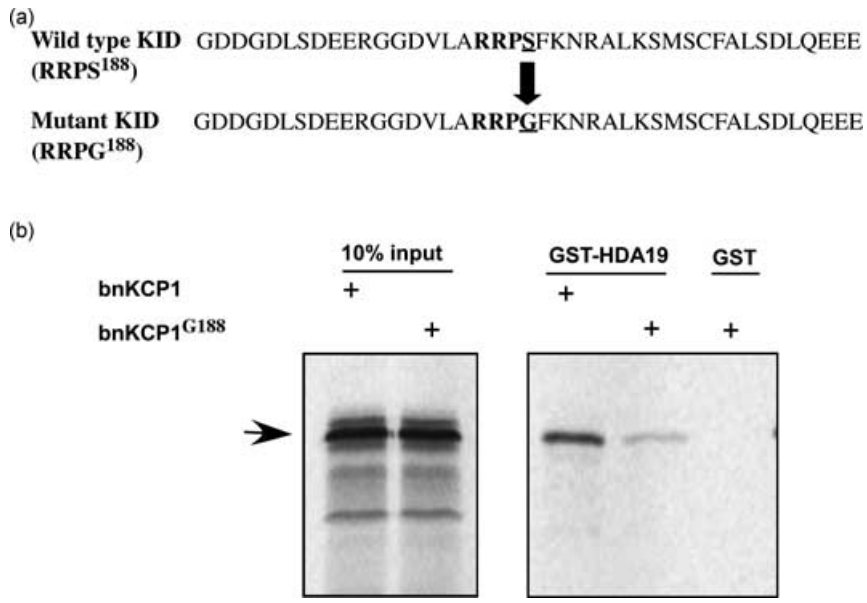

Figure 6. Effect of S^{188} on the interaction between bnKCP1 and GST–HDA19 fusion protein. A glycine residue (G^{188}) was introduced by site-directed mutagenesis to replace S^{188}. The binding activities of wild-type bnKCP1 and the mutant ΔbnKCP1G^{188} with GST–HDA19 or GST alone (negative control) were examined with GST pulldown affinity assay as described in Experimental procedures.
(a) Introduction of G^{188} into the KID of bnKCP1.
(b) *In vitro* protein interaction of bnKCP1 and the mutant ΔbnKCP1G^{188} with GST–HDA19 or GST alone.

response to cold treatment. The lower size (0.9 kb) transcript appears to be induced within 4 h of cold treatment and about 4 h earlier than the higher molecular weight one (1.1 kb) (Figure 8a). The *bnKCP1* transcript accumulated in response to low temperature (4°C), but expression was not detected in leaf blades of plants grown under drought conditions for up to 4 days, high salt stress for up to 11 days, or upon exogenous application of ABA for up to 8 h (data not shown). Interestingly, expression of *bnKCP1* in the stems was repressed upon cold treatment (Figure 8a), suggesting an organ-specific regulation of *bnKCP1* expression in response to low temperature.

As cold acclimation is known to be associated with elevated levels of intracellular concentrations of Ca^{2+} (Knight *et al.*, 1996; Monroy and Dhindsa, 1995), we determined whether Ca^{2+} has any effect on *bnKCP1* expression. Northern blot analysis was performed using total RNA isolated from leaves of seedlings grown at room temperature and treated with the Ca^{2+} channel blocker $LaCl_3$ or the Ca^{2+} ionophore inomycin, which are known to inhibit or enahnce Ca^{2+} influx, respectively (Monroy and Dhindsa, 1995). Induction of *bnKCP1* expression upon treatment with inomycin was rapid (0.5 h) but short-lived, suggesting a transient influx of Ca^{2+}. The *bnKCP1* transcript was undetectable in leaves of seedlings treated with the $LaCl_3$ (Figure 8b), which may be due to a block in the Ca^{2+} channels caused by $LaCl_3$. These results are in accordance with the view that cold-induced calcium influx is vital to cold acclimation in plants (Monroy and Dhindsa, 1995).

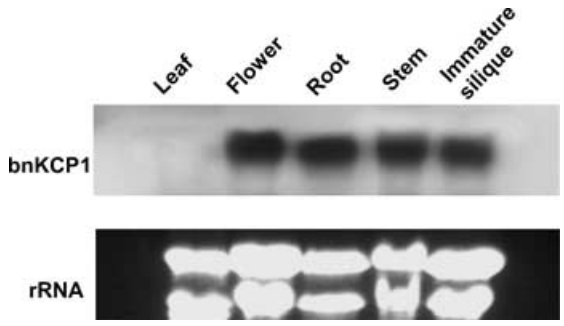

Figure 7. Expression patterns of *bnKCP1* mRNA in different tissues.
Total RNA (20 μg per lane) was isolated from leaves, flowers, roots, stems, and immature siliques at room temperature, electrophoresed through a 1.2% agarose gel containing formaldehyde and probed with the ORF of *bnKCP1* as described in Experimental procedures. EtBr-stained total RNA is shown to indicate even loading.

Transcription activation by bnKCP1

To further characterize the functional role of bnKCP1, it was investigated to determine whether it functions as a transcription activator. Transactivation experiments were carried out in yeast, whereby a yeast strain carrying three reporter genes, *lacZ*, *HIS3*, and *URA3*, driven by promoters fused to GAL4 DNA-binding sites and independently integrated into the yeast genome were transfected with the effector plasmid pDBLeu–bnKCP1 comprising bnKCP1 fused to the GAL4 DB under the control of the ADH promoter. Transformation with the effector plasmid resulted in increasing β-galactosidase activity about eightfold relative to either vector pDBLeu alone or yeast control strain A, which contains plasmid pairs expressing fusion proteins without protein–protein interaction (Figure 9). Reporter genes *HIS3* and *URA3* were also modestly activated by bnKCP1 (data not shown). These results demonstrate that bnKCP1 shows transcription activator or co-activator activity in yeast.

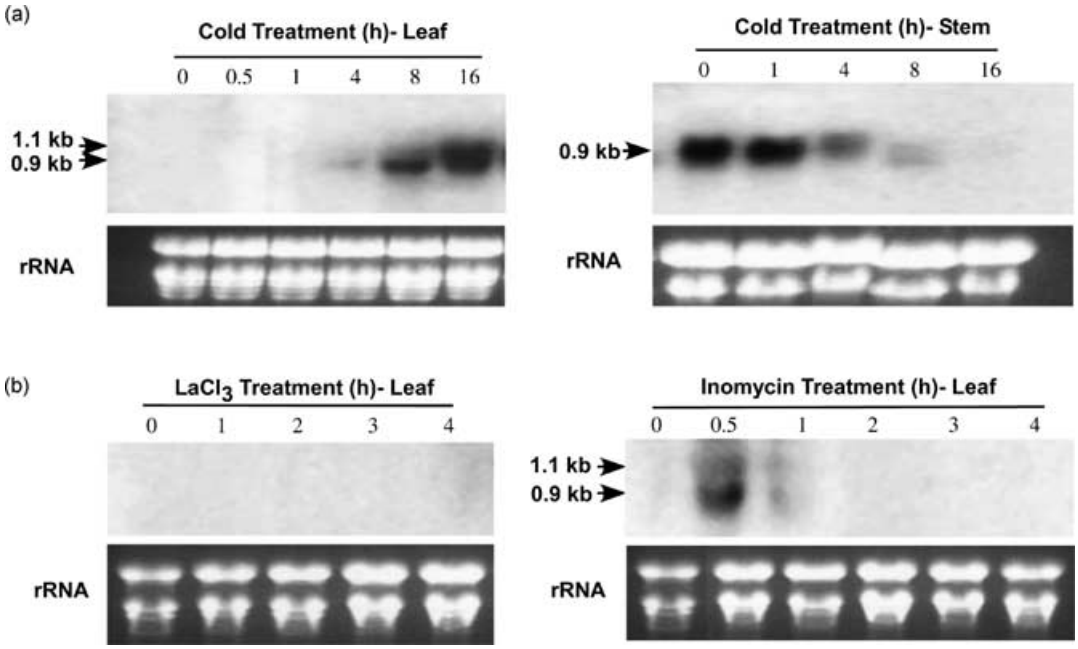

Figure 8. Expression of *bnKCP1* gene in response to low temperature, $LaCl_3$ and inomycin treatments. Total RNA (20 μg per lane) was isolated from leaf blades of four-leaf-stage *B. napus* cv. Westar seedlings after exposure to different stress conditions and analyzed by Northern blotting using the *bnKCP1* ORF as probe.
(a) *bnKCP1* transcript accumulation in leaves and stems of seedlings exposed to cold (4°C).
(b) Expression pattern of *bnKCP1* gene after treatment with $LaCl_3$ and inomycin at room temperature.

To map the transactivation domain of the bnKCP1 protein, one N-terminal truncated mutant of bnKCP1, ΔbnKCP1$^{81–215}$, and two C-terminal truncated mutants, ΔbnKCP1$^{1–160}$ and ΔbnKCP1$^{1–80}$ (Figure 9a) were generated and used in *in vivo* transactivation assays in yeast. As shown in Figure 9(b), deletion of the KID or GKSKS domains had no significant influence on β-galactosidase activity, whereas deletion of the N-terminus resulted in approxi-

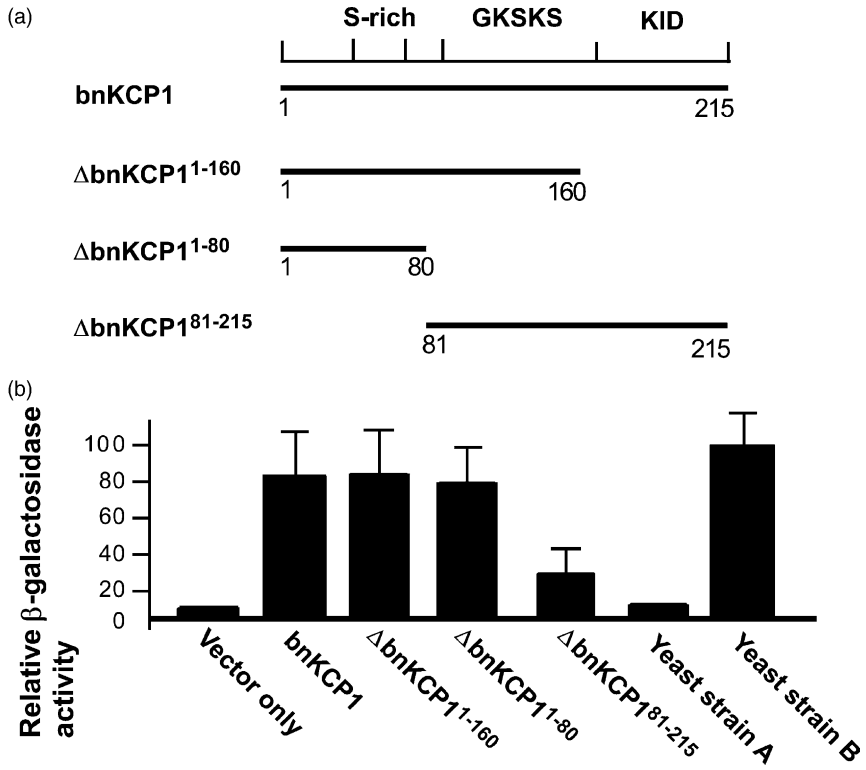

Figure 9. Transactivation of the *lac*Z gene by bnKCP1 in yeast.
The *lac*Z reporter gene was driven by a promoter containing GAL4 DNA-binding sites and integrated into the genome of yeast MaV203 cell. Yeast cells carrying the reporter gene were transfected with the effector plasmids pDBLeu–bnKCP1, pDBLeu–bnKCP1$^{1–160}$, pDBLeu–bnKCP1$^{1–80}$, and pDBLeu–bnKCP1$^{81–215}$ or the vector pDBLeu only. Yeast strains A and B (GibcoL BRL, Life Technologies) were used as negative and positive controls, respectively. The β-galactosidase activity was assayed using CPRG and was expressed as a percentage of activity conveyed by the positive control (yeast strain B). Bars indicate SE of three replicates.

mately 65% reduction in β-galactosidase activity. This demonstrates that the transactivation domain of bnKCP1 may reside in the N-terminal region, and is independent of KID.

Nuclear localization of the bnKCP1 protein

Structural and functional analyses showed bnKCP1 to have features typical of transcription factors. To confirm that bnKCP1 is a nuclear protein, we transformed onion epidermal cell layers with constructs for the expression of either a GUS–bnKCP1 fusion or GUS alone (Figure 10a) using an *Agrobacterium*-mediated transformation method (Kapila *et al.*, 1997). As shown in Figure 10(b), GUS activity was visualized exclusively in the cytoplasm of control onion cell layers. In contrast, a blue precipitate was localized in the nuclei of cell layers transformed with GUS–bnKCP1 fusion construct, although there was still a certain amount of cytoplasm staining, indicating that at least some targeting to the nucleus occurs with the fusion protein.

Discussion

Isolation of proteins that interact with nuclear HAT and HDAC is a direct approach to identifying factors that direct these chromatin re-modeling agents to specific promoters to influence the expression of the associated genes. Here, we described the isolation of the first plant protein, bnKCP1, containing a putative KID domain required for interaction with HDA19. We demonstrated that *bnKCP1* was expressed in all organs tested with the exception of leaf blades, where it was induced in response to cold acclimation; this also resulted in the repression of its expression in stems of seedlings. Furthermore, we showed that bnKCP1 exerts modest transcription activation of the *lac*Z reporter gene via its N-terminal region in yeast, indicating the function of bnKCP1 as a transcription factor.

Given the high genetic and molecular collinearity between the genomes of *Arabidopsis* and *Brassica* species (Cavell *et al.*, 1997; Parkin *et al.*, 2002; Paterson *et al.*, 2001; Ryder *et al.*, 2001; Vanoosthuyse *et al.*, 2001), genes encoding chromatin re-modeling complexes are expected to be highly conserved between *Arabidopsis* and *B. napus*. Indeed, this was confirmed by Southern blot analysis in which the *Arabidopsis* HDA19 bound specifically to a distinct band on a *B. napus* DNA blot under high stringency conditions (data not shown). Furthermore, we recently identified an expressed sequence tag (EST) clone of *B. napus* HDAC among the EST collection generated at Agriculture and Agri-Food Canada, Saskatoon Research Centre (http://www.brassica.ca). This EST was found to code for a polypeptide that shares 85% amino acid identity with the *Arabidopsis* HDA19 (data not shown). These findings validate the use of the *Arabidopsis* HDA19 for screening the *B. napus* yeast two-hybrid cDNA library and for conducting protein-binding assays *in vivo* and *in vitro*.

BnKCP1 has characteristic features of transcription factors

Several pieces of evidence point to the function of bnKCP1 as a transcription factor. These include:

(1) bnKCP1 contains a putative NLS and can target at least a portion of the GUS protein to the nucleus, suggesting that it may be a nuclear protein;

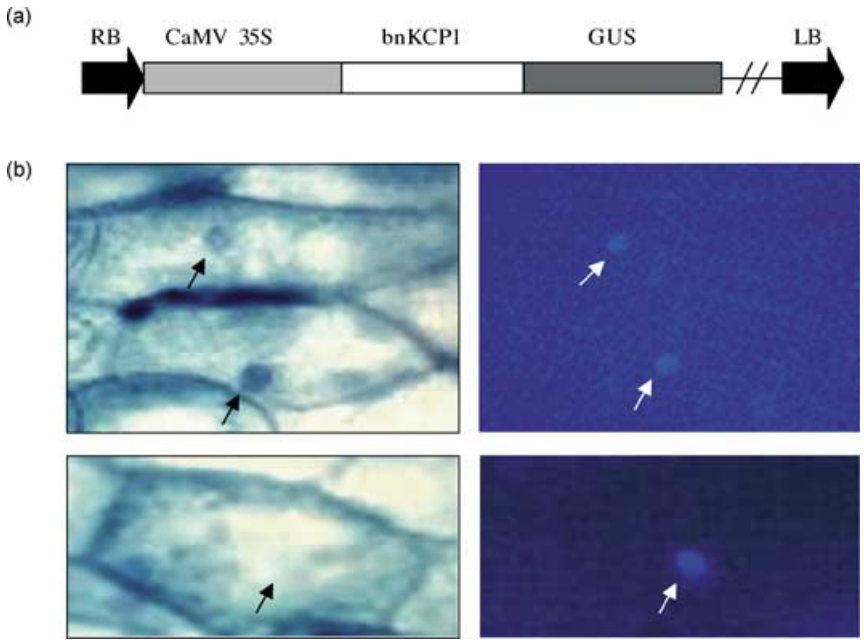

Figure 10. Nuclear localization of GUS–bnKCP1 protein in onion cells.
(a) Schematic diagram of the GUS–bnKCP1 fusion construct containing the CaMV 35S promoter. The *bnKCP1* was fused in-frame to the GUS reporter gene.
(b) Transient expression of GUS–bnKCP1 fusion protein (top) and GUS alone (bottom) in onion cells. Onion tissues were simultaneously analyzed using histochemical GUS assay (left) and nucleus-specific staining with DAPI (right) as described in Experimental procedures.

(2) bnKCP1 has a putative KID domain, which to our knowledge is present only in the CREB family of transcription factors;
(3) bnKCP1 interacts with HDA19, which may be a nuclear protein and a transcriptional regulator; and
(4) bnKCP1 activates transcription in yeast cells and at least part of this activation requires an amino-terminal region.

The most important structural feature of bnKCP1 is the presence of a putative kinase-inducible domain (KID) with alpha secondary structure at the C-terminal region. The KID domain was first identified in the mammalian CREB family of transcription factors, which includes CREB, CREM, and ATF-1. The KID domain in mammalian CREB is involved in at least two functions, interaction with CBP/p300, and site for PK-A phosphorylation of S^{133} (Chrivia *et al.*, 1993; Gonzalez *et al.*, 1991; Montminy, 1997; Quinn, 1993; Shaywitz *et al.*, 2000). Similar to its counterpart in CREB, which is involved in protein binding, the KID domain of bnKCP1 is required for binding to HDA19 *in vivo* and *in vitro*. Moreover, the requirement of S^{188} residue of bnKCP1 for interaction with HDA19 parallels the involvement of S^{133} of CREB in interaction with CBP/p300 (Montminy, 1997). It is interesting to note that bnKCP1, being involved in transcription activation, interacts with HDA19, which is generally associated with transcription repression. This interaction may allow for bnKCP1 to relieve repression exerted by HDAC. A similar finding was reported for the transcription co-activator PIASxß, which was found to interact with HDAC3 to relieve repression exerted by HDAC3 upon TFII–I-mediated gene activation (Tussié-Luna *et al.*, 2002).

Expression of bnKCP1 *is organ specific*

The *bnKCP1* gene appears to be part of a multigene family of at least three members based on Southern blot hybridization. Northern blot analyses showed that two members of this gene family have similar transcript sizes and expression patterns. In *Arabidopsis*, there is one homolog, atKCP (AY088175, At5g24890), sharing high similarity with bnKCP1 and two *atKCP*-like members (At4g31510 and At2g24550) of similar sizes ranging from 1 to 1.2 kb. Northern blot analysis revealed that the *bnKCP1* transcript was expressed in flowers, roots, stems, and immature siliques (Figure 7). The transcript accumulation, however, was undetectable in leaf blades of *B. napus* seedlings, suggesting tissue-/organ-specific expression of the *bnKCP1* gene.

Cold treatment induced *bnKCP1* expression in leaves, but repressed it in stems. The fact that *bnKCP1* expression is regulated differentially in the stems and leaves in response to cold treatment is significant, and may indicate the presence of a regulatory mechanism to allow for its expression in the stems, but not in the leaves, and to induce and repress its expression under cold stress in the leaves and stems, respectively. Characterization of regulatory factors that affect the expression of *bnKCP1* should shed some light on this regulatory mechanism.

Intracellular level of Ca^{2+} *affects* bnKCP1 *expression*

In mammalian cells, external stimuli that increase intracellular concentrations of Ca^{2+} or cAMP induce the expression of not only PK-A, but also the *CREB* gene (Meyer *et al.*, 1993). Therefore, we examined whether conditions that increase intracellular concentrations of Ca^{2+} would also induce *bnKCP1* expression. *B. napus* seedlings were exposed to cold or treated with inomycin. Cold acclimation is known to increase intracellular Ca^{2+} concentrations (Knight *et al.*, 1996; Monroy and Dhindsa, 1995), and inomycin is a known calcium ionophore that increases Ca^{2+} influx (Hurley *et al.*, 1996). Both of these treatments resulted in the induction of *bnKCP1* expression to varying degrees, which indicates that *bnKCP1* is induced by high intracellular Ca^{2+} concentrations. Future determination of the actual intracellular levels of Ca^{2+} caused by these treatments will be required to directly relate Ca^{2+} levels to *bnKCP1* expression. Moreover, it will be important to determine the effects of cold and inomycin treatments on the phosphorylation state of S^{188} of bnKCP1, and how that affects its ability to bind to HDA19 and exert transcription activation.

bnKCP1 *expression may be regulated by other stress conditions*

Although the genomic sequence of *bnKCP1* is currently unknown, suggestions can be made as to its structure and function based on conserved motifs present in its *Arabidopsis* homolog, *atKCP,* which shares 83% amino acid identity with bnKCP1. Several regulatory motifs were identified in *atKCP* by searching the PLACE database of plant *cis*-acting elements (Higo *et al.*, 1998). Two groups of *cis*-acting elements for the regulation of plant genes induced by various environmental stresses or pathogens were found. The core sequence of the DNA regulatory element, CCGAC, designated the low temperature-responsive element (LTRE) (Jiang *et al.*, 1996; Ouellet *et al.*, 1998) or C-repeat (CRT)/dehydration-responsive element (DRE) (Baker *et al.*, 1994; Yamaguchi-Shinozaki and Shinozaki, 1994) is present in the 5′-untranscribed and untranslated region of *atKCP*. The LTRE/CRT/DRE recognized by the transcriptional activators CBFs (Stockinger *et al.*, 1997) is present in the promoters of many cold- and dehydration-responsive genes in *Arabidopsis* and *Brassica*. The second group of *cis*-acting elements present in *atKCP* gene included TCA1 (Goldsbrough *et al.*, 1993), W-box (Rushton *et al.*, 1996; Yu *et al.*, 2001), ASF-1 (Lam *et al.*, 1989) and SEBF (Boyle and Brisson, 2001), which are present in the 5′-untranscribed or -untranslated region of a number of plant genes induced by

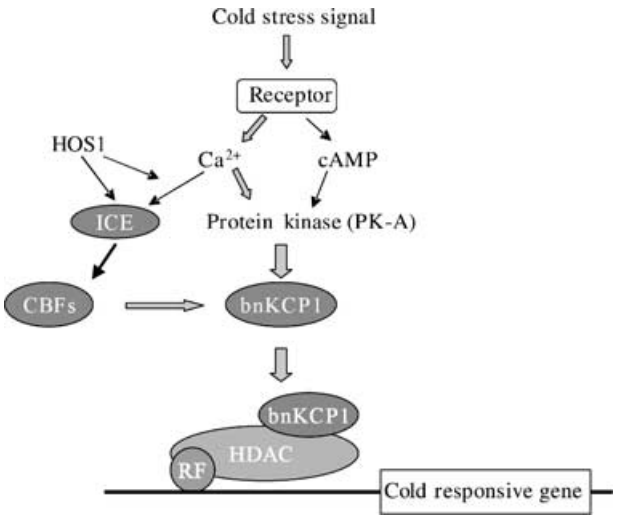

Figure 11. A model for secondary messenger-dependent cold signal transduction pathway in plants.
The intracellular levels of secondary messengers Ca^{2+} and cAMP increase after receiving the cold stress signal resulting in the activation of cAMP-dependent protein kinase (PK-A). bnKCP1 expression may be regulated by the transcriptional activators CBFs. The phosphorylation of S^{188} of bnKCP1 by PK-A enhances bnKCP1 binding to HDAC. This interaction leads to the activation of cold-inducible genes through the relief of repression exerted by HDAC recruited to these genes by recruiting factors under non-inducing conditions, i.e. at normal temperature. An unidentified transcriptional activator, ICE (inducer of CBF expression) (Thomashow, 2001) and a cold signaling negative regulator HOS1 which may target ICE or upstream signaling components (Lee *et al.*, 2001; Xiong *et al.*, 2002) are indicated.

salicylic acid (SA), pathogen or some other forms of stress, suggesting that, in addition to low temperature, *bnKCP1* may also be implicated in the plant's response to SA and pathogenesis.

Do plants have a CREB/CBP-like gene regulation pathway?

In addition to the present findings, there are reports in literature suggesting the presence of plant proteins with similarity to mammalian CREB. Previously, two DNA-binding proteins, TGA1a and TGA1b, containing a basic region homologous to the DNA-binding domain of CREB were isolated from *Nicotiana tabacum* (Katagirl *et al.*, 1989) and a DNA-binding protein, VBP1, with similarity to mammalian CREB in a basic region was isolated from *Vicia faba* (Ehrlich *et al.*, 1992). However, these plant proteins do not have a KID-like domain, nor have they been shown to interact with members of the chromatin re-modeling machinery. Recently, plant orthologs of the CBP/p300 proteins were cloned from *Arabidopsis*. These orthologs shared several functional domains and properties of their counterparts in animals (Bordoli *et al.*, 2001; Pandey *et al.*, 2002).

Taken together, these findings suggest the presence of a secondary messenger Ca^{2+}/cAMP signal transduction pathway in plants similar to the pathway involving CREB and CBP/p300 proteins in mammalian cells. However, unlike CREB, bnKCP1 appears to play a role in this pathway by activating transcription through interaction with HDAC to relieve repression exerted by the HDAC on target genes in response to external stimuli, such as cold stress, that increase intracellular concentrations of secondary messengers. A proposed model for secondary messenger-dependent cold signal transduction pathway involving interaction of bnKCP1 with HDAC is summarized in Figure 11.

Experimental procedures

Plant materials and treatments

Brassica napus L. cvs Cascade (winter type), Westar (spring type) and DES010 (spring type) were used for the isolation of genomic DNA and total RNA. Leaves, flowers, stems, and immature siliques were harvested from plants grown in a controlled-environment greenhouse programmed for a temperature of 22°C and a photoperiod of 16 h : 8 h light:dark. Roots were obtained by culturing sterilized seeds in 0.8% agar plates containing 0.5 MS medium (Sigma–Aldrich, Oakville, Canada) and 1% sucrose. For cold acclimation (4°C), abscisic acid (250 μM), drought, and high salt (850 mM NaCl) treatments, four-leaf-stage seedlings were treated and fourth fully expanded leaf blades were harvested as described by Gao *et al.* (2002). $LaCl_3$ and inomycin treatments were carried out by watering four-leaf-stage plants with 20 mM $LaCl_3$ and 10 μM inomycin, respectively. Plants were wrapped with plastic to slow evaporation.

Yeast two-hybrid screening and cloning

A yeast two-hybrid cDNA library was constructed from poly(A) mRNA isolated from the above-ground parts of the four-leaf-stage seedlings of *B. napus* L. cv. DH12075 and cloned into a GAL4 AD vector pPC86 using the SuperScript Plasmid System (GibcoL BRL, Burlington, Canada). To generate the construct pDBLeu–HDA19, the entire coding region of the *A. thaliana RPD3*-type *HDA19* (*AtRPD3A*) cDNA (Accession no. AY093153; At4g38130) was amplified by PCR using PWO DNA polymerase (Roche, Germany) with primers 5′-HDA1a and 3′-HDA1a (Table 1). The PCR product was analyzed by DNA sequencing and cloned into the *Sal*I and *Not*I sites of the vector pDBLeu in-frame with the GAL4 DB sequence and used as a bait to screen the *B. napus* cDNA library using the PROQUEST Two-Hybrid System (GibcoL BRL). Approximately 1×10^6 transformants were subjected to the two-hybrid selection on synthetic complete (SC) medium lacking leucine, tryptophan, and histidine but containing 15 mM 3-amino-1,2,4-triazole (3AT). The expression of the *HIS*3 reporter gene allowed colonies to grow on the selective medium and the putative His^+ ($3AT^{®}$)-positive transformants were tested for the induction of the two other reporter genes, *URA*3 and *lac*Z. The positive colonies were re-assessed by re-transformation assays in yeast and the cDNA inserts were identified by PCR and DNA sequencing.

Southern blot analysis

Total genomic DNA was isolated from the leaves of *B. napus* L. cv. Westar using a modified cetyltriethylammonium bromide (CTAB) extraction method (Gao *et al.*, 2002). Briefly, 10 μg total genomic

Table 1 List of primers used for plasmid construction

Primer name	Sequence
5′-HDA1a	5′-GCGTCGACGATGGATACTGGCGG CAATTCGC-3′
3′-HDA1a	5′-AGGCGGCCGCTTATGTTTTAGGA GGAAACGCC-3′
5′-HDA1b	5′-GCGTCGACGATGGATACTGGCGG CAATTCGC-3′
3′-HDA1b	5′-GCGCGGCCGCTTATGTTTTAGGA GGAAACGC-3′
5′-bnKCP1a	5′-GCAAGCTTATGGCAGGAGGAGGA CCAACT-3′
3′- bnKCP1a	5′-CGCTCGAGCTCaTCTTCTTCTTCT TCTTC-3′
3′- bnKCP1b	5′-CGCTCGAGATGAACAGGCAAAAGAG GCAT-3′
3′- bnKCP1c	5′-CGCTCGAGCTCCTCCTCATCATTGTC TTC-3′
5′-bnKCP1d	5′-GCGTCGACGATGGCAGGAGGAG GACCAACT-3′
3′-bnKCP1d	5′-GCGCGGCCGCCTCATCTTCTTCTT CTTCCTC-3′
3′-bnKCP1e	5′-GCGCGGCCGCATGAACAGGCAA AAGAGGCAT-3′
3′-bnKCP1f	5′-GCGCGGCCGCCTCCTCCTCATCA TTGTCTTC-3′
5′-bnKCP1g	5′-GATGTTCTTGCGAGGAGACCAGGATTCAAGAACAGAGCATTGAAG-3′
3′-bnKCP1g	5′-CTTCAATGCTCTGTTCTTGAATCCTGGTCTCCTCGCAAGAACATC-3′
5′-bnKCP1h	5′-GCGTCGACGCTAGGGTTGGCTTC ATTG AGA-3′
3′-bnKCP1h	5′-GCGCGGCCGCCTCATCTTCTTCTT CTTCCTC-3′
5′-bnKCP1i	5′-GCGAATTCATGGCAGGAGGAGG ACCAACT-3′
3′-bnKCP1i	5′-CGGAGCTCCTCaTCTTCTTCTTCT TCTTC-3′

DNA was digested with *Eco*RI, *Xba*I, *Hin*dIII, *Pst*I, *Eco*RV and *Kpn*I restriction endonucleases, separated on 0.8% agarose gels, transferred to Hybond-XL membranes (Amersham Phamacia, Baie d'Urfe, Canada) and hybridized with the *bnKCP1* open-reading frame (ORF) labeled with [α-^{32}P]dCTP using the random primer labeling procedure. The DNA fragment used as a probe was isolated from a 0.8% agarose gel and purified with a QIAquick Gel Extraction Kit (Qiagen, Alameda, CA), and the probe was purified with a ProbeQuant G-50 Micro Column (Amersham Phamacia). Hybridization was performed under high stringency conditions as described previously (Gao *et al.*, 2002; Sambrook *et al.*, 1989).

Expression and purification of recombinant HDA19

HDA19 was expressed in *E. coli* as a fusion with glutathione-*S*-transferase (GST) using the pGEX-6P-2 vector (Amersham Pharmacia, Baie d'Urfe, Canada). The coding region of the *Arabidopsis HDA*19 (Accession no. AF195547) was amplified by PCR using 5′-HDA1b and 3′-HDA1b primers (Table 1), sequenced and inserted in-frame with GST into the *Sal*I and *Not*I sites of vector pGEX-6P-2 (Amersham Pharmacia).

The recombinant pGEX-6P-2 plasmid expressing HDA19 was used to transform *E. coli* BL21-CodonPlus (DE3)-RP competent cells (Stratagene, La Jolla, CA). Expression and purification under non-denaturing conditions was carried out as described by Gao *et al.* (2002). The GST–HDA19 fusion protein was analyzed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting with rabbit anti-GST-Pi polyclonal anti-serum (Chemicon, Mississauga, Canada) using the ECL Western blotting analysis system (Amersham Pharmacia).

In vitro *protein interaction assays*

The two fragments, *bnKCP1*$^{1–160}$ and *bnKCP1*$^{1–80}$, and the entire coding region of *bnKCP1* DNA encoding amino acids 1–160, 1–80, and 1–215, respectively, were amplified by PCR and cloned into the *Hin*dIII and *Xho*I sites of the expression vector pET-28b (Novagen, Madison, WI) in-frame with the His-Tag sequence to generate pET-bnKCP1$^{1–160}$, pET-bnKCP1$^{1–80}$, and pET-bnKCP1, respectively. PCR amplification was carried out using the forward primer 5′-bnKCP1a and either of the reverse primers: 3′-bnKCP1a for *bnKCP1*, 3′-bnKCP1b for *bnKCP1*$^{1–160}$, and 3′-bnKCP1c for *bnKCP1*$^{1–80}$ (Table 1). The full-length bnKCP1 protein and the truncated mutants ΔbnKCP1$^{1–160}$ and ΔbnKCP1$^{1–80}$ labeled with [^{35}S]methionine were produced using TNT–Quick Coupled Transcription/Translation System (Promega, Madison, WI) according to the manufacture's instructions, with some modifications. A total of 1 μl RNase inhibitor (GibcoL BRL) and 1 μl protease inhibitors cocktail (Roche, Germany) were added to the lysate reaction. After incubation for 90 min at 30°C, RNase A was added to the reaction mixture to a final concentration of 0.2 mg ml^{-1} and incubated for 5 min at the same temperature.

In vitro protein interaction was detected with GST pulldown affinity assays as described by Ahmad *et al.* (1999) with some modifications. Briefly, 6 μg GST or 4 μg GST fusion protein was incubated with 5 μl [^{35}S]Met-labeled translation mixture in 200 μl bead-binding buffer (50 mM K-phosphate (pH 7.6), 450 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1% BSA, and 1 μl diluted (1 : 12) protease inhibitors set) for 1 h at room temperature. After incubation, 20 μl 50% slurry of glutathione-Sepharose beads containing 10 mg ml^{-1} BSA and 4 μg EtBr was mixed with the reaction mixture followed by gentle rotation for 1 h at 4°C. After washing six times with 1.2 ml bead-binding buffer without BSA and EtBr but containing 12 μl protease inhibitors set (Roche, Germany), the bound proteins were eluted with 30 μl 2× SDS loading buffer, boiled for 2 min and analyzed by 12% SDS–PAGE. After electrophoresis, the gels were dried, treated with Amplify (Amersham Pharmacia) and subjected to fluorography.

In vivo *protein interaction assays*

The entire coding region of *bnKCP1* and the two fragments, *bnKCP1*$^{1–160}$ and *bnKCP1*$^{1–80}$, were PCR amplified and cloned into the *Sal*I and *Not*I sites of pPC86 vector (GibcoL BRL) in-frame with the *GAL*4 AD sequences to generate constructs pPC86–bnKCP1, pPC86–bnKCP1$^{1–160}$, and pPC86–bnKCP1$^{1–80}$. PCR amplification was carried out using the forward primer 5′-bnKCP1d and either

of the reverse primers: 3′-bnKCP1d for *bnKCP1*, 3′-bnKCP1e for *bnKCP1*$^{1–160}$, and 3′-bnKCP1f for *bnKCP1*$^{1–80}$ (Table 1). For *in vivo* protein interaction assays, the MaV203 yeast cells carrying the reporter gene *lac*Z and the construct pDBLeu–HDA19, in which the HDA19 was fused in-frame with GAL4 DB, were transfected with either of the plasmids pPC86–bnKCP1, pPC86–bnKCP1$^{1–160}$, and pPC86–bnKCP1$^{1–80}$ or the vector pPC86 alone. The expression of *lac*Z reporter gene was quantified by measuring the β-galactosidase activity using chlorophenol red-β-D-galactopyranoside (CPRG) according to the manufacturer's instructions (GibcoL BRL). Two yeast control strains A and B (GibcoL BRL) were used as negative and positive controls, respectively.

Site-directed mutagenesis (SDM)

The QuickChange site-directed mutagenesis kit (Stratagene) was used to replace the serine residue in the PK-A phosphorylation site (RRPS^{188}) within the KID domain with a glycine residue to generate bnKCP1G^{188} according to the manufacture's instructions. The two oligonucleotide primers used in SDM were 5′-bnKCP1g and 3′-bnKCP1g (Table 1). The introduced mutation was confirmed by DNA sequencing, and the mutated *bnKCP1G*^{188} was cloned into the *Hin*dIII and *Xho*I sites of pET-28b vector to generate pET-bnKCP1G^{188}, which was then used for *in vitro* protein interaction assays as described above.

Northern blot analysis

Total RNA was isolated from the tissues of *B. napus* L. cv. DES010 as described by Gao *et al.* (2001). These included leaves and stems of four-leaf-stage seedlings, flowers, and immature siliques of adult plants, and roots of cultured seedlings. Probe labelling, hybridization, washing, and membrane stripping were performed as described above for the Southern blot analysis.

Transactivation assay

MaV203 yeast cells expressing the *lac*Z reporter gene driven by a promoter containing GAL4 DNA-binding sites (GibcoL BRL) were transformed with the effector plasmids pDBLeu–bnKCP1$^{1–160}$, pDBLeu–bnKCP1$^{1–80}$, pDBLeu–bnKCP1$^{81–215}$, and pDBLeu–bnKCP1. These vectors were constructed by ligating the PCR-amplified fragments, Δ*bnKCP1*$^{1–160}$, Δ*bnKCP1*$^{1–80}$, and Δ*bnKCP1*$^{81–215}$ and the coding region of *bnKCP1*, respectively, into the *Sal*I and *Not*I sites of the vector pDBLeu (GibcoL BRL) in-frame with the GAL4 DB sequence. The oligonucleotide primers used for the amplification were as follows: for *bnKCP1*$^{1–160}$, 5′-bnKCP1d and 3′-bnKCP1e; for *bnKCP1*$^{1–80}$, 5′-bnKCP1d and 3′-bnKCP1f; for *bnKCP1*$^{81–215}$, 5′-bnKCP1h and 3′-bnKCP1h;and for *bnKCP1*, 5′-bnKCP1d 3′-bnKCP1d (Table 1). The β-galactosidase activity was measured using CPRG according to the manufacturer's instructions (GibcoL BRL). The two yeast strains A and B (GibcoL BRL) were used as negative and positive controls, respectively.

Transient expression of the GUS–bnKCP1 fusion protein

The entire coding region of *bnKCP1* was amplified using primers 5′- and 3′-bnKCP1i (Table 1) and cloned into the *Eco*RI and *Sac*I sites of the binary vector p79–637, a derivative of the vector CB301 (Hegedus, personal communication), to generate construct p77–132, which contains GUS–bnKCP1 fusion under the control of the CaMV35S promoter. The onion epidermal layers were transformed with *Agrobacterium* culture prepared as described by Kapila *et al.* (1997) with a few modifications. Briefly, the onion inner epidermal layers were peeled, placed into a culture of *Agrobacterium tumefaciens* strain GV3101 pMP90 containing either p79–637, for GUS expression only, or p77–132 and subjected to continuous vacuum of −85 kPa for 20 min. After incubation at 22°C under 16-h light condition for 7 days the tissues were placed into GUS staining solution [100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)], vacuum infiltrated for 20 min at −85 kPa and incubated overnight at 37°C. To determine the intercellular location of nuclei, tissues were stained with the nucleus-specific 4′,6-diamidino-2-phenylindole (DAPI) solution (14 μg ml^{-1} DAPI, 0.1× PBS, 90% glycerol) (Varagona *et al.*, 1991) and viewed under a Zeiss microscope using both fluorescence and bright-field optics.

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GenBank accession number for bnKCP1 is AY211985.