

Characterization of a novel *Brassica napus* kinase, *BNK1*

Nancy F. Silva¹, Loraine N. Christie, Maria Mazzurco, Daphne R. Goring*

Biology Department, York University, Toronto, Ont., Canada M3J 1P3

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Abstract

A novel plant protein kinase, designated *Brassica napus* kinase 1 (*BNK1*), was isolated from a λ -pistil cDNA library. The deduced *BNK1* protein contains all eleven conserved subdomains of a kinase and encodes a functional serine/threonine protein kinase. Phylogenetic analysis of several plant protein kinase subfamilies showed that *BNK1* is most closely related to the NAK subfamily of protein kinases. Genomic Southern blot analysis revealed that *BNK1* is a single copy gene in the *B. napus* genome and does not appear to be a member of a multigene family. Expression studies revealed that the *BNK1* transcript was ubiquitously expressed throughout the plant, with highest levels in stem and pistil tissues. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Brassica napus*; Signal transduction; Serine/threonine protein kinase; NAK subfamily

1. Introduction

Organisms are subjected to a variety of external stimuli and endogenous signals that must be recognized and translated into cellular responses. Protein kinases constitute one of the major classes of signal transducers involved in mediating a cell's response to external stimuli. These important regulatory enzymes can be comprised of several integral components in a signaling cascade, from the initial receptor protein to the final effector protein, and form the basis for controlling cellular events in response to environmental, metabolic and/or developmental signals [1,2]. More specifically in plants, protein kinases have been implicated in mediating responses to a variety of signals including light, hormones, pathogen invasion, temperature stress and nutrient deprivation [1]. Given that they are involved in many aspects of cellular regulation, it is no surprise that an estimated 1–3% of

functional eukaryotic genes are predicted to encode protein kinases [1].

Typically, 'conventional' members of the eukaryotic protein kinase superfamily have been subdivided into those that phosphorylate serine/threonine and those that phosphorylate tyrosine residues [2]. The 'non-conventional' protein kinases encompass histidine kinases, which function in two-component sensory regulatory systems and do not share any protein sequence homology to 'conventional' protein kinases [3]. The catalytic domain of 'conventional' protein kinases consists of 250–300 amino acids with alternating regions of high and low conservation. This domain is further divided into eleven conserved subdomains, some of which contain invariant or nearly invariant residues [4].

Phylogenetic analysis derived from an alignment of protein kinase catalytic domains serves as the basis for classification of eukaryotic protein kinases into four groups, but has been modified slightly to include other plant protein kinases [4,5]. First of all, the 'AGC' group consists of the cyclic nucleotide dependent family (PKA and PKG), the PKC subfamily, the PVPK, and the S6 kinase

* Corresponding author. Tel.: +1-416-7362100, ext. 40512; fax: +1-416-7365698.

E-mail address: dgoring@yorku.ca (D.R. Goring).

¹ This author and L.N. Christie contributed equally to this work.

subfamily [6,7]. Secondly, the ‘CaMK’ group includes the calcium dependent protein kinases (CDPKs) and the SNF1 kinase subfamilies [8–10]. Thirdly, the ‘CMGC’ group contains the cyclin-dependent kinase subfamily (CDKs), the MAP kinases, GSK3/Shaggy, and the casein kinase II (CKII) subfamilies [11–20]. Lastly, the ‘other’ group consists of the receptor-like protein kinases (RLKs), Lammer CTR1/Raf-like, and NAK subfamilies of protein kinases [5,11,21–23].

Many protein kinases present in plants have homologues in other eukaryotic organisms (CKIIs, MAPKs and CDKs), while some such as the RLKs, CDPKs and NAKs remain unique to higher plants. In turn, some kinases such as protein tyrosine kinases (PTKs) have not been identified in plants. The existence of homologous animal and plant protein kinases suggests that their signal transduction mechanisms share common themes and may reflect fundamental functions required for all eukaryotic cells [2]. Further identification and characterization of protein kinases that are unique to plants will provide important insight into signaling pathways and mechanisms that are specific for controlling plant growth and development. In this study, we report the cloning and characterization of a novel kinase from *Brassica napus* designated *B. napus* kinase 1 (*BNK1*). Both sequence and phylogenetic analyses indicate that *BNK1* is most closely related to the NAK subfamily of plant protein kinases.

2. Materials and methods

2.1. Construction of λ -pistil cDNA library

Pistils were collected from floral buds of *B. napus* Westar and W1 cultivars 1–2 days before anthesis. Total RNA was isolated as described by Jones et al. [24] and enriched for poly(A)⁺ RNA using pre-packed oligo (dT)₂₅-cellulose beads (New England Biolabs). Five micrograms of pistil poly(A)⁺ RNA was used for the construction of a cDNA library using the ZAP-cDNA[®] synthesis kit as described by the manufacturer’s procedures (Stratagene). Infection of *Escherichia coli* host strain XLI-Blue yielded a primary library with an average titer of 1.0×10^6 plaque forming units. The primary library was subsequently amplified to obtain an average total of 6.6×10^{10} plaque form-

ing units. In vivo mass excision of the pBluescript phagemids from the library was carried out as outlined by the manufacturer (Stratagene).

2.2. Generation of putative novel protein kinase clones

To isolate novel *B. napus* protein kinases, two oligonucleotide combinations were used in a polymerase chain reaction (PCR) to amplify conserved regions from the protein kinase catalytic domains: RK1 (5′ GGiGGTTTCGGiAT^T_{c/A}GTiTT^A_T/_cAA^A_GGG 3′) and RK2 (5′ AAiATiC^T_GiGCCATiCC^A_GAA^A_G/C 3′), or RK1 and RK3 (5′ AAiATiC^T_GiGCCATiCC^A_GAA^A_G/C 3′). The RK1 primer was constructed based upon a conserved amino acid consensus (GGFGIV^F_YKG) within subdomain I of the catalytic domain. The degeneracy of the RK2 primer reflects a conserved amino acid consensus (DFGMARIF) of subdomain VII which closely resembles the *S* receptor kinase in *Brassica*. The RK3 primer was generated based upon conserved amino acids (DFGLAKLL) within subdomain VII prevalent among the RLKs isolated in *Arabidopsis thaliana*. Standard PCR reactions on mass excised pBluescript phagemid DNA were set up using Tsg polymerase (Biobasics). Amplifications were carried out for 35 cycles of 1 min at 95°C, 1 min 30 s at 50°C, and 1 min at 72°C. PCR products of the expected size (~450 bp) were gel purified, cloned into the pT7Blue plasmid (Novagen) and transformed into *E. coli* DH5 α cells [25]. Positive clones were sequenced with an ABI automated sequencer (Core Facility, York University) using the dideoxychain-terminating method described by Sanger et al. [26]. Sequence analyses were performed using the DNasis[®] software (Hitachi), and PCR products showing less than 85 and 70% sequence identity at the nucleotide and amino acid levels, respectively, to protein kinases in the database were further pursued, one of these being *BNK1*.

2.3. Screening of λ -pistil cDNA library

The original 384 bp *BNK1* PCR product was used to screen the λ -pistil cDNA library. Approximately 2×10^6 plaques from the amplified library were screened and plated at a density of 1×10^5 pfu/plate. Duplicate colony lifts were performed and prehybridized for 2 h at 42°C in 50% formamide, 5 × Denhardt’s solution, 5 × SSC, 0.1%

SDS, 1 mM EDTA and 100 µg/ml salmon sperm DNA. Filters were subsequently hybridized overnight in the same solution containing the *BNK1* PCR product labeled with [α - 32 P]dATP by random priming [27]. The filters were then washed twice with $2 \times$ SSC, 0.1% SDS at room temperature for 15 min, followed by two 25 min washes with $0.5 \times$ SSC, 0.1% SDS at 55°C. Plaques containing putative positive clones were cored and subjected to several rounds of screening until single isolates representing the *BNK1* clone were obtained. Single clone excisions to liberate the pBluescript phagemids was performed on each isolate according to the procedure recommended by the manufacturer (Stratagene). Phagemid DNA digested with *EcoRI/XhoI* to release the cloned cDNA was subjected to standard plasmid Southern blot analysis and probed with the [α - 32 P]dATP-labeled *BNK1* PCR product. The membrane was prehybridized at 42°C in $5 \times$ SSPE, $10 \times$ Denhardt's solution and 0.5% SDS for 2 h and hybridized overnight at the same temperature in a buffer containing 50% formamide, $5 \times$ SSPE and 0.5% SDS. Washes were performed twice at room temperature for 15 min in $2 \times$ SSC, 0.1% SDS followed by 30 min washes at 60°C in $0.1 \times$ SSC, 0.1% SDS. Several positive clones were detected by autoradiography and were sequenced using both universal and sequence specific primers to generate the 1400 bp consensus sequence representing the partial *BNK1* cDNA isolated from the λ -pistil cDNA library.

2.4. Rapid amplification of cDNA ends (5' RACE)

The 5' end of the *BNK1* cDNA was obtained by the procedure for the rapid amplification of cDNA ends, originally described by Frohman et al. [28] using the 5' RACE System, version 2.0 kit (Gibco-BRL). First strand cDNA was synthesized from ~300 µg of mixed Westar and W1 pistil total RNA using a gene specific primer GSP1 (5'-AGCACAGTAACCAAT-3') designed to anneal ~390 bp from the 5' end of the partial 1400 bp *BNK1* cDNA. A homopolymeric tail was added to the 5' end of the cDNA using TdT (terminal deoxynucleotidyl transferase) and dCTP. This tailed cDNA was then amplified using a second gene specific primer GSP2 (5'-AACTCTACCGAATCCTCCT-3') designed to anneal 3' to the

GSP1 primer, and an abridged anchor primer (Gibco-BRL) which annealed to the homopolymeric tail. Amplification was carried using the following cycling protocol: 1 min initial denaturation at 94°C, followed by 35 cycles of 1 min/94°C, 30 s/63°C, and 2 min/72°C. A PCR product of the expected size corresponding to the 5' end of *BNK1* was gel purified, cloned into the pT7Blue plasmid (Novagen) and transformed into *E. coli* DH5 α . Confirmation of the 5' RACE product was obtained by plasmid Southern blot analysis as described above. A full length *BNK1* cDNA was generated by digesting both the library and race clones with *EcoRI* and *Bg/II*. The full-length *BNK1* cDNA sequence was confirmed by sequencing using gene specific primers. All DNA and protein sequence analyses were performed using the DNasis[®] Software (Hitachi).

2.5. Genomic DNA isolation and Southern blot analysis

Genomic DNA was extracted from one gram of young *B. napus* leaf tissue [29]. Five micrograms of genomic DNA was digested with several restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Xba*I, *Xho*I), fractionated through a 0.8% agarose gel and transferred overnight in $10 \times$ SSC onto Zetaprobe membrane (BioRad). This was performed in duplicate to test hybridization conditions under low and high stringency conditions. After drying, the membranes were prewashed in $0.1 \times$ SSC, 0.5% SDS for 25 min at 60°C. The membranes were then prehybridized and hybridized as described previously for plasmid Southern blots with the inclusion of 10% dextran sulfate and 50 µg/ml salmon sperm DNA in the hybridization buffer. Washing conditions for genomic Southern blots varied depending on the stringency tested. One membrane was washed under conditions of low stringency for 15 min at room temperature in $2 \times$ SSC, 0.1% SDS followed by a second 15 min room temperature wash in $1 \times$ SSC, 0.1% SDS and three final washes at 50°C in $1 \times$ SSC, 0.1% SDS. The second membrane was washed under conditions of high stringency by lowering the salt concentration to $0.1 \times$ SSC, 0.1% SDS and increasing the temperature to 65°C. Membranes were probed with the [α - 32 P]dATP-labeled *BNK1* cDNA generated by random priming [26] and subjected to autoradiography (XAR-5 film, Kodak) overnight at -80°C .

2.6. Isolation and Northern blot analysis of multiple tissue RNA

B. napus Westar plants were soil grown in a growth chamber at 22°C with a 16 h light period followed by an 8 h dark period at 16°C. Tissue was harvested from floral buds 1–2 days before anthesis, and total RNA extraction from petal, anther and pistil tissues was performed as described previously [24]. Total RNA was isolated from root, leaf and stem tissues using a protocol optimized for high carbohydrate content tissues as described by Cock et al. [30]. Poly(A)⁺ RNA was then isolated using the polyA Spin™ mRNA isolation kit (New England Biolabs) as outlined by the manufacturer's procedure. Five micrograms of poly(A)⁺ RNA was fractionated on a 1.2% formaldehyde gel and transferred to Zetaprobe membrane (BioRad) in 10 × SSC. Hybridization and high stringency wash conditions were conducted as described previously for genomic Southern blot analysis. The membrane was subsequently probed with a cyclophilin EST clone (no. mBN086) as an internal control for even loading.

2.7. Synthesis and purification of *BNK1* wild-type and mutant recombinant proteins

A mutant *BNK1* cDNA was generated by mutating the invariant lysine (amino acid 99) of subdomain II, involved in phosphotransfer, to an alanine using the QuickChange™ site-directed mutagenesis kit (Stratagene). Both the wild-type and the mutant kinases were cloned inframe into the pGEX4T-1 expression plasmid (Amersham Pharmacia Biotech). TOP10 *E. coli* cells carrying pGEX4T1-*BNK1* were grown in a 50-ml culture with shaking at 37°C until an OD₆₀₀ of 0.6. Iso-propyl β-thiogalactoside was added to a final concentration of 0.1 mM to induce the production of the recombinant protein, and the culture was incubated overnight at 20°C. TOP10 cells were then harvested and the pellet was resuspended in 5 ml of lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 200 μM PMSF). After sonication, the insoluble proteins were removed by centrifugation at 12 000 g for 10 min and the solubilized protein extract was incubated with a 10% (v/v) glutathione agarose bead (Sigma) solution for 30 min at 4°C. After extensive washing to remove unbound proteins, the recom-

binant proteins were eluted from the resin using 100 μM elution buffer (50 mM Hepes, pH 7.4, 15 mM glutathione). Purified fusion proteins were subjected to SDS-PAGE and western blot analysis using an anti-GST antibody (MJS Biolynx Inc.) was then performed to confirm the identity of the wild-type and mutant recombinant *BNK1* proteins (data not shown).

2.8. Kinase assay and phosphoamino acid analysis

The purified *BNK1* wild-type and mutant recombinant proteins (1 μg) were mixed with 10 × kinase buffer (200 mM Pipes, pH 7.0, 100 mM MgCl₂, 20 mM MnCl₂, 100 μg/ml aprotinin) and 10 μCi of [γ-³²P]dATP in a 12 μl final volume reaction and incubated at room temperature for 30 min. The kinase reaction was stopped by the addition of 6 μl of 3 × SDS sample buffer (187.5 mM Tris-HCl, 6% (w/v) SDS, 0.03% (w/v) phenol red), boiled for 5 min, and electrophoresed through a 10% SDS-PAGE gel [31]. The SDS-PAGE gel was subsequently stained with Coomassie blue, dried down, and exposed for 10 min to X-ray film. The fusion protein on the Coomassie stained gel corresponding to the phosphorylated wild-type protein detected on the autoradiogram was excised from the gel and subjected to phosphoamino acid analysis [32] using a Hunter thin layer peptide mapping electrophoresis system 700 (C.B.S. Scientific Co., Inc.).

3. Results

3.1. Isolation and sequence analysis of *BNK1* cDNA

We were interested in isolating novel protein kinases expressed in *B. napus* pistil tissue. To this end, a λ-pistil cDNA library was used as a template for PCR amplification with a combination of degenerate oligonucleotide primers designed against the conserved kinase subdomains I and VII [4]. PCR products of the expected lengths were cloned, and sequence analysis led to the identification of a novel kinase named *B. napus* kinase 1 (*BNK1*-accession no. AY007545). The 384 bp *BNK1* PCR product was then used to screen the λ-pistil cDNA library. Several positive clones ob-

tained from the screen were sequenced to generate a partial 1400 bp consensus sequence, and 5' RACE was performed to isolate the remaining 5' end of the *BNK1* cDNA.

The full length *BNK1* cDNA is 1486 bp in length and consists of one open reading frame of 1132 bp. It is predicted to encode a protein of 377 amino acids with an estimated molecular weight of 42.5 kDa. The first methionine is flanked by sequences similar to the plant initiation consensus sequence [33]. Two potential N-linked glycosylation sites (Asn-X-Ser/Thr) corresponding to amino acid residues 23 and 27 were also identified [34]; however, *BNK1* is not predicted to be a secreted protein. The kinase domain of this protein is flanked by short non-kinase domains and does not contain any membrane-spanning region or extracellular domain. Therefore, it is likely that *BNK1* is a cytoplasmic kinase.

All protein kinases characterized to date display sequence similarity in their catalytic domains, which are comprised of eleven subdomains containing some invariant residues important for catalysis [4]. The overall features of this organization are present in the catalytic domain (residues 68–344) of the *BNK1* protein since all the absolutely conserved amino acids as well as the highly conserved amino acid groups were identified. Furthermore, amino acid sequence analysis of subdomains VI (DLKCSN) and VIII (GTYGYCAPE) indicates that *BNK1* has a consensus sequence prevalent among serine/threonine kinases [4]. The predicted *BNK1* amino acid sequence is most closely related to the NAK subfamily of non-receptor type protein kinases (Fig. 1). The sequence identity for the most part is confined to the catalytic domains, and the region between amino acids 58–344 of *BNK1* shares between 50 and 54% amino acid sequence identity to the *Arabidopsis* NAK [11], APK1a [22], APK2a [23], and ARSK1 [35] amino acid sequences (Fig. 1). There is also a predicted gene in the *Arabidopsis* genome (CAB86034) which appears to be orthologous to *BNK1* showing 91% amino acid sequence identity to *BNK1* (Fig. 1).

In order to confirm the relatedness between *BNK1* and members of the NAK subfamily of protein kinases, a phylogenetic tree of several *Arabidopsis* protein kinases belonging to selected subfamilies was constructed using the amino acid sequences from the corresponding catalytic do-

main (Fig. 2). The phylogenetic tree shows that the *BNK1* amino acid sequence clusters with the NAK subfamily. This clustering may also reflect related functions and clearly distinguishes this subfamily from other unrelated groups of protein kinases.

3.2. Genomic Southern blot analysis

The copy number of the *BNK1* gene in the *B. napus* genome was estimated by genomic Southern blot analysis. Genomic DNA was digested with *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, *Xba*I and *Xho*I, probed with the full length *BNK1* cDNA, and washed under conditions of varying stringency. Fig. 3 shows the hybridization pattern obtained for *BNK1* under high stringency conditions which is identical to that obtained for conditions of low stringency (data not shown). In the case of *Bam*HI digested DNA, two fragments hybridized to the *BNK1* probe which is expected given that an internal *Bam*HI site is present in the *BNK1* cDNA sequence. In addition, since no internal sites within the *BNK1* cDNA are present for the other restriction enzymes, only one fragment is expected to hybridize to the *BNK1* probe. This evidence suggests that *BNK1* appears to exist as a single copy gene in the *B. napus* genome and there are no other closely related genes.

3.3. Expression pattern of *BNK1*

Northern blot analysis was performed using poly(A)⁺ RNA isolated from a variety of *B. napus* tissues as shown in Fig. 4. Following hybridization with the full length *BNK1* cDNA, a single transcript of ~1.5 kb in size was detected and found to be expressed ubiquitously in all tissues tested. However, there were differences in the levels of the *BNK1* transcript, with highest levels detected in stem and pistil tissues and significantly lower levels detected in root, leaf, petal and anther tissues (Fig. 4, upper panel). The poly(A)⁺ blot was reprobed with a cyclophilin cDNA as an internal control for loading. A 700 bp cyclophilin transcript was detected in all lanes, though the root sample lane was underloaded (Fig. 4, lower panel). The difference in the intensity of the cyclophilin signal in the anther sample is thought to be attributed to the lower abundance of cyclophilin mRNA present in anther tissue as reported by Gasser et al. [36].

3.4. Kinase activity and phosphoamino acid analysis of the *BNK1* protein

In order to ascertain whether the *BNK1* protein encodes a functional kinase and to determine the specificity of this kinase activity, the full length *BNK1* cDNA was expressed in *E. coli* and the kinase activity of the recombinant protein was examined (Fig. 5A). To ensure that the phosphorylation of the fusion protein was not a direct result of bacterial kinase contamination, a mutant

BNK1 kinase was constructed in which the invariant lysine of subdomain II, involved in phosphotransfer, was mutated to an alanine. Substitution mutations involving this amino acid should render the kinase inactive [4]. In Fig. 5A, an autoradiogram of the protein gel shows that only the wild-type *BNK1* fusion protein demonstrates autophosphorylation activity in the presence of [γ - 32 P]dATP (lane 1) when compared with the mutant kinase which did not show any detectable kinase activity (lane 2). Therefore, this evidence

BNK1		MGWIPCS	GKSSGKTKKR	SDSDENLSR	NCSVSASE	RSKA	KSSV	42
CAB86034		-----	-----RN-T-	RNG-HK-D-K	SSD----T--	K-R-	---L	45
NAK			MGGCFSNRK	T-IASSTWLS	SKFL-RDG-K	G-STASF-YM		40
APK1A			MGICLSAQVK	AE-SGASTKY	DAKDIG-LGS	KASSVSVRPS		40
APK2A	MGNCLDSS	AKVDNSNHSP	HANSASSGSK	VS-KTSR-TG	PSGL-TTSYS	TDSSFGLPPT		58
ARSK1	MAVFKKKKTS	LTSLFLGICYK	AKNAS-YEGG	EKAVMKIRTC	PAFKRL-L-D	I-DPSSPM--		60
BNK1	SESRSRGSND	IVAQTFTFSE	LATATRNFRK	ECLIGEGGFG	RVYKGYLAST	GQTA		96
CAB86034	---K-K---H	-----	-----	-----	-----AS-	S---		99
NAK	PRTEGEILQN	ANLKN-SL--	-KS-----P	DSVV-----	C-F--WIDES	SLAPSKPGTG		100
APK1A	PRTEGEILQS	PNLKS-S-A-	-KS-----P	DSVL-----	C-F--WIDEK	SL--SRPGTG		100
APK2A	LRTEGEILSS	PNLKA---N-	-KN--K---Q	DN-L-----	C-F--WIDQ-	SL--SRPGSG		118
ARSK1	MDDL-HSFTS	QKLRL--L--	-RVI-H--SR	SNML-----	P----FIDDK	VKPGIEAQP		119
BNK1	AIKQLDH	NGLQGNREFL	VEVLMLSLLH	HPNLVNLIGY	CADGDQRLLV	YEYMPGLSLE		153
CAB86034	A-----	-----	-----	-----	-----	-----		156
NAK	IVI-V-R-NQ	E-F--H--W-	A-INY-GQ-D	-----K----	-LEEEH----	--F-TR----		160
APK1A	LVI-V-K-NQ	D-W--HQ-W-	A--NY-GQFS	-RH--K----	-LEDEH----	--F--R----		160
APK2A	IVV-V---KP	E-F--HK-W-	T--NY-GQ-S	-----L-V--	--EGEN----	--F--K----		178
ARSK1	V-V-A--L	H-H--IENGW	RRYFSGQ-S	NKH--K---F	-CEEE--V--	-----R----		177
BNK1	DHLHDISPSK	QPLDWNTRMK	IAAGAAKGLE	YLHDKTMPPV	IYRDLKCSNI	LLGDDYFPKL		213
CAB86034	-----G-	-----	-----	-----	-----	--D-----		216
NAK	N--FRRGTFY	---S---VR	M-L---R--A	F--N AQ-Q-	----F-A--	--DSN-NA--		219
APK1A	N--FRRLYF	---S-KL-L-	V-L-----A	F--S SETR-	----F-T--	--DSE-NA--		219
APK2A	N--FRRGA	---T-AI---	V-V-----T	F--E AKSQ-	----F-AA--	--DA-FNA--		235
ARSK1	NQ-FRRNSL	AMAYVGI---	--L-----A	F--E AEK--	----F-T--	--DS--NA--		235
BNK1	SDFGLAKLGP	VGDKSHVSTR	VMGTYGYCAP	EYAMTGQLTL	KSDVYSFGVV	LLEIITGRKA		273
CAB86034	-----	-----	-----	-----	-----	-----		276
NAK	-----RD--	M--N-----	---Q--A--	--LA--H-SV	-----	---LLS--R-		279
APK1A	-----D--	I-----	---H--A--	--LA--H--T	-----	---LLS--R-		279
APK2A	-----A--	T--NT---K-	-I--H--A-	--VA--R--A	-----	---L-S--R-		295
ARSK1	-----D--	E-EHT--T--	---Q--R--	--I---H--T	MN-----	---L--KRS		295
BNK1	IDNSRCTGEQ	NLVAWARPLF	KDRRFQSOMA	DPMIQQQYPP	RGLYQALAVA	AMCVQEQPNL		333
CAB86034	--S--S---	-----	-----	---L-----	-----	-----		336
NAK	--KNQPVV-H	---D-----YL	TNK-RLLRVM	--RL----SL	TRALKIAVL-	LD-ISIDAKS		339
APK1A	V-KN-PS--R	---E--K-YL	VNK--IFRVI	-NRL-D--SM	EEACKVATLS	LR-LTTEIK-		339
APK2A	M---NGGN-Y	S--D--T-YL	G-K--LFRIM	-TKLG---Q	K-AFT-ANL-	LQ-LNPDAK-		355
ARSK1	M--T-TRR--	S--E---ML	R-Q--LERII	-PRLAN-HKT	EAAQV-ASL-	YK-LSQH-KY		355
BNK1	RPVIADVVT	LTYLASQRFD	PMSQPVQGS	FGPGTPPRSK	RVV			376
CAB86034	--L-----	-S-----	-LA-----	-A-----	-ERWMIFRLS	VNVWLKSYL		395
NAK	--TMNEI-KT	MEE-HI--EA	SKE-QNPQIS	IDNIINKSPQ	A-NYPRPSIM			389
APK1A	--NMSE--SH	-EHIQ-LNAA	IGGNMDKTD	RMRRRSDSVV	SKKVNAGFAR	QTAVGSTVVA		399
APK2A	--KMSE-LVT	-EQLE-VAKP	GKHTQME-P	RFHSSVMQ-	SP-RYSHDRP	LLHMTPGASP		415
ARSK1	--TMCE--KV	-ESIQEVDIR	KHDGNNKEG	KKFVDINKFR	HHRGKRRVN	IAYSDSLVIK		415
APK1A	YPRPSASPLY	V						410
APK2A	LPSYTQSPRV	R						426
ARSK1	ESKAKQNDGI							425

Fig. 1. Amino acid sequence comparisons of *BNK1* to members of the NAK subfamily of protein kinases. The predicted *BNK1* amino acid sequence was compared with the predicted amino acid sequences from four *Arabidopsis* protein kinases, *NAK* [11], *APK1a* [22], *APK2a* [23], and *ARSK1* [35], and as well as a predicted gene from the *Arabidopsis* genome sequence (CAB86034). Identical amino acids are represented by hyphens and spaces indicate gaps introduced to maximize alignment. The arrows indicate the start and end of the catalytic domain.

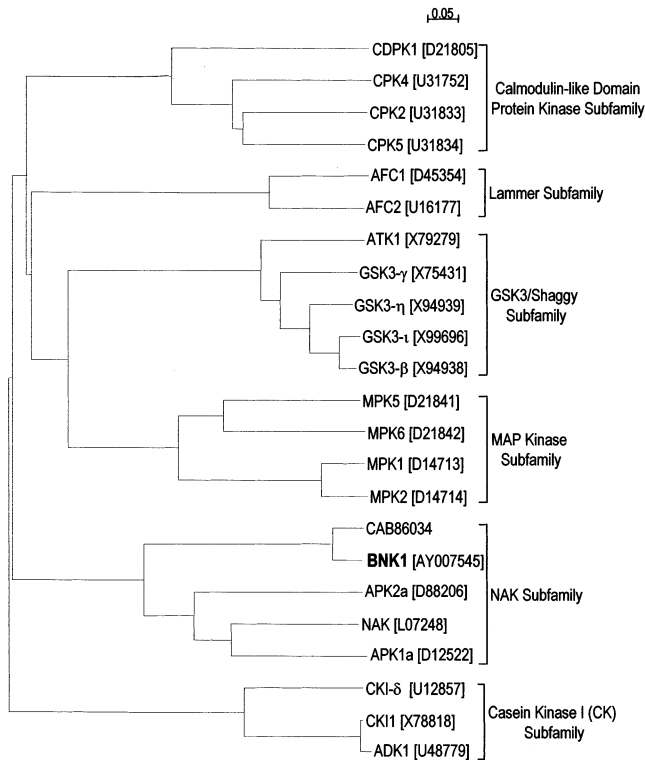


Fig. 2. Phylogenetic tree showing the relationship between BNK1 and *Arabidopsis* protein kinases from a variety of subfamilies. The amino acid sequences encoding the catalytic domains of these protein kinases were aligned using CLUSTAL W [37] and used to construct a phylogenetic tree. The Genbank accession numbers for these protein kinases are indicated in brackets.

strongly suggests that the BNK1 protein encodes a functional kinase as its sequence predicts and that mutation of the invariant lysine to an alanine successfully abolished kinase activity.

In order to determine the amino acid specificity of this autophosphorylating activity, the phosphorylated wild-type BNK1 fusion protein was extracted from the gel and subjected to phosphoamino acid analysis. As expected, only phosphorylated serine and threonine residues were detected (Fig. 5B), therefore indicating that BNK1 encodes a functional serine/threonine kinase.

4. Discussion

In this study, we report the isolation of the BNK1 cDNA encoding a novel protein kinase in *B. napus*. Genomic Southern blot analysis under low and high stringency conditions revealed that BNK1 is present as a single copy gene in the

Brassica genome and does not appear to be a member of a closely related multigene family. The deduced amino acid sequence of BNK1 shows all the characteristics of a protein kinase, in that the catalytic domain of this protein possesses all of the conserved residues necessary for kinase activity. BNK1 does encode a functional kinase capable of autophosphorylation, and phosphoamino acid analysis performed on the phosphorylated wild-type protein demonstrated that BNK1 is a serine/threonine kinase.

Database searches showed that BNK1 is most similar to the NAK subfamily of protein kinases, which include APK2a, APK1a, NAK and ARSK1. Phylogenetic analysis performed using only the kinase domain region of several *Arabidopsis* protein kinases confirmed this relationship, since BNK1 was clearly shown to cluster with this group of protein kinases. All the NAK family members including BNK1 are predicted to encode small kinases, and there appears to be a number of serines in the region N-terminal to the

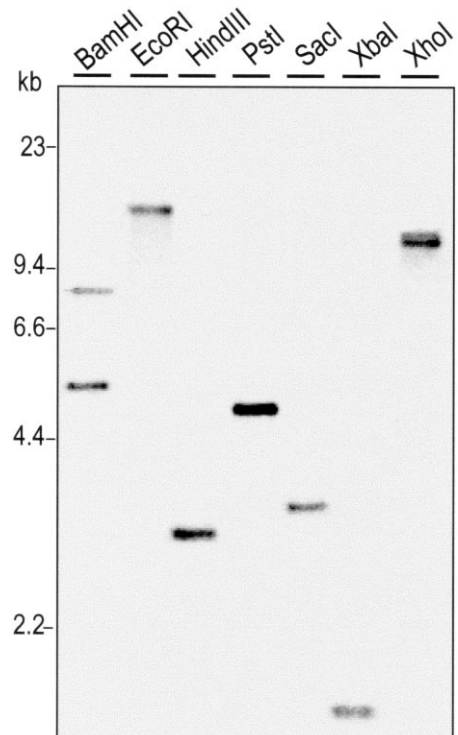


Fig. 3. Genomic DNA Southern blot analysis of BNK1. Genomic DNA isolated from young *B. napus* leaf tissue was digested with *Bam*HI, *Eco*RI, *Hind*III, *Pst*I, *Sac*I, *Xba*I and *Xho*I. Digested DNA was hybridized with the partial 1400 bp BNK1 cDNA under conditions of high stringency. The sizes (in kilobases) of the λ /*Hind* III molecular weight markers are given on the left.

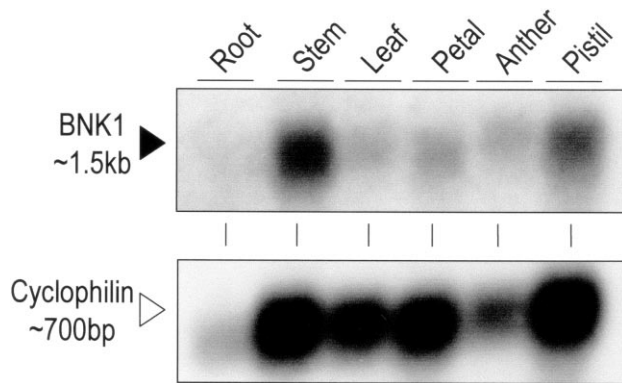


Fig. 4. RNA blot analysis of *BNK1* mRNA in different tissues of *B. napus*. Five micrograms of poly(A)⁺ RNA prepared from root, stem, leaf, petal, anther and pistil tissues was electrophoresed, blotted and hybridized with the full length *BNK1* cDNA (solid arrow). A cyclophilin probe was used as a control for loading (open arrow); however, cyclophilin appears to be expressed at lower levels in anther tissue. The root lane is underloaded, but *BNK1* mRNA can be detected after a longer exposure.

catalytic kinase domain (Fig. 1). However, outside of the catalytic domain, there is very little sequence identity between *BNK1* and the other NAK family members, with the exception of a predicted *Arabidopsis* gene (*CAB86034*) which shows a high degree of sequence identity to *BNK1* and likely represents a *BNK1* orthologue (Fig. 1).

The variety of roles protein kinases play in mediating a multitude of cellular events is reflected by the strikingly different patterns of expression exhibited by the members within the NAK subfamily of protein kinases. *APK2a* is differentially expressed with high levels of mRNA in leaf tissue, moderate levels in roots and at very low levels in floral bud tissue [23]; *ARSK1* exhibits tissue specific root expression [35]; and *BNK1* mRNA levels are highest in the stem and pistil tissues and detected at lower levels in the root, leaf, petal and anther tissues. Furthermore, although members of this subfamily share a significant degree of sequence similarity within the kinase domain, the divergence exhibited in the flanking non-kinase domain regions makes this subfamily of protein kinases interesting. Sequence comparisons and motif searches of the N- and C-terminal non-kinase domain regions did not yield any striking similarities, nevertheless these short domains would be expected to be involved in determining the specificity of action for a particular protein ki-

nase. Although the exact function of the NAK protein kinases remains unknown, it has been suggested that some of these genes may play a role in the regulation of plant growth and development. While much work has been done on protein kinases in yeast and animal systems, relatively little is known about the biological functions of many protein kinases in plants. Continued research is necessary to characterize novel plant protein kinases which may then provide important insights into fundamental signaling pathways in plants.

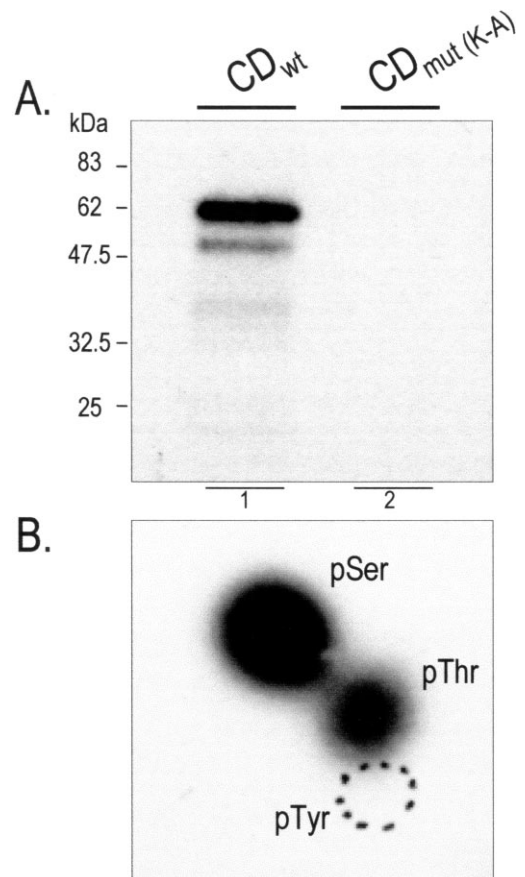


Fig. 5. *BNK1* kinase activity. (A) Analysis of kinase activity of recombinant *BNK1* purified from *E. coli*. The *BNK1* kinase was tested for kinase activity in the presence of [γ -³²P]dATP, separated on a 10% SDS-PAGE gel, and subjected to autoradiography. *CD*_{wt} = wild-type catalytic domain from *BNK1*; *CD*_{mut(K-A)} = kinase inactive form of *BNK1*. The sizes of protein markers in kDa are given on the left. (B) Phosphoamino acid analysis of autophosphorylated *BNK1*. [γ -³²P]dATP labeled *BNK1* was hydrolyzed with HCl and subjected to two-dimensional thin-layer electrophoresis. The position of phosphotyrosine (pTyr) is indicated by the punctate circle, however only radiolabeled phosphoserine (pSer) and phosphothreonine (pThr) residues were detected.

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