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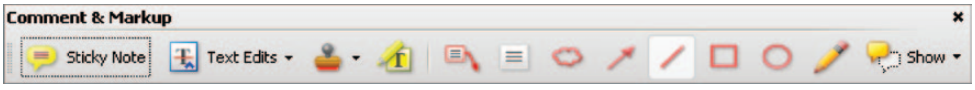

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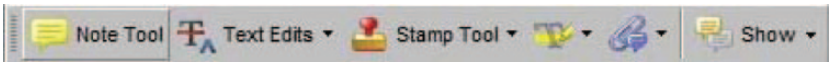

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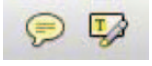

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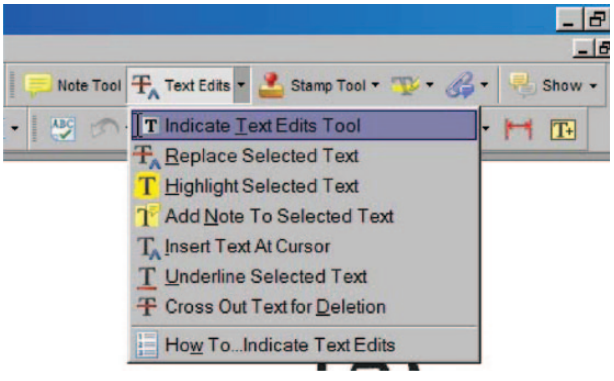

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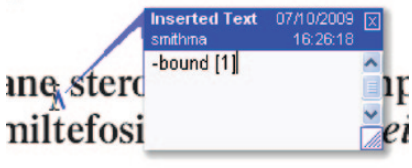

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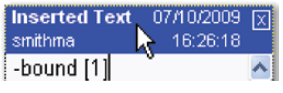

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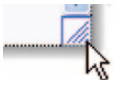

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RESEARCH PAPER

Identification of AHK2- and AHK3-like cytokinin receptors in *Brassica napus* reveals two subfamilies of AHK2 orthologues

Alena Kuderová[1,*], Lucia Gallová[2,*], Katarína Kuricová[1], Eliška Nejedlá[1], Anna Čurdová[1], Lenka Micenková[3], Ondřej Plíhal[2], David Šmajs[3], Lukáš Spíchal[2] and Jan Hejátko[1,†]

[1] Functional Genomics and Proteomics of Plants, Central European Institute of Technology (CEITEC), Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic
[2] Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic
[3] Faculty of Medicine, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: hejatko@sci.muni.cz

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Abstract

Cytokinin (CK) signalling is known to play key roles in the regulation of plant growth and development, crop yields, and tolerance to both abiotic stress and pathogen defences, but the mechanisms involved are poorly characterized in dicotyledonous crops. Here the identification and functional characterization of sensor histidine kinases homologous to *Arabidopsis* CK receptors AHK2 and AHK3 in winter oilseed rape are presented. Five CHASE-containing His kinases were identified in *Brassica napus* var. Tapidor (BnCHK1–BnCHK5) by heterologous hybridization of its genomic library with gene-specific probes from *Arabidopsis*. The identified bacterial artificial chromosome (BAC) clones were fingerprinted and representative clones in five distinct groups were sequenced. Using a bioinformatic approach and cDNA cloning, the precise gene and putative protein domain structures were determined. Based on phylogenetic analysis, four *AHK2* (*BnCHK1–BnCHK4*) homologues and one *AHK3* (*BnCHK5*) homologue were defined. It is further suggested that *BnCHK1* and *BnCHK3*, and *BnCHK5* are orthologues of *AHK2* and *AHK3*, originally from the *B. rapa* A genome, respectively. BnCHK1, BnCHK3, and BnCHK5 displayed high affinity for *trans*-zeatin (1–3 nM) in a live-cell competitive receptor assay, but not with other plant hormones (indole acetic acid, GA_3, and abscisic acid), confirming the prediction that they are genuine CK receptors. It is shown that BnCHK1 and BnCHK3, and BnCHK5 display distinct preferences for various CK bases and metabolites, characteristic of their AHK counterparts, AHK2 and AHK3, respectively. Interestingly, the AHK2 homologues could be divided into two subfamilies (BnCHK1/BnCK2 and BnCHK3/BnCHK4) that differ in putative transmembrane domain topology and CK binding specificity, thus implying potential functional divergence.

Key words: CHASE-containing His kinase, *E. coli*-based live-cell competitive receptor assay; gene structure, JBnB library, modular protein architecture, phylogenetic analysis

Introduction

Cytokinin (CK) phytohormones regulate or participate in complex hormonal interactions involved in the control over numerous physiological and developmental processes such as cell division and differentiation, chloroplast maturation, leaf senescence, gametophyte formation, vascular tissue development, clock-related responses, responses to light, stresses (biotic and abiotic), and availability of macronutrients (for reviews, see Argueso *et al.*, 2009; Werner and Schmülling, 2009). CK signals are perceived and transduced through a multistep histidyl-aspartyl (His–Asp)

phosphorelay (MSP). The MSP pathway is intrinsic to ~20% of bacteria and is exclusively used by yeast and plants but not animals (Schaller *et al.*, 2011). According to the current model of CK signalling, CK molecules are perceived by a membrane-bound hybrid His-kinase (HK) receptor. Upon CK binding, the receptor autophosphorylates at a conserved histidine in the HK domain and the phosphate residue is transferred to the receptor's receiver (REC) domain. The phosphate is then relayed to a His-containing phosphotransfer protein (HPt), which translocates to the nucleus and activates type-B response regulators (RRs). The type-B RRs act as Myb-type transcription factors, inducing expression of primary response genes. The CK primary response genes include abundant type-A RRs, acting as negative feedback regulators of the CK signalling pathway. Generally, the CK receptors represent a small proportion of a plant's complements of sensor HKs, through which (*inter alia*) they also perceive ethylene (Chang *et al.*, 1993) and changes in osmotic conditions (Urao *et al.*, 1999). A feature that distinguishes CK receptors from other sensor HKs is their N-terminal ligand-binding cyclase/histidine kinase-associated sensory extracellular (CHASE) domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001). The CHASE domain is flanked by transmembrane domains and associated with a cytoplasmic HK domain and a C-terminal REC domain (Ueguchi *et al.*, 2001).

The properties and functions of CK receptors are best described in the model dicot *Arabidopsis thaliana.* Among eight identified HKs of this species, three transmembrane ARABIDOPSIS HIS KINASES (AHKs), designated AHK2, AHK3, and AHK4, have been shown to act as genuine CK receptors (Inoue *et al*, 2001; Ueguchi *et al.*, 2001). Studies of single and higher order *ahk* mutants (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004; Riefler *et al.* 2006) have revealed partially redundant but differentiated functions for the individual receptors and prominent roles for the AHK2/AHK3 receptor combination in quantitative control of organ growth, with opposite regulatory functions in roots and shoots (Riefler *et al.*, 2006). Specific roles have been identified for single receptors. Notably, AHK4 and AHK3, respectively, play key roles in early vascular development (Mähönen *et al.*, 2000) and cell differentiation in the cell division/cell differentiation zone of the root meristem (Dello Ioio *et al.*, 2007). *Arabidopsis* loss-of-function mutants of CK receptors have also shown strong tolerance to drought and salt stress (Tran *et al.*, 2007, 2010), increased freezing tolerance (Jeon *et al.*, 2010), and resistance to clubroot disease (Galfe *et al.*, 2009). Using heterologous expression systems, it has been shown that various CK compounds have signalling functions, but only via specific receptors, and that CK binding activities are in the nanomolar range, in agreement with CK concentrations *in planta* (Spíchal *et al.*, 2004; Romanov *et al.*, 2005, 2006). AHK3 and AHK4 show different ligand preferences (Spíchal *et al.*, 2004; Romanov *et al*, 2006), while AHK2 functionally resembles AHK4 (Stoltz *et al.*, 2011). The structural basis for CK recognition by AHK4 was recently unravelled and the crucial amino acids in its CHASE domain for the receptor function were identified (Hothorn *et al.*, 2011).

Owing to the implications of CK signalling in economically important traits, such as nodulation, wood formation, drought, salt, and pathogen resistance (Tran *et al.*, 2007; Nieminen *et al.*, 2008; Choi *et al.*, 2010; Argueso *et al.*, 2012), detailed knowledge of CK signalling components in cultivated plant species including crops has valuable potential applications. However, current knowledge of CK signalling mechanisms in crops is mostly limited to monocotyledonous plants, particularly maize (Asakura *et al.*, 2003; Giulini *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004) and rice (Ito and Kurata, 2006; Jain *et al.*, 2006; Pareek *et al.*, 2006; Du *et al.*, 2007; Choi *et al.*, 2012; Tsai *et al.*, 2012). Thus, there is a clear need to characterize the receptors and downstream signalling components in dicotyledonous crop species. The most important of these species globally include various members of the genus *Brassica*. Oilseed rape, *Brassica napus*, a recently formed allotetraploid containing *B. rapa* (A) and *B. oleracea* (C) genomes, is a valuable crop that is widely used in food and feed industries. It also plays a significant role in arable rotations by improving yields of subsequent cereal crops. However, there is little or no knowledge of CK perception and signalling mechanisms in *B. napus* and other cultivated *Brassica* species. Here, the first identification of five members of the CK receptor gene family homologous to AHK2 and AHK3 and detailed CK binding studies of the encoded proteins from *Brassica napus* (var. Tapidor) are presented. Exploiting the phylogenetically close relationship of *B. napus* to *Arabidopsis thaliana*, five *Arabidopsis* homologues of *B. napus* CHASE-containing His kinase (*BnCHK*) genes were identified. Based on genomic DNA and cDNA sequencing results and bioinformatic predictions, it is shown that all five BnCHKs share typical molecular characteristics of CK receptors and they are phylogenetically compared with *B. rapa* counterparts annotated to date. Detailed binding and recognition characteristics of CK metabolites by BnCHK1, BnCHK3 (homologues of AHK2), and BnCHK5 (homologue of AHK3) are also presented.

Materials and methods

Probe preparation

Hybridization probes, used for identification of JBnB clones (and subsequently subcloned restriction fragments), carrying characteristic sequences of *BnCHK* genes, were prepared by PCR amplification of cDNA regions of *AHK2*, using the following primers: 5′-ACTGAGAGAACAAACTTTGAGAGG-3′ and 5′-CATGGT TCCTTGATGGATCAC-3′ (CHASE probe), 5′-GAATGC TGAAAATGCTGA-3′ and 5′-TGCCAGTTCCACCATAAG-3′ (HK probe), 5′-GGTTGTGGATGATAATCTTGTG-3′, and 5′-CTTGCTACCGCTGTGTAGAG-3′ (REC probe).

Filter hybridizations

The JBnB library and individual bacterial artificial chromosome (BAC) clones were purchased from the John Innes Centre (JIC) Genome Laboratory, Norwich, UK. Filters were pre-processed by

soaking for 2 h at 42 °C in 5× SSC, 0.5% SDS, 1 mM EDTA (pH 8). Bacterial debris was removed using a paper towel; membranes were then rinsed in 2× SSC and hybridized with 100 ng of ^{32}P-labelled probes under low stringency conditions, as described by O'Neill and Bancroft (2000). A DecaLabel™ DNA Labelling Kit (Fermentas) was used to prepare [α-^{32}P]dCTP random-primed labelled probes according to the manufacturer's instructions. Before the next hybridization step with another of the three probes, filters were stripped for re-use by soaking twice in 0.4 M NaOH at 50 °C for 30 min, then neutralized in 0.1× SSC, 0.1% SDS, 0.2 M TRIS (pH7.5) at 50 °C for at least 20 min according to JIC protocols. Restricted DNA, isolated from BAC clones, was Southern-blotted under alkaline conditions to HyBondTM-N+ membrane (GE Healthcare) and hybridized under low stringency conditions as outlined above. Autoradiography was performed using a STORM 840 Phospho-imager (GE Healthcare).

DNA preparation, vectors, and bacterial strains

BAC clones from the JBnB library, constructed in the pBAC/SACB1 vector (Bendahmane, 1999), were propagated in LB medium supplemented with 12.5 μg ml^{-1} chloramphenicol. BAC DNA was isolated using QIAGEN®Plasmid Midi Kit columns according to the manufacturer's protocol. Restricted DNA isolated from BAC clones was subcloned in the pBluescript SK vector and propagated in *Escherichia coli* strain DH10B.

cDNA cloning

RNA was isolated from shoots, hypocotyls, and roots of 6-day-old *B. napus* var. Tapidor seedlings using an RNeasy Plant Mini Kit (QIAGEN) or Trizol reagent (Invitrogen) and treated with RNase-free DNase I (QIAGEN) prior to reverese transcription–PCR (RT–PCR). RNA template (3 μg, consisting of 1 μg from each of the shoot, hypocotyl, and root RNA samples) was used in first-strand cDNA synthesis, catalysed by SuperScript™ III Reverse Transcriptase following the manufacturer's instructions (Invitrogen, Life Technologies, Czech Republic). PCR was performed using Phusion High-Fidelity DNA Polymerase (Finnzymes). 5' and 3' cDNA ends were cloned using a GeneRacer™ (RLM-RACE) Kit, TOPO TA Cloning® Kit for Sequencing, or Zero Blunt® TOPO® PCR Cloning Kit and One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) according to the manufacturers' protocols. Gene-specific primers used for cDNA cloning are listed in Supplementary Table S1 available at *JXB* online.

Sequence and phylogenetic analysis

Raw sequencing data were obtained from Macrogen (http://dna.macrogen.com). Next-generation sequencing (NGS) was performed using the GS Junior system from ROCHE with the company's assistance. Most analytical steps, including primer walking, contig assembly, reading frame definition, sequence alignments, and database searches were performed using DNASTAR software (http://www.dnastar.com). Coding sequences of the defined genomic DNAs were predicted by GENSCAN (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1997), Eucaryotic GeneMark.hmm (http://exon.gatech.edu/eukhmm.cgi; Lomsadze *et al.*, 2005), and FGENESH (http://linux1.softberry.com/all.htm; Yao *et al.*, 2005; Solovyev *et al.*, 2006). Gene structures were visualized in FancyGene (http://bio.ieo.eu/fancygene; Rambaldi and Ciccarelli, 2009). To confirm the presence of conserved domains, motifs were identified by PROSITE (http://prosite.expasy.org; Sigrist *et al.*, 2012) and SMART (http://smart.embl.de/; Schultz *et al.*, 2000). Transmembrane (TM) segments were predicted by SMART, TMAP (Persson and Argos, 1994) and TMHMM (Eddy, 1998; Sonnhammer *et al.*, 1998) algorithms at http://workbench.sdsc.edu/, TMpred (http://www.ch.embnet.org/software/TMPRED_form.html; Hofmann and Stoffel, 1993), TopPred 0.01 (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred; von Heijne, 1992; Claros and von Heijne, 1994), SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html; Hirokawa *et al.*, 1998), and PHYRE2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index; Kelley and Sternberg, 2009). Phylogenetic analysis of the sequences was carried out by Phylogeny.fr (http://www.phylogeny.fr; Dereeper *et al.*, 2008), using the following 'a la Carte' applications: (i) multiple alignment by MUSCLE (Edgar, 2004) under default mode; (ii) Gblocks treatment of alignments (Castresana, 2000); and (iii) phylogenetic tree construction by the Neighbor–Joining method (Saitou and Nei, 1987) with 500 bootstrap replicates to obtain branch-support values (Felsenstein, 1989).

CK binding assay

Non-labelled phytohormones used in the binding assays were obtained from OlChemIm Ltd. (Olomouc, Czech Republic). Radiolabelled *trans*-zeatin ([2-^{3}H]zeatin, 592 GBq/mmol) was obtained from the Isotope Laboratory, Institute of Experimental Botany, AS CR, Prague, Czech Republic. Live-cell CK-binding assays were performed with intact *E. coli* strain KMI001 carrying the pINIIIΔEH vector (Suzuki *et al.*, 2001; Yamada *et al.*, 2001). BnCHK1 and BnCHK5 coding sequences were obtained by gene synthesis at Life Technologies and subcloned in pINIIIΔEH using *Bam*HI and *Sal*I restriction sites. *BnCHK3* was obtained from pBluescript vector by PCR introducing an *Eco*RI restriction site and subcloned to the same restriction site of the empty vector pINIIIdEH. The binding assay was performed according to the method described by Romanov *et al.* (2005, 2006) with slight modifications. Bacterial cultures were grown in liquid M9 medium supplemented with 100 μg ml^{-1} ampicillin and 0.1% (w/v) casamino acids at 25 °C overnight, with shaking (200 rpm), to OD_{600} ~0.7–0.8. The culture density was then increased to OD_{600} ~0.9–1.2 by centrifugation (1000 *g*, 7 min, 4 °C). For assays with each probe, 1 ml portions of the cell suspension were transferred to Eppendorf tubes, then 3 pmol of [^{3}H]tZ, with or without unlabelled tZ or other tested competitors at various concentrations, and 0.1% (v/v) dimethylsulphoxide (DMSO; solvent) was added. After at least 30 min incubation at 4 °C, the sample was centrifuged (6000 *g*, 6 min, 4 °C), the supernatant was carefully removed, and the bacterial pellet was resuspended in 1 ml of scintillation cocktail (Beckman, Ramsey, MN, USA) in an ultrasonic bath. Radioactivity was measured by a Hidex 300 SL scintillation counter (Hidex, Finland). To discriminate between specific and non-specific binding, a high excess of unlabelled tZ (at least 3000-fold) was used for competition, as described by Romanov *et al.* (2006). K_d values were determined as average values from three independent Scatchard analyses (Scatchard, 1949), using GraphPad Prism 5.1 (http://www.graphpad.com/scientific-software/prism/).

Results

Redundant numbers of BAC clones carrying putative CHASE-containing His-kinases were identified by screening the B. napus *genomic library*

To obtain genomic DNA encoding potential CK receptors, a BAC library prepared from genomic DNA of *B. napus* var. Tapidor (JBnB) was experimentally screened (Rana *et al.*, 2004). The choice of hybridization probes was based on a preliminary assessment of frequencies of identical nucleotides between aligned *AHK2*/*AHK3*, *AHK2*/*AHK4*, and *AHK3*/*AHK4* pairs of genes (differing by not more than 2%, Supplementary Fig. S1 at *JXB* online) and knowledge of the three characteristic domains of AHK proteins (Ueguchi *et al.*, 2001). The PCR-generated probes were thus derived from nucleotide sequences corresponding to the phylogenetically conserved CHASE, HK, and REC domains of one of the three *AHK* genes, namely *AHK2*.

In total, 58, 41, and 115 BAC clones that hybridized with the CHASE, HK, and REC probes, respectively, were identified. Of all the BAC clones giving positive signals with at least one probe, 39 hybridized with each probe (Supplementary Table S2 at *JXB* online). In further experiments, most clones, which hybridized weakly with only the CHASE or REC probe, were omitted to avoid sequencing BAC inserts carrying incomplete HK genes. The final list of JBnB clones detected in the hybridization experiments and chosen for further analyses is presented in Supplementary Table S3.

Fingerprinting sorted overlapping BAC clones into five distinct groups

The JBnB library contains 73 728 clones, of which 88% are recombinant, with a mean insert size of 145 kb. Thus, it should represent the 1200 Mb genome of *B. napus* with 7.8-fold redundancy (Rana *et al.*, 2004). To sort out BAC clones carrying overlapping genome fragments, restriction analysis, followed by hybridization of Southern blots of the restriction digests with the CHASE probe, was used. *Sal*I, *Bam*HI, and *Hin*dIII restriction digests and their hybridization patterns with the CHASE probe are presented in Supplementary Fig. S2 at *JXB* online.

In total, five distinct groups were defined among the identified BAC clones. This experimental outcome supported the theoretical estimate of 5.5 *BnCHK* genes among the 43 analysed BAC clones, based on the reported 7.8-fold redundancy in the JBnB library. However, five clones could not be assigned to any of the defined groups and the results of their analysis were ambiguous, mainly because they yielded very weak or no hybridization signals (Supplementary Table S3, Fig. S1 at *JXB* online).

Genomic DNA sequencing and analysis of the candidate BAC clones defined five distinct genes predicted to encode cytokinin receptors

A BAC clone representing each group was chosen for sequencing (Supplementary Table S3 at *JXB* online). Sequencing by primer walking and NGS was used to acquire information about genomic DNA sequences carrying putative genes encoding CK receptors in the identified BAC clones (see the Materials and methods). The first round of sequencing by primer walking of DNA templates, isolated from JBnB025B19, JBnB162F14, JBnB041A21, and JBnB047D2, was successfully initiated by primers derived from *AHK2*. As mispriming of JBnB002E4 occurred, it was finally sequenced by the NGS approach.

In addition to 'BAC walking', in which the whole BAC inserts served as templates, their subcloned restriction fragments giving positive signals with CHASE, HK, and REC probes were also sequenced by primer walking. The subcloned genomic DNA regions of JBnB002E4, JBnB025B19, and JBnB162F14 were stable in *E. coli* (Supplementary Fig. S3 at *JXB* online), and these templates helped verify the outcome of BAC sequencing. Based on the sequencing results, five distinct genomic sequences were defined (accession nos KF621024, KF621025, KF621028, KF621026, and KF621027) with lengths ranging from 8.8 kb to 13.9 kb.

Within these genomic sequences, uninterrupted open reading frames, 3.0–3.6 kb long, were predicted by *ab initio* gene structure prediction software (see also the following section). In addition, preliminary analysis by PROSITE (Sigrist *et al.*, 2012) revealed the presence of three domain profiles typical of TM sensor HKs (CHASE, HK, and REC) within each translated reading frame (not shown). Thus, it was concluded that the five distinct genomic DNA sequences identified by the experimental approach used here encoded hybrid CHASE-containing HKs (CHKs) (Heyl *et al.*, 2013), members of a multistep phosphorelay system and putative CK receptors, which were designated *BnCHK1*–*BnCHK5* (Table 1).

Determination of the structure of the BnCHK *genes by cDNA cloning confirmed bioinformatic predictions*

cDNAs of all identified *BnCHK* genes were cloned by total RNA isolation from *B. napus* seedlings followed by RT–PCR (see the Materials and methods for more details). Coding sequence (CDS) predictions (Supplementary Table S4 at *JXB* online) were confirmed or corrected by sequencing the respective cDNA clones (accession nos KF621029, KF621030, KF621031, KF621032, and KF621033), followed by alignment of the cDNA sequencing output with the corresponding genomic DNA sequences and manual curation of donor and acceptor splice sites. Numbers, positions, and lengths of exons and introns in the genes, together with 5'- and 3'-untranslated regions (UTRs), where identified, are schematically presented in Fig. 1. Both *BnCHK1* and *BnCHK2* have 13 exons (of almost identical sizes) and 12 introns. *BnCHK3* and *BnCHK4* are also very similar, both having 11 exons and 10 introns. However, the 11th exon of *BnCHK3* is much shorter (70 bp) than the corresponding exon of *BnCHK4* (214 bp) due to single nucleotide polymorphism (SNP; G for A substitution) at position 3225 in the *BnCHK3* CDS (corresponding to position 3231 in the *BnCHK4* CDS), introducing a 'premature' termination TGA codon in *BnCHK3*. The CDS close to the 3' end and the 3'-UTR of *BnCHK4* is otherwise almost identical to the genomic sequence flanking the 3' end of *BnCHK3* (not shown). Complete CDS regions of both couples of homologous HKs *BnCHK1*/*BnCHK2* and *BnCHK3*/*BnCHK4* share a high level of identity (97.7% and 96.6%, respectively) (Supplementary Fig. S4 at *JXB* online). The cDNA of *BnCHK5* is shorter than those of any of the other four *BnCHK* genes. It contains only 10 exons and nine introns, and the ninth exon is significantly longer than the other exons of this gene. The pairwise identities of aligned cDNA regions corresponding to the conserved CHASE, HK and REC domains are also presented in Supplementary Fig. S4.

In summary, five distinct genomic sequences of putative *BnCHK* genes, each representing one of the defined groups of BAC clones, carrying overlapping inserts of genomic DNA and encoding putative homologues of *Arabidopsis* CK receptors, have been identified. The five genes form two highly similar pairs BnCHK1/BnCHK2 and BnCHK3/BnCHK4 and one distinct sequence of BnCHK5.

Table 1. *List of sequenced JBnB clones and assigned* BnCHK *genes, identified within their inserts*

BAC identification		Genomic DNA sequencing		Predicted (FGENESH)			Cloned			
Clone ID	Group	Method	No. of bp	No. of exons	CDS length (bp)	Protein length (no. of amino acids)	No. of exons	CDS length (bp)	Protein length (no. of amino acids)	Name
JBnB002E4	I	NGS	13 944	11	3225	1074	11	3225	1074	BnCHK3
JBnB025B19	II	Primer walking	9767	11	3375	1124	11	3375	1124	BnCHK4
JBnB162F14	III	primer walking	8819	10	3054	1017	10	3054	1017	BnCHK5
JBnB041A21	IV	Primer walking	9776	14	3,450	1197	13	3450	1149	BnCHK2
JBnB047D2	V	Primer walking	9013	13	3,450	1149	13	3450	1149	BnCHK1

Multiple alignments of BnCHK proteins revealed modular architecture typical of cytokinin receptors

Reading frames of the cloned *BnCHK* genes were subjected to computational analysis (see the Materials and methods) to examine the modular architecture of the encoded BnCHK proteins. All five proteins have three or four TM domains (Supplementary Fig. S6 at *JXB* online) and three basic domains of conserved tertiary structure: CHASE, HK, and REC domains (Fig. 2).

Multiple sequence alignments of BnCHK and AHK proteins revealed high homology and determined consensual amino acid motifs at conserved positions within the identified domains (Fig. 3). Within the CHASE domain (Anantharaman and Aravind, 2001; Mougel and Zhulin, 2001), there are four amino acid positions at which mutations reportedly cause a complete loss of function (Mähönen *et al.*, 2000), or either complete loss or decrease of CK binding to the AHK4 CHASE domain, and thus are essential for CK sensing (Heyl *et al.*, 2007; Hothorn *et al.*, 2011). With respect to amino acid numbering in the longer AHK4 isoform CRE1b (Q9C5U0-1), these are Trp244, Asp285, Thr301, Phe304, and Thr317. It was found that all these amino acids are conserved in all the identified BnCHKs (Fig. 3A).

Furthermore, each BnCHK possesses an H, N, G1, F, G2 motif within the HK domain (Fig. 3B) and a DD, D, K motif within the REC domain (Fig. 3C) (phosphorelay-mediating histidine and aspartate are underlined). Both of these motifs are characteristic of canonical HKs (West and Stock, 2001). More detailed analysis of the BnCHK protein sequences by the SMART program (Schultz *et al.*, 2000) defined two separate modules within the HK domain: an HK dimerization and phosphoacceptor domain (HisKA) and an HK catalytic domain, called the HK-like ATPase domain (HATPase) (Fig. 2).

CHASE, HK, and REC domains were predicted with high confidence for all proteins except BnCHK3, for which the reliability of the REC domain's prediction was rather low (Table 2). This may reflect the shorter terminal exon of *BnCHK3* described above and although the REC domain of BnCHK3 contains the complete DD, D, K AA motif, it may eventually prove to be a paralogue with a changed or limited HK function.

The amino acid region from the HK to REC domain, previously called the REC-like domain in CK-recognizing AHKs (Ueguchi *et al.*, 2001; Heyl and Schmülling, 2003), was also examined. Prediction programs defined this amino acid region of all BnCHKs as a REC domain with significantly higher than threshold e-values (Table 2). BnCHK2, 3, and 4 all have DS, D, K motifs within this region, while BnCHK1 and BnCHK5 contain DT, D, K and DH, E, K motifs, respectively (Figs 2, 3C). Notably, the e-value was lowest for the REC-like domain of BnCHK2 (Table 2).

TM domains of the BnCHK proteins deserve closer attention. Seven different programs were used to predict α-helixes in BnCHKs (and in AHKs in comparison) potentially spanning through the plasma membrane and flanking the CHASE domain (Supplementary Table S5, Fig. S6 at *JXB* online).

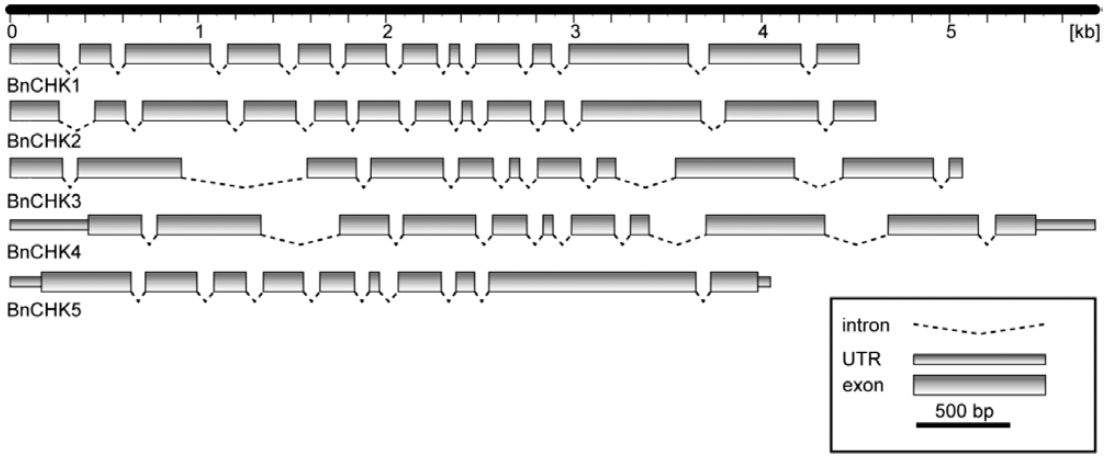

Fig. 1. Schematic structures of the five identified *BnCHK* genes, based on comparison of cloned and sequenced genomic DNA and cDNA sequences. See also Supplementary Fig. S5 at *JXB* online.

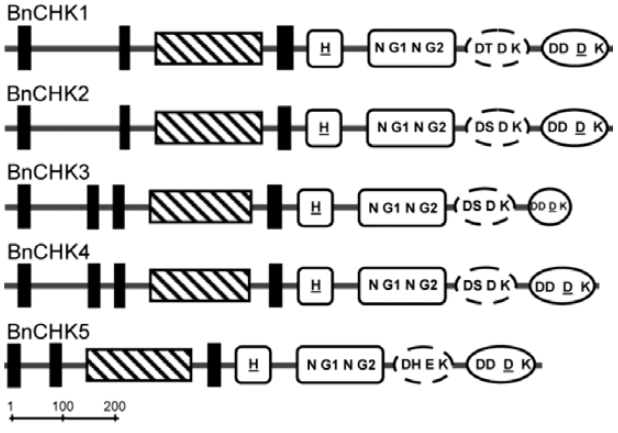

Fig. 2. Domain structure of the five identified *B. napus* CHASE-containing hybrid sensor His kinases. Transmembrane domain (filled rectangle), CHASE domain (striped rectangle), His kinase domain, consisting of HisKA (H) and HATPase (N G1 N G2) modules (open rectangle), putative receiver-like domain (open dotted oval), and receiver domain (open oval).

This analysis again defines two AHK2-like BnCHK pairs: BnCHK1/BnCHK2 and BnCHK3/BnCHK4. Interestingly, these two pairs differ in the putative TM domain topology. All used programs reliably identified three TM segments in BnCHK1 and BnCHK2, which resembles AHK2 (with respect to both the number and the relative position of putative TM segments). In contrast, BnCHK3 and BnCHK4 have four predicted TM domains, with very high probability of forming TM segments at three putative locations upstream of the CHASE domain. BnCHK5 strictly resembles AHK3, again in both the number and relative position of predicted TM domains (Supplementary Table S5, Fig. S6).

Designation of annotated B. rapa *CHASE-containing His kinase genes and* in silico *identification of EST sequences homologous to* BnCHK *genes*

To incorporate CK receptors of *B. rapa*, contributing to the A-part of the allotetraploid genome of *B. napus* (not available when this study was begun) in the phylogenetic analyses (see the next section), sequences of all (four) CHASE-containing sensor HK genes of *B. rapa* listed in the http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase database, which compiles all sequences of the *B. rapa* genome released and annotated to date (Wang *et al.*, 2011; http://www.gramene.org/genome_browser/index.html), were acquired. Based on the information in the *B. rapa* database and the present alignment with sequences of *Arabidopsis* CK receptors (see below), the listed AHK2-related receptor genes were designated as BrCHK1 (Bra035381) and BrCHK2 (Bra013186), and those related to AHK3 and AHK4 as BrCHK3 (Bra030037) and BrCHK4 (Bra024849), respectively (Table 3).

After identifying the *BnCHK* genes, a BLAST search of expressed sequence tag (EST) databases was performed, using the *BnCHK* genes and *BrCHK4* as queries. A total of 31 significant hits among the identified EST sequences were obtained, and are listed in Supplementary Table S6 at *JXB* online. All the EST sequences show significant similarity to the *BnCHK* and *BrCHK4* genes and are expressed in various plant tissues under various conditions (Suplementary Table S6). It is difficult to assign the identified ESTs to the respective *BnCHK* genes, because of the high similarity among *BnCHK* genes (see Supplementary Fig. S4) and numerous SNPs in *BnCHK* orthologues of different varieties compiled in the EST databases. However, links can be at least partially deduced from the score values (Supplementary Table S6). For instance, the EST yielding the highest BLAST score for *BrCHK4*, homologous to *AHK4*, was ES900704 from *B. napus*. This EST represents the HK domain and shares the same contig (#5, Supplementaary Table S6) as ES904730, the only identified *B. napus* EST carrying the CHASE domain. This indicates that ES900704 may represent a CHASE-containing HK homologous to AHK4. However, the cloning of genuine *BnCHK* homologous to AHK4 and its detailed sequence analysis remains to be done.

Phylogenetic analysis of the isolated BnCHK *genes identified four homologues of* AHK2 *and one homologue of* AHK3

Phylogenetic analysis was performed primarily to distinguish between *BnCHK* genes related to *AHK2*, *AHK3*, and *AHK4*. However, not only well-studied sequences from *Arabidopsis*,

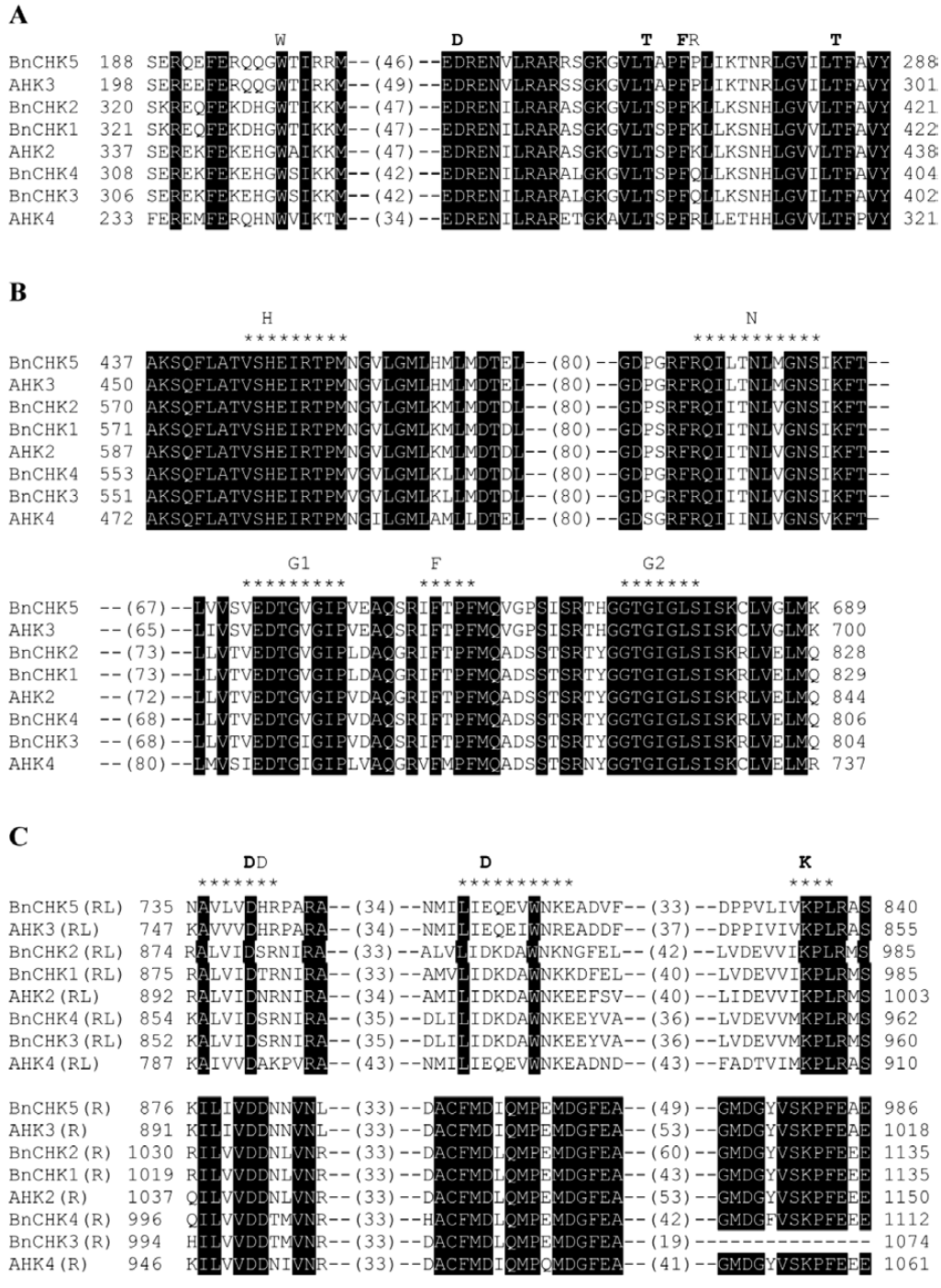

Fig. 3. Consensus amino acid motifs within conserved domains of the five identified *B. napus* CHASE-containing hybrid His kinases, based on multiple alignment with CHASE (A), His kinase (B), receiver-like (RL), and receiver (R) (C) domain sequences of related AHKs by ClustalW (Ueguchi *et al.*, 2001).

but also well-classified sequences from rice representing monocotyledonous CK receptors (Choi *et al.*, 2012; Tsai *et al.*, 2012; Heyl *et al.*, 2013), the four recently annotated sequences encoding putative CK receptors in *B. rapa* and designated here as BrCHK1–BrCHK4, two EST sequences of *B. napus* homologous to *BrCHK4*, and one EST sequence of *B. oleracea* (see the previous section) were incorporated.

First, phylogenetic analysis was performed based on alignments of conserved HK domains. The alignment report, displaying all the sequences used in the analysis, is presented in Supplementary Fig. S7 at *JXB* online. Based on the analysis and constructed phylogenetic tree (Fig. 4), it was determined that the five identified *BnCHK* genes include four *AHK2* homologues (*BnCHK1*, *2*, *3*, and *4*) and one *AHK3* homologue (*BnCHK5*). The ES900704 sequence represents a *B. napus* homologue of AHK4. Amino acid identities for putative HK domains of BnCHK1/BnCHK2 and BnCHK3/BnCHK4 pairs are 99.4% and 98.9%, respectively, pointing to very high similarity between these pairs (see the distance matrix in Supplementary Fig. S7D). Furthermore, pairwise amino acid identities between sequences of each of the BnCHK1/BrCHK1, BnCHK3/BrCHK2, BnCHK5/BrCHK3, and

Table 2. *Probabilities, expressed by e-values, of conserved protein domains identified in BnCHK sequences by SMART (http://smart.embl.de)*

Gene	CHASE	HisKA	HATPase	REC-like	REC
BnCHK1	2.17e-29	1.86e-22	1.91e-33	0.363	1.24e-33
BnCHK2	1.46e-30	1.86e-22	1.1e-33	0.0723	8.76e-34
BnCHK3	1.25e-29	1.19e-20	6.17e-37	0.23	0.00549
BnCHK4	1.25e-29	1.19e-20	4.67e-37	0.243	1.54e-29
BnCHK5	3.34e-30	7.55e-23	4.79e-34	91.1	1.38e-31

Table 3. *Database accessions of* BnCHK *genes identified by screening the JBnB library, and* BrCHK *genes listed in the http://www.gramene.org/genome_browser/index.html, http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase database*

Gene name	*AHK* homologue	Gene ID	Transcript ID	Protein ID
BnCHK1	*AHK2*	KF621024	KF621029	
BnCHK2	*AHK2*	KF621025	KF621030	
BnCHK3	*AHK2*	KF621028	KF621031	
BnCHK4	*AHK2*	KF621026	KF621032	
BnCHK5	*AHK3*	KF621027	KF621033	
BrCHK1 (s)	*AHK2*	Bra035381	Bra035381.1	Bra035381.1-P
BrCHK2	*AHK2*	Bra013186	Bra013186.1	Bra013186.1-P
BrCHK3 (s)	*AHK3*	Bra030037	Bra030037.1	Bra030037.1-P
BrCHK4 (s)	*AHK4*	Bra024849	Bra024849.1	Bra024849.1-P

s, mapped syntenic to the *AHK* chromosome location; syntenic orthologue of *AHK*.

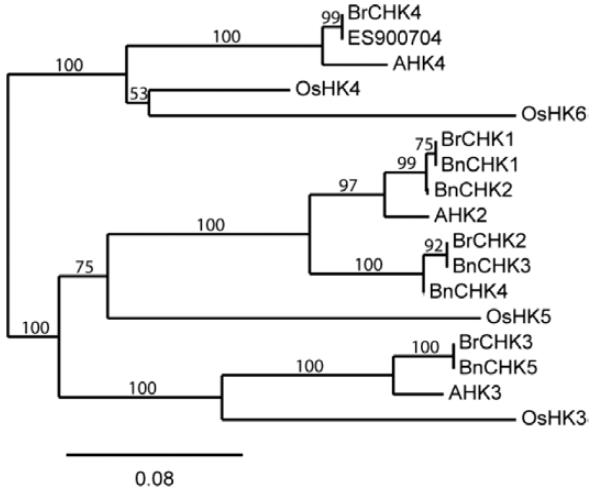

Fig. 4. Phylogenetic relationships of CHASE-containing His kinases from *Brassica napus* (BnCHKs—for gene IDs see Table 3—and gi|150870246|gb|ES900704), *Brassica rapa* (BrCHKs—for gene IDs see Table 3), Arabidopsis (AHKs—At1g27320, At2g01830, and At5g35750), and rice (OsHKs—Os01g69920, Os02g50480, Os03g50860, and Os10g21810). Alignments are based on His kinase domains.

ES900704/BrCHK4 pairs are 100%, indicating that the corresponding *B. napus* and *B. rapa* CHKs are very closely related.

A multiple sequence alignment of conserved CHASE domains (Supplementary Fig. S8 at *JXB* online) was also performed, in which two more EST hits from the database search described in the previous section were included: ES904730 from *B. napus* and asmbl48579 from *B. oleracea*, both representing novel putative CHASE domains, as predicted by SMART (not shown) (Supplementary Table S6). Here, three groups with 100% amino acid identity have been defined: (i) BnCHK1/BrCHK1; (ii) BnCHK3/BnCHK4/BrCHK2/ES904730/asmbl48579; and (iii) BrCHK3/BnCHK5 (Supplementary Fig. S8D), suggesting that the CHASE domains within each group might have similar CK recognition characteristics (Supplementary Fig. S8E). Notably, BnCHK3 has the same CHASE domain as BnCHK4, but both proteins differ in their HK domains (see above). The CHASE alignment further confirms the conclusion from the HK alignment that BrCHK1 and BrCHK5 are homologues of AHK2 and AHK3, respectively (Supplementary Fig. S8E).

Taken together, the phylogenetic analyses indicate that most of the identified *BnCHK* genes are homologues of *AHK2* and *AHK3*, but at least one homologue of *AHK4*, missed by the sequence hybridization screen, also seems to be present in the *B. napus* genome.

Functional analysis in E. coli *reveals different CK binding characteristics of BnCHK1, BnCHK3, and BnCHK5*

To confirm that the cloned BnCHK protein-encoding genes with conserved CHASE domains can function as genuine CK receptors and specifically bind CKs, functional analyses was performed. BnCHK1 and BnCHK3, as representatives of the two groups of the identified BnCHKs homologous to AHK2, and BnCHK5 homologous to AHK3 were tested for their ligand specificity and affinity using an *E. coli*-based direct binding assay (Romanov *et al.*, 2005). First, the CK-binding capacity of the proteins was determined in a dose-dependent assay, using a range of concentrations of tritium-labelled

trans-zeatin ([^{3}H]tZ), followed by Scatchard analysis. As shown in Fig. 5A, in all cases classical saturation curves were obtained. Analysis of the data gave apparent affinity constants (K_d) in nanomolar orders, indicating high affinity binding typical of hormone receptors. Estimated K_d values for BnCHK1, BnCHK3, and BnCHK5 were 2.9 ± 1.8, 1.9 ± 0.4, and 1.5 ± 0.8 nM, respectively. These values correspond well to dissociation constants published for *Arabidopsis* and maize receptors (Romanov *et al*., 2005, 2006; Lomin *et al*, 2011; Stolz *et al.*, 2011). The capacity of representatives of various other low molecular weight phytohormones to compete with [^{3}H]tZ for binding in the assays, including auxin (IAA), gibberellin (GA_3), abscisic acid (ABA), and adenine (Ade) was also tested (Fig. 5B). All three BnCHKs showed high specificity for the CK: unlabelled tZ (but not IAA, GA_3, ABA, or Ade) effectively reduced binding of [^{3}H]tZ (Fig. 5B). Based on these findings, it is concluded that BnCHK1, BnCHK3, and BnCHK5 can specifically recognize CKs.

As a next step, the ligand specificities of all three receptors was investigated in a series of binding experiments with several isoprenoid and aromatic Ade-type CK bases and the synthetic phenylurea-derived CK thidiazuron (TDZ). The apparent affinity constants of the active compounds obtained ranged from 6.6 nM to 5.5 µM (Table 4). The strongest recognized CK for all receptors was tZ, followed by TDZ (Fig. 5D; Table 4). All BnCHKs had much weaker affinity for the *cis*-isoform of zeatin (cZ), dihydrozeatin (DHZ), and kinetin (Kin), and bound the non-substituted aromatic CK N^6-benzyladenine (BA) only weakly. Notably, by far the weakest affinity for kinetin was observed for BnCHK3. On the other hand, BnCHK3 had the highest affinity of all the tested BnCHKs for the aromatic CK BA, which was almost not recognized by BnCHK5 (Fig. 5C, D; Table 4). Interestingly, the hydroxylated BA, *meta*-topolin (mT), was effectively recognized, again particularly by BnCHK3 (K_d=45 nM) but also by BnCHK1 and BnCHK5 (K_d ~100 nM). This indicates that the presence of the OH-group at the *meta*-position of the aromatic side chain (a feature resembling tZ) is important for binding to the receptor. However, the most striking difference in the ligand specificity of the BnCHK receptors was in the binding of N^6-isopenteyladenine (iP), which was strongly recognized by BnCHK1 and BnCHK3 (K_d only 8-fold and 12-fold higher than that for tZ, respectively), but not by BnCHK5 (K_d 90-fold higher than that for tZ; Fig. 5C; Table 4). No significant differences were found in the binding capacities of BnCHKs for various CK metabolites, with the only exception of tZ riboside (tZR) (Fig. 5E; Table 4). While BnCHK1 and BnCHK5 showed very high affinity for tZR with similar K_d values to those of TDZ (Table 4), BnCHK3 sensed tZR significantly less well (Fig. 5E; Table 4). As expected, the other tested tZ metabolites (N^9-glucoside, N^7-glucoside, and *O*-glucoside) were not effectively recognized by either receptor, indicating that modifications of the CK core structure negatively influence binding to the receptor active site. In summary, the tested BnCHKs reveal specificity in terms of their ability to recognize individual CK types and their metabolites. The order of CK preferences obtained by comparison of the relative binding affinities is tZ>TDZ>tZR>iP>mT>DHZ>cZ>BA~Kin>>Ade~tZ7G~tZ9G~tZOG for BnCHK1; tZ>TDZ>iP>tZR>mT>DHZ>cZ~BA>Kin>>Ade~tZ7G~tZ9G~tZOG for BnCHK3; and tZ>tZR~TDZ>mT~DHZ>iP>cZ>Kin>BA>>Ade~tZ7G~tZ9G~tZOG for BnCHK5.

Discussion

Most of the identified BnCHKs are homologous to AHK2 and originate from both B. oleracea *and* B. rapa *genomes*

Phylogenetic analysis groups the *Brassica* species into two, *nigra* and *rapa*/*oleracea*, lineages (Warwick and Black, 1991), which apparently diverged ~8 million years agio (Lysak *et al.*, 2005), while *B. rapa* and *B. oleracea* diverged ~4 million years ago (Inaba and Nishio, 2002). The *B. napus* genome seems to have resulted from the hybridization of *B. rapa* (A) and *B. oleracea* (C) genomes ~10 000 years ago (Nagaharu, 1935).

The phylogenetic analysis presented here is based on multiple alignment of amino acid sequences corresponding to the conserved HK domains from *Arabidopsis*, *B. napus*, *B. rapa*, and rice, and its outcome is consistent with the present gene and protein domain structure findings (see also next section): two pairs of very closely related *BnCHK* genes, *BnCHK1*/*BnCHK2* and *BnCHK3*/*BnCHK4*, are homologues of *AHK2*, while the structurally more distinct *BnCHK5* is a homologue of *AHK3*. As there is 100% amino acid identity between aligned conserved HK domains within the BnCHK1/BrCHK1, BnCHK3/BrCHK2, and BnCHK5/BrCHK3 pairs (Fig. 4; Supplementary Fig. S7D at *JXB* online), it is concluded that *BnCHK1*, *BnCHK3*, and *BnCHK5* originated from the *B. rapa* A genome. As *BrCHK1* and *BrCHK3* have been mapped to syntenic chromosomal regions of *AHK2* and *AHK3*, respectively (http://brassicadb.org/brad/geneFamily.php?fam=Histidine%20Kinase) (Table 3), it is concluded that *BnCHK1* and *BnCHK5* are orthologues of *AHK2* and *AHK3*, respectively. The lower similarity of BrCHK1 to BnCHK2 (99.4%) and BrCHK2 to BnCHK4 (98.9%), respectively, indicates that *BnCHK2* and *BnCHK4* may be inter-homoeologues that originated from the *B. oleracea* C genome.

Comparative physical mapping of the recently sequenced and annotated *B. rapa* genome (Wang *et al*., 2011; http://www.gramene.org/genome_browser/index.html) has indicated that it underwent a whole-genome triplication after divergence of the *Arabidopsis* and *Brassica* lineages (Lysak *et al.*, 2005; Wang *et al*., 2011). The reported final number of identified *B. rapa* protein-coding genes is 41 174, lower than theoretically expected for the triplicated genome (90 000), confirming previous observations of substantial gene losses, which typically occur following polyploidization events (Town *et al.*, 2006). The finding of four predicted *BrCHK* genes, out of a theoretical nine (in analogy to three *AHK* genes), is consistent with this phenomenon. Thus, the number of *BnCHK* genes identified might be close to the true number, particularly the number of identified *BnCHK* homologues of *AHK2*. However, the experimental approach used here will have missed putative *B. napus* orthologues of *AHK4*, as indicated by the position of the ES900704 EST sequence of another *B. napus* line in the

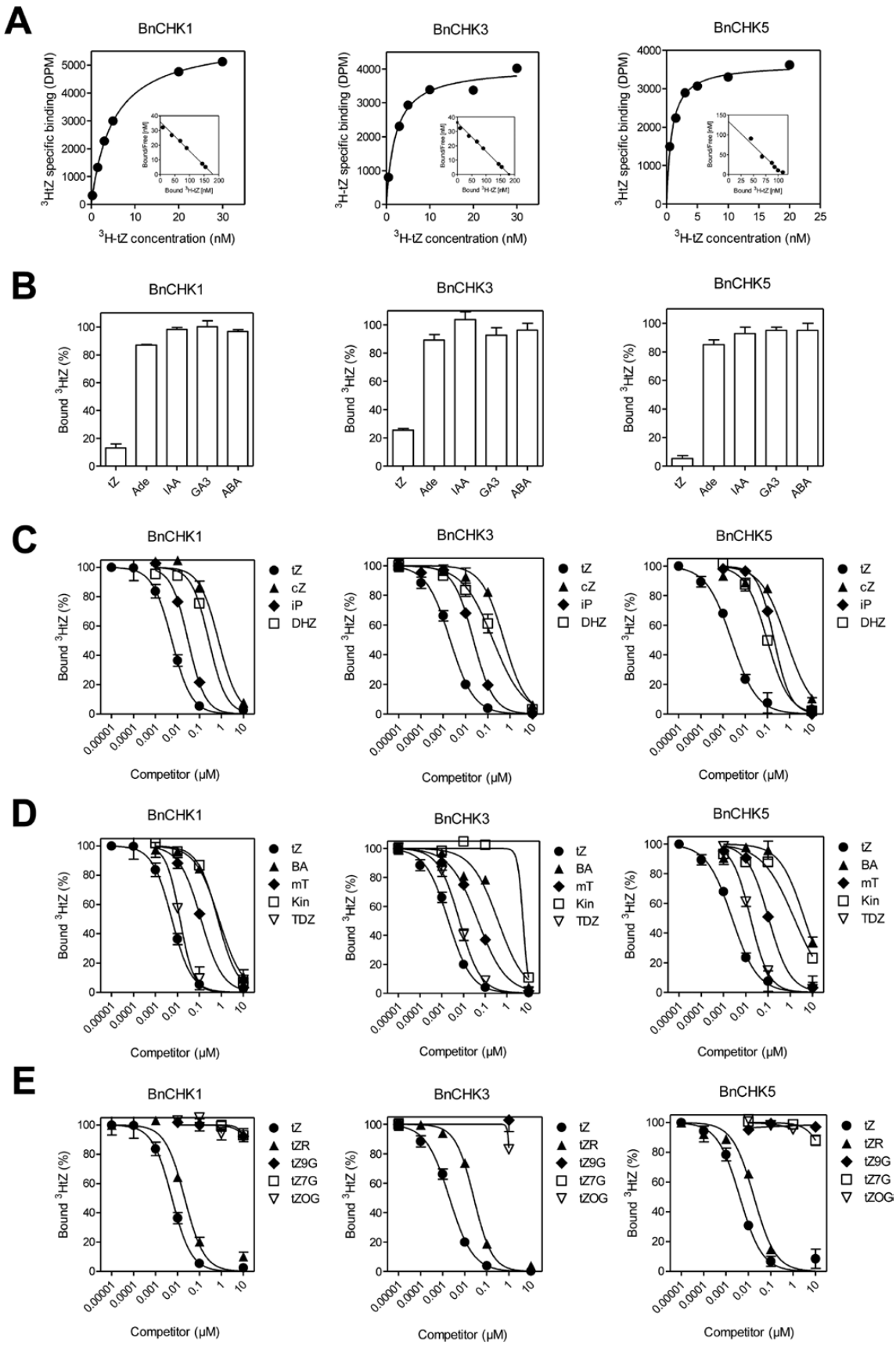

Fig. 5. Dose-dependent binding of [^{3}H]tZ to cytokinin (CK) receptor-expressing *E. coli* clones. Original data from specific binding and Scatchard plots (insets) are shown (A). Competition by non-CK compounds (B), CK metabolites (C, D), and various tZ derivatives (E) with [^{3}H]tZ for binding to the CK receptor-expressing *E. coli* clones. The bound radioactivity corresponding to 100% was 3338, 2383, and 4076 dpm in the case of binding experiments with BnCHK1, BnCHK3, and BnCHK5, respectively.

Table 4. Comparison of the cytokinin affinity of BnCHK1, BnCHK3, and BnCHK5

The apparent K_d values were calculated as ligand concentrations that displaced 50% of the bound [^{3}H]tZ (means and standard deviations obtained from two independent experiments, with three technical replicates per experiment).

Cytokinin	Abbreviation	Apparent K_d (nM)		
		BnCHK1	BnCHK3	BnCHK5
trans-Zeatin	tZ	4.6 ± 1.9	1.9 ± 0.4	2.42 ± 0.3
trans-Zeatin riboside	tZR	17.1 ± 1.6	27.1 ± 1.4	13.5 ± 1.0
trans-Zeatin-7-glucoside	tZ7G	>10 000	>10 000	>10 000
trans-Zeatin-9-glucoside	tZ9G	>10 000	>10 000	>10 000
trans-Zeatin-*O*-glucoside	tZOG	>10 000	>10 000	>10 000
cis-Zeatin	cZ	662 ± 85	440 ± 110	694 ± 28
Dihydrozeatin	DHZ	382 ± 70	174 ± 5	103 ± 5.1
N^6-Isopenteyladenine	iP	35.3 ± 4.6	22.8 ± 0.5	219 ± 18
N^6-Benzyladenine	BA	855 ± 77	440 ± 70	4794 ± 382
meta-Tpoline	mT	119 ± 1.5	44.7 ± 2.0	97.0 ± 13
Kinetin	Kin	895 ± 205	5456 ± 79	1695 ± 195
Thidiazuron	TDZ	11.1 ± 1.9	6.6 ± 0.1	13.9 ± 2.4
Adenine	Ade	>10 000	>10 000	>10 000

phylogram (Fig. 4). The possibility cannot be excluded that the 1–2% difference in nucleotide identity between the *AHK2* and *AHK4* probes (Supplementary Fig. S1 at *JXB* online) may have contributed to a lower T_m of the hybrid molecules between the *AHK2* probe and potential *BnCHK* homologues of *AHK4* during the hybridization experiment, explaining why *BnCHK* homologues of *AHK4* were not retrieved. Further systematic analyses of the completely sequenced *B. napus* and *B. oleracea* genomes, when released, are required to determine definitively whether or not there is more than one *BnCHK* homologue of *AHK3* and *AHK4*.

The characterization of the first five *BnCHK* sequences presented here also confirms previous findings from comparative analyses of genome segments from *B. rapa*, *B. oleracea*, *B. napus*, and *A. thaliana* (O'Neill and Bancroft, 2000; Rana *et al.*, 2004) and comparative analysis of rice and *Arabidopsis* (Liu *et al.*, 2001). Although extensive divergence of gene contents was observed in the studied species, the examined genes showed highly conserved collinearity with their putative orthologues. This high degree of conservation is further corroborated by the detection of very few, or no, SNPs at the nucleotide level between aligned CDS pairs of *BnCHK1*/*BrCHK1* (100% identity), *BnCHK5*/*BrCHK3* (99.7% identity), and *ES900704*/*BrCHK4* (99.9% identity) (not shown), and reflects the recent polyploidization event of *B. rapa* and *B. oleracea* genomes ~10 000 years ago (Nagaharu, 1935).

Predicted conserved protein domains of the encoded BnCHK proteins reveal conserved motifs of two pairs of closely related CK receptors and another more distinct putative CK receptor

All five identified BnCHKs contain the typical conserved functional domains and motifs of CK receptors in *Arabidopsis*: CHASE, HK, REC-like, and REC domains. All five CHASE domains contain identical amino acids at conserved positions that are reportedly crucial for CK binding and signalling of AHK4 (Mähönen *et al.*, 2000; Heyl *et al.*, 2007; Hothorn *et al.*, 2011). In addition, all five HK domains contain the conserved H, N, G1, F, G2 motif and all five REC domains contain the DD, D, K motif, characteristic of canonical HKs (West and Stock, 2001). The most pronounced difference in the conserved structure of functional domains among the BnCHKs is in the REC-like domains. BnCHK1, 2, 3, and 4 have highly similar amino acid motifs within their REC-like domains, with the typical DD, D, K motif of a regular REC domain, as they only have single amino acid substitutions at the second position of the motifs (threonine in DT, D, K of BnCHK1 and serine in DS, D, K, of BnCHK2, 3, and 4, both amino acids with polar, uncharged side chains, Figs 2, 3C, variable residues underlined). In contrast, BnCHK5 displays two substitutions in its REC-like motif: a positively charged histidine at the variable second position and a negatively charged glutamate replacing the conserved aspartate at the third position (DH, E, K, Figs 2, 3C). The latter could be functionally significant, as aspartate to glutamate mutation reportedly abolishes phosphorylation of the REC domain in the *Arabidopsis* response regulator ARR2 (Hass *et al.*, 2004). However, at the moment it can only be speculated whether the REC-like domains of BnCHKs meet the requirements of potentially functional cytoplasmic modules and their specific catalytic functions. Nevertheless, compared with the REC-like amino acid motifs of AHKs, BnCHK1, 2, 3, and 4 resemble AHK2, containing a polar uncharged asparagine at the second position of its DN, D, K motif, while the DH, E, K motif of the REC-like domain of BnCHK5 is identical to the same motif of the REC-like domain of AHK3. This again is in good agreement with the observed similarity between *BnCHK* and *AHK* genes at the nucleotide level and further implies that BnCHK1 and BnCHK5 could be orthologous to AHK2 and AHK3, respectively.

BnCHK1, BnCHK3, and BnCHK5 proteins specifically bind CKs and differ in their ligand specificity

As mentioned above, all five identified BnCHKs contain identical amino acids at conserved positions of their CHASE domains that are known to be crucial for receptor function. These include: Asp285, which forms hydrogen bonds with the adenine ring; Thr301, which restricts the overall size of the binding pocket; and Thr317, which contributes to high-affinity recognition of tZ (Hothorn *et al.*, 2011) (numbering according to the longer AHK4 isoform CRE1b, Q9C5U0-1, used in this study). Results of the functional direct binding assay presented here confirm the predictions that BnCHK1, BnCHK3, and BnCHK5 are CK receptors, as they indeed specifically recognize known adenine-type isoprenoid and aromatic CKs, as well as urea-derived TDZ, with binding affinities similar to those of other, previously described CK-binding HKs (Fig. 5). K_d values of ~1–3 nM obtained in this study with the most active natural CK, tZ, are consistent with reported values for CRE1/AHK4 [2.5 nM, Romanov *et al.* (2005); 3.9 nM, Romanov *et al.* (2006); 4.4 nM, Stolz *et al.* (2011)]. Both receptors also effectively bind tZR, whereas other metabolites, including *O*- and *N*-glucosides, are not recognized (Tab. 4).

CK receptors in *Arabidopsis* form two subgroups in terms of their specificity towards individual CK types. The subgroup formed by AHK2/AHK4 recognizes iP with high affinity; in the case of AHK2, even comparably with tZ. In contrast, AHK3 reveals affinity for tZ similar to that of AHK2/AHK4, but iP is recognized much less efficiently (Romanov *et al.*, 2006; Stolz *et al.*, 2011). The quantitative binding data obtained in this study support the presented phylogenetic data, which suggest that *BnCHK1* and *BnCHK3* are orthologous to *AHK2*, and *BnCHK5* to *AHK3*. Both BnCHK1 and BnCHK3 have ~6.5- and 9.5-fold, respectively, higher affinity for iP than BnCHK5 (Table 4), just as AHK2 has ~10- to 100-fold higher affinity for iP than AHK3 (Romanov *et al.*, 2006; Stolz *et al.*, 2011). In the relative comparison with tZ, BnCHK1 and BnCHK3 bind iP ~8-fold and 12-fold more weakly than tZ, respectively, compared with BnCHK5, which binds iP 90-fold less well than tZ (Table 4). In comparison, AHK2 binds iP even better than tZ (Stolz *et al.*, 2011) while AHK3 recognizes iP with about 100-fold lower affinity then tZ (Romanov *et al.*, 2006). The relatively weak preference of BnCHK5 for iP versus tZ may reflect a specific role in root-to-shoot communication, similar to that of AHK3 (Romanov *et al.*, 2006). Like AHK3, BnCHK5 may therefore be tuned to respond to long-distance signals transported from the roots via the xylem (Romanov *et al.*, 2006), in which tZ is the main CK (Takei *et al.*, 2001). To verify this hypothesis, demonstration that BnCHK5 is predominantly expressed in shoots is required.

Two subfamilies of AHK2 orthologues in B. napus *differ in the number of putative transmembrane regions and CK binding specificity*

AHK2 was originally shown to form two TM domains upstream of the CHASE domain (U-TM) (Ueguchi *et al.*, 2001). Some prediction programs used in this work defined another putative α-helix in this region, although with lower probability (Supplementary Fig. S6 at *JXB* online). In a more phylogenetically distant subgroup of AHK2 orthologues BnCHK3 and BnCHK4, this TM domain was identified by each of the programs used in this study and with probability comparable with that of other TM domains. This result presenting four TM cytokinin receptors, three U-TM and one downstream TM, in *B. napus* is in good agreement with recently published data. Steklov *et al.* (2013) accomplished an analysis of the number and positions of TM domains based on structures of 100 putative CK receptors from different species. In their study, Steklov *et al*. demonstrate that all CREI/AHK4 orthologues have only one U-TM domain, whereas AHK2 orthologues possess three or four TM helices in this possition. In this respect, the 2–3 U-TM domains in BnCHK1 to the four identified in the present study could be considered as additional evidence that these HKs are genuine AHK2 orthologues.

Whether there is any functional importance of the putative structural heterogenity, however, remains unclear. AHK2 and AHK3 possessing two and AHK4 possessing one U-TM domain have been shown to be dominantly located in membranes of the endoplasmic reticulum (ER) (Caesar *et al.*, 2011; Wulfetange *et al*., 2011). The individual AHKs of *Arabidopsis* seedlings have nevertheless been observed to be present at endomembranes or in the plasma membrane (PM) to a slightly different extent with a relatively higher outer-to-endo membrane ratio for AHK4 and a lower ratio for AHK2 (Wulfetange *et al.*, 2013). This might imply that the higher number of U-TM domains the less probable is the receptor occurrence in the outer membrane. However, the potential impact of the structure of the respective HK on its subcellular localization as well as the functional importance of both localization types (PM versus ER) remains to be shown.

The functional analyses also revealed that representatives of both groups of AHK2 orthologues are truly functional CK receptors. In maize, two splice versions of the AHK2 orthologue have been identified, termed ZmHK3a and ZmHK3b (Yonekura-Sakakibara *et al.*, 2004). Compared with ZmHK3a which responded to cytokinins, the shorter isoform ZmHK3b lacking a part of the input domain including one TM domain did not respond to any CK tested (Yonekura-Sakakibara *et al.*, 2004). In comparison, both BnCHK1 and BnCHK3 representing two subgroups of AHK2 homologues (BnCHK1 most probably being a syntenic AHK2 orthologue, and BnCHK3 being a duplicated paralogue) bind CKs and reveal binding specificity (Table 4), which is in agreement with differences in the amino acid composition of their CHASE domains Supplementary Fig. S8D at *JXB* online). The most pronounced difference is the higher affinity of BnCHK3 for tZ (the highest affinity for tZ of all BnCHKs tested). Interestingly, compared with both BnCHK1 and BnCHK5, BnCHK3 also reveals higher affinity for aromatic CKs (both BA and mT). On the other hand, BnCHK3 shows lower affinity for tZR and almost no affinity for kinetin. What the physiological meaning of the presence of these two functional versions with certain ligand preference can be and whether

different TM domain topology could contribute to that specificity remains a matter for further studies.

Conclusion

Where complete sets of CK receptor genes can be identified *in silico*, because genomes have been completely sequenced, comprehensive genomic and proteomic studies can be carried out, which greatly facilitates molecular and genetic identifications of members of gene families. However, annotated transcripts and translated reading frames are bioinformatic predictions, and specific regions hosting genes of interest must be confirmed by sequencing. To the authors' knowledge, no cloned full-length *B. napus* CK receptor gene has been previously described and characterized. Here, high-quality sequences (with accompanying database annotations), gene structure determinations of five CHASE-containing HK subfamily genes, and functional ligand-binding analyses of three encoded proteins from *B. napus* are presented. These data extend our knowledge of CK receptors in dicotyledonous crops and provide foundations for more detailed studies on CK perception and signalling pathways in *B. napus* and other *Brassica* species. As CK receptors play major roles in diverse developmental and physiological processes that govern crop yields, including germination, root formation, tolerance to abiotic stress, and pathogen defences, the findings of such studies should have valuable potential applications. Furthermore, the specific sequences of several members of the gene family obtained should aid further phylogenetic studies of this evolutionarily interesting polyploid species.

Supplementary data

Supplementary data are available from *JXB* online.

Figure S1. Calculation of percentage identity and divergence of aligned CDS of *AHK* genes or their nucleotide regions corresponding to conserved CHASE, His kinase, and receiver protein domains.

Figure S2. Grouping of overlapping BAC clones.

Figure S3. Subcloning of restriction fragments carrying putative *BnCHK* genes.

Figure S4. Calculation of percentage identity and divergence of aligned cDNAs of *BnCHK* genes or their nucleotide regions corresponding to the conserved CHASE, His kinase, and receiver protein domains.

Figure S5. Gene structure analysis of *BnCHK* genes.

Figure S6. Predicted transmembrane segments within the BnCHK amino acid sequences.

Figure S7. FASTA format, multiple alignment in Clustal format, cured alignment in Phylip format, and sequence distances of His kinase protein domains from *Brassica napus* (BnCHK and ES900704), *Brassica rapa* (BrCHK), Arabidopsis (AHK), and rice (OsHK) used in phylogenetic analysis.

Figure S8. FASTA format, multiple sequence alignment in Clustal format, cured alignment in Phylip format, sequence distances, and phylogram of CHASE protein domains from *Brassica napus* (BnCHK and ES904730), *Brassica rapa* (BrCHK), and *Brassica oleracea* (asmbl48579).

Table S1. Primers used for cDNA cloning of *BnCHK* genes.

Table S2. Four filters, each containing six fields with individual clones of the BAC library of *Brassica napus*, var. Tapidor (JBnB library), giving positive signals with DNA probes, derived from sequences of the *AHK2* gene and corresponding to the conserved CHASE, His kinase (HK), and receiver (REC) protein domains.

Table S3. Fingerprinted JBnB clones.

Table S4. Exon prediction in *BnCHK* genes by GENSCAN.

Table S5. Positions of transmembrane (TM) segments predicted in BnCHK and AHK proteins by seven different programs.

Table S6. EST database search outcome of BLASTN using CDS of *BnCHK* genes and *BrCHK4* as query.

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