Plant Molecular Biology **53:** 383–397, 2003.

Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress

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Received 22 July 2003; accepted in revised form 9 October 2003

Key words: Brassica napus, NAC domain, stress, transcriptional activation, yeast 2-hybrid

Abstract

Subtractive expressed sequence tag analysis and screening of cDNA libraries derived from *Brassica napus* leaves subjected to mechanical wounding, flea beetle feeding or cold temperatures revealed eight genes encoding NAC-domain transcription factors. The genes were found to be differentially regulated in response to biotic and abiotic stresses including wounding, insect feeding, *Sclerotinia sclerotiorum* infection, cold shock and dehydration. Five BnNAC proteins were orthologous to *Arabidopsis thaliana* ATAF1 or ATAF2 and gave rise to developmental abnormalities similar to the *A. thaliana nam* and *cuc* mutants when expressed ectopically in *A. thaliana*. Transgenic lines expressing *BnNAC14*, exhibited large leaves, thickened stems and hyper-developed lateral root systems similar to that observed with *A. thaliana NAC1*, but also were delayed in bolting and lacked an apical dominant tap root. Several of the BnNAC proteins were capable of activating gene expression in yeast and recognized an element within the CaMV35S promoter. A yeast two-hybrid screen revealed that BnNAC14 interacted with other select BnNAC proteins *in vitro* and identified an additional BnNAC gene, BnNAC485. The protein interaction and transcriptional activation domains were mapped by deletion analysis.

Introduction

Plants are under constant threat from environmental stress and attack from pathogens and vertebrate and invertebrate herbivores. To cope with this onslaught, they have evolved elaborate mechanisms to perceive the attack and a responsive signaling language to tie the reception of information to the induction of appropriate defense strategies. These mechanisms encompass direct physical and chemical defenses, as well as indirect defenses, such as the release of predator-attracting volatiles (Dicke and Van Poecke, 2002). Specific metabolites, proteins and carbohydrates shed by the pathogen or insect (Hammond-Kosack and Jones, 1996; Baker *et al.*, 1997) and polygalacturonide fragments derived from damaged plant cell walls (Thain *et al.*, 1990) interact with specific plant receptors. These serve to initiate signaling cascades leading to local and systemic defense responses that typically consist of changes to plant architecture (e.g. wax accumulation, trichome formation) and the induction of defense proteins, hyperoxidized chemicals and secondary metabolites (Walling, 2000). One of the least understood resistance mechanisms is tolerance, the ability to compensate for herbivore attack by sustaining and re-growing damaged tissue (Strauss and Agrawal, 1999; Stowe *et al.*, 2000). The plant alters its developmental pattern, establishing an eco-physiological equilibrium with the attacker to limit any decrease in fitness (Baldwin and Preston, 1999; Kessler and Baldwin, 2002). Indeed, one of the larger groups of genes affected by herbivore and pathogen interactions are those involved in plant primary metabolism and growth (Reymond *et al.*, 2000; Stotz *et al.*, 2000; Schenk *et al.*, 2000; Hermsmeier *et al.*, 2001).

The regulation of defense gene expression is largely governed by specific transcription factors. A

common theme that has emerged with the sequencing of whole plant genomes is that these factors often belong to large gene families of more than 100 members. Members of the ERF, bZIP, WRKY (Singh *et al.*, 2002) and MYB (Jin and Martin, 1999) families are the best characterized of the plant defense response activators. While some members of these families are specifically involved in the regulation of defense responses to biotic and environmental stress, others appear to coordinate plant developmental pathways (Jin and Martin, 1999; Chen and Singh, 1999; Robatzek and Somssich, 2001).

Another such family of transcription factors are the NAC-domain proteins. The name derives from a conserved domain originally associated with the *NO APICAL MERISTEM* (*NAM*) gene in *Petunia* (Souer *et al.*, 1996) and the *ATAF1*, *ATAF2* and *CUP-SHAPED COTYLEDON* (*CUC*) genes of *Arabidopsis thaliana* (Aida *et al.*, 1997). NAC-domain proteins are unique to plants and comprise large gene families (Kikuchi *et al.*, 2000); 103 members are present within the *A. thaliana* genome and have been implicated in various aspects of plant development. In *Petunia*, *NAM* was expressed at the primordia and meristem boundaries and mutants failed to develop apical shoots (Souer *et al.*, 1996). However, additional *NAM* genes were believed to be involved since co-suppression studies gave rise to plants with further defects including thicker stems, larger leaves and the absence of axillary meristems (Souer *et al.*, 1998). Similarly, *A. thaliana cuc1* and *cuc2* double-mutant lines lacked shoot apical meristem development and also exhibited fused cotyledons, sepals and stamens (Aida *et al.*, 1997, 1999). *NAC1*, another *A. thaliana* NAC-domain gene, was found to be involved in the auxin-dependent formation of the lateral root system (Xie *et al.*, 2000). More recently, NAC-domain genes have been implicated in the plant defense response as the potato *StNAC* gene and *A. thaliana ATAF1* and *ATAF2* genes were induced by pathogen attack and wounding (Collinge and Boller, 2001). Moreover, the geminivirus DNA replication protein, RepA, was found to interact with two NAC-domain proteins, GRAB1 and GRAB2; however, their effect on viral DNA replication or plant development has not been determined (Xie *et al.*, 1999).

All NAC-domain proteins have a common configuration consisting of a conserved amino-terminal NAC domain region proceeded by a highly variable carboxy terminus. Two lines of evidence support the notion that NAC proteins are involved in transcriptional regulation. First, *A. thaliana* lines over-expressing *NAC1* exhibited up-regulation of the auxin-responsive genes, *AIR3* and *DBP*, and a corresponding decrease was observed in anti-sense lines. NAC1 also localized to the nucleus (Xie *et al.*, 2000). Second, early reports indicated that *ATAF1* and *ATAF2* were able to trans-activate the CaMV 35S promoter (Hirt, original GenBank annotation). This was substantiated by Duval *et al.* (2002) who showed that the *A. thaliana NAM* NAC domain recognized a 7 bp region within the CaMV 35S *as-1* (activation sequence 1) element.

In an effort of better understand plant defenses and regulation of such we conducted subtractive expressed sequence tag analysis of *B. napus* subjected to mechanical wounding, flea beetle feeding or cold temperatures. Here we report the isolation and characterization of nine members of the *B. napus* NAC (*BnNAC*) gene family and show that they are differentially regulated in response to biotic and abiotic stress, form heterodimers and give rise to developmental abnormalities when expressed ectopically in *A. thaliana.*

Materials and methods

Plant, fungal and insect materials

Brassica napus line DH12075 and *Arabidopsis thaliana* ecotype Columbia were grown in soil under a 16 h photoperiod with a daytime temperature of 22 °C and a night temperature of 16 °C. Root development in transgenic *A. thaliana* lines was assessed in MS medium with 3% sucrose according to Xie *et al.* (2000). *A. thaliana* Columbia line SALK 030702.55.50.X containing a T-DNA insertion in the At5g63790 locus encoding ATAF2 was obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). Insertion mutant information was obtained from the SIGNAL website at http://signal.salk.edu. The insertion was verified by PCR using the ATAF2-specific primer SALK030702 (5′-TGGCGTTGTACGGTGAGAAAG-3′) and either one of two T-DNA specific primers, LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) or LBb1 (5′-CGCTGGACCGCTTGCTGCAACT-3′) as well as by Southern blotting. A line homozygous for the insertion was used for phenotypic assessment.

Sclerotinia sclerotiorum isolate '100' was originally collected on *B. napus*. Fungal mycelia were grown on potato-dextrose agar (PDA) at 20 °C and stored at −80 °C in 25% glycerol.

Adult flea beetles were collected in sweep nets from a winter canola field (*B. napus* cv. Casino) at the Saskatoon Research Centre farm. Beetles were maintained on a diet of cabbage leaves in mesh cages in a controlled environment chamber (23 °C, 16 h photoperiod with fluorescent lighting). Beetles were starved for 48 h prior to conducting the feeding bioassays.

Construction of normal and subtractive cDNA libraries

Total RNA was extracted from 8-week old *B. napus* DH12075 leaves 2 h after crushing with forceps, after 30 h of constant flea beetle feeding as described below or from plants that had been grown at 5 °C until they reached the expanded six-leaf stage. Poly(A)$^+$ RNA was isolated with Oligotex (Qiagen) following the manufacturer's instructions. cDNA subtraction was performed with the PCR-Select cDNA Subtraction Kit (Clontech). The PCR products were inserted directly into pGEM-T Easy (Promega) and ca. 1500 expressed sequence tags (EST) generated from each library. Normal cDNA libraries were constructed with the ZAPII cDNA synthesis kit (Stratagene). An EST corresponding to the amino-terminus of *B. napus* NAC1-1 (*BnNAC1-1*) was used to screen the cDNA libraries to isolate additional full-length *BnNAC* cDNAs. The *BnNAC* cDNAs were excised *in vivo* from the lambda ZAP Express vector as pBluescript SK phagemids using ExAssist helper phage (Stratagene).

Expression analysis

B. napus leaves were infected with *S. sclerotiorum* according to the method of Li *et al.* (2003). To simulate wounding, leaves were mechanically damaged by crushing with sterile forceps. For flea beetle feeding, plants were placed in cages with starved flea beetles at a density of 100–150 insects per plant. To examine the effect of low temperature and dehydration, 8-week old plants were placed at 5 °C for 24 h or had moisture withheld until the point of wilting.

Tissues were collected at various time intervals and immediately frozen in liquid nitrogen upon removal. Total RNA was isolated by dispensing 200 mg (wet weight) of ground plant tissue into a 1.5 ml microcentrifuge tube containing 1 ml Trizol Reagent (Invitrogen) and RNA extracted according to the manufacturer's protocol. The RNA pellet was washed with 70% ethanol, dried for 5 min and re-suspended in 50 μl RNase-free double-distilled H_2O. To detect *BnNAC* mRNA, 20 μg of total RNA was separated on 1.2% agarose gels in 10 mM phosphate buffer (pH 7.0) and blotted onto a nylon membrane with 10× SSC. Hybridizations were conducted in 0.5 M phosphate buffer (pH 7.2) with 7% SDS, 1% bovine serum albumin and 1 mM EDTA at 65 °C overnight. Membranes were washed twice with 2× SSC at 65 °C for 10 min, twice with 1× SSC/0.5% SDS at 65 °C for 20 min and finally with 0.1× SSC at 20 °C for 10 min. DNA probes consisted of PCR-amplified fragments corresponding to either the conserved amino-terminal region of *BnNAC*1-1 or carboxyl-terminal regions unique to each *BnNAC* and labeled with [α-^{32}P] dCTP with the Prime-A-Gene Labeling System (Invitrogen).

Computational analysis

EST sequences were annotated according to BLAST nucleotide and protein alignments (www.ncbi.nlm.nih.gov). Multiple peptide sequence alignments were performed with ClustalW1.2 and 100 permutations of the multiple alignment generated using Seqboot. Distance matrices were calculated from the permutated alignment with the PAM method in Protdist. Unrooted, neighbor-joined trees were generated with Neighbor and a consensus tree was determined with Consense. Sequence assembly, analysis and phylogenetic tools were accessed through the Canadian Bioinformatics Resource (www.cbr.nrc.ca). Protein secondary structure was determined by SSPRO, PSIpred version 2.4, PFRMAT SS and PHDsec available in the PredictProtein server (cubic.bioc.columbia.edu/predictprotein) and COILS (www.ch.embnet.org/software/COILS). Determination of nuclear localization sequences was performed by PredictNLS (maple.bioc.columbia.edu/predictNLS) with the method described by Cokol *et al.* (2000). Peptide sequences common to proteins that are targeted for rapid turnover were identified with PEST-FIND (www.at.embnet.org/embnet/tools/bio/PESTfind).

Plant transformation

The entire coding regions of six *BnNAC* cDNAs (1-1, 5-1, 5-7, 5-8, 5-11 and 14) were amplified by PCR with PWO DNA polymerase (Stratagene) and oligonucleotide primers containing restriction enzyme sites suitable for cloning into pBI121 (Clontech) downstream of the CaMV 35S promoter. Sense and antisense *BnNAC5-1* were inserted into the *Bam*HI/*Sac*I sites, *BnNAC5-7*, 5 − 8 and 5 − 11 into the *Xba*I/*Sac*I

sites and *BnNAC1-1* and 14 into the *Bam*HI/*Eco*ICRI sites. Plant transformation was carried out according to the floral dip procedure (Clough and Bent, 1998).

Mapping of BnNAC transcription activation domains

The entire coding regions of nine *BnNAC* cDNAs (*BnNAC* 1-1, 3, 485, 5-1, 5-7, 5-8, 5-11, 8 and 14) were amplified by PCR with PWO DNA polymerase and inserted into the *Sal*I and *Not*I sites of pDBLeu (Invitrogen) which fused the BnNAC open reading frame to the GAL4 DNA-binding domain (DB). Each construct was introduced into *Saccharomyces cerevisiae* MaV203 and tested in a one-hybrid system for the ability to activate transcription of the *LacZ* reporter. The *BnNAC* genes were divided into regions encoding five NAC subdomains according to the scheme of Duval *et al.* (2002) and two carboxyl-terminal subdomains. Various combinations of the subdomains derived from *BnNAC14*, *BnNAC5-8* and *BnNAC485* were amplified by PCR, inserted into pDBLeu and examined for transcriptional activation of the *LacZ* reporter gene.

Identification of BnNAC-interacting proteins with a yeast 2-hybrid screen

A yeast two-hybrid cDNA library was constructed from poly(A) mRNA isolated from the aboveground parts of four-leaf stage seedlings of *B. napus* DH12075 and cloned into the GAL4 activation domain (AD) vector, pPC86, using the SuperScript Plasmid System (Invitrogen). The entire coding regions of the *BnNAC5-1* and *BnNAC14*, which encoded the only two BnNAC proteins that do not activate transcription directly, were used as a bait to screen the *B. napus* cDNA library with the PROQUEST Two-Hybrid System (Invitrogen). About 1.8×10^6 *S. cerevisiae* transformants were subjected to two-hybrid selection on synthetic complete (SC) medium lacking leucine, tryptophan and histidine but containing 15 mM 3-amino-1,2,4-triazole (3AT). Putative His+ (3AT-resistant) transformants were tested for the induction of two other reporter genes, *URA3* and *LacZ*, and reassessed by a second transformation into yeast.

Regions involved in protein interaction were mapped by fusing the NAC or carboxyl-terminal domains of *BnNAC5-7, BnNAC5-8* and *BnNAC485* to the GAL4 AD in pPC86. These were tested for interaction with whole BnNAC14 or constructs encoding various combinations of BnNAC14 NAC and carboxyl-terminal domains in pDBLeu.

Interaction of BnNAC proteins with CaMV 35S promoter elements

Plasmid pFL759-1 containing the CaMV 35S promoter, and resident *as-1* element, fused to β-glucurondiase(GUS) was obtained from P. Fobert (NRCC, Saskatoon, Canada) and has been described by Stonehouse (2002). Three independent *S. cerevisiae* MaV203 colonies transformed with pDBLeu or pDBLeuBnNAC and pFL759-1 were assessed. Total protein in each sample was determined using Bradford reagent (BioRad Laboratories) and GUS specific activity expressed in units equivalent to pmol umbelliferyl released per μg protein per hour. The data were normalized by log (x + 1.0) transformation, analysis of variance was performed with general linear models (Proc GLM) and the differences between means were determined with the Waller-Duncan K-ratio t-test (P <0.05) (SAS Institute Inc. 2000, version 8.2.0).

Results

Isolation of B. napus *NAC domain-containing genes*

About 1500 ESTs were generated from each of three subtractive cDNA libraries developed from plants that had been grown at 5 °C, mechanically wounded or subjected to herbivory by flea beetles. About 5% of the ESTs encoded proteins involved in signal transduction pathways (G-proteins, MAP kinases and transcription factors) or contained domains indicative of DNA interaction (helix-turn-helix and zinc finger). Proteins containing a NAC domain similar to the wound-inducible *ATAF1* and *ATAF2* were encoded by a small proportion of these and were investigated further. Since the PCR-based subtractive library procedure generated only small cDNA fragments, *BnNAC1-1* was used to screen normal cDNA libraries derived from *B. napus* leaf tissue treated as above. cDNAs encoding eight distinct types of BnNAC proteins ranging in size from 252 to 285 amino acids were identified in this manner and denoted BnNAC1-1, 3, 5-1, 5-7, 5-8, 5-11, 8 and 14. Each protein possessed a 160 amino acid amino-terminal NAC domain consisting of five highly conserved regions, denoted A–E as per Kikuchi *et al.* (2000), which were interspersed by short, variable, spacers (Figure 1A). The region encoding subdomain C and extending through the first half of subdomain D contained a high proportion of conserved basic

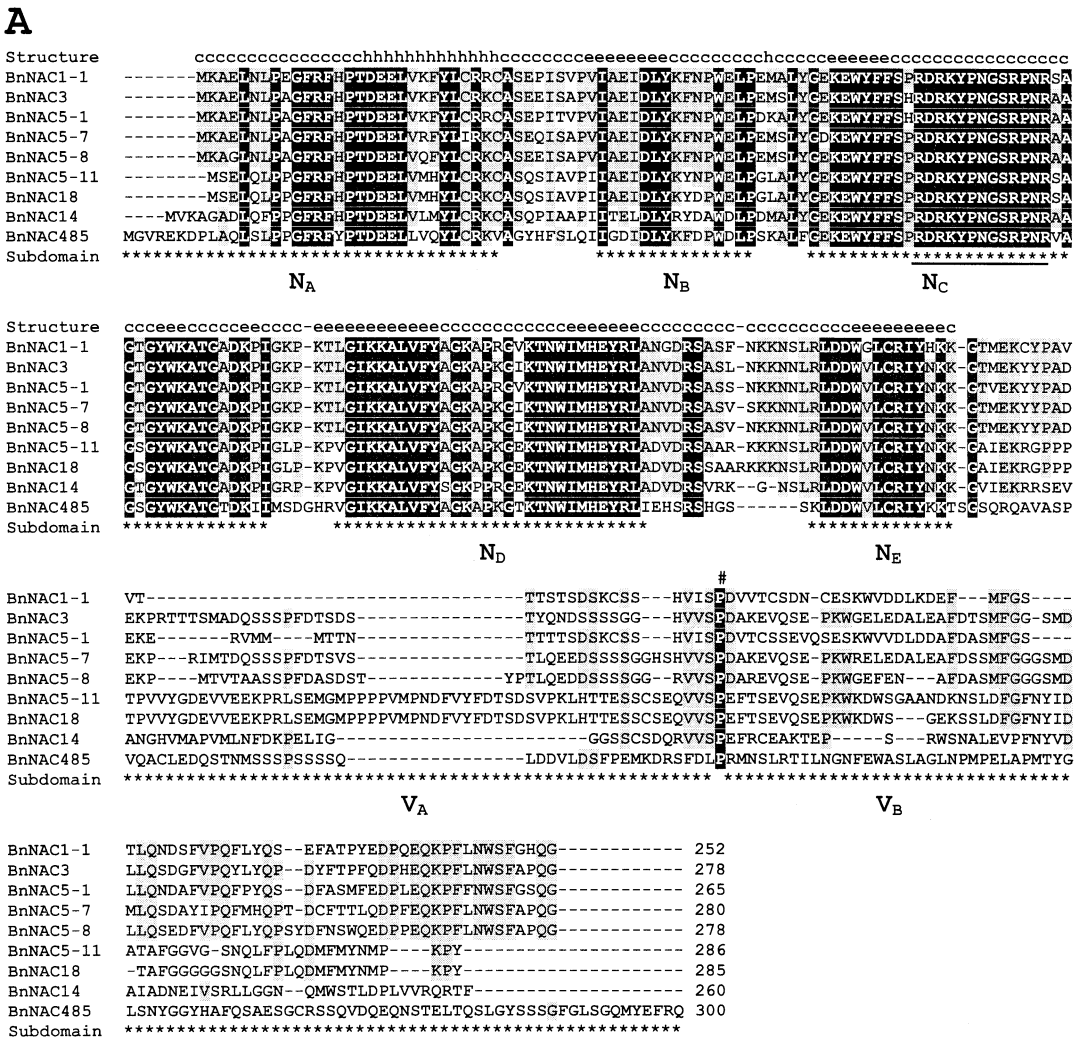

Figure 1. Multiple sequence alignment of *B. napus* NAC domain-containing proteins. Residues that are identical in all (white on black background) or most (black on gray background) proteins are highlighted. A. Translated amino acid sequence of nine BnNAC cDNAs showing residues (*) within the amino-terminal NAC domains comprising five subdomains denoted N_{A-E}. A common Pro residue (#) used to divide the variable carboxy-terminal domain into two subdomains V_{A-B} and a putative nuclear localization signal (underline) are shown. Consensus secondary structure prediction of BnNAC NAC domains as determined using SSPRO, PSIpred, PFRMAT SS and PHDsec is shown above and consists of α-helix (h), extended β-sheet (e) and coiled/loop (c) regions. All BnNAC NAC domains had essentially the same secondary structure. B. Alignment of translated BnNAC variable carboxy-terminal domains with corresponding regions of the most closely related plant homologue. Amino acid residues that may contribute to transcription activation and that are present in all (capitals) or most (lower case) proteins are shown above each alignment. These include Asp (D), Glu (E), Gln (Q), Pro (P), Ser (S) and Thr (T). Predicted PEST domains (boxed) associated with protein turnover are also shown. B1. Alignment of BnNAC14 (AY245885), *A. thaliana* AtGRAB1 (AC010704_4) and *Triticum sativa* TrGRAB1 (AJ010829). B2. Alignment of BnNAC5-11 (AY245884), BnNAC18 (AY245886) and *A. thaliana* ATAF1 (X74755). B3. Alignment of BnNAC1-1 (AY245879), BnNAC3 (AY245880), BnNAC5-1 (AY245881), BnNAC5-7 (AY245882), BnNAC5-8 (AY245883) and *A. thaliana* ATAF2 (AK118910). B4. Alignment of BnNAC485 (AY245887) and *A. thaliana* NAC domain proteins at locus At4g27410 (NM_118875) and locus At1g52890 (NM_104167).

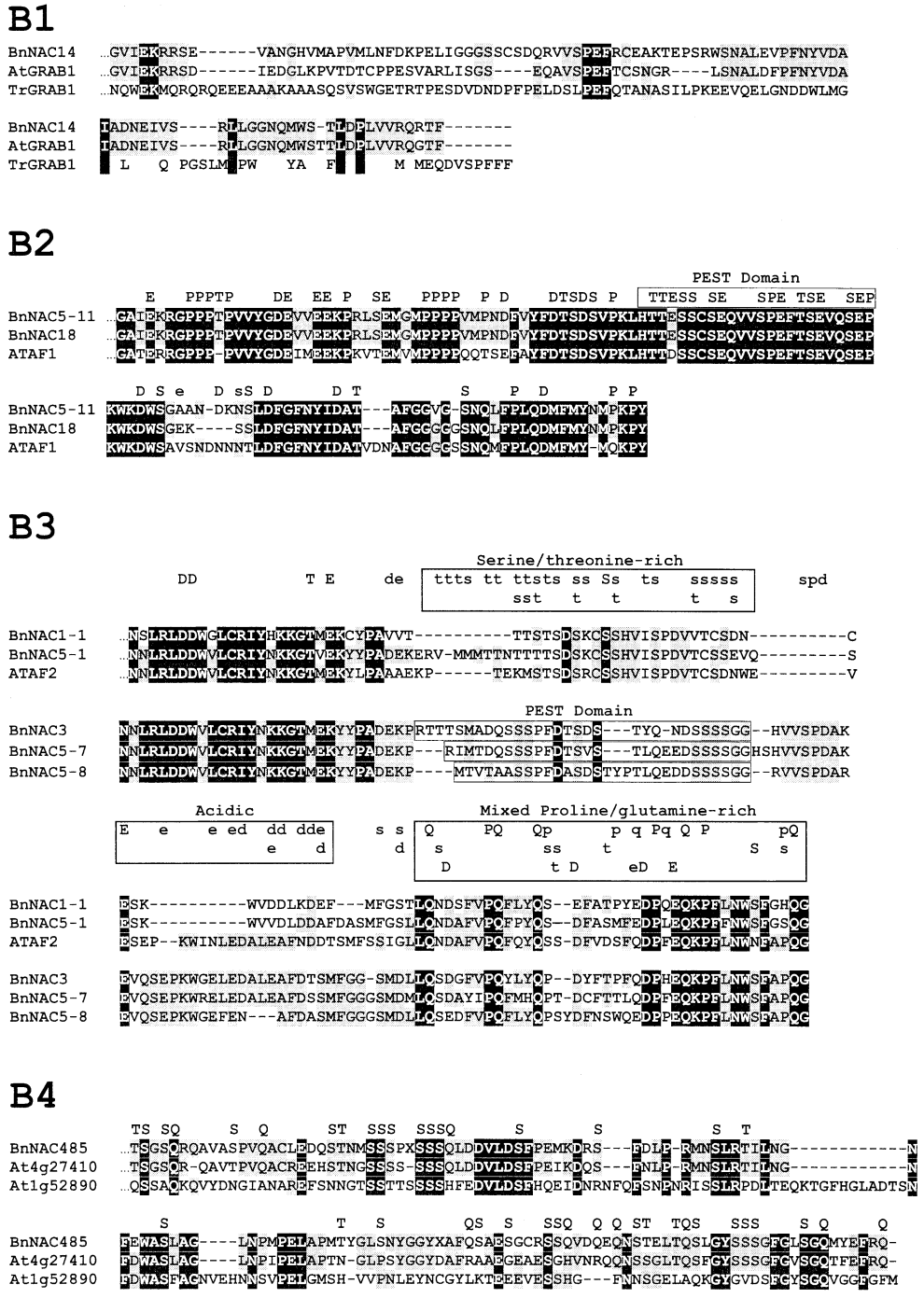

Figure 1. (Continued.)

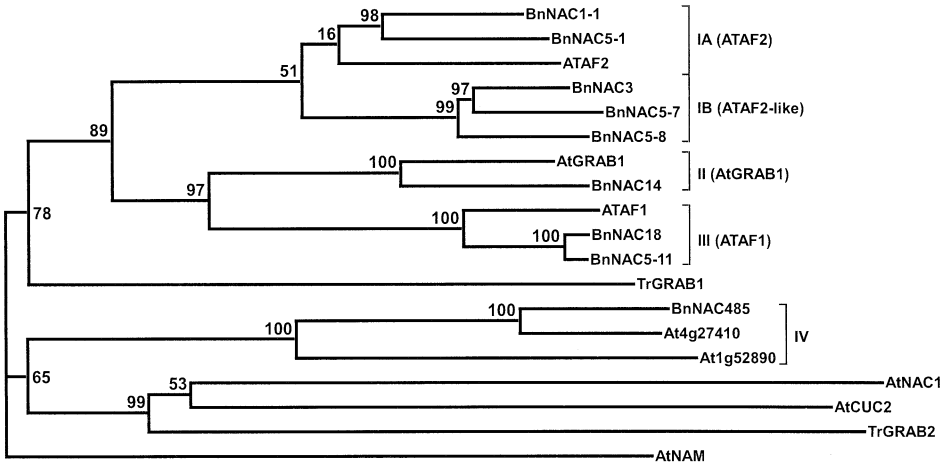

Figure 2. Phylogenetic relationship between *B. napus* (BnNAC) and other plant NAC-domain proteins. The translated sequences used for alignment were as follows: BnNAC1-1, BnNAC3, BnNAC5-1, BnNAC5-7, BnNAC5-8, BnNAC5-11, BnNAC14, BnNAC18, BnNAC485, *A. thaliana* AtGRAB1 and *Triticum sativa* TrGRAB1, *A. thaliana* ATAF1, *A. thaliana* ATAF2, *A. thaliana* NAC-domain proteins at locus At4g27410 and locus At1g52890, *A. thaliana* AtNAC1 (AF198054) and *A. thaliana* AtNAM (AF123311). Groups were assigned based on similarity to ATAF2 (Group I), AtGRAB1 (Group II), ATAF1 (Group III) and BnNAC485 (Group IV). The tree is a consensus of 100 neighbor-joined trees and numbers at the nodes indicate bootstrap values.

amino acids. Previously, a nuclear localization signal was proposed to reside in subdomain C (Kikuchu *et al.*, 2000); however, neither this region nor any other sequence in the NAC domain conformed to canonical monopartite and bipartite nuclear localization elements as determined with PredictNLS software. The latter portion of subdomain A was predicted to form an α-helix, while the remainder of the NAC domain consisted of alternating β-sheet and β-strand (loop) structures.

All of the BnNAC carboxy-terminal domains were predicted to lack extensive α-helix and β-sheet structure and consisted primarily of loops. These domains contained regions that were rich in either serine/threonine, acidic and proline/glutamine residues (Figure 1B). The carboxyl-terminal regions of the Group IB (ATAF2-like) and Group III (ATAF1) BnNAC proteins were predicted to contain PEST domains common to regulatory proteins that are rapidly targeted for degradation by the ubiquitin-26S proteosome pathway (Rechsteiner and Rogers, 1996).

A BLASTX scan of the *A. thaliana* genomic DNA sequence identified 103 open reading frames that could encode NAC domain proteins. The highly variable carboxy-terminal regions of the BnNAC proteins were compared to the corresponding regions of these *A. thaliana* proteins to identify homologues or related gene families (Figure 1B). BnNAC14 was closely related to the AtGRAB1-like protein but neither bore much similarity to the *Triticum aestivum* TrGRAB1 (Figure 1B.1). BnNAC5-11 and BnNAC18 exhibited a high degree of identity to one another and appeared to be homologues of ATAF1 (Figure 1B.2). Two regions within the variable carboxyl domains of BnNAC1-1, 3, 5-1, 5-7 and 5-8 were identical to ATAF2 (Figure 1B.3). Phylogenetic analysis confirmed these relationships with distinct nodes for the ATAF2 (Group IA and IB), AtGRAB1 (Group II) and ATAF1 (Group III) related genes (Figure 2). The *BnNAC485* cDNA isolated during a yeast two-hybrid screen (discussed later) encoded a 300 amino acid protein, the largest of the BnNAC reported here. Analysis of the variable domain identified a putative *A. thaliana* homologue of *BnNAC485* residing at locus At4g27410 with a related gene at At1g52890 (Figure 1B.4). Phylogenetic analysis revealed that these three proteins formed a single clade denoted Group IV (Figure 2). Other well-characterized NAC domain proteins, such as TrGRAB1, TrGRAB2, AtNAC1, AtCUC2 and AtNAM were not closely related to the BnNAC groups.

Expression of BnNAC *genes*

We identified several *B. napus* NAC domain genes amongst ESTs enriched for induction in response to wounding, growth at a reduced temperature and insect feeding. Northern blot analysis with the conserved amino terminus of the *BnNAC1-1* cDNA as a probe

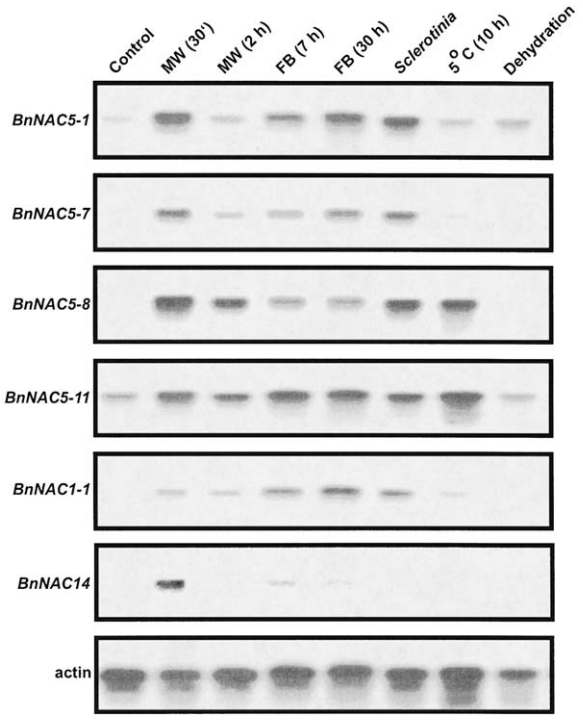

Figure 3. Northern blot analysis of individual *B. napus* NAC gene expression in response to biotic and abiotic stress. Leaves from 8-week old *B. napus* DH12075 plants were sampled at 30 min and 2 h after mechanical wounding by crushing with forceps, being fed upon by flea beetles for 7 and 30 h, being infected with *Sclerotinia sclerotiorum* for 18 h, being subjected to 5 °C for 10 h or dehydrated to the point of wilting. DNA probes used corresponded to the carboxy-terminal region unique to each BnNAC gene. A 5 μg portion of total RNA was loaded and each blot was probed with the *B. napus* actin cDNA to verify loading uniformity.

revealed that *BnNAC* gene expression was induced rapidly (within 30 min) in response to mechanical wounding. This response was transient, returning to basal levels of expression within 10 h. Damage caused by flea beetle herbivory or infection with *S. sclerotiorum* also induced gene expression; however, the level did not dissipate with time as damage to the tissue was continuous. Transferring plants from 22 °C to 5 °C induced expression within 4 h of exposure; however, levels continued to increase for the initial 24 h period and then remained elevated. Conversely, dehydration to the point of wilting did not appear to affect expression.

DNA probes specific to the variable carboxyl-terminal domains were used to determine the expression profiles of six *BnNAC* genes or gene families (Figure 3). *BnNAC1-1* expression was induced by insect and pathogen attack and to a lesser extent by mechanical wounding. Insect, pathogen and mechanical damage also induced the expression of BnNAC5-1 and BnNAC5-7, but both responded more strongly to mechanical wounding than *BnNAC1-1*. *BnNAC14* expression was induced by mechanical wounding, and to a lesser degree by flea beetle damage, but not by pathogen infection, cold or dehydration. *BnNAC5-8* and *BnNAC5-11* were the only genes found to be highly induced by exposure to lower temperature and were responsive to other forms of tissue damage as well. While the basal levels of *BnNAC* expression varied among the individual genes, only *BnNAC5-1* was definitively induced by dehydration.

Expression of BnNAC *genes in* A. thaliana

Six *BnNAC* genes, as well as a *BnNAC5-1* antisense construct, were introduced into *A. thaliana* and expressed under the control of the constitutive CaMV35S promoter. Plants transformed with the antisense *BnNAC5-1* construct did not exhibit any phenotypic difference from either untransformed plants or lines transformed with vector alone. Transgenic plants expressing *BnNAC1-1*, *5-1*, *5-7*, *5-8* and *5-11* sense constructs and representing the ATAF1 and ATAF2 groups were difficult to isolate. Transformed plants, as confirmed by PCR and growth under kanamycin selection, failed to develop adequate root systems. These plants did not generally progress beyond the first true leaf stage and exhibited punctate chlorotic patterns on the first true leaves prior to death. Those lines that survived antibiotic selection exhibited severe developmental abnormalities (Figure 4), with *BnNAC5-7*, *5-8* and *5-11* being more pronounced than *BnNAC1-1* or *BnNAC5-1*. The plants generally formed tight rosettes consisting of small curled or cup-shaped leaves. Buds developed in tight clusters in close proximity to the rosette, bolts were rare and if present were usually less than 1 cm long. The inflorescence possessed poorly developed or fused sepals and petals causing the pistil and stamen to protrude. Siliques formed very rarely and gave rise to small seed that failed to germinate. Due to the small plant size and sterility of the transformed lines, it was not possible to isolate sufficient amounts of mRNA for transcript analysis. As such, it could not be determined if the observed phenotypes were due to ectopic expression of the *BnNAC* genes or co-suppression of the *ATAF1* and *ATAF2* homologues. However, a homozygous *A. thaliana ATAF2* T-DNA insertion line did not exhibit any of the aforementioned abnormalities indicating that the phenotype is likely due to ectopic *BnNAC* expression.

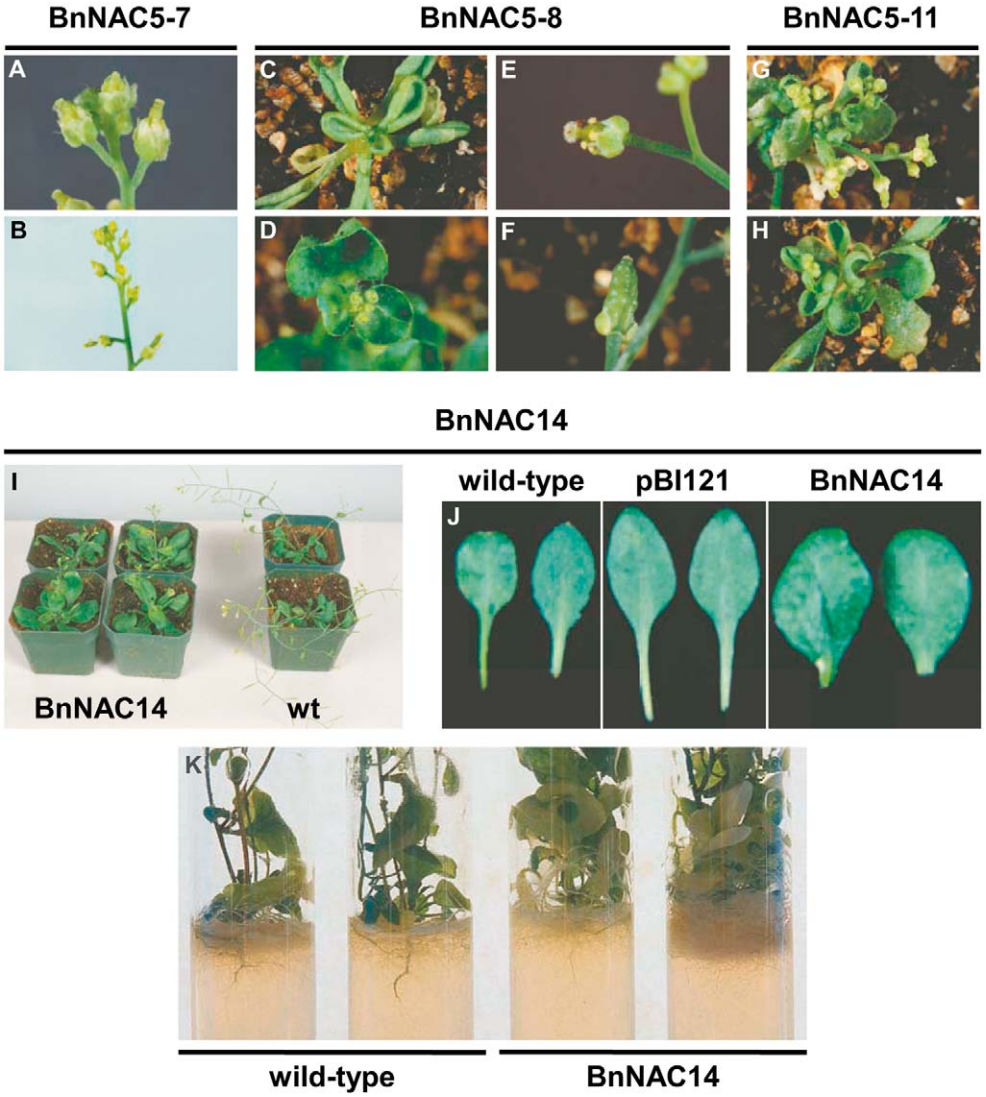

Figure 4. Ectopic expression of BnNAC genes in *A. thaliana*. Expression of BnNAC5-7, BnNAC5-8 and BnNAC5-11 typically resulted in underdeveloped and fused sepals and petals leading to protrusion of the pistil (A, B, E–H) and curled or cup-shaped cotyledons and true leaves (C, D, G and H). Expression of BnNAC14 typically resulted in delay in bolting (I), larger leaves (J) and hyper-developed lateral root formation and absence of root apical dominance (K).

Conversely, plants confirmed to express *BnNAC14* by northern blot analysis exhibited a 'robust' phenotype. Leaves from these lines grew to approximately twice the normal width and were wrinkled or undulate in appearance (Figure 4). In time course experiments, the *BnNAC14* lines germinated and grew at the same rate as non-transformed plants until they reached the rosette stage. At this point, the untransformed plants proceeded to bolt while the *BnNAC14* lines remained in the rosette stage for an additional 3–10 days before the first bolt appeared. During this period leaf size, leaf number and root mass continued to increase. These plants generally possessed thicker stems, although flower development, silique formation and seed set were not affected. The *BnNAC14* lines also lacked an apical-dominant taproot present in untransformed lines and exhibited extensive lateral root system development near the surface (Figure 4).

BnNAC proteins as transcriptional activators

Recent studies demonstrated that a transcription activation domain resided within the carboxy-terminal re-

gion of two *A. thaliana* NAC domain proteins, namely AtNAM and NAC1 (Xie *et al.*, 2000; Duval *et al.*, 2002). Since the carboxy terminus is highly variable among the NAC domain proteins, we used a yeast-one-hybrid system to examine whether the nine BnNAC proteins could also function as transcriptional activators. Each of the BnNAC open reading frames were fused to the GAL4 DNA-binding domain and the chimera placed under the control of the *ADH1* promoter. These constructs were introduced into *S. cerevisiae* MaV203 that contained a *LacZ* reporter gene under the control of the *GAL1* promoter with an associated GAL4 DNA binding site. Beta-galactosidase activity was detected with lines expressing members of the ATAF1 (BnNAC5-11 and BnNAC18) and ATAF2 (BnNAC1-1, BnNAC3, BnNAC5-7 and BnNAC5-8) groups, as well as with BnNAC485 (Figure 5). Yeast lines expressing BnNAC14, belonging to the At-GRAB1 group, exhibited only a very small amount of β-galactosidase activity, whereas BnNAC5-1 was the only ATAF2-like protein that did not activate transcription of the reporter gene. Transcripts corresponding to the *BnNAC5-1* fusion were detected in this yeast line.

Deletion analysis of BnNAC5-8 and BnNAC485 revealed that the variable carboxy-terminal domain was responsible for transcriptional activation, whereas the conserved NAC domain was unable to promote transcription. With BnNAC5-8, the region comprising the acidic and proline/glutamine-rich elements (Figure 1B) was alone responsible for transcription activation, while the serine/threonine-rich region did not function in this regard (Figure 5). Neither of the BnNAC485 carboxy-terminal subdomains was capable of promoting transcription when examined alone. Deletion analysis of BnNAC14 revealed that the slight transcription activation activity observed resided within the first two conserved NAC sub-domains, N_{A-B}. However, this activity was lost or reduced when N_{A-B} was associated with additional NAC subdomains or in the context of the entire protein and may be merely an experimental artifact.

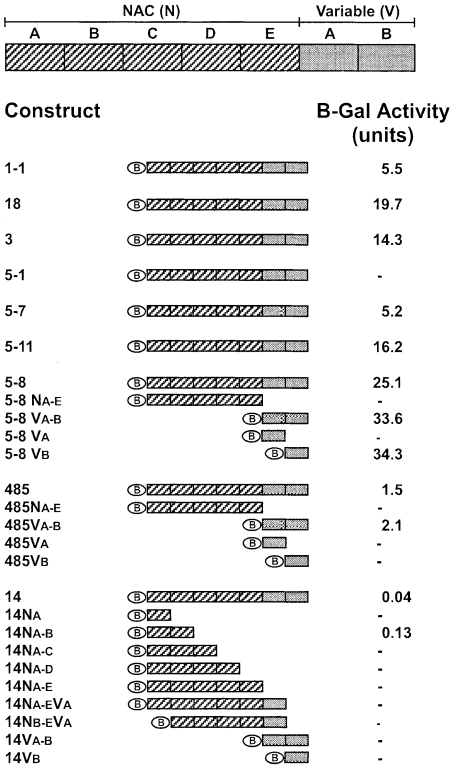

Figure 5. Determination of *B. napus* NAC protein transcription activation potential and mapping of activation domains with the yeast one-hybrid system. The BnNAC proteins were subdivided into five NAC subdomains (N_{A-E}) and two carboxy-terminal variable domains (V_{A-B}) and fused to the GAL4 DNA-binding domain (B). The constructs were introduced into *S. cerevisiae* MaV203 which contained a *LacZ* reporter gene under the control of the *GAL1* promoter with an associated GAL4 DNA-binding site. Beta-galactosidase activity (units) was indicative of transcription activation.

Interaction of BnNAC proteins with the CaMV 35S promoter

The region comprising the N_{D-E} subdomains of AtNAM and NAC1 was shown to recognize the AGGGATG within the *as-1* activation element of the CaMV 35S promoter (Xie *et al.*, 2000; Duval *et al.*, 2002). The N_{D-E} subdomains exhibit a high degree of sequence similarity; however, the region separating the two subdomains of the BnNAC Group I (ATAF2) and Group III (ATAF1) proteins differs extensively from that present in AtNAM, NAC1, BnNAC14 or BnNAC485. The ability of the BnNAC proteins to bind to and trans-activate the CaMV 35S promoter was assessed in yeast using a GUS gene fused to a minimal CaMV 35S promoter but containing the *as-1* activation element. Each of the Group I and Group III BnNAC proteins, with the exception of BnNAC5-1, was able to activate transcription of the minimal CaMV 35S promoter (Figure 6). The amount of GUS activity did not correlate with the strength of the resident activation domain as determined earlier (Figure 5) suggesting that the degree of trans-activation could be a consequence of both the avidity of DNA binding and the efficiency of activation. Neither BnNAC14

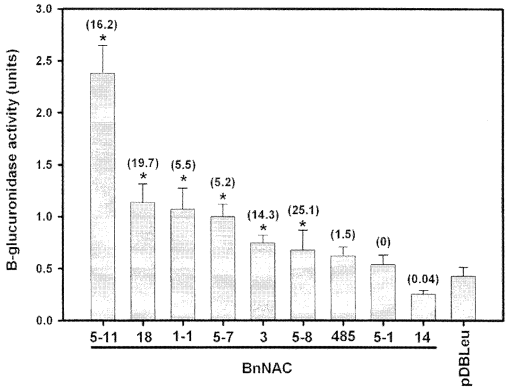

Figure 6. Transactivation of the CaMV 35S promoter by BnNAC proteins. Constructs designed to express individual BnNAC proteins were introduced into *S. cerevisiae* MaV203 in conjunction with a second construct which contained a minimal CaMV 35S promoter and the *as-1* activation sequence fused to the β-glucuronidase gene. Reporter activities significantly greater (analysis of variance, $P < 0.05$) than the control pDBLEU are indicated (*). Values in parenthesis above each bar show amount of β-galactosidase activity from Figure 5 as an indication of activation domain strength.

nor BnNAC485, both of which possessed weak activation domains, were able to activate the CaMV 35S promoter.

Isolation of proteins interacting with BnNAC

A yeast two-hybrid screen was conducted to determine if BnNAC14 and BnNAC5-1 were able to recruit ancillary proteins to the transcription complex. These were the only two BnNAC proteins suitable for use as bait proteins as they were unable to directly activate transcription. The screen performed with BnNAC5-1 did not identify any interacting partners; however, when BnNAC14 was used several independent clones were isolated that encoded either BnNAC5-8 or BnNAC485 (Figure 7). BnNAC14 was subsequently found to interact to a lesser degree with BnNAC3 and BnNAC5-11 and slightly with BnNAC1-1 and BnNAC18 based upon reporter gene expression. BnNAC5-1 did not interact with itself or any of the other BnNAC proteins. Again, transcripts corresponding to the *BnNAC5-1* fusion were detected in this yeast line.

BnNAC14 interacted with the NAC domains alone of BnNAC5-8 or BnNAC485, although several-fold less so than with the intact proteins (Figure 7). This may have been due to either enhanced interaction of the intact proteins with BnNAC14 or increased reporter gene expression due the presence of an additional activation domain from BnNAC5-8 or BnNAC485. Deletion of either the BnNAC14 N_A or V_{A-B} subdomains completely abolished interaction with BnNAC5-8 and BnNAC485 (Figure 7) indicating that both the amino and carboxy-terminal domains are also involved in BnNAC heterodimer formation. The NAC domain of BnNAC5-7 exhibited only a slight interaction with BnNAC14, whereas neither the BnNAC5-8 nor BnNAC485 carboxy-terminal regions alone interacted with BnNAC14.

Discussion

Structural characterization of BnNAC *proteins*

Genes encoding nine BnNAC proteins were isolated from cDNA libraries constructed with tissues subjected to biotic and abiotic stress. Each protein contained a highly conserved amino-terminal NAC domain and a smaller, variable, carboxy-terminal region. Phylogenetic analysis conducted using protein sequences from the nine BnNAC and 103 *A. thaliana* NAC-like proteins revealed that the BnNAC proteins comprised only four distinct groups, each of which corresponded to a sole *A. thaliana* orthologue. This would indicate the presence of a greatly expanded NAC gene family in *B. napus*, arising from genome and gene duplication events during and after speciation.

Xie *et al.* (2000) first reported that NAC1 bound to a region of the CaMV35S promoter encompassed by the *as-1* activation element and distinct from the bZIP recognition sequence. Subsequently, DNAse I footprinting with AtNAM mapped the *cis*-interacting element to the AGGGATG sequence (Duval *et al.*, 2002). In this study, we demonstrated that each of the BnNAC ATAF1 (Group III) and ATAF2-like (Group I) proteins, except BnNAC5-1, were capable of transactivation of a minimal CaMV35S promoter containing the *as-1* element. The *as-1* activation region is a promiscuous element capable of recruiting plant transcription factors, such as the ZIP proteins ASF-1, OBF4, OBF5 and TGA-1, to the CaMV 35S promoter. While the interaction of select NAC proteins with this element is an interesting experimental tool, understanding the biological significance lies in identifying plant genes that are also regulated by this *cis* element.

The NAC N_{D-E} subdomains are required for DNA interaction and were reported to form a helix-turn-helix motif of the type involved in DNA binding (Kikuchi *et al.*, 2000; Duval *et al.*, 2002). However, we were unable to identify this motif using several secondary structure prediction models (SSPRO, PSIpred

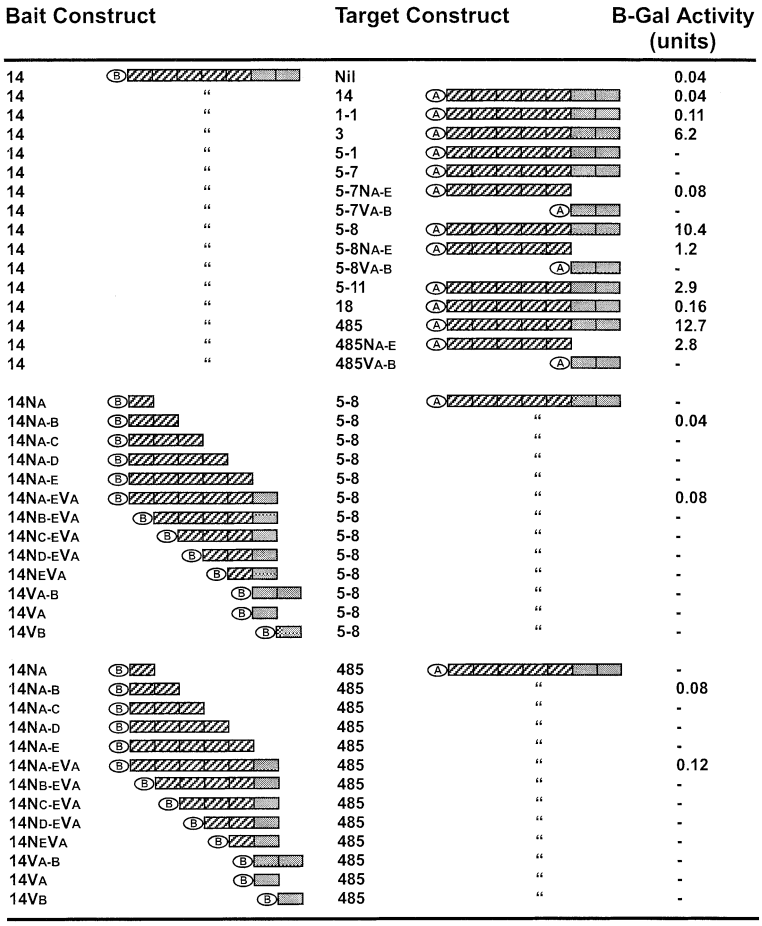

Figure 7. Heterodimer formation between BnNAC14 and other BnNAC proteins and mapping of protein interaction domains. The BnNAC proteins were subdivided into five NAC subdomains (N_{A-E}) and two carboxy-terminal variable domains (V_{A-B}) and fused to the GAL4 activation domain (A) or with BnNAC14 and its derivatives to the GAL DNA-binding domain (B). The constructs were introduced into *S. cerevisiae* MaV203 which contained a *LacZ* reporter gene under the control of the *GAL1* promoter with an associated GAL4 DNA binding site. Beta-galactosidase activity (units) was indicative of protein-protein interaction.

version 2.4, PFRMAT SS, PHDsec and COILS). Rather we predicted that the NAC domains of AtNAM, AtNAC1 and the nine BnNAC proteins comprise an alternating arrangement of primarily β-sheets in the highly conserved region, and β-strands (extended loops) spanning the inter-subdomain spacers as well as the putative nuclear localization sequence. The only α-helical region was found in the latter half of subdomain N_A, that was demonstrated to be involved in protein interactions leading to heterodimer formation. Similarly, the D1 domain of the tobacco bZIP transcription factor, BZI-1, also contained an α-helical region that was thought to provide an interface for interaction with the ankyrin-repeat protein, ANK1; this being required for auxin signaling and response to pathogens (Kuhlmann *et al.*, 2003). It seems more likely that regions within the NAC domains form a unique type of DNA binding motif such as the β-barrel or β-sandwich model proposed for the *A. thaliana* abscisic acid-responsive NAC protein, ANAC (Greve *et al.*, 2003). In this model, conserved residues form a β-sheet scaffold on which the variable inter-subdomain moieties are displayed so as to permit interaction with other proteins or DNA.

Several BnNAC proteins were able to activate transcription in yeast cells and this ability was mapped to the variable carboxy-terminal domain in accordance with that reported for NAC1 (Xie *et al.*, 2000) and AtNAM (Duval *et al.*, 2002). The BnNAC proteins similar to ATAF1 (Group III) and ATAF2 (Group I) exhibited the highest levels of transcription activation. The carboxy-terminal domains of these proteins possessed

distinct regions rich in either serine/threonine, acidic or proline/glutamine residues. This arrangement is similar to that found in the activation domains of other transcriptional regulators (Johnson *et al.*, 1993). The latter half of the BnNAC5-8 carboxy-terminal domain, comprised of the acidic and proline/glutamine-rich regions, was sufficient for full activation. Recently, the proline-rich activation domain of *A. thaliana* bZIP transcription factors was found to interact with two additional proteins, GPRI1 and GPRI2. In addition to containing a GARP DNA-binding domain, these ancillary proteins also possessed acidic and proline-rich transcriptional activation domains (Tamai *et al.*, 2002) suggesting that recruitment of the RNA polymerase transcription complex by proline-rich regions may be mediated by protein interactions at multiple levels.

The NAC domains of AtNAC1 were shown to interact with one another (Xie *et al.*, 2000); however, we saw no evidence of homodimerization with BnNAC14 or BnNAC5-1. Rather, BnNAC14 recruited other BnNAC proteins that were capable of functioning as transcriptional activators despite being unable to activate transcription directly itself. The BnNAC NAC domain was found to be important for heterodimer formation based on the observations that the NAC domain itself was sufficient for interaction, albeit at reduced levels as compared to the intact protein, and deletion of the N_A subdomain abolished interaction. In addition, three of the five amino acid differences between the NAC domains of BnNAC5-8 (strong interaction with BnNAC14) and BnNAC5-7 (limited interaction), reside within or in close proximity to the N_A subdomain. These changes consist of $Gln^{22} > Arg^{22}$ and $Cys^{26} > Ile^{26}$ in the α-helical region of the N_A subdomain and $Glu^{33} > Gln^{33}$ in the region between the N_A and N_B subdomains. Two lines of evidence supported the notion that the variable carboxy-terminal domain may also be involved in protein interaction in addition to their role in transcriptional activation. First, although the variable domains of BnNAC5-8 and BnNAC485 did not interact alone with intact BnNAC14, reporter gene expression increased many fold when associated with their respective NAC domains. Second, deletion of the BnNAC14 V_B subdomain virtually eliminated interaction with both BnNAC5-8 and BnNAC485.

Functional characterization of BnNAC proteins

NAC1 is known to stimulate the expression of auxin-responsive genes, such as *AIR3* and *DBP*, and *NAC1* gene expression is induced upon exposure to auxin (Xie *et al.*, 2000). However, auxin also directs the degradation of the NAC1 protein via the ubiquitin-dependent pathway and the E3 ubiquitin ligase, SINAT5, was found to ubiquitinate NAC1 in an auxin-dependent manner (Xie *et al.*, 2002). ANAC expression was induced exclusively by abscicic acid and was found to interact with another putative E3 ubiquitin ligase, RH2a (Greve *et al.*, 2003). Such opposing mechanisms are believed to finely regulate the hormonal response and consequently plant development. Although we did not find similar interacting partners, ectopic expression of *BnNAC14* in *A. thaliana* gave rise to developmental abnormalities strikingly similar to those observed with *NAC1*, including hyper-development of the lateral root system, large leaves and thicker stems (Xie *et al.*, 2000). It is probable that despite little similarity in their respective variable regions both play similar biological roles in accentuating the auxin response. As well, transgenic *BnNAC14* lines also exhibited a significant delay in bolting and lacked a dominant tap root, suggesting that it may also influence apical meristem formation similar to *AtNAM* (Souer *et al.*, 1998; Sablowski and Meyerowitz, 1998).

Ectopic expression of *BnNAC1-1*, *5-1*, *5-7*, *5-8* and *5-11* representing the ATAF1-like and ATAF2-like groups, resulted in phenotypes more closely associated with *A. thaliana cuc1* and *cuc2* mutants (Aida *et al.*, 1997, 1999). These phenotypes included limited shoot apical meristem formation, cup-shaped or curled cotyledons that in some cases failed to fully separate, and underdeveloped and fused sepals and petals. *CUC1* and *CUC2* are functionally redundant and are expressed in regions bounding the meristem and organ primordia (Ishida *et al.*, 2000; Takada *et al.*, 2001). They are believed to control embryonic shoot apical meristem formation by regulating the expression of the *shoot meristemless* (*STM*) gene since *cuc* mutants do not accumulate *STM* transcripts. Ecoptic expression of *CUC1* (Aida *et al.*, 1999) also leads to ectopic expression of *STM* (Takada *et al.*, 2000); however, we did not observe any phenotypic abnormalities in a homozygous *A. thaliana ATAF2* T-DNA insertion line. It remains to be determined whether the *BnNAC* ATAF-like genes can functionally complement either of the *cuc* mutants.

While the role of certain NAC domain genes in plant development has been established, their significance in defense against biotic and abiotic stress is less clear. *Triticum* GRAB proteins bind to the wheat dwarf

geminivirus RepA protein and prevent viral replication in tissue culture (Xie *et al.*, 1999). Interaction of *A. thaliana* NAC domain protein, TIP, with the turnip crinkle virus capsid protein was required for induction of the hypersensitive response and viral resistance (Ren *et al.*, 2000). While ATAF1 and ATAF2 expression was induced by wounding (Collinge and Boller, 2001), *Alternaria brassicola* infection and ethylene (Schenk *et al.*, 2000), they responded differently to jasmonic and salicylic acid treatment (Schenk *et al.*, 2000). We have shown that BnNAC genes are also subject to differential regulation in response to both biotic (insect herbivory, *S. sclerotiorum* infection) and abiotic (cold, mechanical wounding) stresses. It seems plausible that factors regulating hormonal control of plant growth and development would be also be affected by such stresses as these processes are closely tied to plant health. In fact, some plants are able to tolerate and overcome attack by insect herbivores by increasing growth rate thus compensating for the loss of vital tissue (Hegedus *et al.*, 2002). This relationship between BnNAC gene expression, development and defense responses is currently being examined.

Acknowledgements

This research was made possible with funds from the Canadian Crop Genomics Initiative. We thank the Salk Institute's Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* TDNA insertion mutants. Funding for the SIGnAL indexed insertion mutant collection was provided by the National Science Foundation.

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