Plant Cell Rep
DOI 10.1007/s00299-012-1311-3

ORIGINAL PAPER

Two *Brassica napus* genes encoding NAC transcription factors are involved in response to high-salinity stress

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Received: 17 April 2012 / Revised: 18 June 2012 / Accepted: 1 July 2012

Abstract The NAC protein family is one of the novel classes of plant-specific transcription factors. In this study, two genes (*BnNAC2* and *BnNAC5*) encoding the putative NAC transcription factors were identified in *Brassica napus*. Sequence analysis revealed that the deduced BnNAC proteins contain conserved N-terminal region (NAC domain) and highly divergent C-terminal domain. Yeast transactivation analysis showed that BnNAC2 could activate reporter gene expression, suggesting that BnNAC2 functions as a transcriptional activator. Quantitative RT-PCR analysis revealed that *BnNAC2* was preferentially expressed in flowers, whereas *BnNAC5* mRNAs accumulated at the highest level in stems. Further experimental results indicated that the two genes are high-salinity-, drought- and abscisic acid (ABA)-induced. Overexpression of *BnNAC2* and *BnNAC5* genes in yeast (*Schizosaccharomyces pombe*) remarkably inhibited the growth rate of the host cells, and enhanced the cells sensitive to high-salinity and osmotic stresses. Complementation test indicated that *BnNAC5* could recover the defects such as salt-hypersensitivity and accelerated-leaf senescence of *vni2* T-DNA insertion mutant. Several stress-responsive genes including *COR15A* and *RD29A* were enhanced in the complemented plants. These results suggest that *BnNAC5* may perform the similar function of *VNI2* in response to high-salinity stress and regulation of leaf aging.
Key message *BnNAC2* and *BnNAC5* are salt-, drought- and ABA-induced genes. Overexpression of *BnNAC5* in *Arabidopsis vni2* mutant recovered the mutant defects (salt-hypersensitivity and accelerated-leaf senescence) to the phenotype of wild type.

Keywords *Brassica napus* · NAC domain protein · Gene expression · High-salinity stress · Leaf aging

Communicated by Y. Lu.

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Introduction

Drought and high-salinity are the major abiotic stresses that affect plant growth and agricultural productivity (Chen et al. 2010). In order to respond the external stresses, a lot of genes are induced in plants, leading to physiological and metabolic changes to increase plant stress-tolerance. The expression of these stress-related genes in plants is mainly regulated by specific transcription factors (such as MYB, bZIP, WRKY, AP2/EREBP and NAC, etc.) which play important roles in eliciting stress responses (Singh et al. 2002; Vinocur and Altman 2005; Yamaguchi-Shinozaki and Shinozaki 2006). Previous study reported that *TcWRKY53* was strongly induced by NaCl and drought stresses (Wei et al. 2008). *AtMYB44* transgenic plants displayed the

Published online: 17 July 2012

enhanced tolerance to drought and salt stresses, compared with wild type (Jung et al. 2008). Overexpression of *CBF3* in *Arabidopsis* improved the transgenic plants freezing and dehydration tolerance (Liu et al. 1998). A study reported that ABF2 interacted with the ABA-responsive elements and was involved in ABA/stress responses, and its overexpression altered the expression of ABA/stress-regulated genes, which affected the whole plant survival rate under water-deficit conditions (Kim et al. 2004).

NAC (for *NAM*, *ATAF1/2* and *CUC2*) protein family is a plant-specific transcription factor family. NAC protein has highly conserved DNA-binding domain in N-terminal, and highly variable C-terminal domain, which contains the transcriptional activation site (Souer et al. 1996). It has been reported that 105 and 75 *NAC* genes were identified in Arabidopsis and rice (*Oryza sativa*) genomes, respectively (Ooka et al. 2003). NAC proteins are involved in regulating a lot of plant development processes, such as flower development (Sablowski and Meyerowitz 1998), leaf senescence (John et al. 1997), embryo development (Duval et al. 2002), lateral root formation and development (Xie et al. 2000; He et al. 2005), secondary wall thickenings (Mitsuda et al. 2005). In addition, NAC proteins also participate in plant responses to abiotic and biotic stresses. Previous study revealed that three *NAC* genes (*ANAC019*, *ANAC055*, and *ANAC072*) in *Arabidopsis* were induced by drought, salinity and abscisic acid (ABA). Overexpression of these genes in *Arabidopsis* up-regulated the expression of the stress-inducible genes, resulting in the improved drought- and salt-tolerance of plants (Tran et al. 2004). Similarly, *OsNAC6* is induced by abiotic stresses such as cold, drought, high salinity and jasmonic acid treatment, and overexpression of this gene in rice lead to the increased stress resistance of plants (Nakashima et al. 2007). Recently, a study revealed that an ABA-responsive NAC transcription factor VNI2 integrates ABA-mediated high-salinity stress signals into leaf aging by regulating a subset of COR and RD genes (Yang et al. 2011).

Arabidopsis is considered as a model dicotyledonous plant which genome has been fully sequenced, while rice (*Oryza sativa*) is a very important food crop and also as a good model for studies of monocotyledonous plants because of its small genome (430 Mb). A lot of abiotic stress-induced genes have been well characterized in the two plant species in the past years (Goff et al. 2002; Sasaki et al. 2002; Feng et al. 2002). On the other hand, a little is known about stress-induced genes in *Brassica napus*, especially about *NAC* genes so far. Hegedus et al. (2003) reported that nine *BnNAC* genes were differentially regulated in response to biotic and abiotic stresses including wounding, insect feeding, *Sclerotinia sclerotiorum* infection, cold shock and dehydration. Among them, *BnNAC1-1*, *5-1*, *5-7, 5-8* and *5-11* were orthologous to *Arabidopsis ATAF1* or *ATAF2*. Ectopic expression of these genes in *Arabidopsis* resulted in limited shoot apical meristem formation and cup-shaped or curled cotyledons, similar to Arabidopsis *cuc1* and *cuc2* mutants (Aida et al. 1997, 1999). *BnNAC14* overexpression transgenic plants exhibited the large leaves, thickened stems and hyper-developed lateral root systems, like those observed in *AtNAC1* transgenic plants (Xie et al. 2000). In our study, two *NAC* genes were identified in *Brassica napus*. The two *NAC* genes were induced by high-salinity, drought and ABA (abscisic acid). Overexpression of the two *NAC* genes in yeast (*Schizosaccharomyces pombe*) inhibited the growth rate of the host cells, and enhanced the cells sensitive to high-salinity and osmotic stresses. Overexpression of *BnNAC5* in the T-DNA insertion mutant *vni2* (SALK_143793) conferred a wild-type phenotype to the transgenic plants in response to high-salinity and in regulation of leaf aging.

Materials and methods

Plant growth conditions

Seeds of *Brassica napus* (cv. Zhongyou 821) were surface sterilized and germinated on half-strength Murashige and Skoog (MS) medium (pH 5.8) containing 0.8 % agar under a 16 h light/8 h dark cycle at 25 °C for 7 days. The 7-day-old seedlings were transferred onto MS medium containing 150 mM NaCl, 200 mM mannitol, or 100 μM abscisic acid (ABA) for treatment, respectively.

Quantitative RT-PCR analysis

Total RNA was isolated and purified from different tissues of *B. napus* according to the protocol of the RNeasy plant mini kit (Qiagen). Expression of the selected genes in roots was analyzed by real-time quantitative reverse transcriptase (RT)-PCR using the fluorescent intercalating dye SYBR-Green with a detection system (Opticon 2, MJ Research, Waltham, USA). A *BnACT2* was used as a standard control in RT-PCR reactions. Two-step RT-PCR procedure was performed in all experiments using a method described earlier (Li et al. 2005). In brief, total RNAs, predigested with DNase I for removing any contaminated genomic DNA, were reversely transcribed into cDNAs that were used as templates in PCR reactions. PCR reaction was performed using real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions, with gene-specific primers, *BnNAC2*: up-chain primer, 5′-GTCGAC GAAACTTCCGAGGAC-3′, down-chain primer, 5′-ACA ATCTTCCATGTTCATTTC-3′; *BnNAC5* : up-chain primer, 5′-ATATGATAATGACCATATTGA-3′, down-chain

primer, 5′-GAGTTATTAAAGATAGTCAAC-3′. The C_t (cycle threshold), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, is used as a measure for the starting copy numbers of the target gene. Relative quantity of the target gene expression level was performed using the comparative C_t method. For the accurate amplification of each specific target gene, we carefully designed the primer sets for each gene based on the sequences corresponding to 3′-untranslated region. To achieve optimal amplification, PCR conditions for each primer combination were optimized for annealing temperature, and PCR products were verified by melting curve analysis and confirmed on an agarose gel.

To assay the expression of several stress-responsive marker genes, total RNA was isolated from 2-week-old whole Arabidopsis seedlings, expression levels of COR15A, COR15B, RD29A, RD29B genes were determined by quantitative RT-PCR, using gene-specific primers: COR 15A forward 5′-GCAGATGGTGAGAAAGCGAA-3′ and reverse 5′-GGCATCCTTAGCCTCTCCTG-3′; COR15B forward 5′-ATGACCTCAACGAAGCCACA-3′ and reverse 5′-GTGGCATTCTTAGCCTCTTCT-3′; RD29A forward 5′-TGAAAGGAGGAGGAGGAATGGTTGG-3′ and reverse 5′-ACAAAACACACATAAACATCCAAAGT-3′; RD29B forward 5′-CCAGATAGCGGAGGGGAAAGGACAT-3′ and reverse 5′-AAGTTCACAAACAGAGGCATCATAC-3′. AtACT2 gene was used as a standard control in RT-PCR reactions.

Isolation of *BnNAC* cDNAs

NAC domain proteins from *Arabidopsis thaliana* were used as query sequences for tBLASTn searches of the *B. napus* EST database (http://www.ncbi.nlm.nih.gov/). Vector sequences and low quality sequences were removed manually from the resulting hits. The remaining non-redundant *B. napus* assembled sequences with the highest similarity to the query sequences were kept as putative NAC domain genes.

From these gene predictions, gene-specific primers were designed for PCR to isolate the cDNA and its genomic DNA. *BnNAC2*: up-chain primer, 5′-CTTGGATCCATGGACATGAATCCCAACACT-3′, down-chain primer, 5′-GGGGAGCTCCTAAAACTGAAACATACTAGC-3′; *BnNAC5*: up-chain primer, 5′-GGGGGATCCATGGATAAGGTTAAACTTGTA-3′, down-chain primer, 5′-GGGGAGCTCTTAAAGATTTCGTCTGAAACT-3′. The PCR fragments were sequenced by Shanghai Sunny Biotechnology Co, Ltd.

DNA and protein sequence analysis

Sequences of the isolated *B. napus BnNAC* genes (cDNAs) and their deduced proteins were analyzed using DNAstar software (DNAstar Inc., Madison, WI, USA). Protein sequence homology analysis was performed with ClustalW (http://www.ebi.ac.uk/clustalw/), and phylogenetic analysis was employed to investigate the evolutionary relationships among BnNAC proteins. A Neighbor-Joining tree was generated in MEGA3.1. A bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology.

Isolation of *BnNAC2* promoter and construction of *BnNAC2* promoter: *GUS* reporter vector

BnNAC2 5′-flanking sequence was isolated from *B. napus* using Genome Walking Kit (TaKaRa, Dalian, China). A 1,672 bp 5′-flanking fragment (including putative promoter and 5′-untranslated region) of *BnNAC2* gene was amplified by PCR, using primer pair: up-chain primer, 5′-CTTGTCGACTTAAATAAATCCGCCGTGGCG-3′, down-chain primer, 5′-CTTGGATCCGGTTCTGGGGTTGGTTTAGTG-3′. A *Sal* I site and a *BamH* I site were introduced at 5′-terminal and 3′-terminal of *BnNAC2* promoter, respectively. *BnNAC2* promoter fragment was subcloned into *Sal* I/*BamH* I sites of pBI101 vector to generate the chimeric *BnNAC2:GUS* construct.

Arabidopsis transformation and histochemical assay of GUS activity

Arabidopsis transformation was performed by the floral-dip method. Seeds were harvested and stored at 4 °C. Arabidopsis seeds were sterilized in 75 % (v/v) ethanol for 1 min and then in 10 % NaClO solution for 6 min, followed by washing with sterile water. The seeds germinated on MS medium with 50 mg/L kanamycin for selecting transformants. Transgenic seedlings harboring *BnNAC2p:GUS* were stained in 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) solution overnight according to the method described earlier (Li et al. 2002). The stained plant materials were then cleared and fixed by rinsing with 70 % (v/v) ethanol, and the samples were examined and photographed under a Leica MZ16f stereomicroscope (Leica, Germany).

Transcription activation assay in yeast

To investigate the transcriptional activity of BnNAC proteins, the coding sequences of BnNAC2 and BnNAC5 cDNAs were amplified using a forward primer containing an *EcoR* I site (BnNAC2: 5′-GGGGAATTCATGGACATGAATCCCAACACT-3′, BnNAC5: 5′-GGGGAATTCATGGATAAGGTTAAACTTGTA-3′) and a reverse primer containing a *Sac* I site (BnNAC2: 5′-GGGGAGCTCCTAAAACTGAAACATACTAGC-3′, BnNAC5: 5′-GGGGGATCCATGGATAAGGTTAAACTTGTA-3′), and cloned

into pGBKT7 vector (Clontech, Palo Alto, CA, USA) to generate reporter plasmids, namely, pBD-Bn-NAC2 and pBD-Bn-NAC5. The two reporter plasmids were then individually transformed into yeast strain AH109 containing HIS3 and LacZ reporter genes. Yeast transformants were streaked on selective medium lacking tryptophan and adenine to assay transcriptional activity. β-Galactosidase activity was also assayed by colony-lift filter assay, using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) as substrate (Zhang et al. 2010).

Overexpression of *BnNAC* genes in fission yeast

The coding sequences of *BnNAC2* and *BnNAC5* genes, amplified from their cDNAs by PCR with *Pfu* DNA polymerase, were cloned into yeast vector pREP5N with *Sal* I/*Not* I sites, respectively. Afterwards, the constructed vectors and pREP5N empty vector were transferred into fission yeast (*Schizosaccharomyces pombe*) cells by electroporation (Bio-Rad MicroPulser Electroporation Apparatus, Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. Transformants were then selected on minimal medium (MM) supplemented with 75 mg L^{-1} adenine and 2 μM uracil and 100 mg L^{-1} ampicillin at 30 °C. Ten colonies of each transformant were randomly picked out to grow in liquid MM with 2 μM thiamine, which represses the nmt-1 promoter activity, until mid-log phase in a shaker (220 rpm, 30 °C). Subsequently, yeast cells were harvested and washed three times with MM without thiamine to de-repress the promoter, and then incubated in the same medium without thiamine (induction medium) for 20 h (220 rpm, 30 °C). About 1 ml of the cultured cells was respectively transferred into 20 ml of fresh thiamine-free MM medium (OD600 = 0.1) for further incubation. When the culture grew to stationary phase (OD600 reached 2.0), 5 μl of the culture was spotted onto thiamine-free MM solid medium with 250 mM NaCl and 400 mM mannitol. After 48 h, cell growth status was observed. Meanwhile, 1 ml of the culture was transferred and cultivated in 20 ml of fresh thiamine-free MM medium with 250 mM NaCl, 400 mM mannitol, respectively (OD600 = 0.1). The culture was taken of every 3 h to measure their OD when treated with NaCl and mannitol till 9 h, and then calculated cell growth rate, using empty pREP5N transformants as controls.

Identification of *Arabidopsis vni2* mutant and complementation of BnNAC5

The ***vni2*** mutant (SALK_143793) (also called *vni2-1*, Yang et al. 2011) was obtained from the SALK lines collection. The homozygous ***vni2*** mutant, which was selected by PCR-based genomic identification with gene-specific primers, was confirmed by RT-PCR. The seedlings of ***vni2*** mutant were transferred on MS with 150 mM NaCl for phenotypic confirmation. The coding sequence of BnNAC5 was cloned into the modified pCAMBIA1301 vector with hygromycin resistance and introduced into ***vni2*** mutant. The transformed seeds were selected on MS medium containing 250 mg/L hygromycin.

The *vni2/35S:BnNAC5* transgenic, *vni2*, and wild-type *Arabidopsis* seeds germinated in MS medium for 7 days and then were transferred onto MS with 150 mM NaCl and cultured for 3 days. Subsequently, green leaf rate and chlorophyll content in leaves of vni2/35S:BnNAC5 transgenic, vni2 mutant, and wild-type plants were measured, respectively. In brief, chlorophylls in leaves were extracted with 80 % acetone, and the extracted solution was incubated at 4 °C for 2 h in complete darkness. Chlorophyll content was assayed by measuring absorbance at 645, 652, and 663 nm using a spectrophotometer. To observe the phenotype of adult Arabidopsis plants, the vin2/35S:BnNAC5 transgenic, vni2 mutant, and wild-type seedlings were transplanted in soil for growth to maturation.

Results

Isolation and characterization of *BnNAC2* and *BnNAC5* genes

In order to identify novel members of NAC family in *Brassica napus*, we use *Arabidopsis* NAC proteins as query probes to search the published *B. napus* EST database (http://www.arabidopsis.org/), we obtained six full-length cDNA sequences encoding *B. napus* NAC-like proteins, and consequently designated as *BnNAC1* to *BnNAC6*. Among them, *BnNAC2* and *BnNAC5* (Accession Numbers in GenBank: JF957836 and JF957837) were selected for further study. We amplified the complete sequences of *BnNAC2* and *BnNAC5* cDNAs from total RNAs of *Brassica napus* by RT-PCR, using gene-specific primers. The two cDNAs encodes novel NAC-like proteins in *B. napus*. Subsequently, we isolated *BnNAC2* and *BnNAC5* genes from *B. napus* genome by PCR. By comparing the gene and its cDNA sequences, it was revealed that both *BnNAC* genes contain three extrons and two introns (Fig. 1). The first two exons encodes N-terminal region and the last one encodes C-terminal domain. *BnNAC2* encodes a NAC-like protein with 271 amino acids, and *BnNAC5* deduces a NAC domain polypeptide of 250 amino acids. As shown in Fig. 2, high sequence similarities between BnNAC2/5 and the other plant NAC proteins are found in N-terminus, which contained several distinguishable blocks of heterogeneous amino acids or gaps and are divided into five subdomains. On the other hand, C-terminus of BnNAC2/5

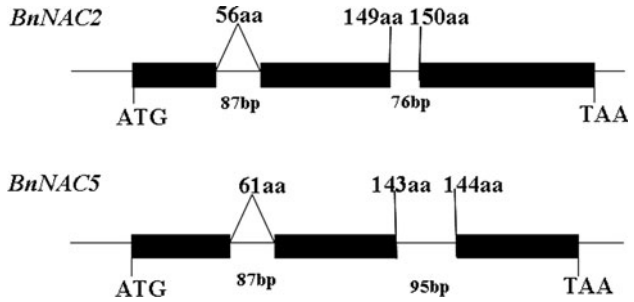

Fig. 1 Structure of *BnNAC2* and *BnNAC5* genes. Exons are denoted by *black boxes*. Introns, 5′-flanking region and 3′-untranslated region are denoted by *lines*. The lengths of the introns in base pairs are indicated. The number at the boundaries of each exon indicates the codon at which the intron is located. The translation initiation and termination codons are shown. *aa* amino acids

proteins, serving as transcription activation domain, showed relatively high diversity from the other plant NAC proteins.

Phylogenetic analysis of BnNAC2 and BnNAC5 proteins

To investigate divergence of BnNAC2/5 proteins with other NAC proteins during evolution, we analyzed phylogenetic relationship of BnNAC2/5 proteins with eight *Arabidopsis* NAC proteins, one tomato NAC protein (SENU5) and five *B. napus* NAC proteins by an MEG3.1 program. As shown in Fig. 3, 16 NACs available could be divided into three subgroups in the tree. BnNAC2 and BnNAC5 locate on the first subgroup of the tree. BnNAC2 occupies a distinct branch that is basal to the NAP and AtNAC2 clade, indicating that divergence in these three proteins occurred relatively late. Similarly, BnNAC5 and ANAC83 (VNI2) form another branch, and SENU5 is basal to this clade, suggesting BnNAC5 are more closely related to ANAC83 (VNI2) and SENU5 than to the others.

Transcriptional activity of BnNAC2 and BnNAC5 proteins

The NAC transcription factors contain conserved N-terminal DNA-binding domain and variable C-terminal transcriptional regulation region (TRR), which can act as either a repressor or activator in regulatory pathways (Tran et al. 2004). In order to determine whether BnNAC2 and BnNAC5 proteins have transcription activation activity, yeast one-hybrid system was employed. The cDNA fragments containing the C-terminal domains of BnNAC2/5 were cloned into pGBKT7 which contains the GAL4-binding domain. To assay their ability to activate transcription of HIS3 and ADE2 reporter genes from GAL4 upstream activation sequence, we transformed AH109 yeast strain with the fusion plasmids pBD-BnNAC2 and pBD-BnNAC5. All the transformed yeast cells grew well in SD medium lacking tryptophan (−Trp) (Fig. 4a). In contrast, pBD-BnNAC2 transformant yeast cells were able to grow well on selection medium lacking tryptophan and adenine (−Trp and −Ade), whereas cells containing pBD-BnNAC5 and pBD did not grow on the same medium (Fig. 4b). Yeast cells with expressing pBD-BnNAC2 on SD medium without histidine and adenine turned blue at the presence of 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (X-Gal), indicating that the reporter gene *LacZ* was activated. However, cells with expressing pBD-BnNAC5 did not show any blue color (Fig. 4c). These results suggest that BnNAC2 protein functions as a transcriptional activator, while BnNAC5 lacks the activity of transcriptional activation.

Expression patterns of *BnNAC2* and *BnNAC5* genes in *Brassica napus*

To investigate the expression patterns of *BnNAC2/5* genes in different tissues of *B. napus*, quantitative RT-PCR analysis was performed. The results showed that *BnNAC2* mRNAs were predominantly accumulated in flowers, whereas the moderate to weak signals of its expression were detected in other tissues such as roots, cotyledons, stems, hypocotyls and leaves. *BnNAC5* transcripts were accumulated at the highest level in stems, at moderate levels in cotyledons and leaves, but at low levels in other tissues (Fig. 5).

To determine the precise expression profiling of the *BnNAC2* gene in *B. napus*, the 1,672 bp *BnNAC2* 5′-flanking region (the putative promoter fragment and 5′-untranslated region) before the translational initiation codon (ATG) was cloned upstream *GUS* reporter gene in pBI101 vector, giving rise to *BnNAC2p:GUS* chimeric gene. *BnNAC2p:GUS* construct was introduced into *Arabidopsis* by *Agrobacterium tumefaciens*-mediated transformation. Expression of *GUS* gene controlled under *BnNAC2* promoter in transgenic *Arabidopsis* plants was examined by histochemical assay (Fig. 6), using non-transformed plants as negative control. In the early seedling development, no GUS expression was detected in the 3-day-old seedlings (Fig. 6a). As seedlings further developed, strong GUS activity was observed clearly in the root tip regions and shoot meristems, but no or weak GUS staining was found in the other tissues of the 7-day-old transgenic seedlings (Fig. 6b). High level of GUS expression was also detected in the veins of rosette leaves of the 15-day-old seedlings (Fig. 6c). As leaves further developed, however, GUS activity was gradually declined to undetectable level in the senescent leaves (Fig. 6d). When

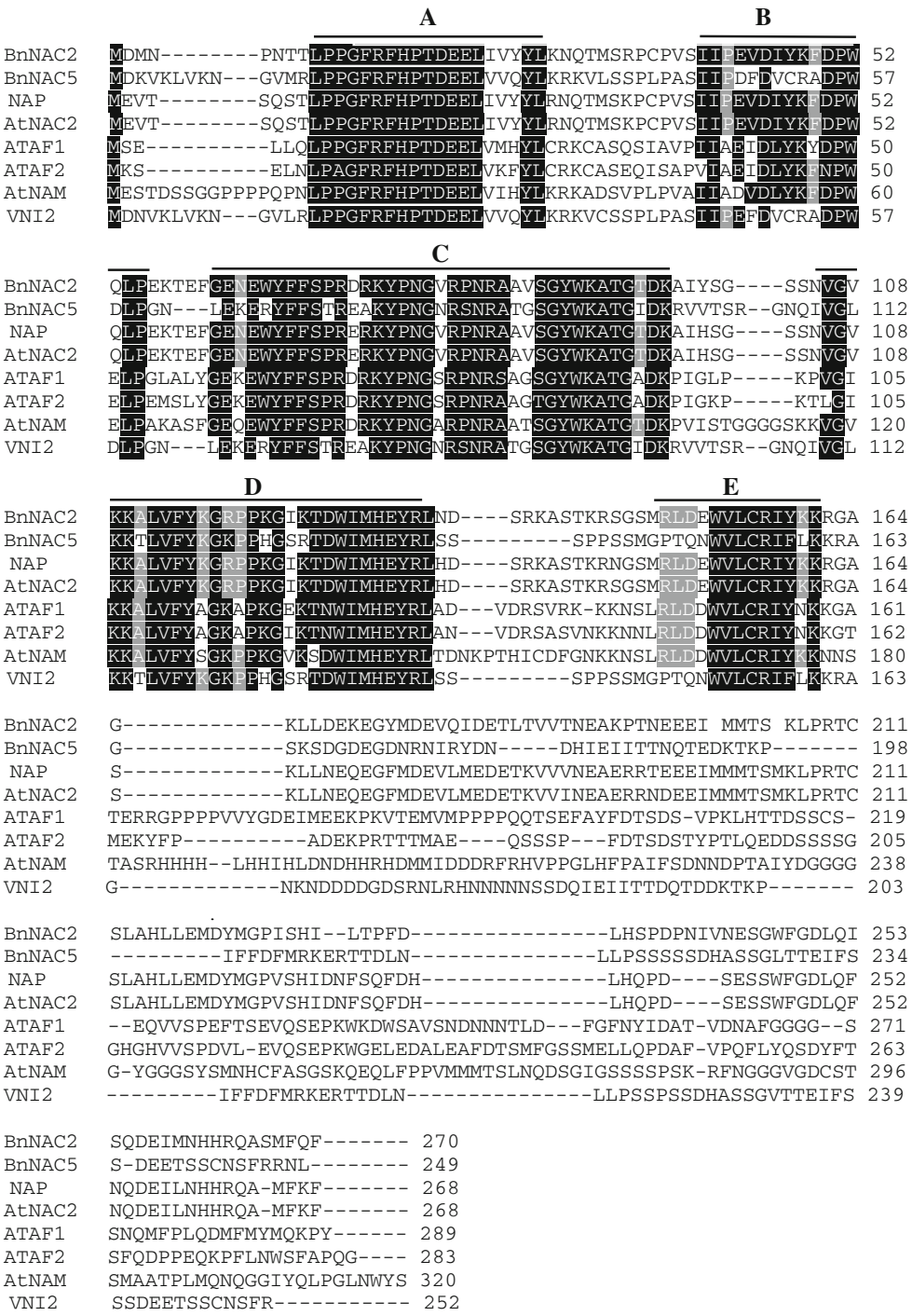

Fig. 2 Sequence alignment of BnNAC2 and BnNAC5 proteins with other NAC domain proteins. BnNAC2 and BnNAC5 are identified in this work, and the other NAC proteins, including *Arabidopsis thaliana* AtNAM (AF123311), ATAF1 (NP_171677), ATAF2 (NP_680161), CUC1 (AB049069), CUC2 (AB002560), AtNAC2 (At3g15510), and VNI2/ ANAC83 (At5g13180) are selected from GenBank. Residues that are identical in all (*white on black background*) or most (*black on gray background*) proteins are highlighted. The five subdomains of NAC domain are denoted by lines and with **a**, **b**, **c**, **d**, and **e**

plants grew to maturation, strong GUS staining was found only in the axils of bracts (Fig. 6e), sepal (Fig. 6f) and abscission zone of mature siliques (Fig. 6f, g). These results are consistent with the RT-PCR data, suggesting that BnNAC2 may mainly function in early seedling development and flower development of *B. napus*.

Expression of *BnNAC2* and *BnNAC5* genes are up-regulated by NaCl, mannitol and abscisic acid (ABA)

To investigate whether the two *BnNAC* genes are induced by high-salinity, drought and abscisic acid (ABA),

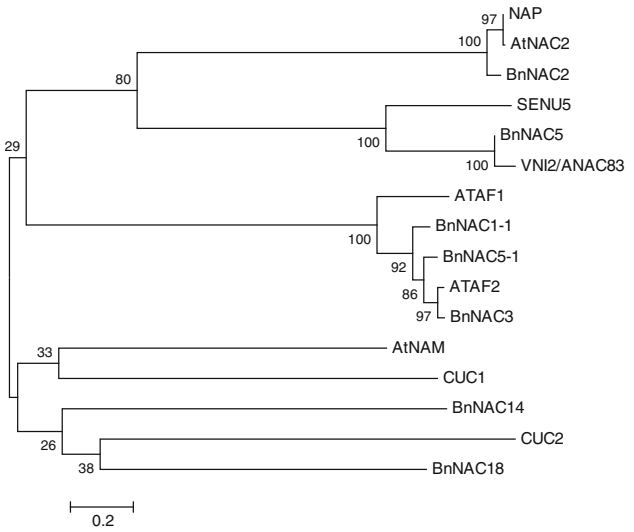

Fig. 3 Phylogenetic relationship of BnNAC2 and BnNAC5 proteins with the other NAC proteins in plants. The minimum evolution tree was constructed in MEGA3.1 from 1,000 bootstrap replicates. BnNAC2 and BnNAC5 in this work; SENU5 from tomato; BnNAC1-1, BnNAC3, BnNAC5-1, BnNAC14, BnNAC18 from *Brassica napus*, the rest proteins from *Arabidopsis thaliana*. The accession numbers of the known NAC proteins in GenBank are as follows: AtNAM (AF123311), ATAF1 (NP_171677), ATAF2 (NP_680161), NAP (AJ222713), CUC1 (AB049069), CUC2 (AB002560), AtNAC2 (At3g15510), VNI2/ANAC83 (At5g13180), BnNAC1-1 (AY245879), BnNAC3 (AY245880), BnNAC5-1 (AY245881), BnNAC14 (AY245885), BnNAC18 (AY245886), SENU5 (Z75524)

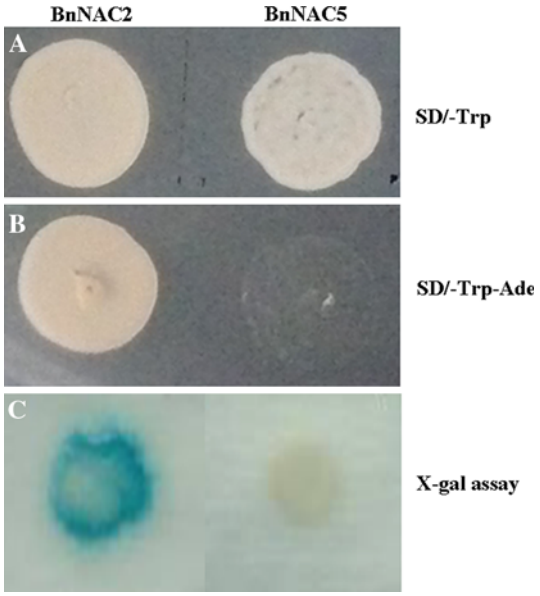

Fig. 4 Analysis of transactivation activity of BnNAC2 and BnNAC5 proteins in yeast. Fusion proteins of pBD-BnNAC2, pBD-BnNAC5 and pBD were expressed in yeast strain AH109. The transformants were streaked on the SD/−Trp−Ade medium. The plates were incubated for 2 days and subjected to an X-gal assay. **a** Yeast transformants were streaked on SD/−Trp medium (SD minimal medium lacking Trp). **b** Yeast transformants were streaked on SD/−Trp−Ade medium (SD minimal medium lacking Trp and Ade). **c** Flash-freezing filter assay of the β-galactosidase activity

quantitative RT-PCR analysis was performed using gene-specific primers. The expression of both *BnNAC* genes was induced by 150 mM NaCl treatment. *BnNAC2* expression was up-regulated at the highest level at 3 h after NaCl treatment, whereas *BnNAC5* reached its peak value at 12 h after NaCl treatment. Similarly, *BnNAC2/5* expression was also up-regulated by 200 mM mannitol treatment. *BnNAC2* activity reached to its peak value at late stage (12 h), whereas *BnNAC5* mRNAs were accumulated at the highest level at early stage (1 h) of the osmotic stress. Furthermore, the expression of the two genes was also induced by ABA. The transcripts of both genes were accumulated at the highest levels at 6 h after ABA treatment (Fig. 7). These results suggested that the *BnNAC*2 and *BnNAC5* genes may be involved in *B. napus* response to salt and osmotic stresses and ABA signaling.

Overexpression of *BnNAC2* and *BnNAC5* in yeast enhances cell sensitivity to high-salinity and osmotic stresses

To investigate whether both genes play roles in response to high-salinity and drought stresses, *BnNAC2* and *BnNAC5* genes were ectopically expressed in fission yeast (*Schizosaccharomyces pombe*). The coding sequences of BnNAC genes were cloned into pREP5N vector, placing the genes under the control of an nmt-1 promoter, which is activated in the absence of exogenous thiamine. The transformed yeast cell lines with expressing *BnNAC2* or *BnNAC5* and control lines containing empty vector (pREP5N) were randomly selected to analyze cell growth under 250 mM NaCl and 400 mM mannitol treatments, respectively, using the same cell lines grown in normal conditions (minimal solid medium without supplemental NaCl, mannitol) as controls. As shown in Fig. 8b, when they grew in non-induction medium, the growth rate of *BnNAC2/5* transformed cell lines was as same as control lines. However, when they grew in the same thiamine-free medium (induction medium), *BnNAC2* and *BnNAC5* transformed cells grew slower than that of control lines (Fig. 8a). As treated with NaCl and mannitol, the transformed cells with expressing *BnNAC2* or *BnNAC5* genes on induction medium were more sensitive than those controls (Fig. 8c, e), whereas they showed similar growth rates on non-induction medium (Fig. 8d, f). Statistical analysis revealed there were significant differences between the transformed cell lines and controls (Fig. 9).

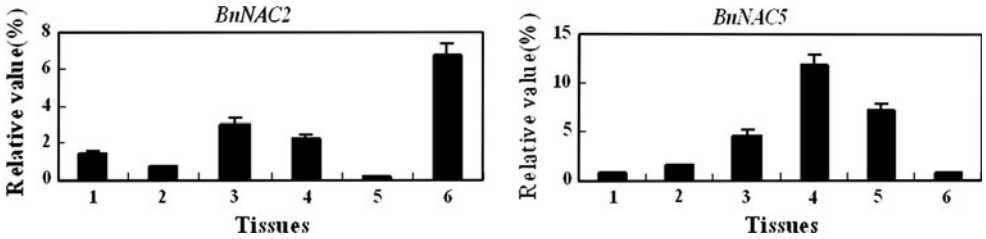

Fig. 5 Quantitative RT-PCR analysis of expression of *BnNAC2* and *BnNAC5* genes in *B. napus* tissues. Total RNAs were isolated from roots (*1*), hypocotyls (*2*), cotyledons (*3*), stems (*4*), leaves (*5*), and flowers (*6*) of *B. napus* respectively. Relative value of the expression of the identified genes in *B. napus* tissues was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (*bar*) were shown from three independent experiments

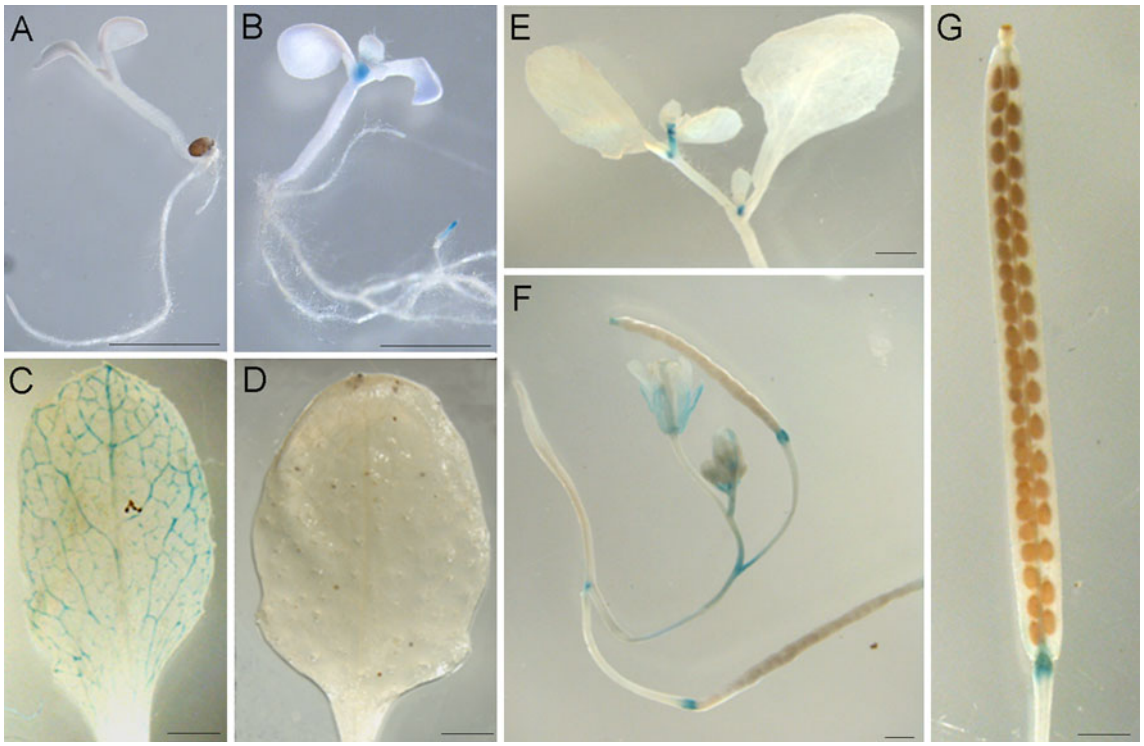

Fig. 6 Histochemical assay of expression of *GUS* gene driven by *BnNAC2* promoter in transgenic *Arabidopsis* plants. GUS staining was not detected in the 3-day-old seedling (**a**), but strong GUS activity was observed clearly in the root tip regions and the shoot meristems of 7-day-old seedling (**b**) and in the veins of rosette leave of a 15-day-old plant (**c**). No GUS activity was detected in the senescent leaf (**d**), whereas relatively high levels of GUS expression were found in the axils of bracts (**e**), sepals (**f**) and the abscission zone of mature siliques (**f**, **g**). *Bar* 5 mm

Phenotypic defects of *vni2* are rescued by *BnNAC5*

The *vni2* knockout mutant (SALK_143793) (also called *vni2-1*, Yang et al. 2011) was obtained from the SALK lines collection. The homozygous *vni2* mutant was identified by PCR and then confirmed that *VNI2* transcripts were undetectable in mutant lines. To evaluate whether *BnNAC5* rescues the phenotypic defects of *vni2*, *BnNAC5* gene was introduced in the homozygous *vni2* mutant. Over 20 homozygous lines (T2 and T3 generations) of the transgenic *vni2/35S:BnNAC5* plants with expressing *BnNAC5* were obtained. The expression levels of *BnNAC5* gene in these *vni2/35S:BnNAC5* transgenic progeny plants were examined by RT-PCR analysis using gene-specific primers (Fig. 10h). As seedlings grew in MS medium, there was no difference among the *vni2/35S:BnNAC5* transgenic plants, wild type, and *vni2* mutant (Fig. 10a). After 7-day-old seedlings were transferred and cultured on MS medium with 150 mM NaCl for 3 days, however, *vni2/35S:BnNAC5* plants displayed a similar phenotype with wild type. In contrast, the *vni2* knockout mutant showed a salt-hypersensitivity phenotype (Fig. 10b). We further determined the

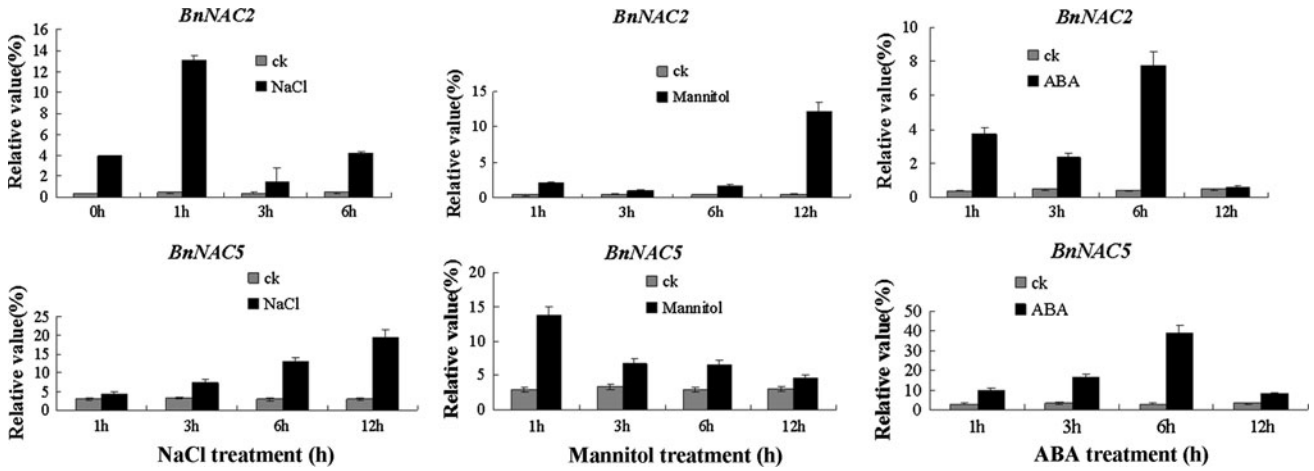

Fig. 7 Quantitative RT-PCR analysis of expression of *BnNAC2* and *BnNAC5* genes under NaCl, mannitol and abscisic acid (ABA) treatments. Total RNAs were isolated from 7-day-old roots treated with 150 mM NaCl, 200 mM mannitol, or 100 μM ABA for 1, 3, 6, 12 h respectively. ck, untreated roots (control). Relative value of the expression of the identified genes in *B. napus* roots was shown as percentage of *BnACT2* expression activity. Mean values and standard errors (*bar*) were shown from three independent experiments

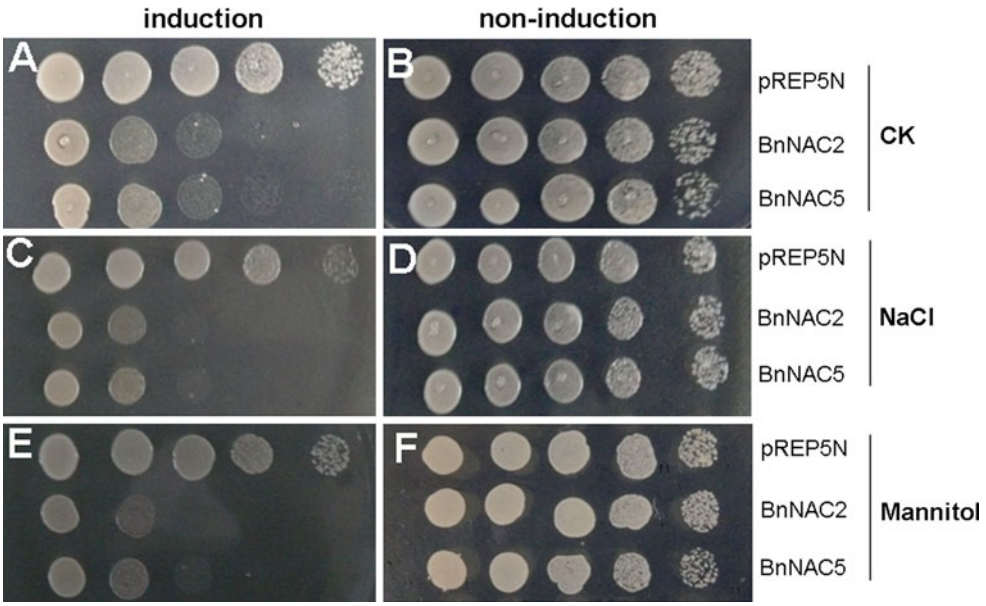

Fig. 8 Analysis of growth rate of yeast cells with Overexpressing *BnNAC2* and *BnNAC5* genes under normal conditions and under NaCl and mannitol treatments. Yeast cells harboring empty pREP5N vector (*upper row*), constructed pREP5N–BnNAC2 (*middle row*) and pREP5N–BnNAC5 (*bottom row*) were cultured on induction medium (**a**, **c**, **e**) or non-induction medium (**b**, **d**, **f**) without or with NaCl and mannitol treatments, respectively. CK, yeast cells were cultured on MM medium without NaCl and mannitol (**a**, **b**); NaCl, yeast cells were cultured on minimal medium with 250 mM NaCl (**c**, **d**); Mannitol, yeast cells were cultured on minimal medium with 400 mM mannitol (**e**, **f**). Five microliters of serial decimal dilutions were spotted onto different medium and incubated at 30 °C for 2 days

total chlorophyll content in leaves and green leaf rate of the *vni2/35S:BnNAC5* transgenic, *vni2* mutant, and wild-type plants under NaCl treatment. Measurement and statistical analysis indicated that the total chlorophyll content and green leaf rate of *vni2* mutant plants were declined more rapidly than those of the *vni2/35S:BnNAC5* transgenic plants and wild type (Fig. 10c, d). When the transgenic plants further developed to maturation, leaf aging was clearly delayed in *vni2/35S:BnNAC5* plants, like wild type, whereas *vni2* mutant showed obvious leaf senescent phenotype (Fig. 10e–g). The above data suggested that *BnNAC5* may perform a similar function of *VNI2* in plant response to high-salinity stress and in regulation of leaf aging.

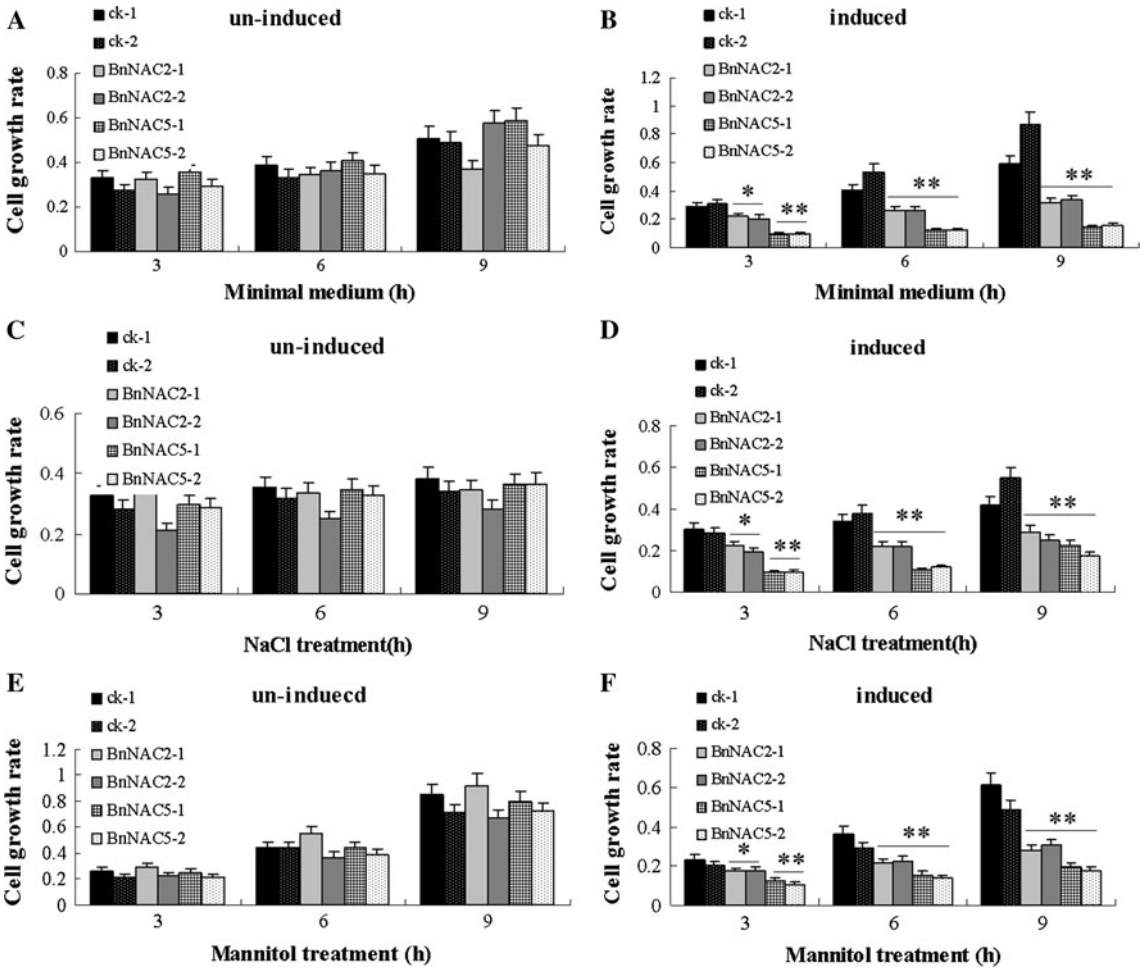

Fig. 9 Statistical analysis of the Cell growth rate of yeast cells by different treated. Cell growth rate of yeast cells, harboring empty plasmid (pREP5N) or constructed vectors (pREP5N–*BnNAC2/5*) cultured in different conditions. Yeast cells were cultured in minimal medium (**a**), 250 mM NaCl (**c**) and 400 μM mannitol (**e**) under non-induction condition. Yeast cells were cultured in minimal medium (**b**), 250 mM NaCl (**d**) and 400 μM mannitol (**f**) under induction condition. Mean values and standard errors (*bar*) from three independent experiments. *Single asterisk* represents significant difference between transformed cell lines and controls ($P < 0.05$) in *t* test. *Double asterisks* represent very significant difference between transformed cell lines and controls ($P < 0.01$) in *t* test

We also analyzed the expression levels of several stress-responsive marker genes, *COR15A, COR15B, RD29A* and *RD29B*, in *vni/35S:BnNAC5* transgenic plants, wild type, and *vni2* mutant. The experimental results indicated that the transcript levels of these genes were significantly enhanced in *vni2/35S:BnNAC5* transgenic plants, compared with those in the *vni2* mutant and wild type (Fig. 11). These data suggest that BnNAC5 may be correlated to the stress-regulation pathway, or directly regulated downstream stress-response genes, as did VNI2 (Yang et al. 2011).

Discussion

NAC proteins are plant-specific transcriptional factors, which are involved in regulating plant development, response to biotic and abiotic stresses, and are also related to cell senescence and death (John et al. 1997; Olsen et al. 2005; Balazadeh et al. 2010; Yang et al. 2011). In this study, we isolated two novel genes (*BnNAC2* and *BnNAC5*) encoding NAC proteins in *B. napus*. Sequence comparison between the genomic DNA and its cDNA revealed that each *BnNAC* gene consists of three exons and two introns. The first two exons encode the conserved NAC domain, whereas the last exon encodes the highly divergent C-terminal transcriptional activation domain, as did the classical NAC genes. *BnNAC2* and *BnNAC5* displayed different tissues expression patterns. *BnNAC2* transcripts were predominantly accumulated in flowers, while *BnNAC5* was mainly expressed in stems, suggesting that the two genes might perform different functions in plant development. In *Arabidopsis*, *AtNAC1* is preferentially expressed in roots and plays an important role in the regulation of lateral root development (Xie et al. 2000). It has been reported that the

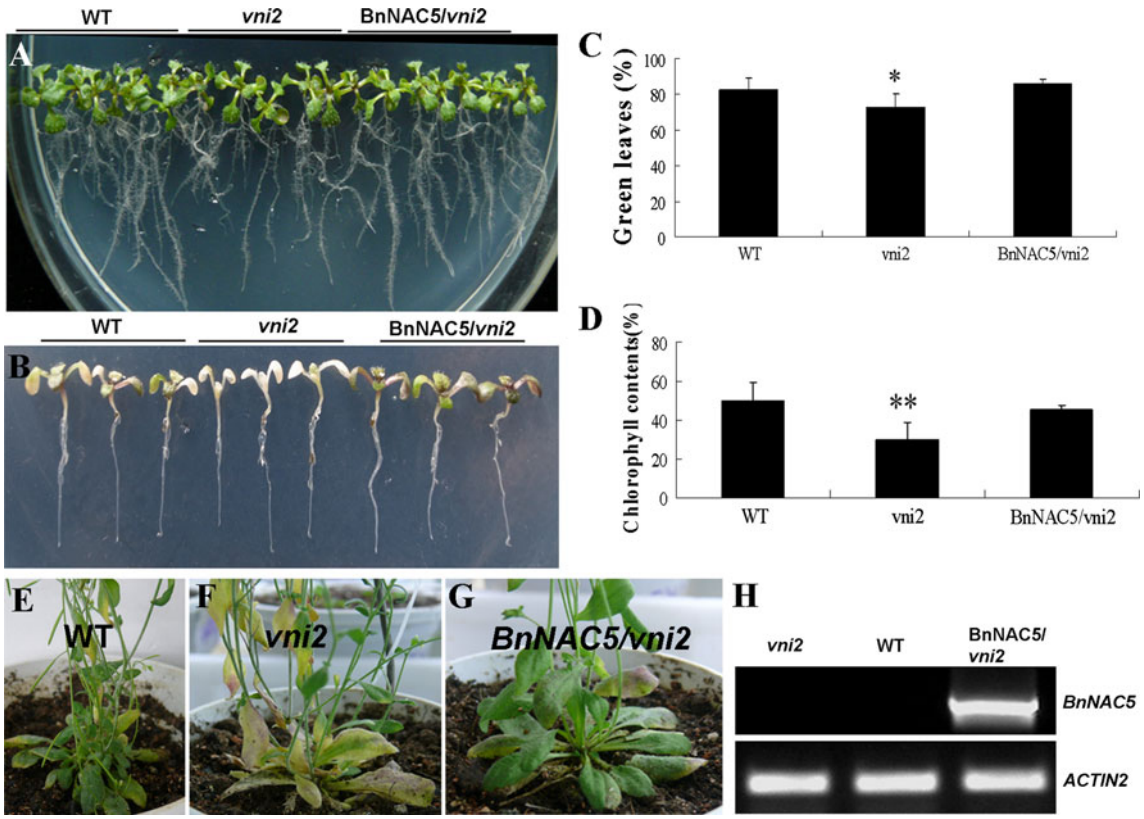

Fig. 10 *BnNAC5* rescues the phenotypic defects of *vni2* mutant (SALK_143793). **a** *vni2/35S:BnNAC5* transgenic, wild type, and *vni2* mutant seedlings were cultured in MS medium. **b** *vni2/35S:BnNAC5* transgenic, wild type, and *vni2* mutant seedlings were cultured in MS medium with 150 mM NaCl. **c** Percentage of green leaves of *vni2/35S:BnNAC5* transgenic, wild type, and *vni2* mutant seedlings with 150 mM NaCl treatment. **d** Chlorophyll content in leaves of *vni2/35S:BnNAC5* transgenic, wild type, and *vni2* mutant seedlings with 150 mM NaCl treatment. **e–g** Phenotype observation of 8-week-old adult *Arabidopsis* plants grown in soil under normal conditions (22 °C, 16 h light/8 h dark). **e** Wild-type plants, **f** *vni2* mutant, **g** *vni2/35S:BnNAC5* transgenic plants. **h** *BnNAC5* expression in the *vni2/35S:BnNAC5* transgenic plants was analyzed by RT-PCR, using RNA samples extracted from 2-week-old seedlings grown on MS medium and Arabidopsis *ACTIN2* as quantification control. *Single asterisk* represents significant difference between the *vni2/35S:BnNAC* transgenic lines and *vni2* mutant ($P < 0.05$) in *t* test. *Double asterisks* represent very significant difference between the *vni2/35S:BnNAC5* transgenic lines and *vni2* mutant ($P < 0.01$) in *t* test. WT, wild type; *vni2*, *vni2* mutant; *BnNAC5/vni2*, the *vni2/35S:BnNAC5* transgenic lines

NAC transcription factors regulate plant response to drought stress through both ABA-dependent and ABA-independent pathways (Tran et al. 2004). *AtNAC2* mRNAs are also accumulated at its highest level in root tissues, and overexpression of this gene in *Arabidopsis* stimulates the development of lateral roots in the transgenic plants (He et al. 2005). A later study revealed that AtNAC2 (also called ANAC092) controls a gene regulatory network during salt-promoted senescence (Balazadeh et al. 2010). *Arabidopsis VNI2* gene encoding a NAC transcription factor is induced by high-salinity in an ABA-dependent manner, and its spatial and temporal expression profiling is correlated with leaf aging and senescence (Yang et al. 2011). In *Brassica napus*, expressions of the nine *NAC* genes are differently regulated by biotic and abiotic stresses (Hegedus et al. 2003). Similarly, our study indicated that both *BnNAC2* and *BnNAC5* were induced by high-salinity, drought and ABA. Overexpression of *BnNAC5* in *vni2* mutant could rescue plant salt-hypersensitive phenotype, suggesting that BnNAC5 may play a VNI2-similar role in response to abiotic stress.

To further characterize the isolated BnNACs, the transcriptional activities of BnNAC2 and BnNAC5 proteins were investigated, using a yeast system. Our results showed BnNAC2 protein functions as a transcriptional activator, whereas BnNAC5 have no such an activity. In order to illuminate this result, we isolated VNI2 (ANAC083), which shared the homology of 91 % in amino acid levels with BnNAC5. By comparing the amino acid sequences of both proteins, we found that there are several amino acid difference in C-terminal between BnNAC5 and VNI2. This amino acid divergence may affect the transcriptional activity. Our experimental results showed that VNI2 protein did not show the transcriptional activity (data not

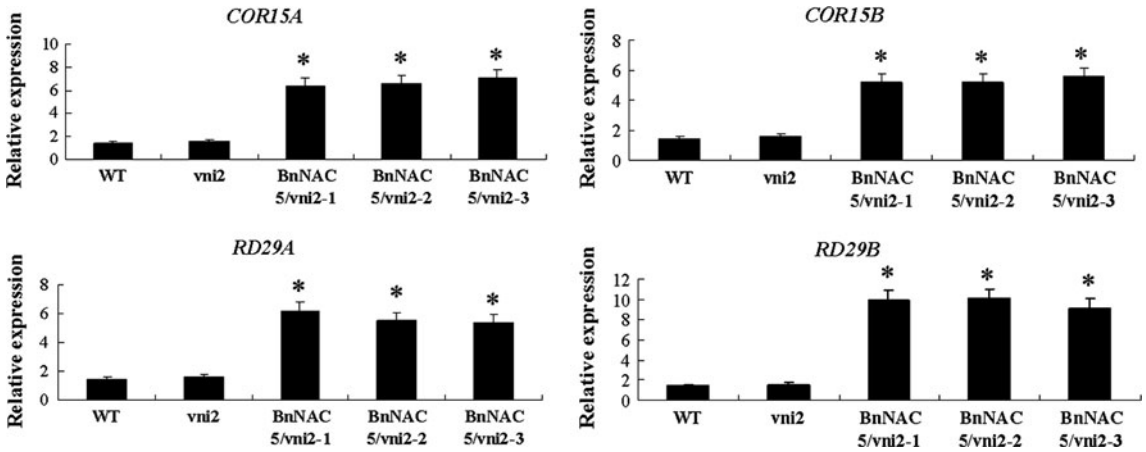

Fig. 11 Quantitative RT-PCR analysis of expression of stress-responsive genes in *vni2/35S:BnNAC5* transgenic Arabidopsis plants. Total RNA was isolated from 2-week-old whole seedlings. Expression levels of *COR15A*, *COR15B*, *RD29A*, *RD29B* genes were determined by quantitative RT-PCR, using ACTIN2 as a quantification control. *Single asterisk* represents significant difference between the *vni2/35S:BnNAC5* transgenic lines and *vni2* mutant ($P < 0.05$) in *t* test. WT, wild type; *vni2*, *vni2* mutant; *BnNAC5/vni2-1, -2* and *-3*, the *vni2/35S:BnNAC5* transgenic lines

shown), similar to BnNAC5. However, the transcriptional activity of BnNAC5 may be enhanced in response to high-salinity stress and in regulation of leaf aging. In NAC family, some members function as transcription activators, while others act as repressors. For example, rice NAC6 is a transcription activator (Nakashima et al. 2007), while CBNAC, a CaM binding NAC protein, has been found to be a transcriptional repressor in *Arabidopsis* (Kim et al. 2007). Recently, a study reported that NARD (NAC Repression Domain, a highly conserved sequence in NAC) not only represses the transactivation ability of NAC proteins, but also influence that of transcription factors from different families, including Dof, WRKY and AP2/DRE. The transcriptional activation ability of the proteins may depend on the interaction between the NARD and activation domain (Hao et al. 2010). Thus, we supposed that the role of NARD might be less than that of activation domain in BnNAC2, leading to this protein as a transcriptional activator. In contrast, the NARD function might be stronger than that of activation domain in BnNAC5, and consequently, this protein would appear like a transcriptional repressor, but it needs further evidence to sustain the hypothesis.

NAC genes play important roles in plant growth, development, and hormone signaling (Olsen et al. 2005). They are also involved in plant stress response. *ANAC019*, *ANAC055*, and *RD26/ANAC072* in *Arabidopsis thaliana* are induced by drought, high salinity and/or abscisic acid (ABA), and overexpression of these genes up-regulates the expression of several stress-related genes, resulting in the enhancement of plant tolerance to drought stress (Tran et al. 2004). To investigate whether BnNAC proteins are involved in high-salinity and osmotic stresses, we chose fission yeast (*S. pombe*) cell, a relative simple system for study. The results showed that overexpression of *BnNAC2/5* in yeast resulted in cells more sensitive to high-salinity and osmotic stresses than those controls. Furthermore, an *Arabidopsis* T-DNA insertion mutant *vni2* (also called *vni2-1*) was employed for investigating the role of *BnNAC5*. Complementation test indicated that *BnNAC5* could recover the phenotypic defects of *vni2* mutant, suggesting that *BnNAC5* is involved in response to high-salinity stress in a similar manner of *VNI2*. It has been demonstrated that *AtNAC2* regulates salt-promoted senescence (Balazadeh et al. 2010), *VNI2* serves as a molecular link to integrate plant response to environmental stresses into modulation of leaf longevity (Yang et al. 2011), and *ANAC083* is involved in the control of senescence processes (John et al. 1997). Likewise, our results showed that growth of yeast cells was remarkably inhibited by overexpression of *BnNAC2/5* genes under normal conditions and high-salinity and osmotic treatments, implying that overexpression of *BnNAC* genes may promote yeast cell senescence. In addition, *BnNAC5* could recover the salt-hypersensitivity and accelerated-leaf senescence of *vni2* mutant to the wild-type phenotype. The *vni2/35S:BnNAC5* transgenic plants showed significantly higher leaf chlorophyll content and green leaf rate than those of *vni2* mutant under NaCl treatment, and displayed a phenotype of the delayed leaf senescence, compared with that of *vni2* mutant. These results suggest that *BnNAC5* may perform the similar function of *VNI2* in regulating leaf longevity against high-salinity stress. Thus, our data will facilitate the understanding of the roles of *B. napus NAC*

genes in response to high-salinity stress and in regulation leaf aging.

Acknowledgments This work was supported by the project from the Ministry of Agriculture of China for transgenic research (Grant No. 2009ZX08009-117B, 2011ZX08009-003), and the Chenguang Project of Wuhan Municipality (Grant No. 200950431185).

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