J. Plant Biol. (2019) 62:147-160
DOI 10.1007/s12374-018-0285-2

ORIGINAL ARTICLE

NAC Transcription Factors from Soybean (*Glycine max* L.) Differentially Regulated by Abiotic Stress

Hyun-Ah So and Jai-Heon Lee*

Department of Genetic Engineering, Dong-A University, Busan 49315, Korea

Received: August 8, 2018 / Accepted: December 27, 2018

Abstract Plant-specific NAC (NAM, ATAF, and CUC)-domain proteins play important roles in plant development and stress responses. This study presents a comprehensive overview of *GmSNAC* genes. Eight of 71 *GmSNAC* genes differentially induced by abiotic stresses were identified. Based on the structural characteristics and phylogenetic relationships of Arabidopsis, rice, and soybean *NAC*s, the *GmSNAC*s were divided into five subgroups. Five GmSNAC proteins were localized in the nucleus and three GmSNAC proteins containing transmembrane domains were found in the nucleus and in other fractions. Based on the transcriptional activities of eight GmSNAC proteins fused to the GAL4 DB, it seems that the respective GmSNAC might play a transcriptional activator or repressor role in yeast. Here, we identify the roles of *GmSNAC49* in stress responses and other processes. The overexpression of *GmSNAC49* in Arabidopsis produced increased tolerance to drought stress compared to WT plants. Real-time (q)RT-PCR analyses suggest that overexpression of *GmSNAC49* resulted in the upregulation of genes involved in the drought and/or ABA signaling pathway. These results suggest that *GmSNAC49* might play a positive role in drought stress tolerance by regulating drought-responsive genes in Arabidopsis. Overall, the results of this systematic analysis of the *GmSNAC* family responsive to abiotic stress will provide novel tools and resources for the development of improved drought tolerant transgenic soybean cultivars.

Keywords: Abiotic stress, *Arabidopsis thaliana*, Expression patterns, *Glycine max*, *NAC* transcription factor, Overexpression transgenic plant

*Corresponding author; Jai-Heon Lee
Tel : +82-51-200-7592
E-mail : jhnlee@dau.ac.kr

Introduction

NAC belongs to a plant-specific transcription factor (TF) family in which the DNA binding domain is highly conserved (Olsen et al. 2005). The name refers to NAM (no apical meristem), ATAF1-2 (Arabidopsis transcription-activating factor 1-2), and CUC2 (cup-shaped cotyledon). It was first reported that *NAC* TF was closely associated with the NAM phenotype in *Petunia* (Souer et al. 1996). A number of *NAC* genes have also been shown to play roles in shoot apical meristem formation and organ separation (Takada et al. 2001; Vroemen et al. 2003; Hibara et al. 2003, 2006). The *NAC* family genes are comprised of 109 members in Arabidopsis (*Arabidopsis thaliana* L.), 151 in rice (*Oryza sativa* L.), and 152 in soybean (*Glycine max* L.) (Kikuchi et al. 2000; Riechmann et al. 2000; Fang et al. 2008; Nuruzzaman et al. 2010; Le et al. 2011).

There is a substantial amount of evidence that *NAC* plays diverse roles, such as shoot apical meristem development as well as developmental response, hormonal control, and defense mechanisms to biotic and abiotic stresses (Olsen et al. 2005). It has been reported that some *NAC* genes are associated with auxin signaling and lateral root formation (Xie et al. 2000, 2002; He et al. 2005; Quach et al. 2014). NAC proteins also participate in biotic and abiotic stress responses. Arabidopsis *ATAF1/ATAF2* and rice *OsNAC6* play roles in both biotic and abiotic stress responses (Delessert et al. 2005; Nakashima et al. 2007; Wu et al. 2009). It has been reported that many stress-inducible *NAC* TFs enhance tolerance to salinity, drought, and/or low-temperature stresses in plants (Nakashima et al. 2012). In Arabidopsis, the ectopic expression of three *NAC* genes, including *ANAC019*, *ANAC055*, and *ANAC072*, was shown to improved tolerance to drought stress (Fujita et al. 2004; Tran et al. 2004, 2007). In addition, transgenic rice plants overexpressing *SNAC1* and *SNAC2* showed increased tolerance to dehydration, high salinity, and/or cold stress (Hu et al. 2006, 2008).

RNA expression analyses of a number of soybean *NAC* genes were carried out recently in response to drought and other

stresses, such as high salinity, cold, and abscisic acid (ABA) treatment (Tran et al. 2009; Hao et al. 2011; Le et al. 2011). Overexpression of *GmNAC11* enhanced only salt tolerance in Arabidopsis, while the ectopic expression of *GmNAC20* increased both salt and freezing tolerance in Arabidopsis (Hao et al. 2011). However, the precise functions of most soybean *NACs* in stress tolerance has not been identified. In this study, we identified soybean Arabidopsis *NAC* genes induced by abiotic stress. Differential RNA expression profiles of *GmSNAC* genes were analyzed under various abiotic stresses, and their transactivation activities and subcellular localization were analyzed. The functions of *GmSNAC49* in seed germination and stress tolerance were examined in overexpression transgenic Arabidopsis. We propose that the *GmSNAC* TFs identified in this study play an important role in gene regulatory networks that serve as an adaptive strategy for soybean to survive under adverse growth conditions.

Results

Identification and Expression Analyses of Soybean *NAC* Genes Induced by Abiotic Stresses

NACs are plant-specific TFs; they are not observed in other organisms. Searching the soybean Transcription Factor Database website (http://soybeantfdb.psc.riken.jp/), 62 *NAC* genes with an ABA-responsive *cis*-acting DNA element (ABRE) or drought-responsive *cis*-acting DNA element (DREB) in the upstream promoter region were identified as *Glycine max* stress-inducible *NAC* (*GmSNAC*) TF and further investigated for their RNA expression under drought stress conditions (Fig. S1A). The expression levels of 22 *GmSNAC* genes showing differential expression by drought stress were monitored in response to the exogenous application of ABA (Fig. S1B).

Real-time quantitative reverse transcription polymerase chain reaction [(q)RT-PCR] analysis was performed to compare the time-course expression of ten *GmSNAC* genes under low-temperature, drought, high-salinity, and ABA stress conditions (Fig. 1). The RNA expression of most of the *GmSNAC* genes was upregulated early by the low temperature stress (Fig. 1A). RNA levels of *GmSNAC23* and *GmSNAC49* peaked significantly 24 h after the high-salinity treatment, while those of the other *GmSNAC* genes were not as high (Fig. 1B). The mRNA levels of *GmSNAC23*, *GmSNAC41*, *GmSNAC49*, and *GmSNAC62* increased greatly up to 24 h after induction of drought stress (Fig. 1C). Much less upregulation was observed in the other four *GmSNAC* genes (Fig. 1C). Exogenous ABA application resulted in a steady

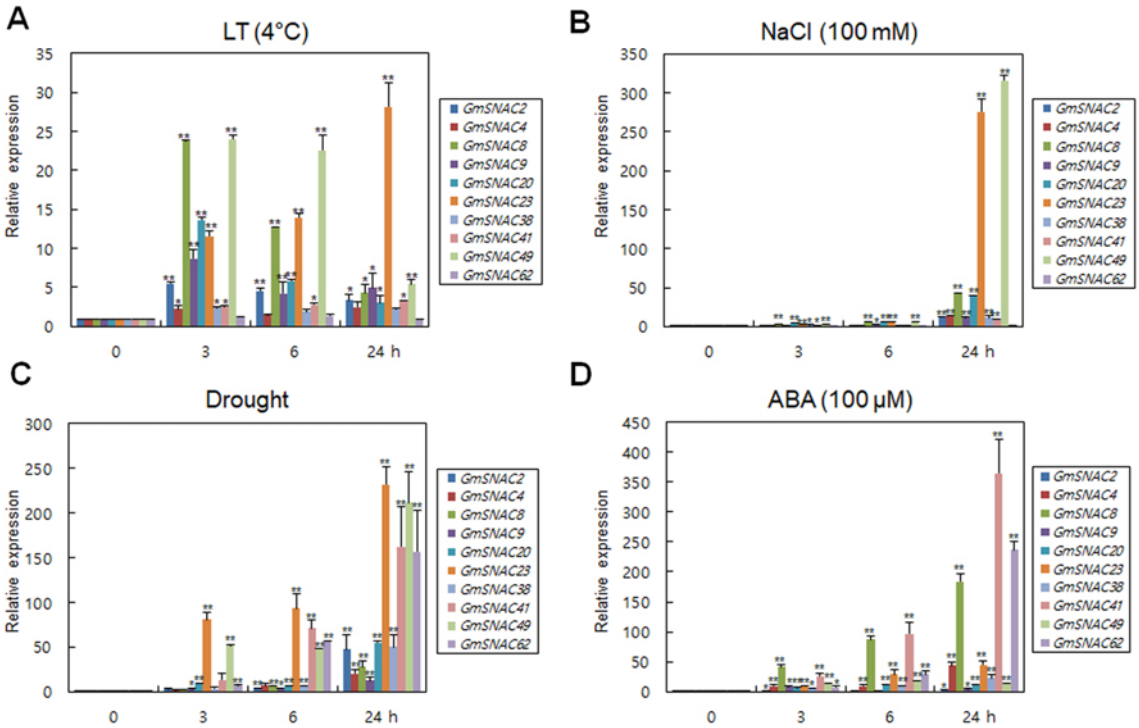

Fig. 1. Real-time quantitative RT-PCR analyses of ten *GmSNAC* genes under abiotic stress conditions. Soybean plants were exposed to low temperature (4°C) (A) high salinity (B) or dehydration stress (C) or subjected exogenously to ABA stock (100 μM) (D). Transcript levels of each *GmSNAC* were quantified by real-time RT-PCR against those of *Tubulin A*. The expression level was assigned a value of 1 under the unstressed condition. Each value is the mean ± SD of three independent experiments. Asterisks indicate significant difference from the corresponding WT values as determined by Student's *t* test (*$0.01 \le P < 0.05$, **$P < 0.01$).

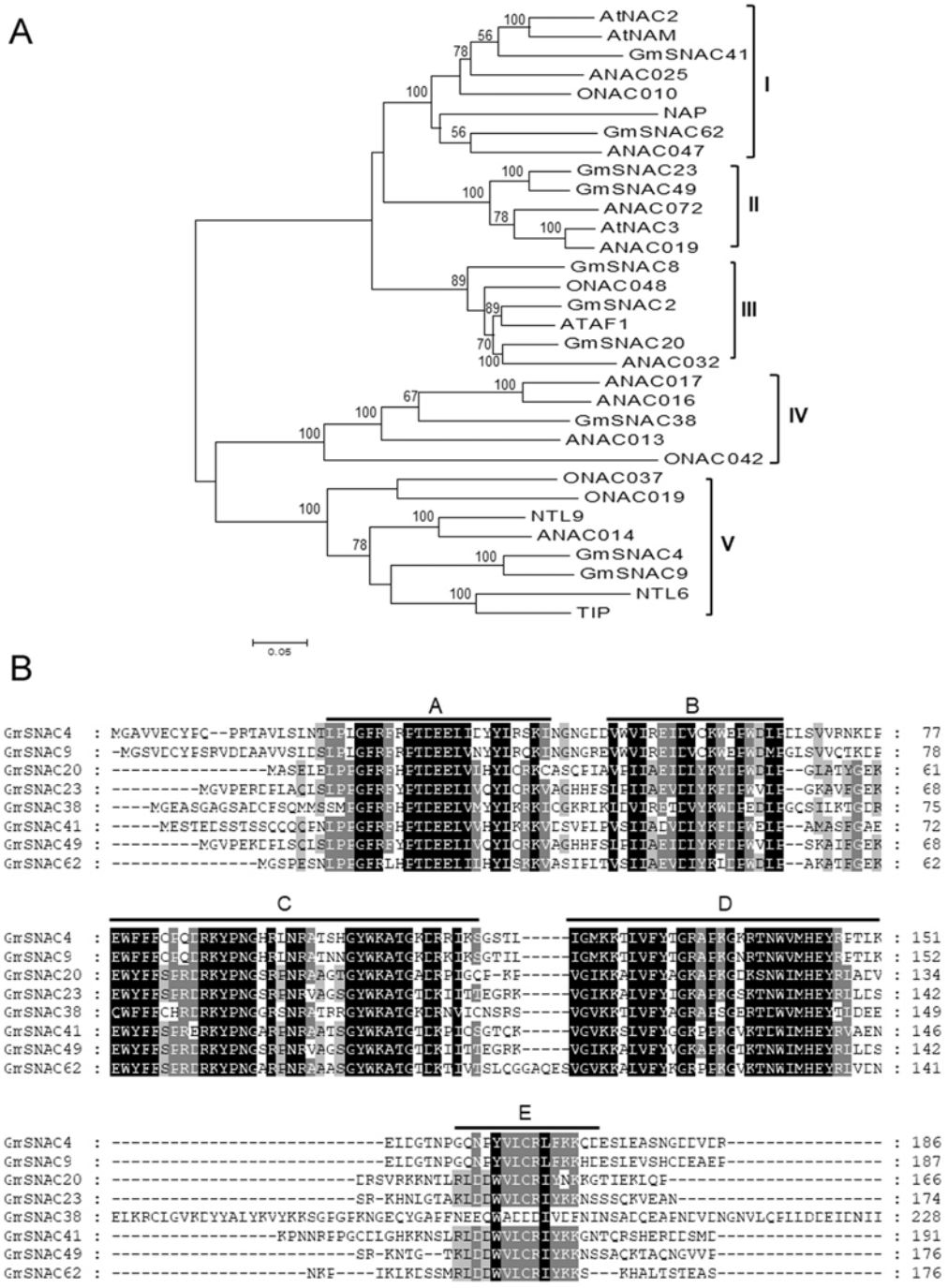

Fig. 2. Neighbor-joining phylogenetic tree and multiple sequence alignment of the soybean GmSNAC proteins. (A) The unrooted tree, constructed with MEGA 3.0, was generated using the amino acid sequence coding for the entire protein from soybean, Arabidopsis, and rice *NAC* genes. Arabidopsis and rice NACs orthologous to GmSNACs (Table 1) were retrieved from databases. The protein sequences used to construct the tree were NTL9 (At4g35580), ATAF1 (ANAC002, At1g01720), ANAC072 (RD26, At4g27410), AtNAC2 (ANAC056, At3g15510), AtNAC3 (ANAC055, At3g15500), ANAC017 (AT1G34190), ANAC047 (At3g04070), ONAC037 (Os08g0157900), ONAC048 (OsNAC6, SNAC2, Os01g0884300), ONAC010 (Os07g0566500), NAP (ANAC029, At1g69490), ANAC025 (At1g61110), AtNAM (ANAC018, At1g52880), ANAC019 (At1g52890), ANAC032 (AT1G77450), ONAC042 (NP_001063547, Os09g0493700), ANAC013 (AT1G32870), ANAC016 (AT1G34180), ONAC019 (NP_001056531, Os06g0101800), ANAC014 (AT1G33060), NTL6 (ANAC062, AT3G49530), TIP (ANAC091, AT5G24590). The tree shows the five5 phylogenetic subfamilies (I–V). The confidence level of the monophyletic groups was estimated by bootstrap analysis of 1,000 replicates. Bootstrap values are shown for each node that had >50% support in a bootstrap analysis of 1,000 replicates. (B) Multiple sequence alignment analysis of the NAC domains from the soybean GmSNAC proteins. The five conserved subdomains A-E of NAC are indicated by lines above the sequence. Proteins were aligned using CLUSTALW at the T-coffee website.

Table 1. Information of the GmSNAC proteins in this study

Gene name	Length (a. a)	pI	MW (kDa)	NAM (a. a.)	TM (a. a.)	Gene accession number	Rice homolog (identity %)	Arabidopsis homolog (Identity, %)
GmSNAC2	268	8.1	30.6	9-134		JX569044	Os01g0884300.1 (60)	At1g01720.1/ATAF1 (71)
GmSNAC4	587	4.88	65.6	21-149	565-587	JX569045	Os08g0157900.1 (31)	At4g35580.1/NTL9[a] (43)
GmSNAC8	295	6.1	34.17	7-132		JX569046	Os01g0884300.1 (66)	At1g01720.1/ATAF1[b] (62)
GmSNAC9	604	5.16	67.67	22-150	582-602	JX569047	Os08g0157900.1 (55)	At4g35580.1/NTL9[a] (71)
GmSNAC20	299	6.25	34.08	7-132		JX569048	Os01g0884300.1 (65)	At1g01720.1/ATAF1[b] (69)
GmSNAC23	337	6.47	37.9	14-140		KF657304	Os01g0816100.1/OsNAC4[c] (62)	At4g27410.1/RD26/ANAC072[d] (71)
GmSNAC38	560	4.7	62.76	19-146	529-551	KF657305	Os05g0426200.1 (55)	AT1G34190.1/NAC017 (44)
GmSNAC41	368	6.26	40.94	18-144		KF657306	Os07g0566500.1/ONAC010 (66)	At3g15510.1/ANAC056[e] (51)
GmSNAC49	343	8.11	38.67	14-140		KF657307	Os01g0816100.1/(OsNAC4[c] (56)	At3g15500.1/ANAC055/AtNAC3[f] (64)
GmSNAC62	353	8.87	39.78	8-144		KF657308	OSJNBb0014I10.5 (72)	At3g04070.1/NAC047 (50)

[a]Yoon et al. (2008); [b]Wu et al. (2009); [c]Kikuchi et al (2000); [d]Fujita et al. (2004); [e]He et al. (2005); [f]Bu et al. (2008)

increase in the transcript level of *GmSNAC8* and a dramatic rise in the transcript levels of *GmSNAC41* and *GmSNAC62* (Fig. 1D). The full-length cDNAs of the ten *GmSNAC genes* were cloned and their predicted amino acid sequences were analyzed (Table 1). When the sequence identities of the *GmSNAC*s were compared, *GmSNAC2*, *GmSNAC4*, and *GmSNAC8* were very similar (Table 1); thus, eight *GmSNAC*s (excluding *GmSNAC2* and *GmSNAC8*) were analyzed further.

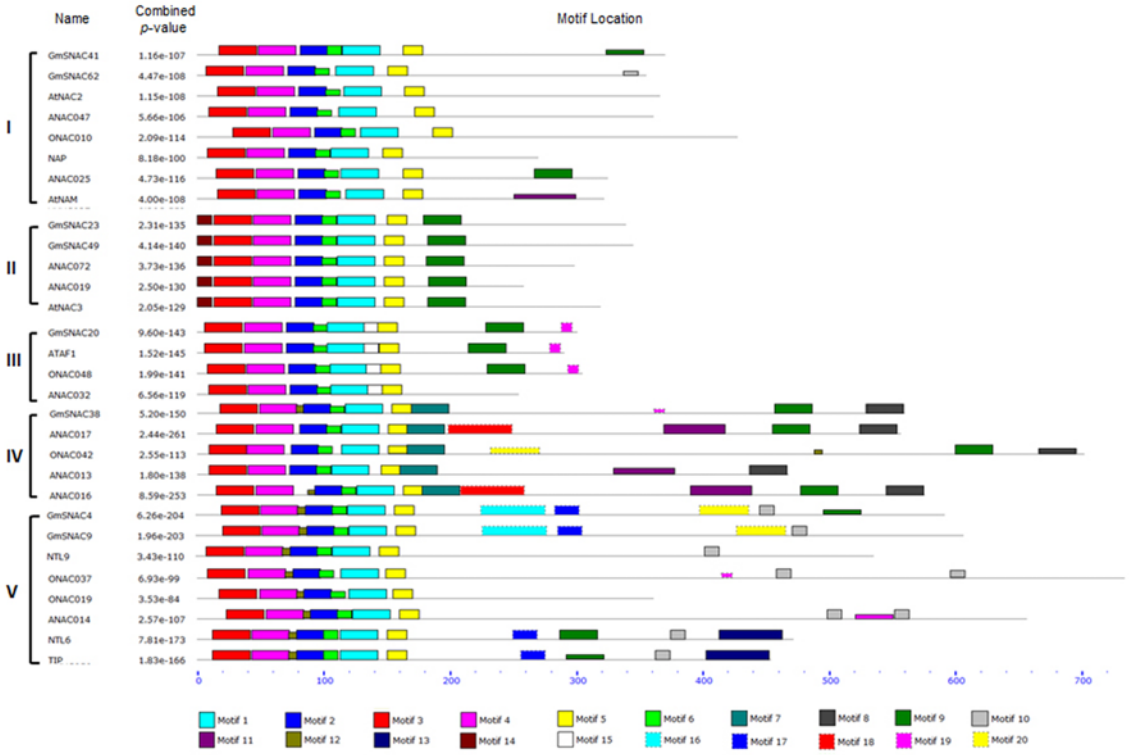

Fig. 3. Schematic representation of the conserved motifs in GmSNAC proteins and their Arabidopsis and rice NACs orthologues. All motifs were identified by MEME using the complete amino acid sequences of the soybean GmSNAC proteins and each Arabidopsis and rice orthologue described in Fig. 3A. The protein sizes are shown at the bottom of the figure. The different motifs are numbered 1–20.

Phylogenetic and Multiple Alignment Analyses of the GmSNAC Proteins

Puranik et al. (2012) classified NAC proteins into six subgroups. Five GmSNACs besides NTLs are classified as type I NAC proteins; they contain the DNA-binding domain present in the N-terminus and the transcriptional regulatory (TR) domain in the C-terminus (Puranik et al. 2012). Based on their structural features, GmSNAC4, GmSNAC9, and GmSNAC38 belong to type II NAC, with the transmembrane (TM) domain in the C-terminus, named NTL. A phylogenetic tree was constructed with the MEGA3 neighbor-joining method, using multiple alignments of ten soybean GmSNACs and their Arabidopsis and rice homologues, with a bootstrap analysis of 1,000 replicates to ensure the statistical reliability (Fig. 2A). The results showed that GmSNACs can be divided into five subgroups (I–V) (Fig. 2A). GmSNAC41 and GmSNAC62, which fall into subgroup I, share the highest homologies, with AtNAC2 (He et al. 2005) and NAP (Guo and Gan 2006) (Fig. 2A). GmSNAC23 and GmSNAC49 are assigned to subgroup II, along with ANAC072 (RD26) (Fujita et al. 2004) and ANAC019 (Bu et al. 2008) (Fig. 2A). Subgroup III includes GmSNAC2, GmSNAC8, and GmSNAC20; their homologues are Arabidopsis ATAF1 (Wu et al. 2009) (Fig. 2A). GmSNAC38 belongs to subgroup IV, which contains ANAC016 (Kim et al. 2013), ANAC017 (Ng et al. 2013), and ANAC013 (Clercq et al. 2013) (Fig. 2A). These *NAC* genes are involved in oxidative stress response (Clercq et al. 2013; Kim et al. 2013; Ng et al. 2013). GmSNAC4 and GmSNAC9 belong to subgroup V, characteristic of membrane-bound NAC, such as NTL6 (Yang et al. 2014), NTL9 (Yoon et al. 2008), and turnip crinkle virus-interacting protein (TIP) (Ren et al. 2000) (Fig. 2A). Because high homologies among *GmSNAC2*, *GmSNAC8*, and *GmSNAC20* indicate that they are redundant, the molecular characterization of *GmSNAC20* was examined further, but those of *GmSNAC2* and *GmSNAC8* were not (Fig. 2A; Table 1).

Multiple alignments of the N-terminal regions containing the NAC domains were carried out with eight GmSNACs (Fig. 2B). All eight proteins contained the NAC domains in the N-terminus, and the NAC domain was further divided into five subdomains—A, B, C, D, and E (Fig. 2B). The domains of the eight GmSNACs were analyzed using the MEME program to gain further insight into the diversification of the GmSNAC proteins (Fig. 3). Twenty distinct motifs were identified (Fig. 3). The N-terminal motifs revealed the conserved NAC motifs (Fig. 3); the details of the 20 putative motifs are presented in Table 2. Motifs 3, 4, 2, 1, and 5 specified the NAC subdomains A–E, respectively (Fig. 3). Closely related genes in the phylogenetic tree share common motif compositions other than the NAC motif, indicating the functional similarity among NAC proteins within the same subfamily (Fig. 3). However, there are also distinctive motifs within the same subfamily, which may play a unique role in the respective *NAC* gene (Fig. 3).

Transcriptional Activation Analyses of Soybean *GmSNAC*

The transactivation activities of the eight GmSNACs were analyzed using a yeast–one hybrid system (Fig. 4). The

Table 2. Motif sequences identified by MEME tools

Motif	Multilevel consensus sequence
1	[VI]G[IV]KK[AT]LVFY[RA]G[KR][AP]PKG[ETV][KR]T[ND]W[IV]MHEYRL
2	RKYPNG[SA]R[PS]NRA[AT]GSGYWKAT
3	LPPGFRF[HR]PTDEEL[VI]V[HY]YL[KCR]RK[IAV][AN][GS]xPL[PS]
4	[IV]IA[ED][IV]D[LV][YC]K[FW][DE]PW[DE]LP[GA][KL][AS][LV]F[GK][ET]K[ED]W[YE][FW]F
5	SL[RGK][LQ]D[DP][WY]VL[CY][RK][IL][YF]KK
6	G[KT]D[KR]PIK[ST]G[GKS]R
7	SG[APS]GPKNGEQYGAPF[QINR]EE[EDQ]W[AVL][DE][DS]D[DINS][EDHV][DH][VAFH][DN]
8	[GP]F[LF][LCF][LF][SP]I[IMV][GI]ALCA[IFL][FC]WV[FIL][VIK][AG][TN][VLM][GR][VGL][SLM]GR[PCS][LV]
9	[QT][ND][SG][NGS][GC]S[TES][SQ][SV][SFV]S[SP][AEQ][LF][DW][DAS][VEL][LV][DEQ]S[IL]P[KSE][IST]P[DAE][RSW][AS][AF]
10	QG[TI]APRR[IL]RLQ
11	N[NY]TS[DG]Q[KR]QQLLY[EQ]QFQ[DY]QTPENQLNN[IY]M[DH]PSTTLNQ[FI]T[DS][DN][IM]WF[EK]DDQA
12	FF[CA]P[RLQ]D
13	[IM][KT]K[GR]CG[KN][FL][MT]RSK[NS][RV]T[GS]F[IV]FKKI[AT][AS][MV][GK]CSY[GR]GLFR[AV][AG]VVAVV[CF][LV]MSVCSL
14	MG[VIL][PQR]E[KLRT]DPL[AST]Q
15	ADVDRS[VA]RKK[NK]
16	I[PS][AE][HN]SEEAISN[IV][IV]TP[GV][DE][IR][CH][ST]DG[CY][DN]A[CP]DAQ[DN]QI[EV][EL]P[TV]AEE[DF]QPLN[FV]DI[FY]YD
17	[KT]LFSP[LV][HL][AS][HQ][VFI][QP][SPT][ED][FL][GHY][SY][QS][AFY]N
18	VAVP[DE][QY]PVVRYE[DN][AG][PR][CR]VD[DE][RT][KR][FL][CF]NPV[IK]LQLEDI[DE][EK]LLN[EG]IP[DN]APGV[NP]QR[CQ]
19	LQD[MI][FL]MY[WM]
20	MGQV[HY]N[MV]V[DN][DY][CY]EQ[PQ]R[IN][CF][DS]A[FV][AV]S[AG][DN]TGI[IR][IR]R[HT]R[EP][GV]RNEQ

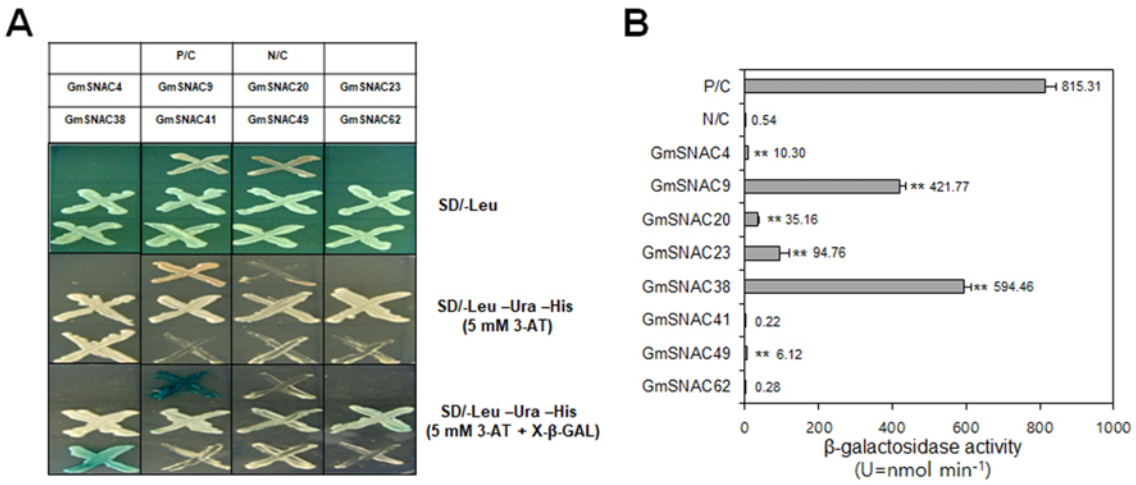

Fig. 4. Transcriptional activity analyses of the GmSNAC proteins. (A) Soybean *GmSNAC* genes were cloned into pDEST32 vector and then transformed into yeast host strain MaV203. For the positive control, MaV203 cells transformed with Krev1 (Rap1A, a member of the Ras family of GTP binding proteins) and RalGDS-wt (the Ral guanine nucleotide dissociator stimulator protein) were used, and cells with Krev1 and RalGDS-m2 were used for the negative controls. Yeast MaV203 strains containing the respective GmSNAC fusion construct were grown on nonselective plates (SD/-Leu) and selective plates (SD/-Leu/-Ura/-His). The β-galactosidase activities were examined by X-gal staining on the selective plates (SD/-Leu/-Ura/-His) supplemented with X-gal. (B) The β-galactosidase activities were examined by ONPG assay, with the MaV203 yeast cells co-transformed with the BD/AD plasmids. Data represent mean ± SD from three independent experiments. All the experiments were carried out in at least three replications. Asterisks indicate significant difference from the corresponding N/C (Negative control) values as determined by Student's *t* test (*$0.01 \le P < 0.05$, **$P < 0.01$).

coding regions of the *GmSNAC* genes were fused to the GAL4 DNA-binding domain, respectively. Yeast cells transformed with each recombinant plasmid were assayed for their abilities to activate transcription from the GAL4 upstream activation sequence and to promote yeast growth in medium absent histidine (His) and uracil (Ura) (Fig. 4A). As shown in Fig. 5A, the growth of all the transformants was fine on the SD media lacking leucine (Leu) (Fig. 4A). On the selection SD medium without His and Ura, the transformants harboring *GmSNAC4*, *GmSNAC9*, *GmSNAC20*, *GmSNAC23*, or *GmSNAC38*, including the positive control (P/C), grew well, while the cells containing *GmNAC41*, *GmSNAC49*, *GmSNAC62*, or the negative control (N/C) did not grow on the same medium (Fig. 4A). Evident blue coloration was observed in the cells containing *GmSNAC38*, including the P/C, and pale blue pigments were also found in the yeasts with *GmSNAC9*, *GmSNAC20*, or *GmSNAC23* (Fig. 4A).

The β-galactosidase activities of GmSNAC proteins were monitored for a more precise estimation (Fig. 4B). As shown in Fig. 4A, the enzyme activities of GmSNAC38 were highest among the GmSNAC proteins (Fig. 4B). The levels of GmSNAC9 were shown to be relatively high, and those of GmSNAC20 and GmSNAC23 were moderate (Fig. 4B). By contrast, other GmSNAC proteins, including GmSNAC4, GmSNAC41, GmSNAC49, and GmSNAC62, displayed very low enzyme activity levels (Fig. 4B). These results indicate that GmSNAC9 and GmNAC38 have high transactivation activity; GmSNAC20 and GmSNAC23 have moderate transactivation activity; and GmSNAC4, GmSNAC41, GmSNAC49, and GmSNAC62 have no transactivation activity.

Subcellular Localization of GmSNAC in Plants

The subcellular localization of the eight GmSNACs was examined by expressing the fusion protein with green fluorescent protein (GFP) in tobacco (Fig. 5). *Agrobacterium tumefaciens* cells transformed with DNA construct carrying GFP-GmSNAC and GFP under the control of the 35S CaMV promoter were introduced into tobacco plants (Fig. 5). As shown in Fig. 6, the GFP-only controls were distributed uniformly throughout the whole cell, whereas the fusion proteins of five GmSNACs (excluding GmSNAC4, GmSNAC9, and GmSNAC38) were restricted to the nucleus (Fig. 5). The GFP signals were not restricted to the nucleus, but were also found in the cytoplasmic region (Fig. 5). Three GmSNAC proteins—GmSNAC4, GmSNAC9, and GmSNAC38—contain the C-terminal TM domain, which is mostly found in NTLs, type II NAC (Fig. 3; Table 1) (Puranik et al. 2012). The TM domain in the C-termini of GmSNAC4, GmSNAC9, and GmSNAC38 was predicted for endoplasmic reticulum (ER) membrane or plasma membrane localization (PSORT, http://psort.hgc.jp/form.html) (Fig. 4A; Table 1). The α-helical TM motif is known to be responsible for the plasma membrane or ER membrane anchoring (Puranik et al. 2012), implying that GmSNACs with TM domains may be anchored to the plasma membrane or ER membrane.

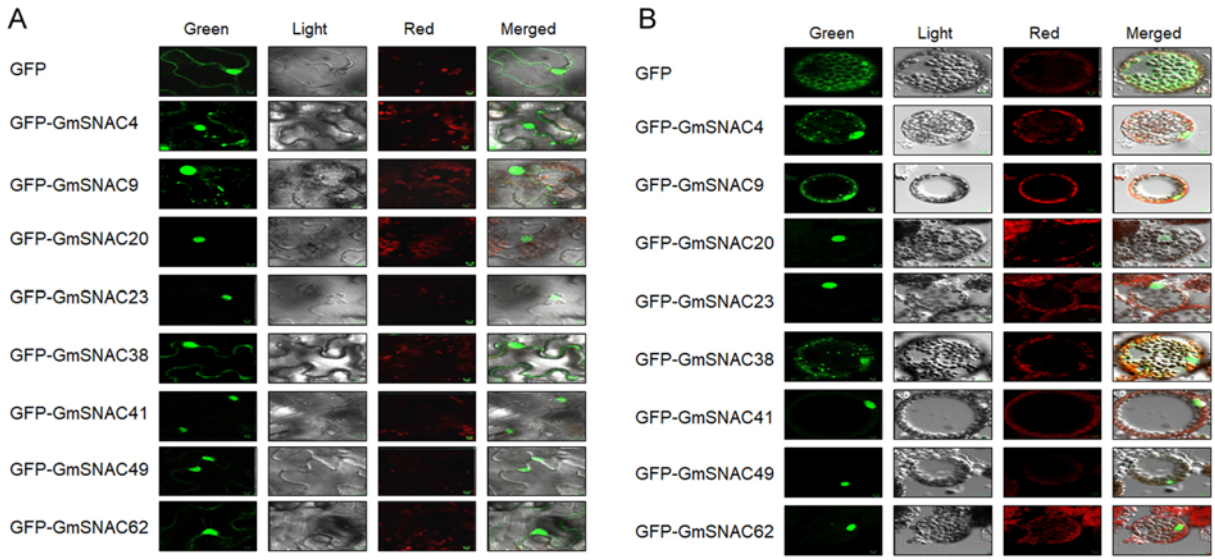

Fig. 5. Subcellular localization of GFP-GmNAC fusion proteins in the tobacco epidermis (A) and protoplast (B). GFP-GmNAC was transiently expressed in *Nicotiana benthamiana* using an *Agrobacterium*-mediated transformation method. The epidermal tissues and the isolated protoplasts were observed with a Zeiss LSM700 confocal microscope. The scale bar represents 10 μm. For each panel, light represents the actual image, the red channel represents chloroplast fluorescence, and the green channel represents GFP fluorescence.

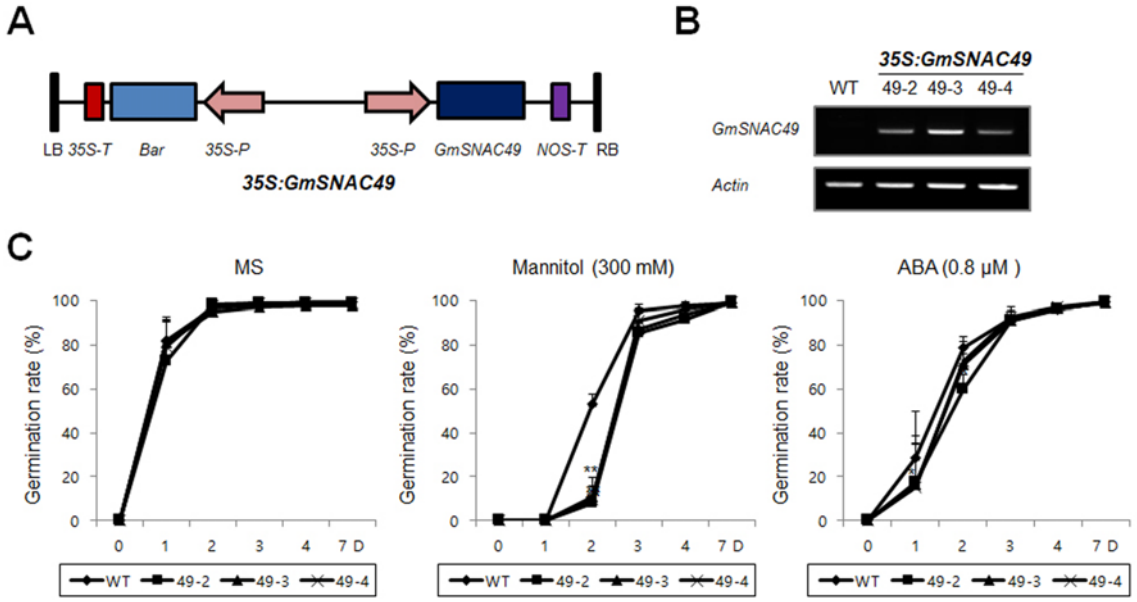

Fig. 6. Germination rate of the WT and *35S:GmSNAC49* transgenic plants under osmotic stress conditions. (A) Schematic representation of the *35S:GmSNAC49* overexpression (OE) construct. (B) RT-PCR was carried out to analyze the constitutive expression of *GmSNAC49* in the OE transgenic plants (#2, #3, #4). Each PCR product was analyzed by agarose gel electrophoresis, along with that of the housekeeping gene, *Actin*. (C) Seed germination rates of the WT and *GmSNAC49* OE lines were analyzed with and without mannitol (300 mM) or ABA (0.8 μM). Seed germination percentages of the indicated lines grown on different concentration of NaCl, mannitol, or ABA were recorded after the end of stratification (4°C, 3 d). The data show the mean ± SD (n=100) of three replicates. Asterisks indicate significant difference from the corresponding WT values as determined by Student's *t* test (*$0.01 \le P < 0.05$, **$P < 0.01$).

Germination and Growth Phenotype of *GmSNAC49* Overexpression Transgenic Arabidopsis in Response to ABA and Osmotic Stress

To better understand the function of *GmSNAC* TFs in plants, we attempted to generate overexpression (OE) transgenic Arabidopsis plants (Fig. 6A). First, transgenic Arabidopsis plants overexpressing *GmSNAC49* were obtained; their functional studies are described in this study. We were able to confirm that three OE plants constitutively express *GmSNAC49* transcript (Fig. 6B). Homozygous T4 transgenic seeds were used for their

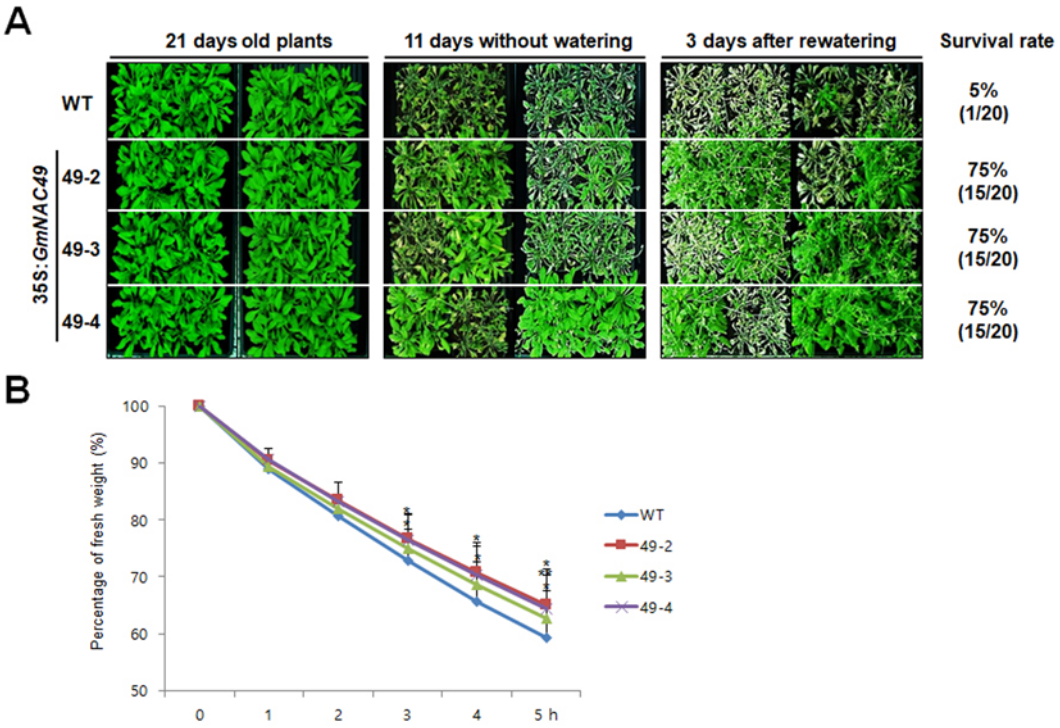

Fig. 7. Overexpression of *GmSNAC49* enhances drought tolerance in Arabidopsis. (A) Three-week-old, soil-grown WT and *35S:GmSNAC49* Arabidopsis transgenic plants were kept in a growth chamber without watering for 11 d. Survival rates were evaluated 3 d after re-watering. (B) Transpiration water loss of the WT and the *35S:GmSNAC49* Arabidopsis transgenic plants. Detached leaves from four-week-old plants grown on soil were incubated at room temperature, and fresh weight (FW) was measured at the time intervals indicated. Water content was calculated from percentage of FW compared with weight at zero time. Error bars, mean ± SD of twenty leaves from each of three replicates. Asterisks indicate significant difference from the corresponding WT values as determined by Student's *t* test (*0.01 ≤ P < 0.05, **P < 0.01).

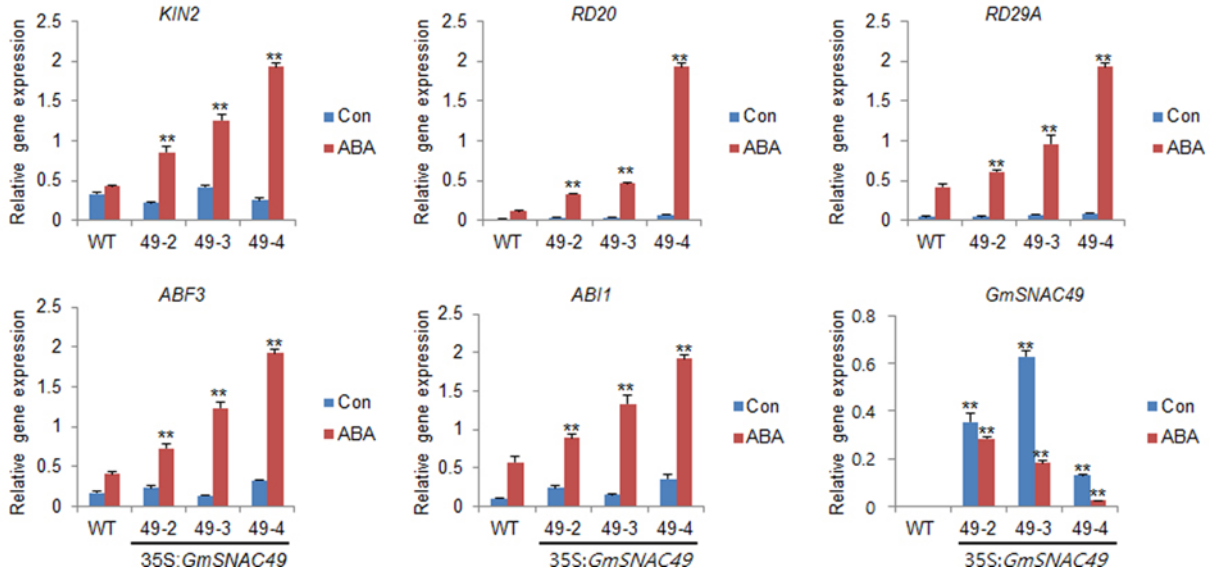

Fig. 8. Analyses of real-time (q)RT-PCR of the WT and *35S: GmSNAC49* transgenic plants in response to ABA. Leaf samples of four-week-old plants without any treatment were used for control (Con). The entire Arabidopsis plants were sprayed with ABA (100 μM), and the leaves were sampled for RNA extraction after 6 h. RT-PCR for each drought-responsive gene (*KIN2*, *RD20*, *RD29A*, *ABF3*, and *ABI1*) and *GmSNAC49* was performed with total RNAs extracted from the harvested samples, as indicated above. Transcript levels of each gene were quantified by real-time (q)RT-PCR against *ACTIN* transcript levels. Mean values from three independent technical replicates were normalized to the levels of an internal control—*ACTIN* transcript level. Data are the mean ± SD from three independent biological replicates. Asterisks indicate significant difference from the corresponding WT values as determined by Student's *t* test (*0.01 ≤ P < 0.05, **P < 0.01).

phenotype and stress tolerance test. Germination rates of the OE lines were examined compared to those of wild type (WT) plants in either normal MS media or supplemented with mannitol or ABA (Fig. 6C). Under the non-stress conditions, the germination rate of the transgenic lines was the same as that of WT (Fig. 6C). In the presence of an osmoticum such as mannitol in the media, the germination rates of OE plants were significantly lower on the second day after germination (DAG) compared to those of WT (Fig. 6C). In the presence of ABA in the media, the OE transgenic seeds germinated more slowly than the WT seeds during the period between 1 and 2 DAG (Fig. 6C). These results indicate that the ectopic expression of *GmSNAC49* might be involved in repressing germination during the early germination process, especially with the addition of mannitol or ABA hormone in Arabidopsis.

Because the RNA expression of *GmSNAC49* was responsive to multiple abiotic stresses, we studied its functions in abiotic stress responses using the OE transgenic plants (Fig. 7). Drought stress was tested in the WT and OE plants (Fig. 7A). Survival rates of the *GmSNAC49* OE transgenic plants were significantly higher than that of the WT exposed to drought stress (Fig. 7A). Leaf water loss was also compared between the WT and the OE transgenic plants (Fig. 7B). Water loss rate was lower in the OE transgenic plant than in the WT, implying that the ectopic expression of *GmSNAC49* resulted in reduced water evaporation, which is closely related to the improved drought tolerance in Arabidopsis.

To investigate the role of *GmSNAC49* in transcriptional regulation, the *35S:GmSNAC49* transgenic plants and the WT were subjected to ABA application or water deficit stress, and real-time (q)RT-PCR analysis was conducted to determine the expression of ABA signaling components and downstream Arabidopsis ABA-responsive genes (Fig. 8). RNA levels of *KIN2*, *RD20*, *RD29A*, *ABF3*, and *ABI1* genes were significantly higher in the *5S:GmSNAC49* plants compared to the WT after ABA application (Fig. 8). The transcript levels of *GmSNAC49* were higher in the 49-2 transgenic lines than in 49-3 and 49-4; there was no detectable amount of *GmSNAC49* in WT (Fig. 8). Although the OE plants displayed higher expression levels of stress marker genes compared to WT, RNA expression level of *GmSNAC49* did not correlate with transcription levels of stress-responsive genes (Fig. 8). These results suggested that *GmSNAC49* regulates transcription of key ABA-dependent upstream signaling factors such as *ABF3* and *ABI1*, indicating that overexpression of *GmSNAC49* thus contributed to the higher sensitivity to osmotic stress during germination and increased tolerance to osmotic stress in *35S:GmSNAC49* transgenic Arabidopsis.

Discussion

In this study, we investigated the expression of 62 soybean *NAC* genes under abiotic stress conditions and carried out the molecular characterization of eight stress-inducible *GmSNAC* genes differentially induced by abiotic stress. Sequence alignment analysis showed that GmSNAC proteins have conserved NAC domains composed of five subdomains (A–E) (Ooka et al. 2003; Puranik et al. 2012). The highly conserved, positively charged subdomains C and D bind to DNA (Ooka et al. 2003; Jensen et al. 2010; Chen et al. 2011; Puranik et al. 2012). It has been proposed that subdomain A might be associated with the formation of a functional dimer, and subdomains B and E might be closely related with the functional diversity of *NAC* genes (Duval et al. 2002; Ooka et al. 2003; Ernst et al. 2004; Jensen et al. 2010; Chen et al. 2011; Puranik et al. 2012). In addition, it has been proposed that interaction between NAC and other proteins is involved in plant–pathogen interaction or stress tolerance (Xie et al. 1999; Tran et al. 2007). In contrast, it is notable that the C-terminus region might be responsible for protein interaction and transactivation repression or activation, which might contribute to diverse functions of the respective *NAC* genes (Ooka et al. 2003; Ernst et al. 2004; Chen et al. 2011; Puranik et al. 2012).

Most TFs, including NAC, consist of a DNA-binding domain and a transcriptional activation domain. In this study, eight GmSNACs showed different transcriptional activities fused to GAL4 DB in yeast (Fig. 4). NACs such as CBNAC (Kim et al. 2007) from Arabidopsis functions as a transcriptional repressor. Although the full length of Arabidopsis NAC1 showed little activation activity, the C-terminus of NAC1 exhibited strong transcriptional activation activity (Xie et al. 2000). Using deletion experiments for GmNAC20, a transcriptional repression domain was identified as NAC repression domain (NARD), with 35 aa in subdomain D of the NAC DNA-binding domain (Hao et al. 2010). The NARD-like sequences are sufficient to suppress even other TF families, such as DNA binding with One Finger (Dof), WRKY, and APETALA 2/dehydration-responsive element (AP2/DRE) (Hao et al. 2010). Hydrophobic "LVFY" residues in NARD were shown to be required for transcriptional repression, possibly by interfering with DNA binding and/or nuclear transport (Hao et al. 2010). To our surprise, the presence of the conserved NARD sequences in the D domains of all eight GmSNAC proteins did not result in transcriptional repression (Fig. 2B; Fig. 4). This finding indicates that there are regions other than the NARD domain controlling transcriptional activities in NAC proteins. The ability of NAC protein to regulate gene expression might depend on the interplay among the NARD, the C-terminus activation domain, and the DNA-binding domain, suggesting that NAC family proteins may have different mechanisms for

transcriptional regulation.

Compared to general TFs, NAC TFs are targeted not only to the nucleus for their transcriptional role, but also to other locations in the cell. Fig. 5 shows that five GmSNAC proteins (excluding GmSNAC4, GmSNAC9, and GmSNAC38) were restricted to the nucleus (Fig. 5). GmSNAC4, GmSNAC9, and GmSNAC38 are predicted for localization to the nucleus, as well as the membrane, such as plasma membrane or ER based on PSORT analysis. Eleven GmNAC TFs were predicted to be localized to the membrane based on the existence of the TMs among the 152 soybean GmNACs (Le et al. 2011), and more than 13 NAC members in Arabidopsis and six NAC members in rice possessed strong a-helical TMs, like the NTM1 structure (Kim et al. 2007). It has been suggested that membrane-bound transcription factors (MTFs) are synthesized as dormant, membrane-bound forms that are anchored to the cellular membranes, such as plasma membranes, ER, and nuclear membranes. Upon stimulation by certain environmental and/or developmental cues, they are processed via a regulated intramembrane proteolysis (RIP) mechanism involving specific intramembrane proteases or via a regulated ubiquitin/proteasome-dependent processing (RUP) mechanism (Brown et al. 2000; Vik and Rine 2000; Hoppe et al. 2001; Kim et al. 2007). The processed, transcriptionally active forms go into the nucleus and control the expression of target genes. Four Arabidopsis NTLs—NTL6, NTL8, NTL9, and NTL12—are activated by membrane-associated proteases in the ER by liberating the TFs from their TM domain by environmental stresses (Kim et al. 2007; Chen et al. 2008; Seo et al. 2008). Three GmSNAC TFs—GmSNAC4, GmSNAC9, and GmSNAC38—are MTFs with a TM (Table 1; Fig. 5) and are closely related to Arabidopsis NTL6 and NTL9 (Fig. 2A). Compared to the nuclear localization of five stress-inducible GmSNAC TFs, localization of those three GmSNAC TFs was not restricted to the nucleus, but was also present in other fractions (Fig. 5). It is predicted that they move to the nucleus after activation of the membrane-associated proteases, similar to other NTLs, although their precise cellular localization needs to be examined further.

Phylogenetic analysis revealed that eight GmSNAC proteins were classified into five subgroups (Fig. 2A). GmSNAC49 exhibited the highest identities (80%) with ANAC072 (RD26) (Fujita et al. 2004) (Fig. 2A). *RD26* significantly reduced Arabidopsis growth in the media supplemented with ABA (Fujita et al. 2004). In our study, the ectopic expression of *GmSNAC49* did not affect the growth of any seedlings in response to ABA (data not shown). Germination rates of *GmSNAC49* OE lines were slightly lower than those of WT in the presence of ABA, while germination of the OE transgenic seeds was considerably delayed by mannitol-inducing osmotic stress compared to WT plants (Fig. 6C). The overexpression of *GmSNAC49* increases drought tolerance and dehydration in Arabidopsis (Fig. 6). To investigate the target genes of *GmSNAC49*, we performed real-time (q)RT-PCR analysis using the *GmSNAC49*-overexpressed plants. As might be expected, several ABA- and drought-responsive genes were upregulated in the *GmSNAC49*-overexpressed plants (Fig. 8). For example, the *RD20* gene, a stress-inducible caleosin, upregulated in *GmSNAC49*-overexpressed plants, functions in stomata control, transpiration, and drought tolerance (Aubert et al. 2010) (Fig. 8). Genes responsive to drought, such as *KIN2* and *RD29A*, (Yamaguchi-Shinozaki and Shinozaki, 1993, 2006) were also upregulated in *GmSNAC49*-overexpressed plants (Fig. 8). Upregulation of *ABF3* and *ABI1* and ABA-signaling upstream genes in the *GmSNAC49*-overexpressed plants is also notable (Fig. 8). Similar to *AREB1* and *AREB2*, *ABF3*is a master bZIP TF, contributing to improved drought stress tolerance thorough ABRE-dependent ABA signaling cascades (Yoshida et al. 2010). Knockout mutant *ABI1,* a protein phosphatase 2C, displayed an insensitive response to ABA during the seed and vegetative stages (Leung et al. 1997). *ABI1* mutant was shown to germinate faster in the presence of ABA and to dehydrate faster than WT, implying that *ABI1* plays an important role in the germination process and in drought stress tolerance in the ABA-dependent pathway (Leung et al. 1997). Therefore, it is presumed that ectopic *GmSNAC49* might upregulate the expression of key ABA-signaling genes, resulting in enhanced drought tolerance in the plants. These findings suggest that *GmSNAC49* functions as a positive regulator in ABA signal transduction in vegetative tissue.

Materials and Methods

Plant Materials, Growth Conditions and Stress Treatments

Soybean (*Glycine max* L. Gwangan) and *Nicotiana benthamiana* were grown in a growth chamber at 28°C/24°C with a photoperiod of 16 h at a photosynthetic flux of 70 μmol photons m^{-2} s^{-1}. Soybean plants (3-4 week-old) were treated with low temperature, dehydration, wounding, high salinity stress (100 mM NaCl), and with abscisic acid (ABA) (100 μM) solution as described previously (Chung et al. 2013). After the treatments, the samples were frozen in liquid nitrogen and stored at -75ºC. Arabidopsis (*Arabidopsis thaliana* ecotype Columbia, Col-0) and transgenic plants were grown at 22°C under long-day conditions (16-h light/8-h dark) or short-day conditions (8-h light /16-h dark).

Database Search, Multiple Sequence Alignment, and Phylogenetic Analysis for NAC Proteins

Soybean Transcription Factor Database website (http://soybeantfdb.psc.riken.jp/) was utilized to retrieve 62 *NAC*s, of which promoter regions contain any ABA responsive *cis*-acting element (ABRE) or drought-responsive *cis*-acing element (DREB). TBLASTN (McGinnis and Madden 2004) provided by NCBI was used to collect the Arabidopsis and rice NAC genes homologous to soybean GmSNACs. The SMART program (Letunic et al. 2004) was used to search for the NAC domain in the GmSNACs.

Alignments of protein sequences were performed by the ClustalW program (Higgins et al. 1996) with default parameters. GeneDoc was also used to align the motif as a secondary method to align sequences and recheck the result. The neighbor-joining tree was constructed based on the obtained sequences of predicted NAC proteins and their most homologous NAC proteins from Arabidopsis and rice. Phylogenetic analysis was based on the neighbor-joining tree using the MAFFT web-based alignment program, with 1,000 bootstrap replications (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/index.html). The conserved motifs in the GmSNAC protein sequences were identified using multiple expectation maximization for motif elicitation (MEME) (http:///www.meme.sdsc.edu) (Bailey et al. 2006). The analysis parameters were set as following: number of repetitions, any; maximum number of motifs, 20; and the optimum motif widths were constrained to between 6 and 50 residues.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative (q)RT-PCR Analyses

Total RNA was isolated from the samples using an RNA extraction kit (Ambion, Chuncheon, Republic of Korea) and treated with DNase I (Promega, Madison, WI, USA). First strand cDNA was synthesized using 2 μg of total RNA, oligo d(T) primer, and M-MLV reverse transcriptase (Invitrogen, Waltham, MA, USA) according to the manufacturer instructions. Samples from each reaction (1 μL) were used in a 20-μL premix PCR mixture containing *Taq* polymerase (Bioneer, Daejeon, Republic of Korea), with soybean *NAC* gene (Table S1) or Arabidopsis stress-marker gene primers (Table S2). For (q)RT-PCR, amplification was performed for 26 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s. PCR product was analyzed by agarose gel electrophoresis and staining with ethidium bromide. (q)RT-PCR was performed for 40 cycles using 1 μL cDNA as a template and CFX-96^{TM} Real Time system with SYBR Premix (Bio-Rad, Foster City, CA, USA). (q)RT-PCR data were analyzed with CFX Manager v2.1 software (Bio-Rad). Data was normalized to *Tubulin* for soybean *NAC* genes or to *ACTIN* mRNA levels for Arabidopsis plant. All the primers used for (q)RT-PCR analysis are listed in Table S1.

Yeast One-hybrid Assay

For the yeast one-hybrid assay, each full-length cDNA of eight *GmSNAC* was PCR-amplified with CACC-forward primer and reverse primer, respectively (Table S3). Each PCR amplified DNA fragment was cloned between *attB1* and *attB2* sites of the pENTR/D TOPO vector (Invitrogen), resulting in pENTR-GmSNAC. The resulting PCR products were cloned into the pENTR vector (Invitrogen) and then recombined into the gateway destination binary vector, pDEST32 (Clontech, Mountain View, CA, USA) yielding pDB-GmSNAC. MaV203 was transformed with each pBD-GmSNAC, selected on SD/-Leu medium, and allowed to grow for 4 d at 30ºC, respectively. Transformants were subsequently grown on SD/-Leu/-Ura/-His medium containing 5 mM 3-AT (3-Amino-1,2,4-Triazole) or supplemented with X-β-Gal (5-bromo-5-chloro-3-indolyl-β-D-galactoside) for 3 days at 30ºC.

Quantification assay was performed by using *o*-nitrophenyl-D-galactopyranoside (ONPG) to test β-galactosidase activity according to the method described in the yeast protocols (Invitrogen). Three transformants for each transfection were selected randomly and subjected to the ONPG assay. MaV203 strain was cotransformed with BD-Krev1/AD-RalGDS-wt for the positive control, and BD-Krev1/AD-RalGDS-m2, as a negative control.

Construct and Subcellular Localization of GFP-fusion Protein

Each *GmSNAC* of pENTR-GmSNAC was then recombined as a C-terminal fusion of GFP into the Gateway destination binary vector, pK7FGW2 (Plant Systems Biology, Belgium; http://www.psb.ugent.be/) yielding *35S:GFP-GmSNAC* by a LR recombination reaction. The GFP fusion constructs of the eight *GmSNAC* genes were transformed into *Agrobacterium* sp. strain C58c1, respectively. For the transient expression of GFP proteins in plant, the positive transformants were inoculated into the leaves of *benthamiana* respectively as described previously (Chung et al. 2004). The protoplasts were isolated from the leaves as described by Abel and Theologis (1994). A Zeiss LSM700 (Carl Zeiss, Oberkochen, Germany) confocal microscope was used to observe the fluorescence as described previously (Chung et al. 2009).

Transformation Vectors and Construction of Transgenic Plants

To produce the *35S:GmSNAC49* transgenic plants, the ORF region of *GmSNAC49* (148 aa) of pENTR-GmSNAC49 was cloned into the pENTR vector (Invitrogen) and then recombined into the gateway destination binary vector, pB7WG2D (Plant Systems Biology), in which transgene expression is under the control for the CaMV 35S promoter. Transformation of Arabidopsis was performed by the vacuum infiltration method using *Agrobacterium tumefaciens* strain C58c1 (Clough and Bent 1998). Transgenic plants were sprayed with basta and the resistant plants were transferred to soil to obtain homozygous T3 seeds. For the phenotypic analysis, T3 homozygous lines were used.

Germination and Osmotic Stress Tolerance Tests

Each plant was grown in the same conditions, and seeds were collected at the same time. Germination (full emergence of radicles) of the wild-type and the *35S:GmSNAC49* transgenic seeds was scored on MS medium (2% Suc and 0.8% agar) without or with different concentrations of ABA (0.8 μM), or mannitol (300 mM) as indicated. Plates were chilled at 4°C in the dark for 3 d (stratified) and moved to 22°C with a 16-h-light/8-h-dark cycle. The percentage of seed germination was scored after 4 d with 3 repetitions. The wild-type and the *35S:GmSNAC49* transgenic seedlings (10-d-old) were transferred to MS media with or without NaCl (150 mM) or mannitol (200 mM) and were subsequently grown for 10 d. Root length of the seedlings grown under normal and osmotic stress conditions was measured with five replications.

For the soil-grown plant drought tolerance test, the 3-week-old plants were subjected to progressive drought by withholding water for 11 d. The test was repeated a minimum of three times. To measure leaf water loss, the fully expanded leaves were removed from 4-week-old plants, placed abaxial side up in open Petri dishes at room temperature, and weighed at different time intervals. Leaves of similar developmental stages (third to fifth true rosette leaves) from 4-week-old soil-grown plants were used.

Statistical Analysis

Statistical testing was performed with SPSS software (V13.0, SPSS Inc. USA).

Acknowledgements

This research was supported by Research Program through Dong-A University in Korea.

Author's Contributions

HAS Designed and executed the experiment; JHL Designed and wrote initial manuscript draft, critically edited and finalized the

manuscript. All the authors agreed on the contents of the paper and post no conflicting interest.

Supporting Information

Fig. S1. RT-PCR analyses of soybean *NAC* genes in response to dehydration stress or ABA treatment.
Table S1. Information of the primer sets of GmSNACs for RT-PCR and real-time qRT-PCR.
Table S2. Information of the primer sets of GmSNACs for pENTR vector cloning.
Table S3. Primer sequences used for real-time (q)RT-PCR.

References

Abel S, Theologis A (1994). Transient transformation of Arabidopsis leaf protoplasts: a versatile system to study gene expression. Plant J 5:421−427

Aubert Y, Vile D, Pervent M, Aldon D, Ranty B, Simonneau T, Vavasseur A, Galaud JP (2010) RD20, a stress-inducible caleosin, participates in stomata control, transpiration and drought tolerance in *Arabidopsis thaliana*. Plant Cell Physiol 51:1975−1987

Bailey TL, Williams N, Misleh C, Li WW (2006) MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res 34:W369–W373

Brown MS, Ye J, Rawson RB, Goldstein JL (2000) Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 100:391−398

Bu Q, Jiang H, Li C-B, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C (2008) Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Res 18:756−767

Chen YN, Slabaugh E, Brandizzi F (2008) Membrane-tethered transcription factors in *Arabidopsis thaliana*: novel regulators in stress response and development. Curr Opin Plant Biol 11:695–701

Chen Q, Wang Q, Xiong L, Lou Z (2011) A structural view of the conserved domain of rice stress-responsive NAC1. Protein Cell 2:55–63

Chung E, Seong E, Kim Y-C, Chung EJ, Oh S-K, Lee S, Park JM, Joung YH, Choi D (2004) A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annum* L. cv. Bukang). Mol Cells 17:377−380

Chung E, Cho CW, Yun BH, Choi HK, So HA, Lee SW, Lee J-H (2009) Molecular cloning and characterization of the soybean DEAD-box RNA helicase gene induced by low temperature stress. Gene 443:91−99

Chung E, Cho C-W, So H-A, Kang J-S, Chung YS, Lee J-H (2013) Overexpression of *VrUBC1*, a Mung Bean E2 Ubiquitin-Conjugating Enzyme, Enhances Osmotic Stress Tolerance in Arabidopsis. PLoS One 8:e66056

Clercq DI, Vermeirssen V, Aken OV, Vandepoele K, Murcha M W, Law SR, Inzé A, Ng S, Ivanova A, Rombaut D, van de Cotte B, Jaspers P, de Peer YV, Kangasjärvi J, Whelan J, Breusegem FV (2013) The Membrane-Bound NAC Transcription Factor ANAC013 Functions in Mitochondrial Retrograde Regulation of the Oxidative Stress Response in Arabidopsis. Plant Cell 25:3472–3490

Delessert C, Kazan K, Wilson IW, Van Der Straeten D, Manners J, Dennis ES, Dolferus R (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in Arabidopsis. Plant J 43:745–757

Duval M, Hsieh TF, Kim SY, Thomas TL (2002) Molecular characterization of AtNAM: a member of the Arabidopsis NAC domain superfamily. Plant Mol Biol 50:237–248

Ernst HA, Olsen AN, Larsen S, Lo Leggio L (2004) Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. EMBO Rep 5:297–303

Fang Y, You J, Xie K, Xie W, Xiong L (2008) Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics 280:547–563

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 39:863–876

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601−612

Hao Y-J, Song Q-X, Chen H-W, Zou H-F, Wei W, Kang X-S, Ma B, Zhang W-K, Zhang J-S, Chen S-Y (2010) Plant NAC-type transcription factor proteins contain a NARD domain for repression of transcriptional activation. Planta 232:1033–1043

Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY (2011). Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. Plant J 68:302–313

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J 44:903–916

Hibara K, Takada S, Tasaka M (2003) CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. Plant J 36:687–696

Hibara K, Karim MR, Takada S, Taoka K, Furutani M, Aida M, Tasaka M (2006) Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. Plant Cell 18:2946–2957

Higgins DG, Thompson JD, Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. Methods Enzymol 266:383−402

Hoppe T, Rape M, Jentsch S (2001) Membrane-bound transcription factors: regulated release by RIP and RUP. Curr Opinion Cell Biol 13:344–348

Hu HH, Dai MQ, Yao JL, Xiao BZ, Li XH, Zhang QF, Xiong LZ (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103:12987–12992

Hu HH, You J, Fang YJ, Zhu XY, Qi ZY, Xiong LZ (2008) Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. Plant Mol Biol 67:169–181

Jensen MK, Kjaersgaard T, Nielsen MM, Galberg P, Petersen K, O'Shea C, Skriver K (2010) The *Arabidopsis thaliana* NAC transcription factor family: structure-function relationships and determinants of ANAC019 stress signalling. Biochem J 426:183–196

Kikuchi K, Ueguchi-Tanaka M, Yoshida KT, Nagato Y, Matsusoka M, Hirano HY (2000) Molecular analysis of the NAC gene family in rice. Mol Gen Genet 262:1047–1051

Kim SY, Kim SG, Kim YS, Seo PJ, Bae M, Yoon HK, Park CM (2007) Exploring membrane-associated NAC transcription factors in Arabidopsis: implications for membrane biology in genome regulation. Nucleic Acids Res 35:203–213

Kim YS, Sakuraba Y, Han SH, Yoo SC, Paek NC (2013) Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence. Plant Cell Physiol 54:1660–1672

Le DT, Nishiyama R, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS (2011) Genome-wide survey

and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. DNA Res 18:263–296

Le DT, Nishiyama R, Watanabe Y, Tanaka M, Seki M, Ham LH, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP (2012) Late developmental stages under drought stress revealed by genome-wide transcription analysis. PLoS One 7:e49522

Letunic I, Copley RR, Schmidt S, Ciccarelli FD, Doerks T, Schultz J, Ponting CP, Bork P (2004) SMART 4.0: towards genomic data integration. Nucleic Acids Res 32:D142−D144

Leung J, Merlot S, Giraudat J (1997) The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatase 2C involved in abscisic acid signal transduction. Plant Cell 9:759−771

McGinnis S, Madden TL (2004) BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32:W20−W25

Meng Q, Zhang C, Gai J, Yu JG (2007) Molecular cloning, sequence characterization and tissue-specific expression of six NAC-like genes in soybean (Glycine max (L.) Merr.). J Plant Physiol 164:1002−1012

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) NAC transcription factors in plant abiotic stress responses. Biochim Biophys Acta 1819:97–103

Ng S, Ivanova A, Duncan O, Law SR, Aken OV, Clercq ID, Wang Y, Carrie C, Xu L, Kmiec B, Walker H, Breusegem FV, Whelan J, Giraud E (2013) A Membrane-Bound NAC Transcription Factor, ANAC017, Mediates Mitochondrial Retrograde Signaling in Arabidopsis. Plant Cell 25:3450–3471

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S (2010) Genome-wide analysis of NAC transcription factor family in rice. Gene 465:30–44

Nuruzzaman M, Sharoni AM, Kikuchi S (2013) Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front Microbiol 4:248

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) DNA-binding specificity and molecular functions of NAC transcription factors. Plant Sci 169:785–797

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S (2003) Comprehensive analysis of NAC family genes in Oryza sativa and *Arabidopsis thaliana.* DNA Res 10:239–247

Puranik S, Sahu PP, Srivastava PS, Prasad M (2012) NAC proteins: regulation and role in stress tolerance. Trends Plant Sci 17:369–381

Quach TN, Tran LS, Valliyodan B, Nguyen HT, Kumar R, Neclakandan AK, Guttikonda SK, Sharp RE, Nguyen HT (2014) Functional analysis of water stress-reponsive soybean GmNAC003 and GmNAC004 transcription factors in lateral root development in Arabidopsis. PLoS One 9:e844886

Ren T Qu F, Morris TJ (2000) *HRT* Gene Function Requires Interaction between a NAC Protein and Viral Capsid Protein to Confer Resistance to Turnip Crinkle Virus. Plant Cell 12:1917–1925

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Jang JZ, Ghandehari D, Sherman BK, Yu G (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science 290: 2105–2110

Schmutz J, Cannon SB, Schlueter J, Ma JX, Mitros T, Nelson W, Hyten DL, Song QJ, Thelen JJ, Cheng JL, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu SQ, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du JC, Tian ZX, Zhu LC, Gill N, Joshi T, Libault M, Sethuraman A, Zhang XC, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J, Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178–183

Seo PJ, Kim SG, Park CM (2008) Membrane-bound transcription factors in plants. Trends Plant Sci 13:550–556

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 85:159–170

Takada S, Hibara K, Ishida T, Tasaka M (2001) The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development 128:1127–1135

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16:2481–2498

Tran LS, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, Maruyama K, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K (2007) Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. Plant J 49:46–63

Tran LS, Quach TN, Guttikonda SK, Aldrich DL, Kumar R, Neelakandan A, Valliyodan B, Nguyen HT (2009) Molecular characterization of stress-inducible GmNAC genes in soybean. Mol Genet Genomics 281:647–664

Vik A, Rine J (2000) Membrane biology: membrane-regulated transcription. Curr Biol 10:R869–R871.

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaai MA, de Vries SC (2003) The cup-shaped cotyledon 3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell 15:1563–1577

Wang Z, Libault M, Joshi T, Valliyodan B, Nguyen HT, Xu D, Stacey G, Cheng J (2010) Soy DB: a knowledge database of soybean transcription factors. BMC Plant Biol 10:14

Wu Y, Deng Z, Lai J, Zhang Y, Yang C, Yin B, Zhao Q, Zhang L, Li Y, Yang C, Xie Q (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. Cell Res 19:1279–1290

Xie Q, Sanz-Burgos AP, Guo H, García JA, Gutiérrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol 39:647–656

Xie Q, Frugis G, Colgan D, Chua NH (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14:3024–3036

Xie Q, Guo HS, Dallman G, Fang S, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature 419:167–170

Yamaguchi-Shinozaki K, Shinozaki K (1993) Arabidopsis DNA encoding two desiccation-responsive rd29 genes. Plant Physiol 101:1119–1120

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803

Yang Z-T, Lu S-J, Wang M-J, Bi D-L, Sun L, Zhou S-F, Song Z-T, Liu J-X (2014) A plasma membrane-tethered transcription factor, NAC062/ANAC062/NTL6, mediates the unfolded protein response in Arabidopsis. Plant J 79:1033–1043

Yoon H-K, Kim S-G, Kim S-Y, Park C-M (2008) Regulation of Leaf

Senescence by NTL9-mediated Osmotic Stress Signaling in Arabidopsis. Mol Cells 25:438–445

Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. Plant J 61:672–685