

ATAF2, a NAC Transcription Factor, Binds to the Promoter and Regulates *NIT2* Gene Expression Involved in Auxin Biosynthesis

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The transcription factor ATAF2, one of the plant specific NAC family genes, is known as repressor of pathogenesis-related genes and responsive to the diverse defense-related hormones, pathogen infection, and wounding stress. Furthermore, it is important to consider that tryptophan-dependant IAA biosynthesis pathway can be activated by wounding and pathogen. We found that *ATAF2pro::GUS* reporter was induced upon indole-3-acetonitrile (IAN) treatments. And *ataf2* mutant showed reduced sensitivity to IAN whereas *35S::ATAF2* plants showed hyper-sensitivity to IAN. IAN biosynthesis required nitrilase involved in the conversion of IAN to an auxin, indole-3-acetic acid (IAA). We found that the *NIT2* gene was repressed in *ataf2* knockout plants. Expression of both *ATAF2* and *NIT2* genes was induced by IAN treatment. Transgenic plants overexpressing *ATAF2* showed up-regulated *NIT2* expression. *ATAF2* activated promoter of the *NIT2* gene in *Arabidopsis* protoplasts. Electrophoretic mobility shift assay revealed that *NIT2* promoter region from position -117 to -82 contains an ATAF2 binding site where an imperfect palindrome sequence was critical to the protein-DNA interaction. These findings indicate that ATAF2 regulates *NIT2* gene expression via *NIT2* promoter binding.

INTRODUCTION

Plants are under constant threat from environmental stress and attack from pathogens, vertebrates, and invertebrates. To cope with this, plants have evolved elaborate mechanisms in perceiving the attack and responding to the signaling to unite the reception of information to the induction of appropriate defense gene expression (Hegedus et al., 2003). Upon stress, gene expression is tightly regulated in a coordinated and timely manner, and the regulation of gene expression is largely governed by specific transcription factors (Wray et al., 2003).

Plant-specific NAC transcription factor containing the NAC domain, namely *NAM* (*no apical meristem*), *ATAF1,2* and *CUC2* (*cup-shaped cotyledon*) is one of the largest plant-specific families (Ernst et al., 2004; Riechmann et al., 2000). The members

of the NAC protein family are identifiable by the presence of a highly conserved N-terminal NAC domain accompanied by diverse C-terminal domains (Ernst et al., 2004). The DNA binding ability of this protein family has been assigned to the NAC domain (Duval et al., 2002; Ernst et al., 2004; Xie et al., 2000). Only a portion of the NAC genes has been studied to date and the family has been implicated in diverse processes (Fang et al., 2008). Some NAC proteins have a role in biotic and abiotic stress as well as in plant development (Fang et al., 2008). For example, transgenic plants overexpressing one of three different *Arabidopsis* NAC proteins (ANAC019, ANAC055, and ANAC072) display significantly increased drought tolerance (Tran et al., 2004). The TM2/*Arabidopsis* NAC domain-containing protein 69 is involved with auxin and salt signaling in seed germination (Park et al., 2011). Thus, NAC proteins are emerging as important proteins in plant development and stress response.

Although NAC family members are very numerous and are known to be transcription factors, little is known about the direct target genes and the DNA-binding specificity of NAC proteins (Olsen et al., 2005). *Arabidopsis* ATAF1 and ATAF2 were isolated based on their ability to activate the *Cauliflower mosaic virus* (CaMV) 35S promoter in yeast (Souer et al., 1996). However, only some DNA binding sites in a direct target gene of NAC proteins has been clearly identified to date. For example, ANAC019, ANAC055, and ANAC072 bind to the 63 bp promoter region of the *ERD1* gene and the NAC recognition sequence (NACRS), ANNNNTCCNNNNNNNACACGCATGT, containing CACG as the core DNA-binding site was determined using the yeast one-hybrid system (Tran et al., 2004). Furthermore, vascular-related NAC-domain 7 (VND7) and secondary wall-associated NAC domain protein 1 (SND1) directly bind to secondary wall NAC binding element (SNBE) in the target promoters (Zhong et al., 2010).

Previously, *ATAF2* was induced by salt stress and pathogen attack and involved in the regulation of biotic and abiotic stress and development (Delessert et al., 2005; Seki et al., 2002). However, *ATAF2* has no known biological function in auxin pathway. We report here that *ATAF2* directly binds to the promoter of *nitrilase 2* (*NIT2*) that encodes a nitrilase, which converts IAN to auxin, and activates *NIT2* transcription *in vivo*.

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Furthermore, we analyzed the ATAF2 binding sequence within the *NIT2* promoter and demonstrated that the sequence contains an imperfect palindrome, CAAATNNNATTTG.

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana (ecotype Col-0) were grown in soil under a 16 h-light/8 h-dark photoperiod at 23°C. For chemical treatments, plants were grown aseptically on plant nutrient medium (Haughn and Somerville, 1986) supplemented with 0.5% (w/v) sucrose (PNS) and 0.6% agar or PNS supplemented with 10–50 μ M IAN or 0.5–3 μ M IAA.

Construction of transgenic plants

The coding region of *ATAF2* was obtained by PCR amplification. The resulting PCR fragment of *ATAF2* was cloned into *XbaI/SacI* site in pC2300M2 (CAMBIA). The construct was introduced into Col-0 and *ataf2* mutant plants using *Agrobacterium*-mediated transformation as described previously (Clough and Bent, 1998). The *ataf2* mutant was obtained from the Salk mutant collection (SALK_015750).

To generate *ATAF2*promoter::*GUS* transgenic plants, a 2.3 kb fragment upstream from the start codon of *ATAF2* was PCR amplified from genomic DNA of *Arabidopsis*. The 2.3 kb fragment was ligated into *Bam*H1/*Sma*I site of the promoter-less *GUS* expression vector pBI101 (Clontech).

To generate *NIT2*-RNAi transgenic plants, fragment of *NIT2* was PCR amplified from cDNA of *Arabidopsis*, cloned, and inserted into the pHANNIBAL vector in inverted repeat manner. And then, a *NotI* fragment containing CaMV 35S promoter with which repeated sequence fused was subjected to cloning into the pART27 binary vector. The resulting vector was subjected into *Agrobacterium*-mediated transformation as described previously (Clough and Bent, 1998).

Construction of deleted promoter regions fused to β -glucuronidase or luciferase reporter genes

For the construction of various chimeric genes with the *NIT2* promoter fused to the *GUS* or *LUC* reporter genes, initially the 2.4 kb upstream sequence of *NIT2* was obtained with PCR using the NIT2F2.4K and NIT2R2.4K primers (Supplementary Table S1). With the 2.4 kb PCR fragment as a template, a series of 5' deletion constructs of the *NIT2* promoter were generated using the same reverse PCR primer, NIT2PROR and specific forward primer set: P1 primer for the 1,467 bp P1 construct (-1,505 to -38); P2 primer for the 665 bp P2 construct (-703 to -38); P3 primer for the 234 bp P3 construct (-272 to -38); P4 primer for the 230 bp P4 construct (-268 to -38); P5 primer for the 199 bp P5 construct (-237 to -38); P6 primer for the 148 bp P6 construct (-186 to -38); P7 primer for the 117 bp P7 construct (-155 to -38); P8 primer for the 90 bp P8 construct (-128 to -38); P9 primer for the 80 bp P9 construct (-118 to -38). The resulting constructs were ligated into a *Bam*HI/*Nco*I site of the promoter-less pC2300X vector. For the two 3' deletion constructs, PCR was performed with P2 primer and P10 primer for the 584 bp P10 construct (-665 to -81) or with P2 primer and P11 primer for the 548 bp P11 construct (-665 to -117). The constructs were ligated into the *Bam*HI site of pBKM2 vector in which 35S minimal promoter was fused with the *LUC* coding region.

RNA blot analysis and qRT-PCR

For expression pattern analyses, three-week-old plants were

harvested from PNS agar plates, and then grown hydroponically in a solution containing 300 mM NaCl, 100 μ M ABA, 200 μ M IAN, 1 mM SNP, and 1 mM salicylic acid (SA). For dehydration stress, plants were transferred on Whatman 3MM paper at 23°C with approximately 60% humidity condition. The total RNA was extracted with TRI REAGENT as described in the manufacturer's protocol (Molecular Research Center). RNA blot was performed according to a published protocol (Church and Gilbert, 1984).

qRT-PCR was performed with SYBR Green (KapaBiosystems) according to the manufacturer's instructions and as previously described (Huh et al., 2012). Three independent biological replicate cDNA samples were tested for each experiment. Relative mRNA levels for each transcript were determined by the comparative cycle-threshold (CT) method (Appliedbiosystems). Expression data was normalized with an endogenous *Actin7* gene (internal reference). The following primer sets were used: *ATAF2F* 5'-TCTTCTACGCAGGGAAAGCTCC-3' and *ATAF2R* 5'-AGCCATTGTCGTGGTCCCTCG-3', *NIT1F* 5'-CGTTGCCCATCCACCAC-3' and *NIT1R* 5'-TATGAACGCCAACCGCTAAACC-3', *NIT2F* 5'-TCG TTA ATA ATG GAA GAG A-3' and *NIT2R* 5'-AAA AAG AAA AGA ACA CAA CTG-3', *NIT3F* 5'-CCCCGTTTACGACACTCCTA-3' and *NIT3R* 5'-CATCCACCTTCTACCGCAA-3', *Actin7F* 5'-AATGGTGAAGGCTGGTTTTG-3' and *Actin7R* 5'-TGCCTCTGTGAGTAGAACTG-3'.

Transient expression assay using *Arabidopsis* protoplasts

For transactivation analysis in *Arabidopsis* protoplasts, 2×10^4 protoplasts were transfected with 21 μ g plasmid DNA with different combinations of reporter, effector, and internal control. This procedure was performed as described previously (Jin et al., 2001).

Bacterial expression of glutathione S-transferase (GST)-ATAF2 fusion protein

ATAF2 fragment was prepared by PCR amplification and cloned into the *XbaI* and *SacI* sites of the pGEX-3X vector (Amersham). The resulting in-frame fusion protein plasmid was transformed into *Escherichia coli* strain BL21 (DE3). The GST fusion protein was recovered by affinity chromatography on glutathione-sepharose beads (Pharmacia).

Electrophoretic mobility shift assay (EMSA)

DNA binding reactions containing specified concentrations of protein (50–150 ng), 5 μ l of 4 \times reaction buffer [200 mM KCl, 100 mM HEPES/KOH at pH 7.9, 2 mM EDTA, 2 mM dithio-threitol (DTT), 20% glycerol, 20 μ g/ μ l bovine serum albumin (BSA)], 1 μ g poly dIdC (Pharmacia), and 1 μ l 1% Nonidet P-40 in a final volume of 18 μ l. The labeled DNA probe (25 ng) was then added to the reaction mixture. After incubation for 30 min at room temperature, 10 μ l of the mixture was loaded on a 6% non-denatured polyacrylamide gel. Electrophoresis was performed in 0.5 \times TBE (54 mM Tris-borate, pH 8.3, 1 mM EDTA) for 2.5 h. Gels were dried and exposed for autoradiography. The DNA probes used in the analyses were: F1S, 5'-AAAT AAGAAGGCAAATATAATTTGATAACAACACTATT-3'; F1AS, 5'-AATAGTTGTTATCAAATTATATTTTGCCTTCTTATT-3'; F2S, 5'-ATATTTGGGTGAATATTTTTCCAAATTAAGTGTAGC-3'; F2AS, 5'-GCTACAGTTAATTTGGAAAAATATTCACCCAAATA T-3'; F3S, 5'-ATGGAGTAATAATATATTTTGTAAATTGTCA GTCT-3'; F3AS, 5'-AGACTGACAATTAACAAAATATATTAT TACTCCAT-3'.

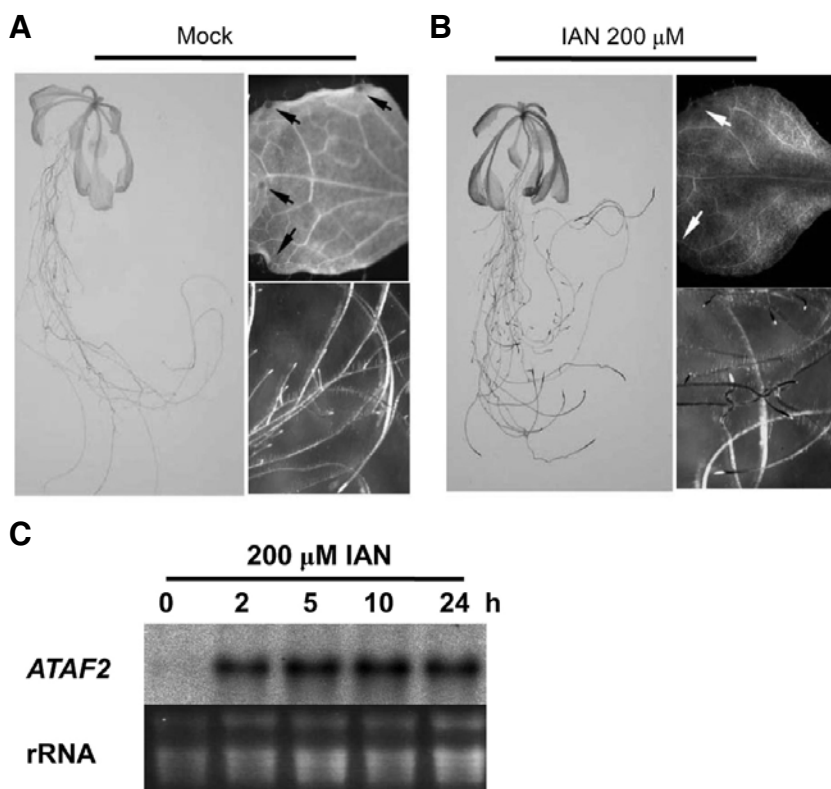


Fig. 1. Histochemical assay of the *GUS* reporter gene under the control of *ATAF2* promoter in transgenic plants upon IAN treatment. (A) Histochemical assay was done with 18 days old *ATAF2pro::GUS* transgenic plants treated with water for 5 h as control. Enlarged images show leaf and root tissue. Arrows indicate the staining of the hydathodes. (B) *ATAF2pro::GUS* transgenic plants were treated with 200 μ M IAN for 5 h and histochemical assay was performed. (C) Expression pattern of *ATAF2* under IAN treatment. Three-week-old *Arabidopsis* seedlings grown vertically on PNS medium were used and 200 μ M IAN treatment was conducted for indicated time periods. RNA blot analysis was performed with *ATAF2* gene-specific probe. Ribosomal RNA (rRNA) was used as a loading control.

RESULTS

ATAF2pro::GUS reporter is activated by IAN treatment

Previously, it was shown that gene expression of *ATAF2* was increased upon salicylic acid (SA) and methyl jasmonic acid (MeJA) treatments but was not changed upon abscisic acid (ABA) treatment (Delessert et al., 2005). We performed microarray experiment using *ataf2* mutant. Interestingly, expression of some of auxin-related genes was repressed in *ataf2* mutant (data not shown). However, biological functions of *ATAF2* related to plant auxin hormones are still not clear. To identify biological functions of *ATAF2* in auxin hormone response, we first generated *ATAF2pro::GUS* reporter system which allowed to reveal gene expression patterns by histochemical staining of plant tissue upon plant hormone treatments. Weak expression of the *ATAF2pro::GUS* fusion gene was observed in several regions of rosette leaf while strong expression was revealed in the hydathodes under normal growth conditions (Fig. 1A). By contrast, *ATAF2pro::GUS* reporter was activated by IAN treatment (Fig. 1B). By IAN treatment, intense staining was shown in rosette leaves and roots (Fig. 1B). *ATAF2* function of auxin pathway has not been clearly identified although *ATAF2* is involved in the plant defense *via* repressing the expression of *pathogenesis-related* genes and is known as target of replicase protein of *Tobacco mosaic virus* (TMV) (Delessert et al., 2005; Wang et al., 2009). To confirm the expression result, we also performed RNA blot analysis upon IAN treatment. And *ATAF2* was strongly induced within 2 h in response to IAN treatment (Fig. 1C). These results indicated that *ATAF2* might be involved in auxin signaling.

ATAF2 is required for full induction of *NIT2* in *Arabidopsis*

Arabidopsis nitrilases (*NIT1*, *NIT2*, and *NIT3*) convert IAN to IAA and indole-3-acetamide (IAM) (Pollmann et al., 2002). IAA is an auxin and some microarrays analyses have demonstrated up-regulation of *NIT1* and *NIT2* in response to IAN and NaCl treatment (Grsic et al., 1998; Jiang et al., 2007; Woodward and Bartel, 2005). Furthermore, NAC transcription factor also controls salinity stress via the auxin pathway (He et al., 2005; Park et al., 2011). Therefore, we wanted to understand whether *ATAF2* might be related to the auxin pathway by regulating *NIT* genes at the transcriptional level under environmental stress conditions. We first analyzed gene expression of *NIT1*, *NIT2*, and *NIT3* in *ataf2* mutant upon IAN treatment. *NIT1* and *NIT3* transcript levels were not affected in *ataf2* mutant upon IAN treatment (Figs. 2B and 2D). However, *NIT2* gene expression in the *ataf2* mutant was repressed (by about 2-fold) compared to Col-0 plants upon IAN treatment (Fig. 2C), suggesting that *ATAF2* specifically affects one of three *NIT* genes (Fig. 2C). *ATAF2* transcripts were induced within 2 h after IAN treatment (Fig. 2A). *NIT2* transcripts were also elevated within 2-5 h after IAN treatment (Fig. 2C), suggesting that expressions of both genes are related each other (Fig. 2C). We also performed RNA blot analysis. Transcript levels of *ATAF2* and *NIT2* almost did not change under this condition (Supplementary Fig. S1F). The transcripts of *ATAF2* started to accumulate within 1 h after NaCl, SNP, SA, and dehydration treatment and most of time gradually increased further (Supplementary Figs. S1A, S1C, S1D, and S1E). *NIT2* gene expression upon diverse stress conditions was increased within 5 h, later than that of *ATAF2* (Supplementary Figs. S1A-S1E). For the ABA treatment, the transcript level of *NIT2* was increased but *ATAF2* gene did not respond (Supplementary Fig. S1B). These results suggest that

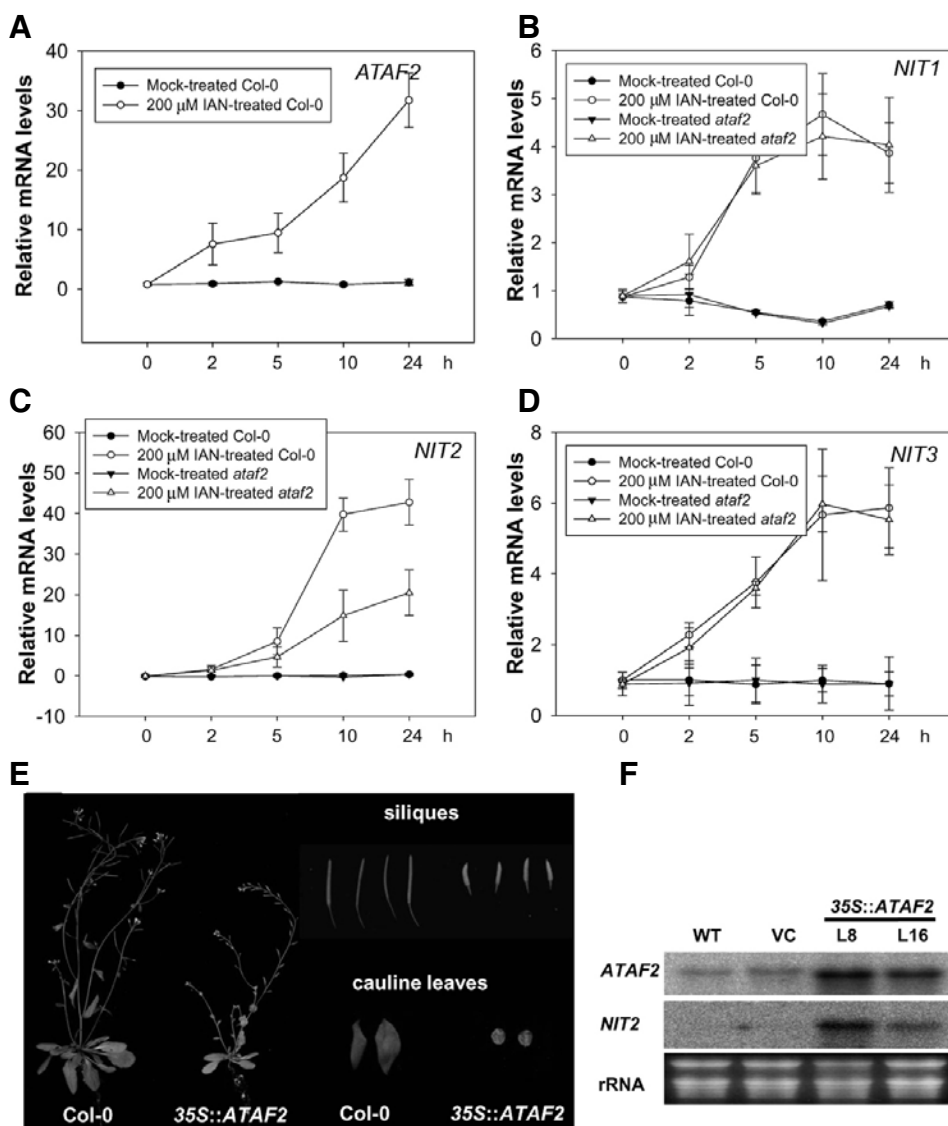


Fig. 2. ATAF2 requirement for full expression of *NIT2* but not for *NIT1* and *NIT3* upon IAN treatment. (A) Induction of *ATAF2* upon IAN treatment was confirmed by qRT-PCR. (B-D) Expression pattern of *NIT1*, *NIT2*, and *NIT3* in *ataf2* knock-out mutant and Col-0 plants upon IAN treatment. Samples were harvested at the indicated time points following mock and 200 μ M IAN treatments. Three independent biological replicate cDNA samples were tested for each experiment. Expression data was normalized with an endogenous *Actin7* gene (internal reference). (E) Phenotypic abnormalities in *35S::ATAF2* plants. Six-week-old *35S::ATAF2* transgenic plants showed reduced size and height and lighter green leaf colors when compared with Col-0 plants. *35S::ATAF2* transgenic plants also exhibit stunted siliques and round-shaped cauline leaves. (F) RNA blot analysis of *ATAF2* and *NIT2* gene in wild type and *35S::ATAF2* transgenic plants. Twelve-day-old seedlings of wild type (WT), vector control (VT), *35S::ATAF2* transgenic (L8 and L16) plants were hydroponically cultured and harvested. The blot was hybridized with *ATAF2* and *NIT2* gene-specific probes. Ribosomal RNA (rRNA) was used as a loading control.

the *ATAF2* induction by osmotic stress might occur through the ABA-independent pathway while the ABA-dependent pathway could involve the *NIT2* induction. And *ATAF2-NIT2* regulation might be closely related to diverse biotic and abiotic stress response.

We also generated *ATAF2*-overexpressing plants under the control of the CaMV *35S* promoter and analyzed the *NIT2* gene expression in the *35S::ATAF2* transgenic plants. *ATAF2*-overexpressing transgenic plants exhibited abnormal developmental phenotypes such as dwarfism, and leaf-yellowing (Fig. 2E). This developmental change does not appear to be associated with *ATAF2* overexpression in the C24 background with respect to the dwarfism, but leaf-yellowing phenotype was similar (Delessert et al., 2005). We do not know why some of the *ATAF2* overexpression phenotypes are different depending on the background. The *NIT2* transcripts were highly expressed in two independent *35S::ATAF2* transgenic plants, compared with wild type and vector control plants (Fig. 2F). This result suggested that *ATAF2* might directly regulate *NIT2* gene expression and

affect developmental processes.

***ataf2* plants exhibit reduced IAN sensitivity and *35S::ATAF2* plants show increased sensitivity to IAN**

A previous study demonstrated the IAN insensitivity of *nit1* mutant plants and increased sensitivity to IAN of *NIT2*-overexpressing transgenic plants (Normanly et al., 1997). We showed that *ATAF2* regulated *NIT2* expression from the study of *ataf2* mutant and *35S::ATAF2* transgenic plants (Figs. 2C and 2F). Based on these results, we tested whether sensitivity to IAN was altered in *ataf2* mutant and *ataf2* complementation lines. When germinated on the medium with 20-40 μ M IAN, *ataf2* mutant plants showed reduced sensitivity to IAN (Figs. 3A and 3B). After 8 days growth in the presence of 30 μ M IAN, the *ataf2* mutant plants exhibited about 30% increased primary root elongation compared with wild type plants, although the difference in root elongation was not as dramatic as the *nit1-3* mutant (Fig. 3C) (Normanly et al., 1997). In contrast, when grown in the presence of 1 μ M IAA, *ataf2* root elongation was not dif-

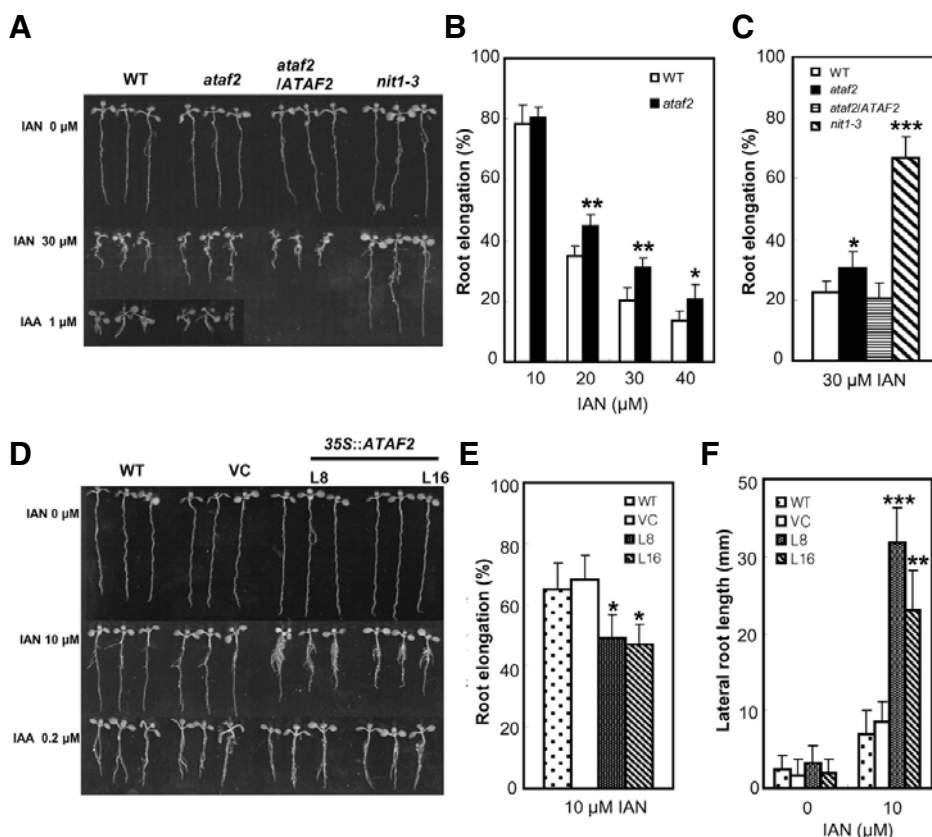


Fig. 3. Indole-3-acetonitrile (IAN) sensitivity test of *ataf2* knockout and *35S::ATAF2* plants. (A) Growth of seedlings on plant nutrient medium with sucrose (PNS) or PNS containing 30 μM IAN or 1 μM IAA. (B) IAN dose response of primary root growth. Shown is the percentage of elongation for IAN treatment at indicated concentration compared with that of the PNS control. Ten seedlings of each genotype were measured at individual IAN concentration. (C) Root elongation of *ataf2*, *ataf2/ATAF2* complementation transgenic line, *nit1-3*, and wild type plants on PNS with 30 μM IAN. Twelve seedlings of each genotype were used. (D) IAN sensitivity of *35S::ATAF2* transgenic plants. Eight-day-old seedlings were grown on PNS or PNS containing 10 μM IAN or 0.2 μM IAA. (E) IAN sensitivity of primary root elongation in WT, VC, and two independent *ATAF2*-overexpressing transgenic line seedlings upon IAN treatment. The percent root growth compared to non-treated control was measured. Twelve seedlings of each genotype were used for each condition. (F) Lateral root length of WT, VC, and two *ATAF2*-overexpressing transgenic line plants on PNS with and without 10 μM IAN. At least 12 seedlings of each genotype were measured for each condition (Student's *t*-tests; * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

type were used for each condition. (F) Lateral root length of WT, VC, and two *ATAF2*-overexpressing transgenic line plants on PNS with and without 10 μM IAN. At least 12 seedlings of each genotype were measured for each condition (Student's *t*-tests; * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

ferent from that of wild type plants (Fig. 3A). This phenomenon also reported (Normanly et al., 1997); that is, nitrilase converts indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) and IAA treatment did not make any difference in root growth in WT and auxin-related mutant plants. Furthermore, *ataf2* complementation transgenic plants showed comparable root elongation with wild type plants on IAN (Fig. 3C), indicating that sensitivity to IAN in the *ataf2* mutant plants resulted from disruption of the *ataf2* gene.

We obtained consistent gain-of-function results from *ATAF2*-overexpressing plants, which displayed increased sensitivity to IAN (Fig. 3D). The primary root elongation in *35S::ATAF2* transgenic plants was inhibited about 30% compared with wild type plants when both were grown on 10 μM IAN (Fig. 3E). However, the *35S::ATAF2* plants showed a 3-4 fold increase in total lateral root length compared with that of the wild type or a vector control transgenic plants (Fig. 3F).

ATAF2 transactivates the *NIT2* gene promoter

From the gene expression and phenotypic analysis in the plants with gain and loss of *ATAF2* function, we speculated that *ATAF2* might activate the *NIT2* gene at the transcriptional level. Thus, we performed the transient transactivation assay with *NIT2* promoter using *Arabidopsis* protoplasts. Reporter constructs containing the region spanning positions from -1459 to +48 of the *NIT2* promoter (P1) and *ATAF2* expression vector or empty vector were co-transfected into *Arabidopsis* protoplasts.

And indeed *ATAF2* induced the expression of the *NIT2* promoter-driven *GUS* reporter gene (Fig. 4A).

To define the region of the *NIT2* promoter that responds to *ATAF2* transactivation, a series of 5' deletion mutants of the *NIT2* promoter construct were generated (from P2 to P9). Deletion of positions from -665 to -117 of the *NIT2* promoter region (P2 to P7) had no significant impact on the response to *ATAF2* (Fig. 4A). Further deletions (P8 and P9) from position -117 to -82 resulted in a complete loss of *ATAF2* responsiveness.

To further confirm the location of the *ATAF2*-responsive *cis*-elements, two 3' deletion mutants (P10 and P11) fused with 35S minimal promoter and *luciferase* (*LUC*) reporter were constructed. Only the P10 construct that contained a sequence from position -117 to -82 was responsive to *ATAF2* (Fig. 4B). Taken together, these results demonstrate the existence of a discrete *ATAF2* responsive element that localizes to the 36 base pair region from position -117 to -82 in the *NIT2* promoter.

ATAF2 binds to the -117 to -82 region of the *NIT2* promoter *in vitro*

To test whether *ATAF2* could bind to the *ATAF2*-responsive *cis*-elements, EMSA was performed. For a labeled probe in EMSA, three tandemly arrayed 36 nucleotide sequences of *NIT2* promoter were chosen to test the specificity of binding (Fig. 5A). Among the three fragments, only the 36-bp segment from -117 to -82 that contains the *ATAF2*-responsive element was bound by the GST-*ATAF2* protein, as also confirmed by a

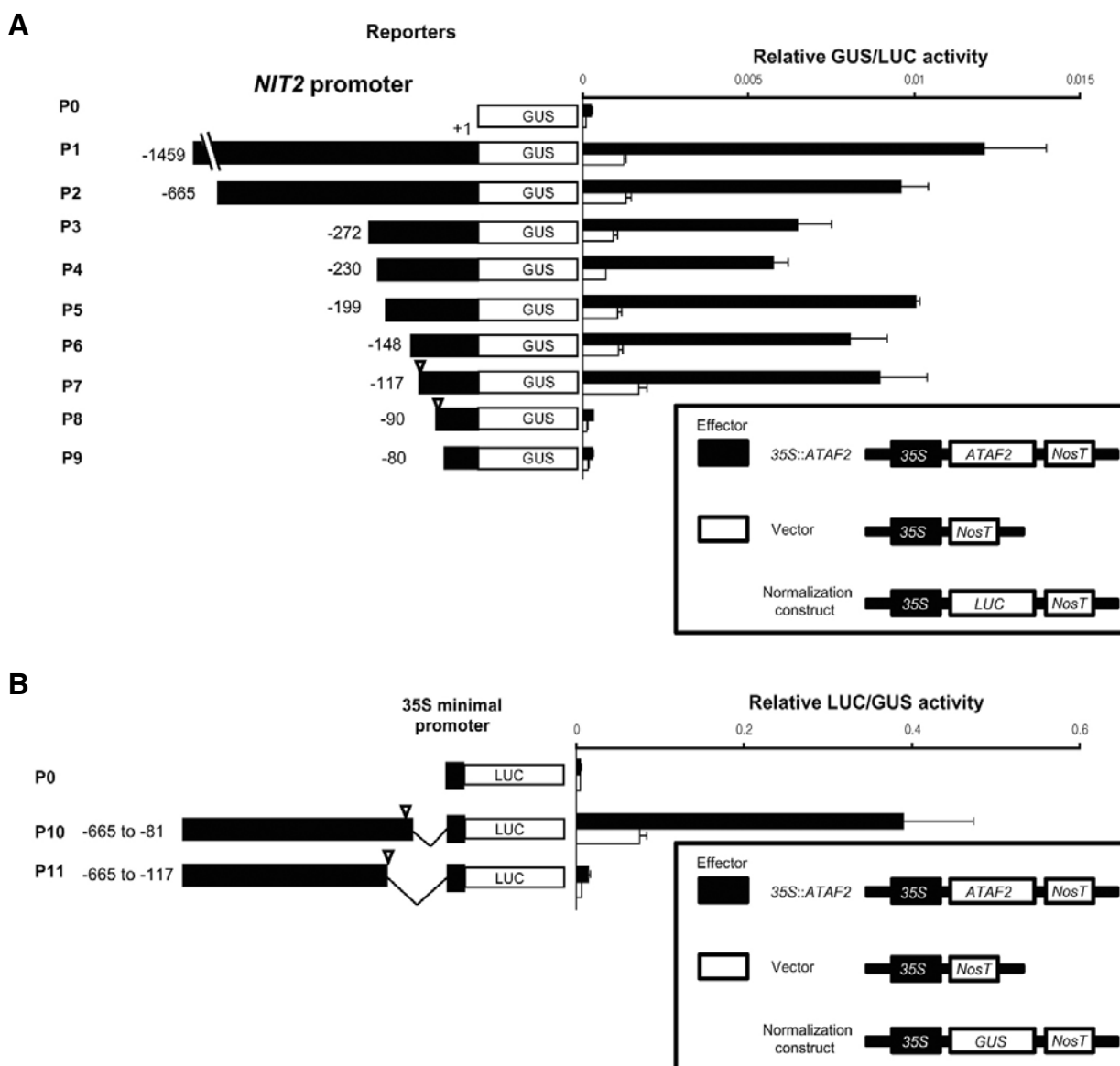


Fig. 4. Activation of the *NIT2* promoter::*GUS* (or *LUC*) fusion gene by ATAF2 protein in *Arabidopsis* protoplasts. (A) and (B) *35S*::*LUC* (or *GUS*) plasmid was co-transfected as a control for cell viability and transfection efficiency analysis. *Arabidopsis* protoplasts were transfected with effector plasmids and various reporter plasmids with different length sets of *NIT2* promoter.

competition assay using unlabeled probe (Fig. 5A). Interestingly, that ATAF2-responsive element was shown to contain an imperfect palindrome sequence (Fig. 5B), as previously shown in a few NAC proteins (Olsen et al., 2005; Zhong et al., 2010). Thus, mutational analysis of the ATAF2-responsive element was performed. Two oligonucleotides (M4 and M6) containing mutations in the palindrome sequence were unable to compete for the binding at a 100-fold molar excess (Fig. 5B, compare lanes 1 and 11 to lanes 5 and 7). Three oligonucleotides carrying mutations M2, M3, and M7 were also unable to compete for the complex formation, but with a slightly increased competition compared to M4 and M6 (Fig. 5B, compare lanes 1 and 11 to lanes 3, 4, and 8). In contrast, mutations M1, M5, M8, or M9 only marginally influenced the ability of the oligonucleotides to

compete for ATAF2 binding to the sequence from position -117 to -82 (Fig. 5B, compare lanes 1 and 11 to lanes 2, 6, 9, and 10). Collectively, these results suggest that the region from positions -117 to -82 contains an important motif for ATAF2 to bind to the *NIT2* promoter.

DISCUSSION

In this study, we found biological function of ATAF2 was involved in auxin biosynthesis *via* *NIT2* gene regulation. The induction level of *NIT2* was repressed in *ataf2* mutant upon IAN treatment. Meanwhile, the stable expressions ATAF2 in *Arabidopsis* Col-0 ecotype plants increase *NIT2* transcript level. To confirm of IAN-sensitivity (of root elongation) in the *nit2* loss-of-

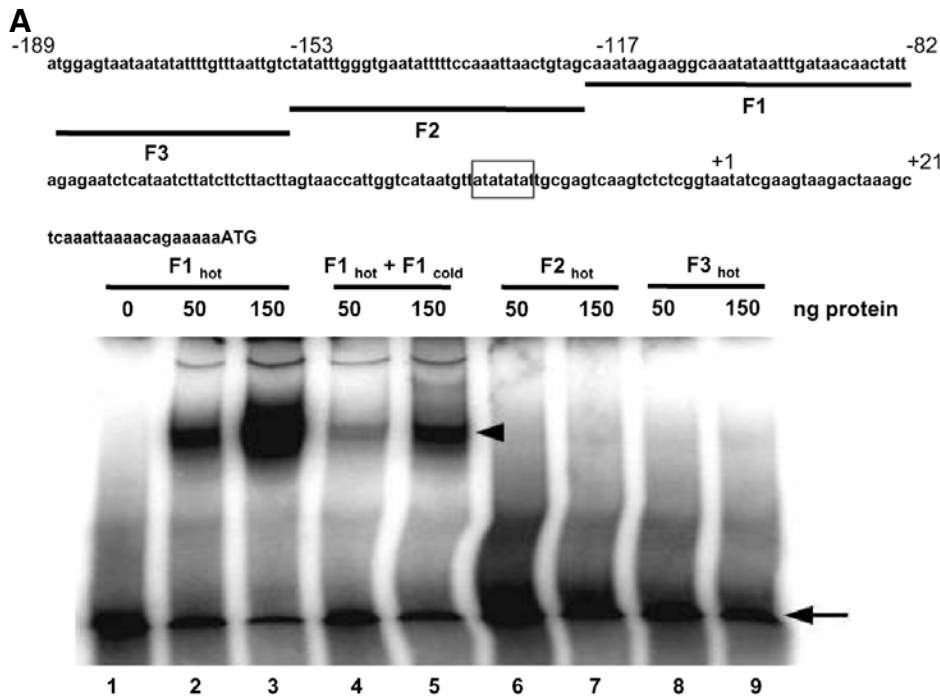
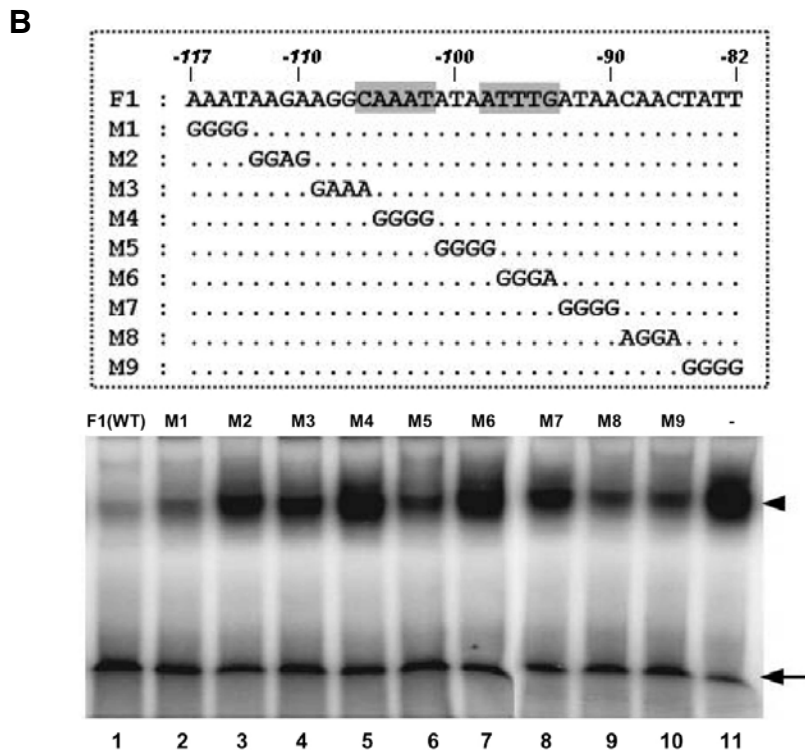


Fig. 5. Binding of recombinant GST-ATAF2 fusion protein to the -117 to -82 fragment of *NIT2* promoter in electrophoretic mobility shift assay (EMSA). (A) For labeled probes preparation in EMSA, three tandemly arrayed 36 nucleotide sequences of *NIT2* promoter (underlined, F1-F3) were selected to examine the specificity of binding. Lane 1, 500 ng of GST; lanes 2, 4, 6, 8, 50 ng of GST-ATAF2; lanes 3, 5, 7, 9, 150 ng of GST-ATAF2; lanes 2 and 3 contained ³²P-labeled F1 fragment as probe; lanes 4 and 5 contained the radio-labeled F1 fragment as probe and 100-fold unlabeled F1 fragment as competitor; lanes 6-7 and 8-9 contained ³²P-labeled F2 and F3 fragments as probes, respectively. (B) EMSA of the -117 to -82 fragment of *NIT2* promoter incubated with the oligonucleotide carrying mutations as competitors. Shown are the sequences of the mutant oligonucleotides (M1-M9) corresponding to the -117 to -82 (F1) of *NIT2* promoter which were used as competitors in EMSA. EMSAs were performed using ³²P-labeled F1 fragment as probe. Lane 1 contained cold-labeled F1 fragment as competitor; lanes 2-10 contained base-substituted mutant competitors shown in the box; lane 11, no competitor.



function mutant, we obtained *NIT2*-RNAi transgenic plants. However, *NIT2*-RNAi plant repressed both *NIT1* and *NIT2* gene expressions because *NIT1* and *NIT2* have highly similar nucleotide sequence (Supplementary Fig. S2A). The *NIT2*-RNAi transgenic plants exhibited slightly more enhanced IAN-insensitivity compared with *nit1-3* mutant (Supplementary Fig. S2B).

Thus, ATAF2-*NIT2* regulation could be involved in auxin signaling. We also performed a microarray with 27K *Arabidopsis* gene chip (Green Gene BioTech Inc.) and *NIT2* expression was repressed about 0.5 fold in *ataf2* mutant (data not shown). ATAF2 was able to transactivate the *NIT2* promoter *in vivo* when protoplasts were used. Moreover, ATAF2 protein specifi-

cally bound to the *NIT2* promoter region where an imperfect palindrome sequence exists.

ATAF2 is responsive to diverse stresses such as plant defense-related hormone, pathogen infection, wounding, and environmental stress (Delessert et al., 2005; Seki et al., 2002; Wang et al., 2009). We also showed *ATAF2* and *NIT2* transcripts were induced under diverse stress conditions (Supplementary Fig. S1). These results mean that *ATAF2* functions could be involved in biotic and abiotic stress via *NIT2* regulation. Interestingly, *ATAF2* was known to repress the pathogenesis-related gene expressions although the exact mechanism of *ATAF2* function in plant defense is not known. It was recently shown that some defense genes were repressed by *ATAF2*-TMV replicase interaction upon TMV infection (Wang et al., 2009). And lesion formation, wounding, and pathogen infection also activate tryptophan-dependent IAA biosynthesis pathways (Normanly and Bartel, 1999; Zhao and Last, 1996). Furthermore, the tryptophan biosynthesis pathway is also involved in the defense responses (Ishihara et al., 2008). Thus, we can postulate that *ATAF2* might regulate auxin biosynthesis pathway and this regulation could be connected with *ATAF2*-mediated plant defense system. And the regulation of *ATAF2*-*NIT2* could affect IAA levels. However, it can be expected that *NIT2* induction via *ATAF2* may not give rise to dramatic changes in IAN sensitivity because *Arabidopsis* has three nitrilase genes and, one of them, *NIT1*, accounts for the significant portions (60% at 30 μ M IAN) of total nitrilase activity (Normanly et al., 1997). Auxin-related mutant showed dwarfism and *ATAF2*-overexpressing transgenic plant also exhibited similar phenotype (Fig. 2E). However, this phenotype itself does not exclude the possibility of *ATAF2* involvement in other plant hormones such as salicylic acid (SA) or jasmonic acid (JA) because *ATAF2* could regulate *pathogenesis-related genes* (Wang et al., 2009). Furthermore, NAC transcription factors interact each other and exhibit diverse functions (Fang et al., 2008). Thus, there is a possibility of *ATAF2* and *NIT2* involvement in complex plant signaling pathway.

NAC domains from NAC1, AtNAM, and ANAC bind specifically to *as-1* like sequence within the CaMV 35S promoter (Duval et al., 2002; Xie et al., 2000). Three dehydration-inducible NAC proteins (ANAC019, ANAC055, and ANAC072) bind specifically to the CATGTG motif of *early response to dehydration 1 (ERD1)* promoter both *in vitro* and *in vivo* (He et al., 2005). It was reported that the NAC proteins recognize a sequence containing CATGT and harboring CACG as the core DNA binding site (Tran et al., 2004). Furthermore, vascular-related NAC-domain 7 (VND7) and secondary wall-associated NAC domain protein 1 (SND1) directly bind to secondary wall NAC binding element (SNBE) in the target promoters (Yamaguchi et al., 2011; Zhong et al., 2010). However, still little is known about the NAC protein binding site in biological target gene of NAC. The present study reports for the first time that the *ATAF2* protein specifically binds to the imperfect palindromic sequence -117 to -82 of the *NIT2* promoter. These results demonstrate that the *ATAF2* protein functions as a transcriptional activator with *NIT2* as an immediate target gene of *ATAF2*, and infer that a NAC domain might be able to recognize more than one sequence apparently unrelated to each other and/or that the sequence preference of a NAC protein depends on the presence of an additional *cis*-regulatory element. For example, *ATAF2* share 95% sequence identity to ANAC102. Furthermore, *anac102* mutant also exhibited less sensitivity to IAN and ANAC102 bound to *ATAF2*-responsive element (data not shown). These results demonstrate that *NIT2* gene could be regulated by

ATAF2 and other NAC proteins via their binding to the imperfect palindromic sequence -117 to -82 of the *NIT2* promoter.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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REFERENCES

- Church, G.M., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81, 1991-1995.
- Delessert, C., Kazan, K., Wilson, I.W., Van Der Straeten, D., Manners, J., Dennis, E.S., and Dolferus, R. (2005). The transcription factor *ATAF2* represses the expression of pathogenesis-related genes in *Arabidopsis*. *Plant J.* 43, 745-757.
- Duval, M., Hsieh, T.F., Kim, S.Y., and Thomas, T.L. (2002). Molecular characterization of AtNAM: a member of the *Arabidopsis* NAC domain superfamily. *Plant Mol. Biol.* 50, 237-248.
- Ernst, H.A., Olsen, A.N., Larsen, S., and 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., and 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.
- Grsic, S., Sauerteig, S., Neuhaus, K., Albrecht, M., Rossiter, J., and Ludwig-Muller, J. (1998). Physiological analysis of transgenic *Arabidopsis thaliana* plants expressing one nitrilase isoform in sense or antisense direction. *J. Plant Physiol.* 153, 446-456.
- He, X.J., Mu, R.L., Cao, W.H., Zhang, Z.G., Zhang, J.S., and Chen, S.Y. (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.
- Hegedus, D., Yu, M., Baldwin, D., Gruber, M., Sharpe, A., Parkin, I., Whitwill, S., and Lydiate, D. (2003). Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. *Plant Mol. Biol.* 53, 383-397.
- Huh, S.U., Lee, I.J., Ham, B.K., and Paek, K.H. (2012). *Nicotiana tabacum* Tsp1-interacting ferredoxin 1 affects biotic and abiotic stress resistance. *Mol. Cells* 34, 43-52.
- Ishihara, A., Hashimoto, Y., Tanaka, C., Dubouzet, J.G., Nakao, T., Matsuda, F., Nishioka, T., Miyagawa, H., and Wakasa, K. (2008). The tryptophan pathway is involved in the defense responses of rice against pathogenic infection via serotonin production. *Plant J.* 54, 481-495.
- Jiang, Y., Yang, B., Harris, N.S., and Deyholos, M.K. (2007). Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *J. Exp. Bot.* 58, 3591-3607.
- Jin, J.B., Kim, Y.A., Kim, S.J., Lee, S.H., Kim, D.H., Cheong, G.W., and Hwang, I. (2001). A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in *Arabidopsis*. *Plant Cell* 13, 1511-1526.
- Normanly, J., and Bartel, B. (1999). Redundancy as a way of life-IAA metabolism. *Curr. Opin. Plant Biol.* 2, 207-213.
- Normanly, J., Grisafi, P., Fink, G.R., and Bartel, B. (1997). *Arabidopsis* mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the *NIT1* gene. *Plant Cell* 9, 1781-1790.
- Olsen, A.N., Ernst, H.A., Leggio, L.L., and Skriver, K. (2005). NAC transcription factors: structurally distinct, functionally diverse. *Trends Plant Sci.* 10, 79-87.
- Park, J., Kim, Y.S., Kim, S.G., Jung, J.H., Woo, J.C., and Park, C.M. (2011). Integration of auxin and salt signals by the NAC transcription factor NIM2 during seed germination in *Arabidopsis*.

- Plant Physiol. 156, 537-549.
- Pollmann, S., Muller, A., Piotrowski, M., and Weiler, E.W. (2002). Occurrence and formation of indole-3-acetamide in *Arabidopsis thaliana*. *Planta* 216, 155-161.
- Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., et al. (2000). *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* 290, 2105-2110.
- Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., et al. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* 31, 279-292.
- Souer, E., van Houwelingen, A., Kloos, D., Mol, J., and 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.
- Tran, L.S., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., and 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.
- Wang, X., Goregaoker, S.P., and Culver, J.N. (2009). Interaction of the *Tobacco mosaic virus* replicase protein with a NAC domain transcription factor is associated with the suppression of systemic host defenses. *J. Virol.* 83, 9720-9730.
- Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. *Ann. Bot.* 95, 707-735.
- Wray, G.A., Hahn, M.W., Abouheif, E., Balhoff, J.P., Pizer, M., Rockman, M.V., and Romano, L.A. (2003). The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* 20, 1377-1419.
- Xie, Q., Frugis, G., Colgan, D., and Chua, N.H. (2000). *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev.* 14, 3024-3036.
- Yamaguchi, M., Mitsuda, N., Ohtani, M., Ohme-Takagi, M., Kato, K., and Demura, T. (2011). VASCULAR-RELATED NAC-DOMAIN7 directly regulates the expression of a broad range of genes for xylem vessel formation. *Plant J.* 66, 579-590.
- Zhao, J., and Last, R.L. (1996). Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis*. *Plant Cell* 8, 2235-2244.
- Zhong, R., Lee, C., and Ye, Z.H. (2010). Global analysis of direct targets of secondary wall NAC master switches in *Arabidopsis*. *Mol. Plant* 3, 1087-1103.

