An NAC domain transcription factor ATAF2 acts as transcriptional activator or repressor dependent on promoter context

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Abstract  The ARABIDOPSIS THALIANA ACTIVATION FACTOR 2 (ATAF2) protein has been demonstrated to be involved in various biological processes including biotic stress responses, photo morphogenesis, and auxin catabolism. However, the transcriptional function of ATAF2 currently remains elusive. Therefore, to further understand the molecular function of ATAF2, we evaluated the transcriptional activities of ATAF2 using a transient assay system in this study. We used an effector consisting of a GAL4-DNA binding domain (GAL4-BD) fused to ATAF2, and observed upregulated reporter gene expression, suggesting that ATAF2 potentially has transcriptional activation activity. ATAF2 has been shown to activate reporter gene expression under the control of the ORE1 promoter. By contrast, ATAF2 significantly repressed reporter gene expression driven by the NIT2 promoter. These data suggest that ATAF2 is a bifunctional transcription factor that can alter target gene expression depending on the promoter sequences.

Key words:  Arabidopsis thaliana, bifunctional transcription factor, NAC domain protein, transcription factor, transient assay.

The NAM/ATAF/CUC (NAC) domain transcription factor family is a plant-specific transcription factor family involved in various biological processes such as seed germination, secondary cell wall formation, senescence, and biotic and abiotic stress responses (Christianson et al. 2009; Nakano et al. 2015; Nuruzzaman et al. 2013; Shao et al. 2015; Yamaguchi and Demura 2010). The N-terminal region of the NAC domain proteins is highly conserved and functions in dimer formation, nuclear localization, and DNA binding, whereas the C-terminal region is highly divergent and confers transcriptional activity (Jensen and Skriver 2014). Although most of the NAC domain transcription factors have been reported as transcriptional activators, some NAC domain transcription factors are known to act as transcriptional repressors, such as CALMODULIN BINDING NAC PROTEIN (CBNAC) and VND-INTERACTING2 (VNI2) (Kim et al. 2007; Yamaguchi et al. 2010).

The ARABIDOPSIS THALIANA ACTIVATION FACTOR 2 (ATAF2) protein has been known to be involved in various biological processes including wounding and biotic stress responses, photo morphogenesis, brassinosteroid, and auxin catabolism (Delessert et al. 2005; Huh et al. 2012; Peng et al. 2015; Wang et al. 2009). Several candidates of the direct target genes of ATAF2 have been identified (Huh et al. 2012; Peng et al. 2015; Wang et al. 2009). A previous study reported that ATAF2 represses the expression of a number of pathogenesis-related genes including PR1, PR2, and PDF1.2 (Delessert et al. 2005). In contrast, another study showed that ATAF2 activates PR1, PR2, and PDF1.2 expression (Wang and Culver 2012). Thus, the molecular function of ATAF2 as a transcription factor, currently remains elusive. Therefore, to characterize ATAF2 function in depth, we performed a dual luciferase assay.

First, to investigate whether ATAF2 has transcriptional activation activity, an effector was created by fusing ATAF2 to a GAL4-DNA binding domain (GAL4-BD-ATAF2) (Figure 1). ATAF2 cDNA was subcloned into the pENTR/D-TOPO vector (Thermo Fisher Scientific, https://www.thermofisher.com), and then integrated into pA35BDG, a GATEWAY destination vector, which contains the GAL4-BD driven by the cauliflower mosaic virus 35S promoter (CaMV35S) (Yamaguchi et al. 2015). The GATEWAY destination vector containing the multi-
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cloning site (MCS) fragment was used as a control (Yamaguchi et al. 2008). The nucleotide sequences of the MCS and primers used in this experiment are described in Supplementary Table S1. The plasmids containing firefly luciferase (luc) linked to GAL4 binding sites and Renilla reinformis luc under the control of the CaMV35S promoter were used as a reporter and reference construct, respectively (Ohta et al. 2000) (Figure 1A). The effector, reporter, and reference plasmids were used to transfect Arabidopsis protoplasts, which were obtained from 3- to 4-week-old leaves of plants that were grown under long-day conditions, via a PEG transformation method (Sakamoto et al. 2016). Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com) using a Mithras LB940 Multimode Microplate Reader (Berthold, http://berthold.com). The GAL4-BD-fused VND7 and VNI2 were used as the controls for the transcriptional activator and repressor, respectively (Figure 1A). As reported previously, GAL4-BD-VND7 but not GAL4-BD-VNI2 was used as the control for the transcriptional activator and repressor, respectively (Figure 1A). As reported previously, GAL4-BD-VND7 but not GAL4-BD-VNI2 was used as the control for the transcriptional activator and repressor, respectively (Figure 1A).
BD-VNI2 upregulated the firefly luciferase activity (Yamaguchi et al. 2008, 2010) (Figure 1B). When GAL4-BD-ATAF2 was used as the effector, the luciferase activity was significantly increased compared to that of the control (Figure 1B), suggesting that ATAF2 potentially has transcriptional activation activity.

The SRDX motif has been demonstrated to be a strong transcriptional repression motif modified from the repression domain of SUPRMAN (SUP; Hiratsu et al. 2003). When this motif is fused to transcriptional activators, the chimeric transcription factors become dominant repressors (Hiratsu et al. 2003). To further examine the transcriptional activity of ATAF2, ATAF2 cDNA was fused to SRDX motif (ATAF2-SRDX), amplified by using a specific primer set (Table S1) and subcloned into pA35BDG (Figure 2A). We used a reporter construct containing luc under the control of the CaMV35S promoter with GAL4 binding sites to increase the basal luciferase activity (Mitsuda et al. 2005) (Figure 2A). As shown in Figure 2B, although GAL4-BD-ATAF2 upregulated luciferase activity, GAL4-BD-ATAF2-SRDX strongly downregulated the reporter activity, suggesting that ATAF2 acts as a repressor instead of a transcriptional activator when fused with SRDX.

As described above, several direct target gene candidates of ATAF2 were isolated (Delessert et al. 2005; Garapati et al. 2015; Huh et al. 2012; Peng et al. 2015; Wang et al. 2009). To examine how ATAF2 regulates the expression of the direct target genes, a dual luciferase assay was carried out using the reporter constructs expressing the firefly luc under the control of various promoter regions of the direct target genes (Figure 3). The entry vectors harboring the coding sequence of ATAF2 and ATAF2-SRDX were integrated into pA35G, a Gateway destination vector, which contains the CaMV35S promoter (Endo et al. 2015) (Figure 3A). The promoter fragments were subcloned into the pENTR/D-TOPO vector and then integrated into the pAGL Gateway destination vector containing the firefly luc (Endo et al. 2015) (Figure 3A).

ORESARA1 (ORE1) has been demonstrated to be a key regulator of senescence (Rauf et al. 2013), and has been reported as a direct target of ATAF1, which is closely related to ATAF2 (Garapati et al. 2015). The reporter gene expression driven by the ORE1 promoter was significantly upregulated by ATAF2 but not by ATAF2-SRDX (Figure 3B), indicating that ATAF2 activates ORE1 expression. It has been reported that the overexpression of ATAF2 exhibited a leaf-yellowing phenotype (Delessert et al. 2005; Huh et al. 2012). Previous work has reported that, ORE1 overexpression accelerated leaf senescence (Qiu et al. 2015). These data together with our transient assay results suggest that ATAF2 is involved in leaf senescence via the regulation of ORE1 expression (Figure 4).

ATAF2 binds to the promoter region of NITRILASE2 (NIT2) encoding a nitrilase that converts indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA), an auxin (Huh et al. 2012). When the NIT2 promoter containing the binding site (CAAATNNNATTG, -148 to -136) (Huh et al. 2012) was used as a reporter, both ATAF2 and ATAF2-SRDX significantly downregulated luciferase activity (Figure 3B). These data strongly suggest that ATAF2 represses NIT2 expression. However, it was previously shown that ATAF2 acts as a transcriptional activator for NIT2 (Huh et al. 2012). As described above, previous reports have shown that overexpression of ATAF2 activated or repressed the expression of the

Figure 3. ATAF2 has bifunctional activities. (A) Schematic diagrams of the constructs. The reporter constructs contain the promoter of ORE1, NIT2, or BAS1 with the firefly luciferase reporter gene. The effector constructs contain the MCS, ATAF2, or ATAF2-SRDX driven by the CaMV35S promoter. (B) Results of the transient transfection assay. Firefly luciferase activities were normalized by Renilla luciferase activities. Error bars indicate SD (n = 4). Asterisks indicate statistically significant differences (Welch’s t-test with Bonferroni–Holm correction, **p < 0.01; *p < 0.05) compared to the vector control.
pathogenesis-related genes (Delessert et al. 2005; Wang et al. 2009). It is possible that ATAF2 upregulates and downregulates the expression of some target genes including NIT2 in response to environmental conditions.

Peng et al. (2015) showed that ATAF2 binds promoter regions of BAS1 and SOB7, encoding brassinosteroid-inactivating enzymes, to suppress their expression. Thus, we also used the BAS1 promoter containing the binding sites (AAA AAT CT, -1021 to -1014, AAA ATA TCT, -717 to -709) (Peng et al. 2015) as a reporter (Figure 3A). When ATAF2 was used as an effector, no significant change was observed when compared with the use of the control effector (Figure 3B). By contrast, interestingly, the reporter expression was significantly upregulated by the ATAF2-SRDX effector (Figure 3B). If ATAF2 could directly upregulate or downregulate BAS1, the expression level of the reporter gene would be significantly decreased by ATAF2-SRDX. Thus, this result suggests a transcriptional cascade for BAS1 expression (Figure 4). Similarly, a previous report demonstrated that TCP3, a member of TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) family, upregulates the expression of miR164, which negatively regulates CUC genes (Baker et al. 2005; Laufs et al. 2004; Mallory et al. 2004). The overexpression of TCP3-SRDX induces ectopic expression of the CUC genes (Koyama et al. 2007). As in the case of TCP3, ATAF2 may activate unknown transcriptional factors or microRNAs that repress BAS1 (Figure 4). Although ATAF2 binds to the BAS1 promoter (Peng et al. 2015), the direct regulation of ATAF2 for BAS1 expression may not largely contribute to the entire regulation of BAS1 expression.

In this study, we demonstrated that ATAF2 has both transcriptional activation activity and repression activity under the same condition (Figure 3). WUSCHEL (WUS), an Arabidopsis thaliana homeodomain transcriptional factor, has been characterized as a bifunctional transcription factor (Ikeda et al. 2009). ATAF1, one of the closest homologs of ATAF2, promotes senescence via the direct activation of ORE1 expression and direct repression of GLK1 expression (Garapati et al. 2015). The NAC domain transcription factors have been demonstrated to form homo- and/or heterodimer complexes (Tran et al. 2007; Xu et al. 2013; Yamaguchi et al. 2008). Thus, ATAF2 may have bifunctional activities by forming heterodimer complexes with different partners depending on the promoter context. In addition, it is likely that ATAF2 forms different protein complexes with other transcription factors, and has different transcriptional activities for some target genes, such as NIT2, BAS1, and the pathogen-related genes, in response to endogenous or exogenous statuses. Further studies need to be carried out to unveil the biological functions of ATAF2, such as isolation of the interacting factors, examination of the relationship between the binding sequences and transcriptional activities of ATAF2, and identification of the unknown factors that are activated by ATAF and repress BAS1.

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References

Baker CC, Sieber P, Wellmer F, Meyerowitz EM (2005) The early petal1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. Curr Biol 15: 303–315
Christianson JA, Wilson IW, Llewellyn DJ, Dennis ES (2009) The low-oxygen-induced NAC domain transcription factor ANAC102 affects viability of Arabidopsis seeds following low-oxygen treatment. Plant Physiol 149: 1724–1738
Delessert C, Kazan K, Wilson IW, Van Der Straeten D, Manners J, Dennis ES, Dolfers R (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in Arabidopsis. Plant J 43: 745–757
Endo H, Yamaguchi M, Tamura T, Nakano Y, Nishikubo N, Yoneda A, Kato K, Kubo M, Kajita S, Katayama Y, et al. (2015) Multiple classes of transcription factors regulate the expression of VASCULAR-RELATED NAC-DOMAIN7, a master switch of xylem vessel differentiation. Plant Cell Physiol 56: 242–254
Garapati P, Xue GP, Munne-Bosch S, Balazadeh S (2015) Transcription factor ATAF1 in Arabidopsis promotes senescence by direct regulation of key chloroplast maintenance and senescence transcriptional cascades. Plant Physiol 168: 1122–1139
Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J 34: 733–739
Huh SU, Lee SB, Kim HH, Paek KH (2012) ATAF2, a NAC transcription factor, binds to the promoter and regulates NIT2 gene expression involved in auxin biosynthesis. Mol Cells 34: 305–313
Ikeda M, Mitsuda N, Ohme-Takagi M (2009) Arabidopsis WUSCHEL is a bifunctional transcription factor that acts as...
a repressor in stem cell regulation and as an activator in floral patterning. *Plant Cell* 21: 3493–3505

Jensen MK, Skriver K (2014) NAC transcription factor gene regulatory and protein-protein interaction networks in plant stress responses and senescence. *IUBMB Life* 66: 156–166

Kim HS, Park BO, Yoo JH, Jung MS, Lee SM, Han HI, Kim KE, Kim SH, Lim CO, Yun DJ, et al. (2007) Identification of a calmodulin-binding NAC protein as a transcriptional repressor in *Arabidopsis*. *J Biol Chem* 282: 36292–36302

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. *Plant Cell* 19: 473–484

Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the *CUC* genes is required for boundary size control in *Arabidopsis* meristems. *Development* 131: 4311–4322

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr Biol* 14: 1035–1046

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of *Arabidopsis* regulate secondary wall thickenings and are required for anther dehiscence. *Plant Cell* 17: 2993–3006

Nakano Y, Yamaguchi M, Endo H, Rejab NA, Ohtani M (2015) NAC-MYB-based transcriptional regulation of secondary cell wall biosynthesis in land plants. *Front Plant Sci* 6: 288

Nuruzzaman M, Sharomi M, Kikuchi S (2013) Roles of NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. *Front Plant Sci* 6: 902

Shao H, Wang H, Tang X (2015) NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. *Front Plant Sci* 6: 902

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

Wang X, Culver JN (2012) DNA binding specificity of ATAF2, a NAC domain transcription factor targeted for degradation by Tobacco mosaic virus. *BMC Plant Biol* 12: 157

Wang X, Goregaoker SP, Culver JN (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

Xu ZY, Kim SY, Hyeon Y, Kim DH, Dong T, Park Y, Jin JB, Joo SH, Kim SK, Hong JC, et al. (2013) The *Arabidopsis* NAC transcription factor ANAC096 cooperates with bZIP-type transcription factors in dehydration and osmotic stress responses. *Plant Cell* 25: 4708–4724

Yamaguchi M, Demura T (2010) Transcriptional regulation of secondary wall formation controlled by NAC domain proteins. *Plant Biotechnol* 27: 237–242

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) VASCULAR-RELATED NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in *Arabidopsis* roots and shoots. *Plant J* 55: 652–664

Yamaguchi M, Nagahage ISP, Ohtani M, Ishikawa T, Uchimiya H, Kawai-Yamada M, Demura T (2015) *Arabidopsis* NAC domain proteins VND-INTERACTING1 and ANAC013 interact with multiple NAC domain proteins. *Plant Biotechnol* 32: 119–123

Yamaguchi M, Ohtani M, Mitsuda N, Kubo M, Ohme-Takagi M, Fukuda H, Demura T (2010) VND-INTERACTING2, a NAC domain transcription factor, negatively regulates xylem vessel formation in *Arabidopsis*. *Plant Cell* 22: 1249–1263