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Note

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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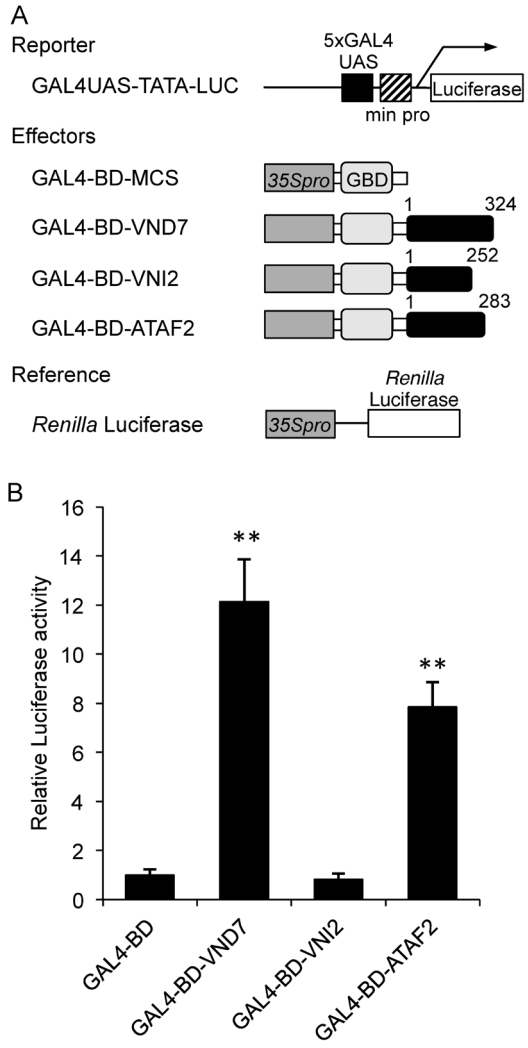

Figure 1. ATAF2 has transcriptional activation activity. (A) Schematic diagrams of the constructs used in the dual luciferase transient assay. The reporter construct contained the firefly luciferase reporter gene under the control of five repeats of the upstream activation sequence of GAL4 (5× GAL4 UAS) fused to a minimal *CaMV35S* promoter (min pro). The effector constructs contained GAL4-BD bound to an empty multiple cloning site (GAL4-BD-MCS) or to coding sequences corresponding to full-length *VND7, VNI2*, and *ATAF2* driven by the *CaMV35S* promoter (*35Spro*). (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, ** $p<0.01$) compared to the GAL4-BD-MCS vector control.

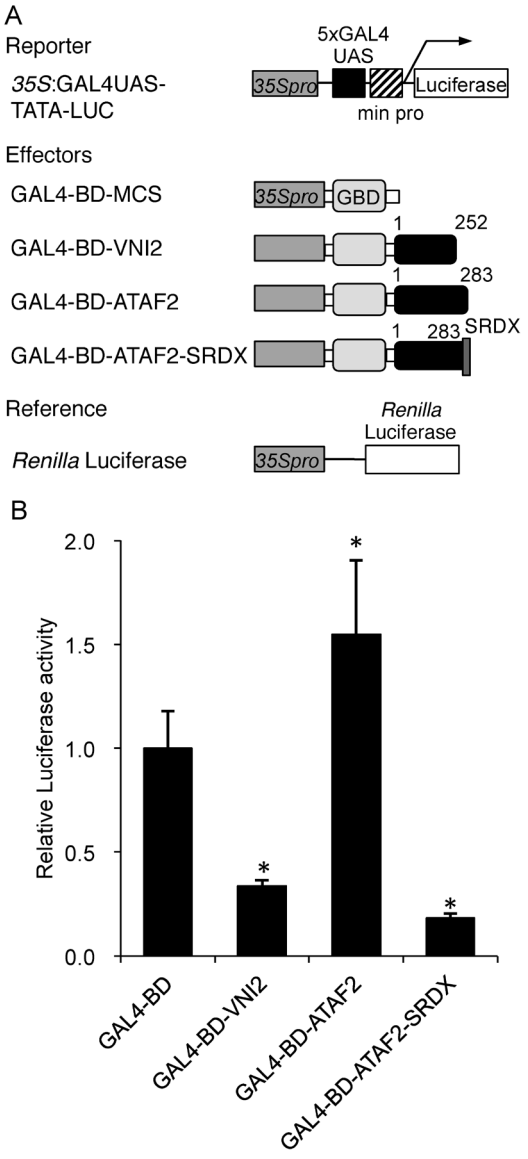

Figure 2. ATAF2-SRDX acts as a transcriptional repressor. (A) Schematic diagrams of the constructs used in the dual luciferase transient assay. The reporter construct contains the 5× GAL4/UAS and the minimal promoter sequence upstream of the firefly luciferase reporter gene, with the enhancer region of the *CaMV35S* promoter (*35Spro*). (B) Results of the transient transfection assay. Firefly luciferase activities were normalized to *Renilla* luciferase activities. Error bars indicate SD (n=3 or 4). Asterisks indicate statistically significant differences (Welch's t-test with Bonferroni–Holm correction, * $p<0.05$) compared to the vector control, GAL4-BD-MCS.

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 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*

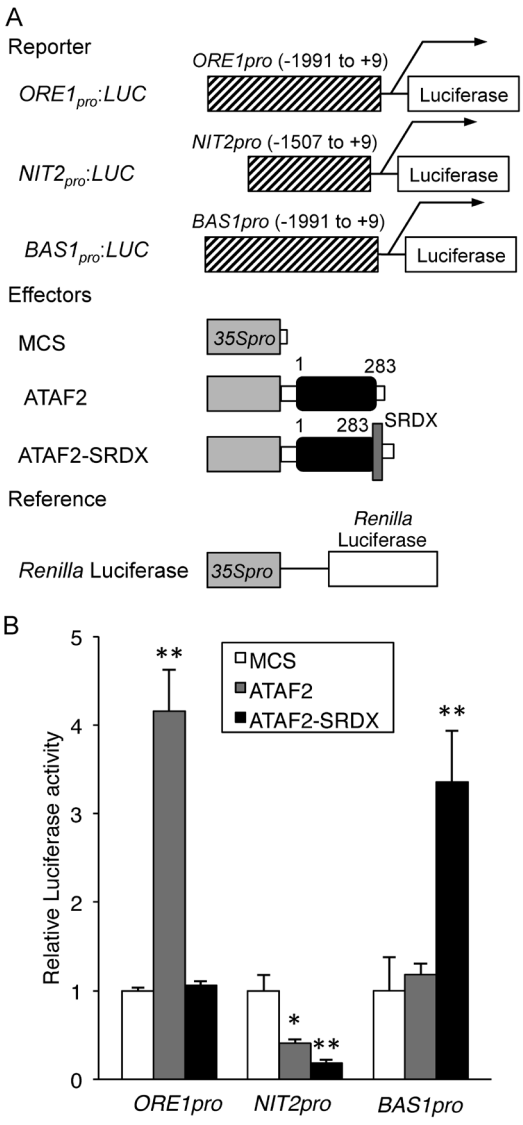

Figure 3. ATAF2 has bifunctional activities. (A) Schematic diagrams of the constructs. The reporter constructs contain the promoter of *ORE1*, *NIT2*, or *BAS1* with the firefly luciferase reporter gene. The effector constructs contain the MCS, *ATAF2*, or *ATAF2-SRDX* driven by the *CaMV35S* promoter. (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.

(*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 (CAA ATNNNATT G, -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

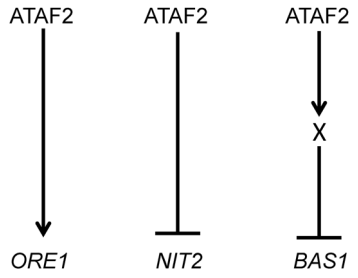

Figure 4. Schematic diagram of the transcriptional activities of ATAF2. Our transient expression assays demonstrated that ATAF2 activates *ORE1* expression, represses *NIT2* expression, and downregulates *BAS1* expression through the upregulation of unknown factors that negatively regulate *BAS1* expression (X).

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 TEOSINETE 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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