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Published in:
FEBS open bio

DOI:
10.1016/j.fob.2013.07.006

Publication date:
2013

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
ATAF1 transcription factor directly regulates abscisic acid biosynthetic gene NCED3 in Arabidopsis thaliana

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A R T I C L E   I N F O
Article history:
Received 20 July 2013
Accepted 23 July 2013

Keywords:
Abscisic acid
ATAF1 transcription factor
ABA biosynthesis
DNA-binding

A B S T R A C T
ATAF1, an Arabidopsis thaliana NAC transcription factor, plays important roles in plant adaptation to environmental stress and development. To search for ATAF1 target genes, we used protein binding microarrays and chromatin-immunoprecipitation (ChIP). This identified T[AC,G]GT[AC] and T[AC,G]GTG as ATAF1 consensus binding sequences. Co-expression analysis across publicly available microarray experiments identified 25 genes co-expressed with ATAF1. The promoter regions of ATAF1 co-expressors were significantly enriched for ATAF1 binding sites, and TITCGTA was identified in the promoter of the key abscisic acid (ABA) phytohormone biosynthetic gene NCED3. ChIP-qPCR and expression analysis showed that ATAF1 binding to the NCED3 promoter correlated with increased NCED3 expression and ABA hormone levels. These results indicate that ATAF1 regulates ABA biosynthesis.

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Introduction
Abscisic acid (ABA) controls numerous physiological processes in plants and is best known for its regulatory role in abiotic stress responses [1,2]. Upon drought and high salinity, ABA promotes desiccation tolerance by stomatal closure, enabling plants to adapt to water stress. ABA also regulates developmental processes such as seed germination, vegetative growth and bud dormancy [3–5]. More recent studies have shown that ABA also impacts plant biotic stress signaling [6,7]. Consequently, although the pathways of ABA biosynthesis and catabolism are largely defined (reviewed by Nambara and Marion-Poll [8]), understanding the regulation of these pathways is important. In particular, the cleavage of 9-cis-epoxycarotenoids to xanthoxin catalyzed by 9-cis-epoxycarotenoid dioxygenases (NCEDs) during ABA biosynthesis, which is believed to be the key regulatory step of ABA biosynthesis [9], deserves elucidation. In rice, Yaish et al. reported that over-expression of the APETALA-2-like transcription factor (TF) OsAP2–39 is associated with the up-regulation of the ABA biosynthetic gene OsNCED-1 leading to an increase in endogenous ABA levels [10]. Among the five NCED genes in Arabidopsis, NCED3 plays a key role in ABA biosynthesis during water deficit [11,12], and nced3 mutants exhibit increased water loss and reduced ABA levels in vegetative tissues [12]. Recently, Jiang et al. reported the identification of a gain-of-function acquired drought tolerance (adt) mutant to be a WRKY TF conferring increased drought tolerance, ABA levels and direct NCED3 promoter binding [13]. Apart from APETALA-2-like OsAP2–39 and adt, no other direct transcriptional regulators of NCED genes have been reported.

We previously highlighted NAC (petunia NAM and Arabidopsis ATAF1, ATAF2, and TC2) TFs as components related to ABA and biotic stress signaling [14]. Overall, NAC genes encode a large, plant-specific family of TFs with roles in many aspects of growth, development and environmental stresses [15,16]. The N-terminal region of NAC proteins contains the highly conserved NAC domain encompassing a homo- and heterodimerization region indispensable for DNA-binding [17]. Moreover, a number of reports have identified core NAC
DNA-binding sites [18–20], and the consensus binding site (BS) [T,A] [T,G][T,A,C,G]CG[T,G,A] has been proposed from studies of Arabidopsis ANAC019 [17]. Indeed, phylogenetically distant members of the NAC TF family bind this NAC-BS in vitro, albeit with various affinities [21]. This suggests that there may be non-canonical k-mers relevant for various NAC TF family members that are not yet defined.

NAC members of the ATA F1 clade of the NAC family exhibit strong and transient expression patterns to ABA treatment and environmental stress [22–24]. Additionally, ATA F1 is ubiquitously expressed in various organs, including stomatal guard cells [23]. We previously reported that ata f1 mutants are ABA-hyposensitive during seedling development and germination [14]. In line with this, plants over-expressing ATA F1 were shown to be ABA hypersensitive [24]. Also, ATA F1 was identified in a yeast two-hybrid screen to interact with Sucrose nonfermenting 1 (SNF1)-related serine/threonine-protein kinase 1 (SnRK1) subunits AKIN10 and AKIN11 [25], positive regulators of ABA metabolism and key integrators of transcription networks in response to stress and energy signaling [26,27].

To improve our understanding of the regulatory potential of ATA F1, we here delineate the DNA-binding specificity of ATA F1 using protein binding microarrays (PBM), co-expression analyses and chromatin-immunoprecipitation (ChIP). This identifies abscisic acid biosynthetic NCED3 as an ATA F1 regulatory target gene. In plants over-expressing ATA F1, this correlates with increased NCED3 transcript abundance and, most importantly, increased ABA phytomone levels. Taken together, our data indicate that ATA F1 is a regulator of ABA biosynthesis in Arabidopsis.

Results

ATAF1 consensus-binding site

We used protein-binding microarrays (PBM) as an unbiased strategy to search for ATA F1 consensus-binding motifs. PBM permits the identification of TF DNA binding specificities at single base resolution [28,29]. Since Arabidopsis NACs only bind DNA as dimers [17], we first established whether ATA F1 can homodimerize. Yeast-2-hybrid experiments showed that ATA F1 homodimerizes independently from the ATA F1 C-terminal transcriptional regulatory domain (Fig. 1A). We then heterologously expressed and purified a GST-tagged version of the ATA F1 DNA-binding NAC domain (residues 1–165; Fig. 1B) and incubated PBMs using this protein. Subsequent PBM analysis identified T[A,C,G]CG[T,A,G] and T[T,A,C,G]CGT 6-mers as the most significant descriptors for ATA F1 binding. The ATA F1 binding specificity position weight matrix (PWM), derived from all relevant 8-mers bound by ATA F1, is graphically illustrated in Fig. 1C (see also Supplementary Fig. S1).

The ATA F1 co-expression cluster is enriched for ATA F1 consensus binding sites

Co-expression occurs among TFs and target genes, and co-expression clusters can be enriched for common TF binding-sites [30,31]. To complement our PBM data to search for direct target genes of ATA F1, we data-mined >8,500 ATH1 microarray samples from the Genevestigator [32] data repository. Using a stringent (>2-fold regulation, P < 0.05) selection criterion for ATA F1 transcript level perturbations, we found 403 microarray slides from 87 perturbations. Using this data set we identified 25 top-ranking genes co-expressed with ATA F1 (r ≥ 0.66) (Table 1). Hierarchical clustering of ATA F1 and its co-expressors identified strong induction by ABA application, drought, osmotic and salt stresses (Fig. 2A), confirming earlier ATA F1 studies [23,24]. Also, the ATA F1 cluster exhibited distinct expression patterns during biotic stress (Fig. 2A). Using the PBM-derived ATA F1 binding oligomers and the POBO program [33], we analyzed whether the promoters of the ATA F1 gene cluster have an over-representation of ATA F1 binding sites. Bootstrapping analysis using 1000 promoter sets of the background Arabidopsis genome and the ATA F1 co-expression cluster, respectively, identified a significant (P < 0.001) enrichment of both T[A,C,G]CG[T,A,G] and T[T,A,C,G]CGT in the ATA F1 cluster compared to background genomic distribution (Fig. 2B).

ATA F1 directly regulates the abscisic acid biosynthetic gene N Ced3

In addition to ABA-inducible expression, the list of ATA F1 co-expressed genes also includes the key regulatory ABA biosynthetic gene N Ced3. We performed two experiments to investigate whether ATA F1 directly regulates ABA biosynthesis. First, to investigate the correlation between ATA F1 and ABA hormone biosynthesis in planta, we produced plants over-expressing ATA F1 (3SS:ATA F1-HA). In agreement with earlier studies [24,25,34], these plants showed stunted growth and delayed flowering (Fig. 3A–C, and Supplementary Fig. S2). The observed phenotype of these plants correlated with increased ATA F1 transcript levels and were not associated with transgene silencing of ATA F1 and other ATA F1 subcluster members, as reported by Kleinow et al. [25], Figs. 3B and 4A. Using these plants, we observed ATA F1 over-expressing plants to have significant (approx. 6- and 7.5-fold) increased ABA levels (P < 0.01) compared to wild-type and ata f1 mutants, respectively (Fig. 3D).

Second, to determine whether ATA F1 binds the promoter of N Ced3 in vivo, we performed ChIP on wild-type and 3SS:ATA F1-HA plants (Fig. 3A). Subsequent qPCR identified a region (position ~1134 to ~1265 bp) including a TGTGCGTA ATA F1 binding motif to be enriched in ChIPs from ATA F1 over-expressing plants (Fig. 3E). ATA F1 did not bind a region between ~120 and ~218 bp (non-binding; NB), confirming ATA F1 binding specificity. As a technical control, the FIT locus targeted by the HA-tagged TFL2 TF was included [35] (Fig. 3E). Finally, to assess the regulatory potential of ATA F1 we determined the transcript levels of N Ced3 and found ~10-fold increase in ATA F1 over-expressing plants compared to wild-type. In contrast, mean N Ced3 levels were significantly lower in ata f1 mutants (Fig. 3F), substantiating the strong positive correlation between ATA F1 and N Ced3 transcript levels (Fig. 2A).

In addition to N Ced3, transcript levels of several other top-ranking ATA F1 co-expressed genes displayed ATA F1-dependent expression perturbations, though with a narrower dynamic range compared to N Ced3 (Fig. 4B).

Fig. 1. ATA F1 homodimerization and consensus binding site. (A) Fusion proteins of Gal4-(DBD)—ATA F1(1–165), Gal4-(AD)—ATA F1(1–289), Gal4-(AD)—ATA F1(1–165), and empty Gal4-(DBD) were co-transformed, expressed in yeast, and screened after 7 days for transactivation activity of HIS3 and ADE2 reporter genes. (B) SDS–PAGE and Coomassie Blue staining of gel molecular-mass-markers (lane 1; molecular masses in kDa at left) and approx. 5 μg of affinity-purified recombinant GST—ATA F1(1–165). (C) Consensus binding site of ATA F1 from duplicate PBM experiments.
Collectively, our data indicate that ATAF1 activates ABA hormone biosynthesis in plants, through transcriptional activation of NCED3.

**Discussion**

Here we identify ATAF1 as a positive regulator of ABA biosynthesis. We show that ATAF1 and NCED3 are co-expressed, and that ATAF1 binds the NCED3 promoter in vivo. Most importantly, ATAF1-mediated induction of NCED3 in plants over-expressing ATAF1 correlates with increased ABA levels. In support of our observations, ATAF1 exhibits strong expression in stomatal guard cells and in the vasculature [23], and plants over-expressing ATAF1 have reduced stomatal aperture [24]. Interestingly, NCED3 has a constrained spatial expression in vascular tissues [36], yet transient expression of NCED3 in guard cells causes a decrease in stomatal aperture [37]. Hence, both at the transcript level and at the physiological level, the overlap between ATAF1 and NCED3 abundances and functionalities is evident. Hypersensitivity to ABA and increased endogenous ABA levels are often associated with increased tolerance to salt and drought stresses [38,39]. Thus, reduced ABA levels in plants may be associated with drought sensitivity [40]. We have previously reported that atafl mutants are ABA-hyposensitive during seedling development and germination [14], and plants over-expressing ATAF1 have been shown to be hypersensitive to ABA and drought tolerant [24]. Moreover, plants over-expressing ATAF1 display stunted growth and delayed flowering, unlike ABA-hypersensitive plants over-expressing the ATAF1 interaction partner SnRK1.1/AKIN10 [25,26,41]. This agrees with reports on ABA-deficient mutants displaying early flowering [42], and exogenous ABA application to delay flowering [43].

In addition to NCED3, several other ATAF1 co-expressors showed displayed ATAF1-dependent expression perturbations. Specifically, significant changes in expression levels of both ABI2 and DREB2A were observed in aaf1–2 and ATAF1-overexpressing plants, respectively, compared to wild-type plants (Fig. 4B). DREB2A encodes a transcription factor regulating drought and osmotic-inducible genes [44,45], and ABI2, and other clade A type 2C protein phosphatases (PP2Cs), are known to be negative regulators of ABA signaling by dephosphorylation of ABA-activated Sucrose nonfermenting 1 (SNF1)-related serine/threonine-protein kinases (SnRKs) [46–48]. We speculate that the positive correlation between ATAF1 and transcript levels of genes encoding PP2Cs (Fig. 2, Table 1, and Fig. 4A) may reflect a negative feed-back loop needed to dampen the increased endogenous ABA.

**Table 1**

<table>
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<th>AGI</th>
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<th>Description</th>
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<th>TVCGTR&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>+</td>
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<td>Zinc-finger protein 2</td>
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<td>Protein phosphatase 2C family protein</td>
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<td>AT5G59220</td>
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<td>Highly ABA-induced PP2C gene 1 (HA1)</td>
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<tr>
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<td>PAS domain-containing tyrosine kinase protein</td>
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</table>

<sup>a</sup> Using 403 microarray samples from 87 different conditions where ATAF1 was >2-fold regulated in treated samples compared to control samples.

<sup>b</sup> V = [A,C,G] and R = [A,G], + indicates number of ATAF1 binding 6-mers in 1 kb promoters.
ATAF1 co-expression gene cluster is enriched for ATAF1 binding sites. (A) Using 403 microarray samples from 87 conditions either significantly inducing or repressing ATAF1 expression, identifies 25 co-expressed genes with a Pearson correlation coefficient > 0.66. (B) Bootstrapping analyses using PDBO [33] show that promoters of ATAF1 co-expressed genes are significantly \( P < 0.001 \) enriched for ATAF1 consensus binding sites \( [\text{T,A,C,G}] \text{CGT}[\text{A,G}] \) and \( [\text{T,A,C,G}] \text{CGT} \).

Fig. 3. Ectopic expression of ATAF1 arrests plant development and activates ABA biosynthesis. (A) Eight week-old short-day grown Col-0 wild-type and afa1–2, compared to plants ectopically expressing HA-tagged ATAF1. Two independent 35S::ATAF1-HA lines (1:3 and 3:1) display growth reduction. (B) Quantitative expression profiles of endogenous and ectopic ATAF1 in Col-0 wild-type, afa1–2 and 35S::ATAF1-HA plants. Mean \( (\pm \text{SEM}) \) relative expression units (log 10 scale) are displayed using ACT2 as reference. Bars represent the mean of three biological replicates. (C) Expression of HA-tagged ATAF1 using SDS-PAGE and Western blot. The blot was probed with an anti-HA antibody. Representative result from three replicates is shown. The unspecific band at 37 kDa serves as a loading control. (D) Endogenous levels of ABA were measured. Bars represent the mean of three biological replicates. Error bars represent \( \pm 1 \) standard deviation. Statistical analyses were performed using Student's \( t \)-test of the differences between individual means compared to Col-0 (\( *P < 0.01 \)). (E) Direct binding of ATAF1 to the NCED3 promoter (between position –1136 and –1265 bp) was analyzed using ChIP–qPCR on 35S::ATAF1-HA over-expressing (ATAF1-HA) and Col-0 wild-type plants. A non-binding control (NB) 1 kb downstream of the ATAF1–NCED3 binding site, and a positive TFL2 TF binding-site control (FLOWERING LOCUS T-1 [FT], [35]) were included. The ChIP results obtained by three independent replicate experiments are represented as percentage of input (\%IP), and the error bars indicate \( \pm 1 \) standard deviation. (F) Expression of ABA biosynthetic gene NCED3 in ATAF1 over-expressing plants compared to wild-type Col-0 and afa1 mutants. Expression level of NCED3 was determined by qPCR in the indicated genotypes. Mean \( (\pm \text{SEM}) \) relative expression units are displayed using ACT2 as reference. Statistical analyses were performed using Student's \( t \)-test of the differences between individual means compared to Col-0 (\( *P < 0.05, **P < 0.01 \)).

levels associated with ATAF1 induction or over-expression.

Finally, we note that our unbiased PBM-approach recovered a binding-site similar to that reported for ANAC019 [17], and that functional redundancy has been reported for NAC TFs [49,50]. Overlapping polymorphisms within NAC binding sites, could also explain why residual NCED3 and ABA levels are observed in afa1 mutant plants (Fig. 3D and F). This also includes the potentially physiologically relevant ATAF1–ATAF2 heterodimerization reported by Wu et al. [24], and Supplementary Fig. S4). However, using PBM to uncover binding site preferences for all basic helix-loop-helix (bHLH) TFs from C. elegans, none of the bHLH proteins that participate in heterodimeric interactions were shown to exhibit significant sequence-specific DNA binding on their own [51]. In our study, ATAF1 on it's own shows sequence specificity to DNA-binding (Fig. 1C). Knowing that dimerization-deficient NAC mutants do not bind DNA [17], this indicates that top-ranking oligomers in this study are bona fide targets of ATAF1 homodimers. In combination with stringent co-expression analysis using hundreds of genome-wide expression data sets, our data emphasize the value of in vitro-defined oligomers in estimating TF binding sites and identification of target genes. Ideally, probing all plant TFs using such analyses should uncover the complex transcriptional imprint required to fine-tune plant hormone homeostasis.
Methods and materials

Plant materials and growth conditions

Arabidopsis thaliana wild-type accession Col-0 and ataf1–2 mutant plants (T-DNA insertion line SALK-057618) [23], were grown on soil in controlled environment chambers under an 8 h light regime (150–170 μE/m² s) at 21 °C and 65% relative humidity.

Plasmid construction and plant transformation

The AtAF1–HA C-terminally tagged gene was generated by amplifying a full-length AtAF1 cDNA obtained from ABRC with forward and reverse primers; AAGAATTCATCAGAAT-TATTCAGTTGCC and CCGGGATCCCTAAGCGTAATCTGGTACGTCG-TATGGGTAAGGCTTCTGCAATGC, respectively, and cloned into pCAMBIA3300. Transformation of Col-0 plants was performed by the floral dip method [52] using Agrobacterium tumefaciens strain GV3101 (pMP90). Transgenic plants were selected by BASTA spraying, and homozygous T3 seeds from transformants expressing transgenes were used for subsequent analyses. The 35S:AtAF1–HA construct used in this study complements the ataf1 mutations [14], indicating that the addition of the HA epitope does not impair AtAF1 function.

Protein expression and purification

N-terminally GST-tagged, recombinant AtAF1(1–165) was cloned, expressed and purified as described [21].

Protein binding microarray

Microarray design, preparation, and PBM experiments were performed as described previously by Berger and Bulyk [53]. All experiments were performed using custom-designed “all 8-mer” arrays synthesized in a “4 × 44K” array format (Agilent Technologies, CA, USA) containing 4 copies of publicly available de Bruijn sequences [28,54]. Briefly, 200 nM of GST-AtAF1(1–165) protein was incubated on the microarray for 60 min at RT. TF–DNA interactions were detected by first incubating the array with a rabbit anti-GST polyclonal antibody (Invitrogen), followed by a Cy5 labeled anti-rabbit antibody (Jackson-Immuno, PA, USA). Blocking, protein binding and washing procedures were identical to standard PBM protocols [53].

Protein binding microarray data normalization and motif analysis

Microarrays were scanned using a SureScan scanner at a 2 μm resolution (Agilent Technologies, CA, USA), and spot intensities retrieved using Feature Extraction Software (Agilent Technologies, CA, USA). Data normalization and analysis were performed as described previously [53]. Resulting PWMs were graphically visualized using enoLOGOS [55].

Western blotting

Nuclear extracts were precipitated overnight with 80% acetone and proteins resuspended and boiled in SDS–PAGE loading buffer. Supernatants were separated by SDS–PAGE and proteins detected by Western blotting using monoclonal mouse anti-HA antibody (Sigma, MO, USA).

Yeast two-hybrid assays and qPCR analyses

Both methods were as described in [21]. Primers used to clone AtAF1(1–165) into pGBK7 were AAGAATTCATCAGAAT-TATTCAGTTGCC and CCGGGATCCCTAAGCGTAATCTGGTACGTCG-TATGGGTAAGGCTTCTGCAATGC. Reverse primers were CCGGGATCCCTAAGCGTAATCTGGTACGTCG-TATGGGTAAGGCTTCTGCAATGC, respectively. For quantitative real-time PCR (qPCR), Actin2 (ACT2) was used as a reference. For NCED3 we used AGCTTCTTACATGCGCCAC and CGCTCTCTGCA AAAATTCACT. For endogenous AtAF1 we used GTGTTTACGGCAGAAATC and
MS–LC for hormone quantifications

Quantifications were performed as described [58]. Statistical analyses were performed using Student’s t-test (**p < 0.01).

Accession numbers

The following accession codes were used: ATAF1, At1g01720; FL, At1g65480; N Ced3, At3g14440.

Funding

This work was supported by the Danish Research Council (09-06410) and Villum-Kann Rasmussen Foundation (VKR09-007) awarded to M.K.J.

Acknowledgments

We thank the Arabidopsis Biological Resource Center at Ohio State University and for cDNA and seed stocks. No conflicts of interest are declared.

Supplemental material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2013.07.006.

References


