ATAF1 transcription factor directly regulates abscisic acid biosynthetic gene NCED3 in Arabidopsis thaliana

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Abstract
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]CGT[AC] and T[TC,T]CGT 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 TTGGCTA 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-epoxyxatotenoids to xanthoxin catalyzed by 9-cis-epoxyxatotenoid 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-I 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 CUC2) 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,C][T,A,C,G]G[T,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 ATAF clade of the NAC family exhibit strong 
and transient expression patterns to ABA treatment and environmental 
stress [22–24]. Additionally, ATAF1 is ubiquitously expressed in 
various organs, including stomatal guard cells [23]. We previously 
reported that ataf1 mutants are ABA-hyposensitive during seedling 
development and germination [14]. In line with this, plants over-
expressing ATAF1 were shown to be ABA hypersensitive [24]. Also, 
ATAF1 was identified in a yeast two-hybrid screen to interact with 
Sucrose nonfermenting 1 (SNF1)–related serine/threonine-protein ki-
nase 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 
ATAF1, we here delineate the DNA-binding specificity of ATAF1 us-
ing protein binding microarrays (PBM), co-expression analyses and 
chromatin-immunoprecipitation (ChIP). This identifies abscisic acid 
biosynthetic NCED3 as an ATAF1 regulatory target gene. In plants 
over-expressing ATAF1, this correlates with increased NCED3 tran-
script abundance and, most importantly, increased ABA phytom-
borne levels. Taken together, our data indicate that ATAF1 is a regu-
lator of ABA biosynthesis in Arabidopsis.

Results

ATAF1 consensus-binding site

We used protein-binding microarrays (PBM) as an unbiased strate-
gy to search for ATAF1 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 ATAF1 can homodimerize. Yeast-2-hybrid 
experiments showed that ATAF1 homodimerizes independently from 
the ATAF1 C-terminal transcriptional regulatory domain (Fig. 1A). We 
then heterologously expressed and purified a GST-tagged version of 
the ATAF1 DNA-binding NAC domain (residues 1–165; Fig. 1B) and in-
cubated PBMs using this protein. Subsequent PBM analysis identified 
T[A,C,G]CGT[A,G] and TT[A,C,G]CGT 6-mers as the most significant 
descriptors for ATAF1 binding. The ATAF1 binding specificity position 
weight matrix (PWM), derived from all relevant 8-mers bound by 
ATAF1, is graphically illustrated in Fig. 1C (see also Supplementary 
Fig. S1).

The ATAF1 co-expression cluster is enriched for ATAF1 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 
ATAF1, we data-mined ~8.500 ATH1 microarray samples from the 
Genevestigator [32] data repository. Using a stringent (>2-fold regu-
lation, P < 0.05) selection criterion for ATAF1 transcript level pertur-
bations, we found 403 microarray slides from 87 perturbations. Using 
this data set we identified 25 top-ranking genes co-expressed with 
ATAF1 (r ≥ 0.66) (Table 1). Hierarchical clustering of ATAF1 and its co-
expressors identified strong induction by ABA application, drought, 
osmotic and salt stresses (Fig. 2A), confirming earlier ATAF1 studies 
[23,24]. Also, the ATAF1 cluster exhibited distinct expression patterns 
during biotic stress (Fig. 2A). Using the PBM-derived ATAF1 bind-
ings oligomers and the POBO program [33], we analyzed whether the 
promoters of the ATAF1 gene cluster have an over-representation of 
ATAF1 binding sites. Bootstrapping analysis using 1000 promoter sets 
of the background Arabidopsis genome and the ATAF1 co-expression 
cluster, respectively, identified a significant (P < 0.001) enrichment of 
to background genomic distribution (Fig. 2B).

ATAF1 directly regulates the abscisic acid biosynthetic gene NCED3

In addition to ABA-inducible expression, the list of ATAF1 co-
expressed genes also includes the key regulatory ABA biosynthetic 
genre NCED3. We performed two experiments to investigate whether 
ATAF1 directly regulates ABA biosynthesis. First, to investigate the 
correlation between ATAF1 and ABA hormone biosynthesis in planta, 
we produced plants over-expressing ATAF1 (35S:ATAF1-HA). In agree-
ment 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 
ATAF1 transcript levels and were not associated with transgene si-
lencing of ATAF1 and other ABA subclade members, as reported by 
Kleinow et al. [25], Figs. 3B and 4A. Using these plants, we observed 
ATAF1 over-expressing plants to have significant (approx. 6- and 7.5-
fold) increased ABA levels (P < 0.01) compared to wild-type and ataf1 
mutants, respectively (Fig. 3D).

Second, to determine whether ATAF1 binds the promoter of NCD3 
in vivo, we performed ChIP on wild-type and 35S:ATAF1-HA plants (Fig. 
3A). Subsequent qPCR identified a region (position ~1134 to ~1265 
bp) including a TTAGCTA ATAF1 binding motif to be enriched in ChiPs 
from ATAF1 over-expressing plants (Fig. 3E). ATAF1 did not bind a re-
region between ~120 and ~218 bp (non-binding; NB), confirming ATAF1 
binding specificity. As a technical control, the F1 locus targeted by the 
HA-tagged TFL2 TF was included [35] (Fig. 3E). Finally, to assess the 
regulatory potential of ATAF1 we determined the transcript levels of 
NCD3 and found ~10-fold increase in ATAF1 over-expressing plants 
compared to wild-type. In contrast, mean NCD3 levels were signifi-
cantly lower in ataf1 mutants (Fig. 3F), substantiating the strong pos-
itive correlation between ATAF1 and NCD3 transcript levels (Fig. 2A). 
In addition to NCD3, transcript levels of several other top-ranking 
ATAF1 co-expressed genes displayed ATAF1-dependent expression 
perturbations, though with a narrower dynamic range compared to 
NCD3 (Fig. 4B).
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 [30], 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 ataf1 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 AB12 and DREB2A were observed in ataf1–2 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 AB12, 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

Top 25 ATAF1 co-expressed genes.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Pearson corr. coeff</th>
<th>Description</th>
<th>TTVCGT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TTVGTR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G61820</td>
<td>0.7855</td>
<td>Unknown protein</td>
<td>+ + + + + + +</td>
<td>+ + + + + + + + + + + +</td>
</tr>
<tr>
<td>AT5G05410</td>
<td>0.7705</td>
<td>DRE-binding protein 2A (DREB2A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G77450</td>
<td>0.7477</td>
<td>NAC domain containing protein 32</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>AT3G19580</td>
<td>0.7384</td>
<td>Zinc-finger protein 2</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>AT3G62260</td>
<td>0.7287</td>
<td>Protein phosphatase 2C family protein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT5G59220</td>
<td>0.7167</td>
<td>Highly ABA-induced PP2C gene 1 (HAI1)</td>
<td>+ + + + + + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>AT5G63790</td>
<td>0.7147</td>
<td>NAC domain containing protein 102</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>AT3G17770</td>
<td>0.7132</td>
<td>Dihydroxyacetone kinase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT1G61340</td>
<td>0.7052</td>
<td>F-box family protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G24140</td>
<td>0.7037</td>
<td>F-box/RNA-like superfamily protein</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>AT5G02020</td>
<td>0.6946</td>
<td>Encodes a protein involved in salt tolerance, names SIS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT5G04080</td>
<td>0.6932</td>
<td>Unknown protein</td>
<td>+ + + + + + +</td>
<td>+ + + + + + + + + + + +</td>
</tr>
<tr>
<td>AT2G33700</td>
<td>0.6925</td>
<td>Protein phosphatase 2C family protein</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>AT4G27410</td>
<td>0.6901</td>
<td>NAC (No Apical Meristem) domain protein</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>AT1G05100</td>
<td>0.6893</td>
<td>Mitogen-activated protein kinase kinase kinase 18</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>AT5G62020</td>
<td>0.6889</td>
<td>Heat shock transcription factor 2B</td>
<td>+</td>
<td>+</td>
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<tr>
<td>AT5G04250</td>
<td>0.6685</td>
<td>Cysteine proteinases superfamily protein</td>
<td>+ + + + + + + + + + + +</td>
<td></td>
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<tr>
<td>AT4G37180</td>
<td>0.6788</td>
<td>Homeodomain-like superfamily protein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT3G14440</td>
<td>0.6773</td>
<td>Nine-cis-epoxytocarotenoid dioxygenase 3 (NCED3)</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>AT5G13810</td>
<td>0.6713</td>
<td>Glutaredoxin family protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT4G34000</td>
<td>0.6675</td>
<td>Abscisic acid responsive elements-binding factor 3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT5G57050</td>
<td>0.6659</td>
<td>Protein phosphatase 2C family protein (ABI2)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT5G04340</td>
<td>0.661</td>
<td>Zinc finger of Arabidopsis thaliana 6</td>
<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>AT1G60190</td>
<td>0.659</td>
<td>ARM repeat superfamily protein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT4G23050</td>
<td>0.6556</td>
<td>PAS domain-containing tyrosine kinase protein</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</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.
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 ataf1 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 Ceanothus 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; AAAAATTCATGGTACAATATTTTACCGTGGCCA and CCGGATCCCTAAGCGTAATCTGGTCGGCTGATGTTACAAAGCTCCTTCGATGTAC, 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

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 [53]. Resulting PWMs were graphically visualized using enoLOGOS [55].

Western blotting

Nuclear extracts were precipitated over-night 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 AAGAATTCATGGTACAATATTTTACCGTGGCCA and CCGGATCCCTAAGCGTAATCTGGTCGGCTGATGTTACAAAGCTCCTTCGATGTAC, respectively. Primers for pGADT7-ATAF1(1–165) and pGADT7-ATAF1(1–289) used forward primer AAGAATTCATGGTACAATATTTTACCGTGGCCA and reverse primers were CCGGATCCCTAAGCGTAATCTGGTCGGCTGATGTTACAAAGCTCCTTCGATGTAC, respectively. For quantitative real-time PCR (qPCR), Actin2 (ACT2) was used as a reference. For NED3 we used AGCTCTTTACATGGCCAG and CCGTCTCTGGAAACAAATTCTAC. For endogenous ATAF1 we used GTGTTTTACCGGCAGAATC and
Five-week old 3SS:ATAF1-HA, 3SS:TF2L-HA and Col-0 wild-type plants were harvested. The ChiP procedure was performed according to Reimer and Turk [57]. The DNA was sheared by sonication using a Mysonix sonicator (CT, USA) set to 3.5 output 10 × 10 s with 20 s interval, and immunoprecipitated using anti-HA antibodies (Sigma, MO, USA). Each of the IPs was performed at least three independent times. For ChiP of N Ced3 we used CAG TTTGCTATATCCGA AACAAC and T GATGTAACACACGCGAC. For the non-binding (NB) control we used; GTGATAGGGAATTAAAAAGGG and GTTCAGTCTGCACTTTGACC. For F I we used GCCTAAATAGTTGGCCGA and TGCGATCGAGAAATTTAAGCA.

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 (VKRO9-007) 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.

Supplementary 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.


