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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[A,C,G]CGT[A,G] and TT[A,C,G]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 TTGCGTA 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 *CUC2*) TFs as components related to ABA and biotic stress signaling [6,14]. 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

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Abbreviations: ABA, abscisic acid; ATAF1, *Arabidopsis thaliana* activating factor 1; DBD, DNA-binding domain; ChIP, chromatin-immunoprecipitation; NAC, NAM, ATAF1/2, *CUC2*; *NCED3*, 9-cis-epoxycarotenoid dioxygenase-3; PBM, protein-binding microarrays; PWM, position weight matrix; SnRK, Sucrose nonfermenting 1 (SNF1)-related serine/threonine-protein kinase; TF, transcription factor.

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DNA-binding sites [18–20], and the consensus binding site (BS) [T,A][T,G][T,A,C,G]CGT[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 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 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 *ATAF1*, we here delineate the DNA-binding specificity of *ATAF1* using 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* transcript abundance and, most importantly, increased ABA phytohormone levels. Taken together, our data indicate that *ATAF1* 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 *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 incubated 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 regulation, $P < 0.05$) selection criterion for *ATAF1* transcript level perturbations, we found 403 microarray slides from 87 perturbations. Using this data set we identified 25 top-ranking genes co-expressed with *ATAF1* ($r \geq 0.66$) (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* binding oligomers and the POBO program [33], we analyzed whether the

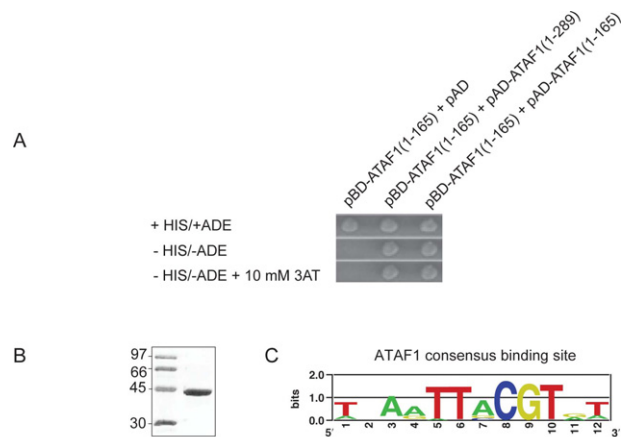


Fig. 1. *ATAF1* homodimerization and consensus binding site. (A) Fusion proteins of Gal4-(DBD)-*ATAF1*(1–165), Gal4-(AD)-*ATAF1*(1–289), Gal4-(AD)-*ATAF1*(1–165), and empty Gal4-(DBD) were co-transformed, expressed in yeast, and screened after 7 days for transactivation activity of *HIS3* and *ADE2* 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 μ g of affinity-purified recombinant GST-*ATAF1*(1–165). (C) Consensus binding site of *ATAF1* from duplicate PBM experiments.

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 both T[A,C,G]CGT[A,G] and TT[A,C,G]CGT in the *ATAF1* cluster compared 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 gene *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 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 *ATAF1* transcript levels and were not associated with transgene silencing of *ATAF1* and other *ATAF* 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 *NCED3* *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 TTGCGTA *ATAF1* binding motif to be enriched in ChIPs from *ATAF1* over-expressing plants (Fig. 3E). *ATAF1* did not bind a region between –120 and –218 bp (non-binding; NB), confirming *ATAF1* binding specificity. As a technical control, the *FT* 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 *NCED3* and found ~10-fold increase in *ATAF1* over-expressing plants compared to wild-type. In contrast, mean *NCED3* levels were significantly lower in *ataf1* mutants (Fig. 3F), substantiating the strong positive correlation between *ATAF1* and *NCED3* transcript levels (Fig. 2A). In addition to *NCED3*, transcript levels of several other top-ranking *ATAF1* co-expressed genes displayed *ATAF1*-dependent expression perturbations, though with a narrower dynamic range compared to *NCED3* (Fig. 4B).

Table 1
Top 25 *ATAF1* co-expressed genes.^a

AGI	Pear. corr. coeff	Description	TTVCGT ^b	TVCGTR ^b
AT5G61820	0.7855	Unknown protein		+
AT5G05410	0.7705	DRE-binding protein 2A (DREB2A)	+++++	+++++
AT1G77450	0.7477	NAC domain containing protein 32	++	+++
AT3G19580	0.7384	Zinc-finger protein 2	++	++
AT3G62260	0.7287	Protein phosphatase 2C family protein	+	++
AT5G59220	0.7167	Highly ABA-induced PP2C gene 1 (HAI1)	+++	+++
AT5G63790	0.7147	NAC domain containing protein 102	++	++
AT3G17770	0.7132	Dihydroxyacetone kinase	+	++
AT1G61340	0.7052	F-box family protein		
AT1G21410	0.7037	F-box/RNI-like superfamily protein	+	+++
AT5G02020	0.6946	Encodes a protein involved in salt tolerance, names SIS		
AT5G04080	0.6932	Unknown protein	++	+++
AT2G33700	0.6925	Protein phosphatase 2C family protein	+	++
AT4G27410	0.6901	NAC (No Apical Meristem) domain protein	+	+++
AT1G05100	0.6893	Mitogen-activated protein kinase kinase kinase 18	++	++++
AT5G62020	0.6889	Heat shock transcription factor B2A	+	+
AT5G04250	0.6865	Cysteine proteinases superfamily protein	++++	+++++
AT4G37180	0.6788	Homeodomain-like superfamily protein	+	
AT3G14440	0.6773	Nine-cis-epoxycarotenoid dioxygenase 3 (NCED3)	+	+++
AT5G13810	0.6713	Glutaredoxin family protein		
AT4G34000	0.6675	Abscisic acid responsive elements-binding factor 3	+	+
AT5G57050	0.6659	Protein phosphatase 2C family protein (ABI2)	+	+
AT5G04340	0.661	Zinc finger of <i>Arabidopsis thaliana</i> 6	++	+
AT1G60190	0.659	ARM repeat superfamily protein	+	+
AT4G23050	0.6556	PAS domain-containing tyrosine kinase protein		

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

^b V = [A,C,G] and R = [A,G], + indicates number of *ATAF1* binding 6-mers in 1 kb promoters.

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 *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, alike 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

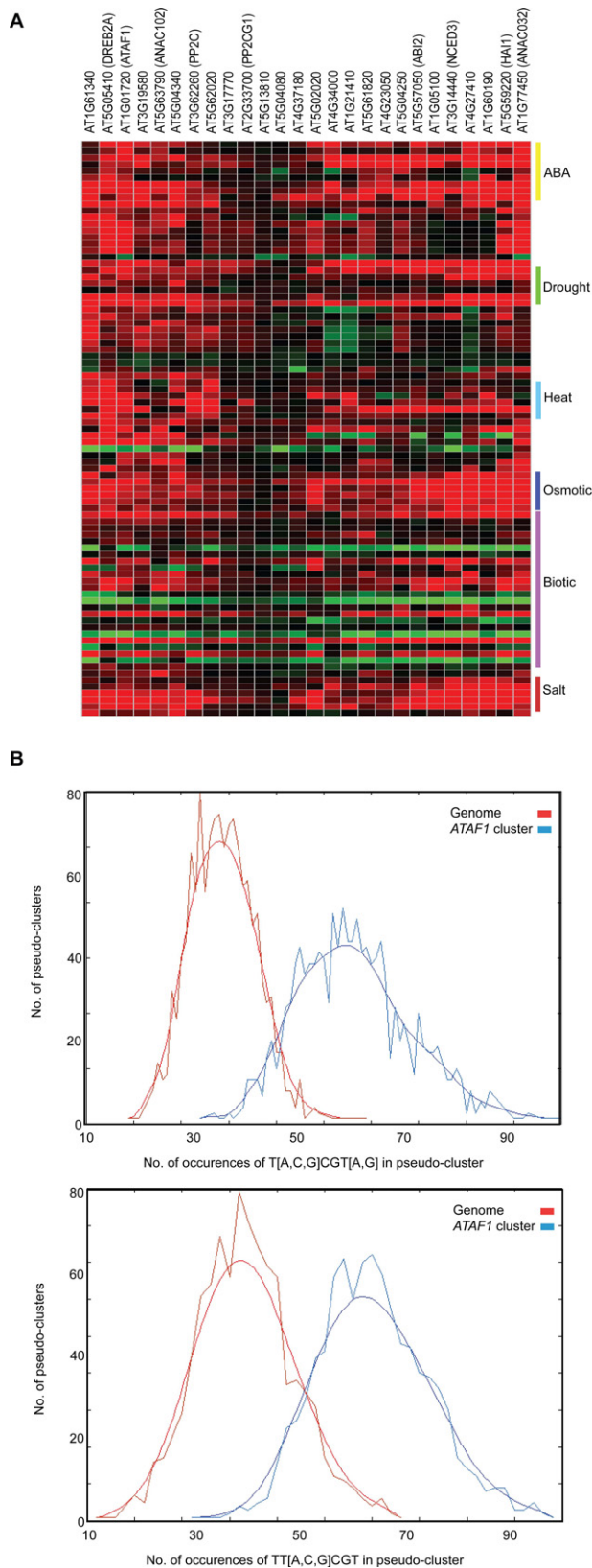


Fig. 2. *ATAF1* co-expression gene cluster is enriched for *ATAF1* binding sites. (A) Using 403 microarray samples from 87 conditions either significantly inducing or repressing *ATAF1* expression, identifies 25 co-expressed genes with a Pearson correlation coefficient >0.66 . (B) Bootstrapping analyses using POBO [33] show that promoters of *ATAF1* co-expressed genes are significantly ($P < 0.001$) enriched for *ATAF1* consensus binding sites T[A,C,G]CGT[A,G] and TT[A,C,G]CGT.

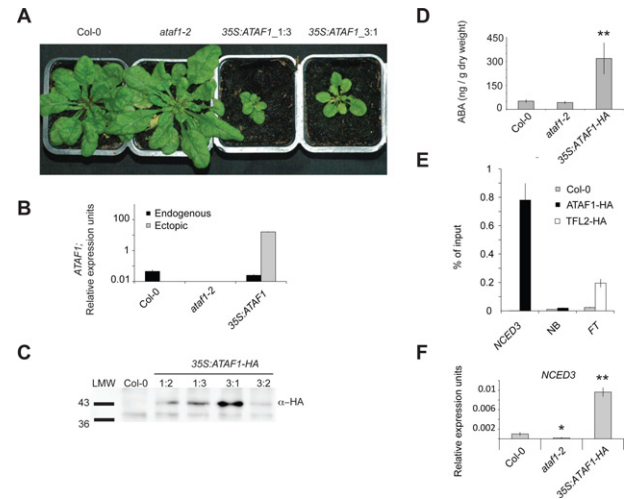


Fig. 3. Ectopic expression of *ATAF1* arrests plant development and activates ABA biosynthesis. (A) Eight week-old short-day grown Col-0 wild-type and *ataf1-2*, compared to plants ectopically expressing HA-tagged *ATAF1*. Two independent *35S:ATAF1-HA* lines (1:3 and 3:1) display growth reduction. (B) Quantitative expression profiles of endogenous and ectopic *ATAF1* in Col-0 wild-type, *ataf1-2* and *35S:ATAF1-HA* plants. Mean (\pm sem) relative expression units (log₁₀ 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 ± 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 (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 ± 1 standard deviation. (F) Expression of ABA biosynthetic gene *NCED3* in *ATAF1* over-expressing plants compared to wild-type Col-0 and *ataf1* mutants. Expression level of *NCED3* was determined by qPCR in the indicated genotypes. Mean (\pm sem) relative expression units are displayed using *ACT2* as reference. Bars represent the mean of three biological replicates. 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 *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 *Caenorhabditis 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 its 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.

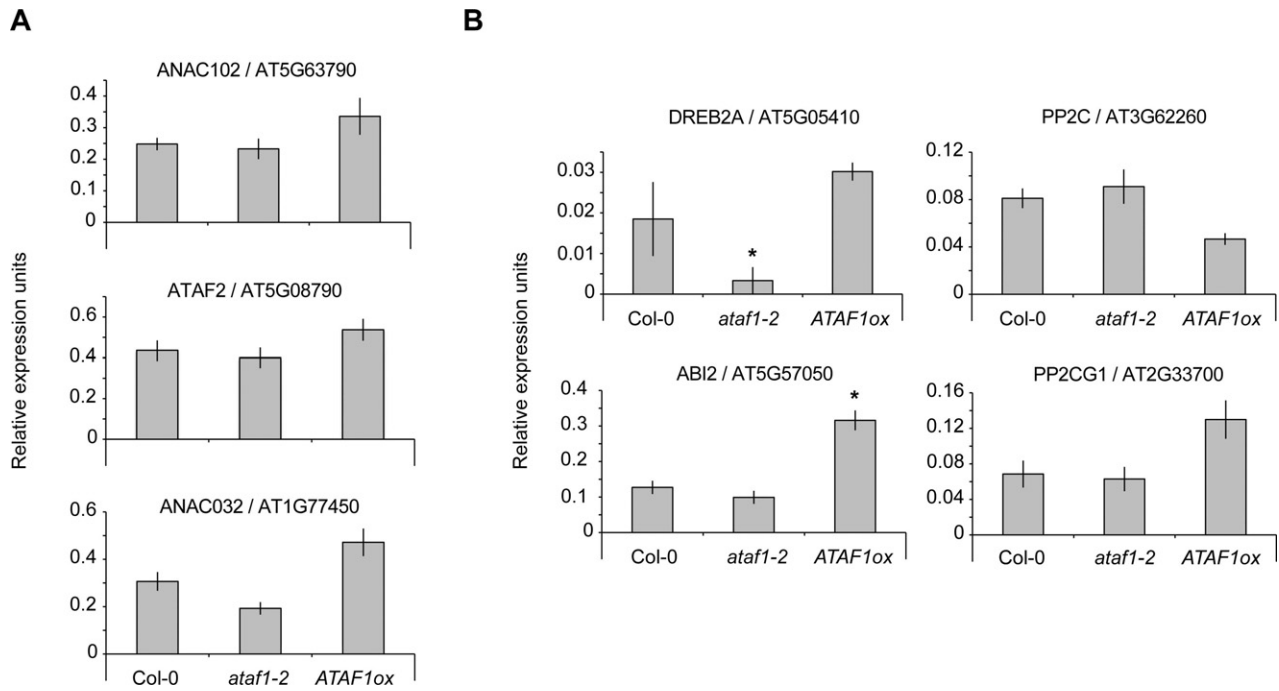


Fig. 4. Expression perturbations of *ATAF* subclade members and *ATAF1* co-expressed genes. (A) Expression of genes encoding *ATAF* subclade NAC TFs. Expression level of candidate genes was determined by qPCR in the indicated genotypes. Mean (\pm sem) relative expression units are displayed using *ACT2* as reference. Bars represent the mean of three biological replicates. (B) Expression of *ATAF1* co-expressed genes in *ATAF1* over-expressing plants compared to wild-type Col-0 and *ataf1* mutants. Statistical analyses were performed using Student's *t*-test of the differences between individual means compared to Col-0 ($*P < 0.05$).

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 $\mu\text{E}/\text{m}^2 \text{ s}$) 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; AAAGAATTCATGTCAGAAT-TATTACAGTTGCC and CCGGGATCCCTAAGCGTAATCTGGTACGTCG-TATGGGTAAGGCTTCTGCATGTAC, 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' 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 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 pGBKT7 were AAGAATTCATGTCAGAAT-TATTACAGTTGCC and CCGGGATCCCGCCTCTCGGTAGCTCC. Primers for pGADT7-*ATAF1*(1–165) and pGADT7-*ATAF1*(1–289) used forward primer AAGAATTCATGTCAGAATTATTACAGTTGCC. Reverse primers were CCGGGATCCCGCCTCTCGGTAGCTCC and CCGGGATCCGTAAG-GCTTCTGCATGTACATGAA, respectively. For quantitative real-time PCR (qPCR), *Actin2* (*ACT2*) was used as a reference. For *NCED3* we used AGTCCTTACCTATGGCCAG and CGTCTCTGGAACAAATTCATC. For endogenous *ATAF1* we used GTTGTTTACGGCGACGAAATC and

TAAACGGTCTCGTGTGCCATAA. For ectopic *ATAF1* we used GTTGT-TACGGCGACGAAATC and CCGCAACAGGATTCAATCTT. QPCRs were performed in triplicate for each individual line using Brilliant II SYBR Green qPCR kit (Stratagene, CA, USA) on an iCycler IQ (Bio-Rad, CA, USA). Quantification of CT (cycle threshold) values was achieved by calculating means of normalized expression using Q-gene software [56].

ChIP assay

Five-week old 35S:*ATAF1-HA*, 35S:*TFL2-HA* and Col-0 wild-type plants were harvested. The ChIP procedure was performed according to Reimer and Turck [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 *NCED3* we used CAGTTGCTATATATCTA-GAAACCA and TGATGTAACACCCGAC. For the non-binding (NB) control we used; GGTATAGAGGGAATTAAGGG and GTCTCAAGTCT-CAACTTTGAACC. For *FT* we used GCTCAACATGTTGCTCGAA and TGC-GATCAGTAAAATACACAGACA.

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; *NCED3*, At3g14440.

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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>.

References

- Hu, X., Wu, X., Li, C., Lu, M., Liu, T., Wang, Y. et al. (2012) Abscisic acid refines the synthesis of chloroplast proteins in maize (*Zea mays*) in response to drought and light. *PLoS One* 7, e49500.
- Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H. et al. (2003) Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. *Plant J.* 34, 137–148.
- Yoshida, T., Nishimura, N., Kitahata, N., Kuromori, T., Ito, T., Asami, T. et al. (2006) ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. *Plant Physiol.* 140, 115–126.
- Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T. and Chua, N.H. (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. *Plant J.* 32, 317–328.
- Barrero, J.M., Piqueras, P., Gonzalez-Guzman, M., Serrano, R., Rodriguez, P.L., Ponce, M.R. et al. (2005) A mutational analysis of the ABA1 gene of *Arabidopsis thaliana* highlights the involvement of ABA in vegetative development. *J. Exp. Bot.* 56, 2071–2083.
- de Torres-Zabala, M., Truman, W., Bennett, M.H., Lafforgue, G., Mansfield, J.W., Rodriguez Egea, P. et al. (2007) *Pseudomonas syringae* pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *EMBO J.* 26, 1434–1443.
- Fan, J., Hill, L., Crooks, C., Doerner, P. and Lamb, C. (2009) Abscisic acid has a key role in modulating diverse plant–pathogen interactions. *Plant Physiol.* 150, 1750–1761.
- Nambara, E. and Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Ann. Rev. Plant Biol.* 56, 165–185.
- Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M. et al. (2006) Functional analysis of Arabidopsis NCED6 and NCED9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *Plant J.* 45, 309–319.
- Yaish, M.W., El-Kereamy, A., Zhu, T., Beatty, P.H., Good, A.G., Bi, Y.M. et al. (2010) The APETALA-2-like transcription factor OsAP2-39 controls key interactions between abscisic acid and gibberellin in rice. *PLoS Genet.* 6.
- Tan, B.C., Joseph, L.M., Deng, W.T., Liu, L., Li, Q.B., Cline, K. et al. (2003) Molecular characterization of the Arabidopsis 9-cis epoxy-carotenoid dioxygenase gene family. *Plant J.* 35, 44–56.
- Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T. et al. (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxy-carotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *Plant J.* 27, 325–333.
- Jiang, Y., Liang, G. and Yu, D. (2012) Activated expression of WRKY57 confers drought tolerance in Arabidopsis. *Mol. Plant* 5, 1375–1388.
- Jensen, M.K., Hagedorn, P.H., de Torres-Zabala, M., Grant, M.R., Rung, J.H., Collinge, D.B. et al. (2008) Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards *Blumeria graminis* f. sp. *hordei* in Arabidopsis. *Plant J.* 56, 867–880.
- Aida, M., Ishida, T., Fukaki, H., Fujisawa, H. and Tasaka, M. (1997) Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *Plant Cell* 9, 841–857.
- Tran, L.S., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K. et al. (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.
- Olsen, A.N., Ernst, H.A., Leggio, L.L. and Skriver, K. (2005) DNA-binding specificity and molecular function of NAC transcription factors. *Plant Sci.* 169, 785–797.
- Kim, H.S., Park, H.C., Kim, K.E., Jung, M.S., Han, H.J., Kim, S.H. et al. (2012) A NAC transcription factor and SN1 cooperatively suppress basal pathogen resistance in *Arabidopsis thaliana*. *Nucleic Acids Res.* 40, 9182–9192.
- Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M. et al. (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant J.* 39, 863–876.
- Wu, A., Allu, A.D., Garapati, P., Siddiqui, H., Dortay, H., Zhan, M.I. et al. (2012) JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. *Plant Cell* 24, 482–506.
- Jensen, M.K., Kjaersgaard, T., Nielsen, M.M., Galberg, P., Petersen, K., O'Shea, C. et al. (2010) The *Arabidopsis thaliana* NAC transcription factor family: structure-function relationships and determinants of ANAC019 stress signalling. *Biochem. J.* 426, 183–196.
- Jensen, M.K., Kjaersgaard, T., Petersen, K. and Skriver, K. (2010) NAC genes: time-specific regulators of hormonal signaling in Arabidopsis. *Plant Signal. Behav.* 5, 907–910.
- Lu, P.L., Chen, N.Z., An, R., Su, Z., Qi, B.S., Ren, F. et al. (2007) A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. *Plant Mol. Biol.* 63, 289–305.
- Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B. et al. (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. *Cell Res.* 19, 1279–1290.
- Kleinow, T., Himbert, S., Krenz, H., Jeske, H. and Koncz, C. (2009) NAC domain transcription factor ATAF1 interacts with SNF1-related kinases and silencing of its subfamily causes severe developmental defects in Arabidopsis. *Plant Sci.* 177, 360–370.
- Jossier, M., Bouly, J.P., Meimoun, P., Arjmand, A., Lessard, P., Hawley, S. et al. (2009) SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in *Arabidopsis thaliana*. *Plant J.* 59, 316–328.
- Baena-Gonzalez, E., Rolland, F., Thevelein, J.M. and Sheen, J. (2007) A central integrator of transcription networks in plant stress and energy signalling. *Nature* 448, 938–942.
- Berger, M.F., Badis, G., Gehrke, A.R., Talukder, S., Philippakis, A.A., Pena-Castillo, L. et al. (2008) Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. *Cell* 133, 1266–1276.
- De Masi, F., Grove, C.A., Vedenko, A., Alibes, A., Gisselbrecht, S.S., Serrano, L. et al. (2011) Using a structural and logics systems approach to infer bHLH-DNA binding specificity determinants. *Nucleic Acids Res.* 39, 4553–4563.
- Vandepoele, K., Quimbaya, M., Casneuf, T., De Veylder, L. and Van de Peer, Y. (2009) Unraveling transcriptional control in Arabidopsis using cis-regulatory elements and coexpression networks. *Plant Physiol.* 150, 535–546.
- Truman, W. and Glazebrook, J. (2012) Co-expression analysis identifies putative targets for CBP60g and SARD1 regulation. *BMC Plant Biol.* 12, 216.
- Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiol.* 136, 2621–2632.

- [33] Kankainen, M. and Holm, L. (2004) POBO, transcription factor binding site verification with bootstrapping. *Nucleic Acids Res.* 32, W222–W229.
- [34] Hegedus, D., Yu, M., Baldwin, D., Gruber, M., Sharpe, A., Parkin, I. et al. (2003) Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. *Plant Mol. Biol.* 53, 383–397.
- [35] Turck, F., Roudier, F., Farrona, S., Martin-Magniette, M.L., Guillaume, E., Buisine, N. et al. (2007) Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. *PLoS Genet.* 3, e86.
- [36] Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H. et al. (2008) Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol.* 147, 1984–1993.
- [37] Melhorn, V., Matsumi, K., Koiwai, H., Ikegami, K., Okamoto, M., Nambara, E. et al. (2008) Transient expression of AtNCED3 and AAO3 genes in guard cells causes stomatal closure in *Vicia faba*. *J. Plant Res.* 121, 125–131.
- [38] Shi, Y., Wang, Z., Meng, P., Tian, S., Zhang, X. and Yang, S. (2013) The glutamate carboxypeptidase AMP1 mediates abscisic acid and abiotic stress responses in Arabidopsis. *New Phytol* 199(1), 135–150.
- [39] Li, W., Cui, X., Meng, Z., Huang, X., Xie, Q., Wu, H. et al. (2012) Transcriptional regulation of Arabidopsis MIR168a and argonaute1 homeostasis in abscisic acid and abiotic stress responses. *Plant Physiol.* 158, 1279–1292.
- [40] Du, H., Wu, N., Fu, J., Wang, S., Li, X., Xiao, J. et al. (2012) A GH3 family member, OsGH3-2, modulates auxin and abscisic acid levels and differentially affects drought and cold tolerance in rice. *J. Exp. Bot.* 63, 6467–6480.
- [41] Tsai, A.Y. and Gazzarrini, S. (2012) AKIN10 and FUSCA3 interact to control lateral organ development and phase transitions in Arabidopsis. *Plant J.* 69, 809–821.
- [42] Domagalska, M.A., Sarnowska, E., Nagy, F. and Davis, S.J. (2010) Genetic analyses of interactions among gibberellin, abscisic acid, and brassinosteroids in the control of flowering time in *Arabidopsis thaliana*. *PLoS One* 5, e14012.
- [43] Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T. et al. (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science* 311, 91–94.
- [44] Sakuma, Y., Maruyama, K., Osakabe, Y., Qin, F., Seki, M., Shinozaki, K. et al. (2006) Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression. *Plant Cell* 18, 1292–1309.
- [45] Kim, J.S., Mizoi, J., Yoshida, T., Fujita, Y., Nakajima, J., Ohori, T. et al. (2011) An ABRE promoter sequence is involved in osmotic stress-responsive expression of the DREB2A gene, which encodes a transcription factor regulating drought-inducible genes in Arabidopsis. *Plant Cell. Physiol.* 52, 2136–2146.
- [46] Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. et al. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324, 1064–1068.
- [47] Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F. et al. (2009) Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant* 60, 575–588.
- [48] Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y. et al. (2009) In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 462, 660–664.
- [49] Zheng, X.Y., Spivey, N.W., Zeng, W., Liu, P.P., Fu, Z.Q., Klessig, D.F. et al. (2012) Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe* 11, 587–596.
- [50] Bu, Q., Jiang, H., Li, C.B., Zhai, Q., Zhang, J., Wu, X. et al. (2008) Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. *Cell Res.* 18, 756–767.
- [51] Grove, C.A., De Masi, F., Barrasa, M.I., Newburger, D.E., Alkema, M.J., Bulyk, M.L. et al. (2009) A multiparameter network reveals extensive divergence between *C. elegans* bHLH transcription factors. *Cell* 138, 314–327.
- [52] Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743.
- [53] Berger, M.F. and Bulyk, M.L. (2009) Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nat. Protoc.* 4, 393–411.
- [54] Badis, G., Chan, E.T., van Bakel, H., Pena-Castillo, L., Tillo, D., Tsui, K. et al. (2008) A library of yeast transcription factor motifs reveals a widespread function for Rsc3 in targeting nucleosome exclusion at promoters. *Mol. Cell.* 32, 878–887.
- [55] Workman, C.T., Yin, Y., Corcoran, D.L., Ideker, T., Stormo, G.D. and Benos, P.V. (2005) enoLOGOS: a versatile web tool for energy normalized sequence logos. *Nucleic Acids Res.* 33, W389–W392.
- [56] Muller, P.Y., Janovjak, H., Miserez, A.R. and Dobbie, Z. (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. *BioTechniques* 32, 1372–4, 1376, 1378–9.
- [57] Reimer, J.J. and Turck, F. (2010) Genome-wide mapping of protein–DNA interaction by chromatin immunoprecipitation and DNA microarray hybridization (ChIP–chip). Part A: ChIP–chip molecular methods. *Methods Mol. Biol.* 631, 139–160.
- [58] Forcat, S., Bennett, M.H., Mansfield, J.W. and Grant, M.R. (2008) A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress. *Plant Methods* 4, 16.