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NAC domain transcription factor ATAF1 interacts with SNF1-related kinases and silencing of its subfamily causes severe developmental defects in *Arabidopsis*

Tatjana Kleinow [a,*], Sarah Himbert [a,1], Björn Krenz [a], Holger Jeske [a], Csaba Koncz [b,c]

[a] Institute of Biology, Department of Molecular Biology and Plant Virology, Universität Stuttgart, Pfaffenwaldring 57, 70550 Stuttgart, Germany
[b] Max Planck Institute for Plant Breeding Research, Carl-von-Linné Weg 10, 50829 Köln, Germany
[c] Institute of Plant Biology, Biological Research Center of the Hungarian Academy of Sciences, Temesvári krt. 62, H6701 Szeged, Hungary

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ABSTRACT

ATAF1, a member of the plant-specific NAC transcription factor family in *Arabidopsis thaliana,* was identified in two-hybrid and *in vitro* binding assays as interacting partner of SNF1-related protein kinase (SnRK1) catalytic subunits. SnRK1s represent essential factors in stress and glucose signal transduction, and are involved in coordinate regulation of metabolic, hormonal and developmental signaling pathways. Transcription profiles of ATAF1 and closely related NACs indicate that their expression is co-regulated in various organs and by wounding, methyl jasmonate, hydrogen peroxide, pathogen infection, abscisic acid, cold, drought, salt and osmotic stress. Transgenic *Arabidopsis* carrying a 35S::ATAF1 construct developed fast senescing curly leaves and showed various grades of dwarfism leading to growth arrest and subsequent seedling death. RT-PCR analysis exhibited a silencing effect of the overexpression construct that down-regulated transcription of endogenous ATAF family members in plants showing severe developmental defects. These results together with the analysis of T-DNA insertion mutants suggest that the ATAF subfamily members perform redundant functions and act as positive regulators of plant development.

1. Introduction

Plants respond to various stress stimuli, causing disturbance in the cellular energy status, by complex regulatory changes in their carbohydrate metabolism, which is required for proper optimization of growth and development under limiting environmental conditions. Sugar signaling plays a pivotal role in the regulation of metabolism and is tightly linked to modulation of development, switch from vegetative to reproductive phase, control of senescence, and responses to abiotic and biotic stresses [1–5]. There is accumulating evidence for an extensive cross-talk between sugar, hormone and light signal transduction networks in plants [1–3,6–8]. Members of the sucrose non-fermenting 1-related (SnRK1)/AMP-activated protein kinase (AMPK) family are important regulators of sugar signal transduction and energy/carbon metabolism [8–11]. SnRK1s occur in heterotrimeric complexes consisting of a catalytic α-subunit, an activating γ-subunit, and a target selective adaptor β-subunit that anchors the α- and γ-subunits [9,10]. Because in *Arabidopsis* and maize the γ-subunit (i.e. termed also βγ-subunit) carries a domain characteristic of the β-subunits in other organisms, it has been suggested that plant SnRK1 enzymes may also be active as heterodimers of α- and βγ-subunits [4,12,13]. Remarkable conservation of biological functions of plant SnRK1 kinases is indicated by the fact that the α, and γ proteins can suppress the sucrose non-fermenting defects of yeast *snf1*/α and *snf4*/γ mutants [9,12–15]. Although plants carry three different subfamilies of yeast Snf1-related SnRK kinases, only the SnRK1 family shows close functional and structural relationship with yeast Snf1 and animal AMPKs [9,10,16]. Analysis of various members of the SnRK1 family in different plant species documents that they are responsible for the regulation of many downstream targets of glucose signal transduction, implicated in e.g. starch biosynthesis, salt stress tolerance, pathogen responses, development and senescence. Biochemical studies indicate that SnRK1 kinases modulate the functions of key metabolic enzymes either directly by phosphorylation or indirectly by controlling gene expression [4,8–10]. Although SnRK1 signaling in *Arabidopsis* is reported to control transcription of over 1200 genes, thus far only few transcription factors are known, which may represent potential downstream targets of SnRK1 kinase regulation [9–11,17].

Here we describe the identification of a novel SnRK1-binding transcription factor, ATAF1, which belongs to the NAC (NAM [no apical meristem], ATAF, CUC2 [cup-shaped cotyledon]) family, one

* Corresponding author. Tel.: +49 711 685 65075; fax: +49 711 685 65096.
E-mail address: tatjana.kleinow@bio.uni-stuttgart.de (T. Kleinow).
[1] Present address: Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, 1030 Vienna, Austria.

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of the largest families of plant-specific transcription factors. The NAC subfamilies are known to control various processes in development, organ formation, abiotic/biotic stress responses, senescence and hormone signal transduction [18]. Nonetheless, the biological function and regulation of the majority of NAC transcription factors are still largely unknown [18]. In their conserved N-terminal regions all NACs possess five highly conserved motifs, representing the family-defining NAC DNA-binding domain [18,19]. Divergent C-terminal domains of characterized NACs function as transcription activators in yeast and plant cell assays [18]. Based on similarities between their NAC domains and short C-terminal motifs, members of the NAC family are divided into several subgroups [18,20,21]. Comparative bioinformatics studies support the assumption that specific regulatory functions of NAC subgroups in *Arabidopsis* and rice correlate with structural differences in their NAC domains [18,20,21]. The expression levels of NAC transcripts are differentially controlled by various pathways, including posttranscriptional regulation by microRNAs [18,22,23]. Because certain classes of NACs show analogous co-regulation by specific sets of stimuli, including phytohormones, dehydration or wounding, a role for NAC proteins in the cross-talk between different signaling pathways is proposed [18]. The activity of some NACs is also modulated posttranslationally either by proteolytic processing and release from membrane-bound state for subsequent nuclear import, or by binding of co-factors like calmodulin, or by posttranslational modification with N-acetyl glucosamine [18,24–28]. In addition, ubiquitination-mediated proteolysis also plays an important role in regulating the stability of NAC1 and ANAC019 factors [29–31].

In this study, we identified a binding between ATAF1 and the SnRK1 kinase catalytic α-subunits AKIN10 and AKIN11 in protein interaction assays. To dissect possible biological functions of ATAF1 and other closely related NAC transcription factors of the ATAF subfamily, we analyzed their transcriptional regulation and characterized transgenic *Arabidopsis* plants carrying an ATAF1 overexpression construct as well as knock-out mutants for all four subfamily members. The sum of the findings suggests for the ATAF subfamily a co-regulated expression pattern, functional redundancy and a role as potent regulators of plant development.

2. Materials and methods

2.1. Plasmid construction

Full-length coding region of *ATAF1* (At1g01720) was PCR-amplified (25 cycles of 30 s 94 °C, 30 s 58 °C, 1 min 72 °C followed by 5 min 72 °C) from an *Arabidopsis* cDNA library made from cell suspension and seedlings [32] using the primer pair: 5′-ACCCGGGAATTCCCATGTCAGAATTATTACAGTT-3′ and 5′-CGA-GAATTCCCGGGCTAGTAAGGCTTCTGCATGT-3′ (added SmaI and EcoRI sites underlined). The PCR product was inserted into pGEM-T (Promega), sequenced and subcloned by EcoRI into the yeast two-hybrid prey vector pACT2 (BD Biosciences/Clontech) in frame with the Gal4 activation domain (GAD), as well as by SmaI in fusion with the Gal4 DNA-binding domain (GBD) of bait vector pAS2-1 (BD Biosciences/Clontech). To generate the GBD–$ATAF1_{108–289}$ fusion in pAS2-1, an *ATAF1* cDNA fragment was excised by NcoI–XhoI from pACT2 and inserted into the NcoI–SalI sites of pAS2-1. For constitutive expression *in planta*, the *ATAF1* coding sequence was excised by BamHI–BglII from pACT2 and inserted into the BamHI site of pPCV002-Gigi (c-Myc epitope) and pPCV812-Menchu (hemagglutinin [HA] epitope) *Agrobacterium* binary vectors [33,34]. By these vectors, ATAF1 was expressed in *Arabidopsis* (Col-0) in fusion with intron-disrupted coding sequences of either HA or c-Myc epitope tags under the control of *Cauliflower mosaic virus* (CaMV) 35S promoter. Other constructs used in this work were previously described [14,15,33,35].

2.2. Plant transformation and segregation analysis

ATAF1 expression vectors verified by restriction endonuclease digestions and sequencing were introduced into *Agrobacterium* GV3101 (pPMP90RK) by conjugation [36]. *Arabidopsis thaliana* (Col-0) plants were transformed by the floral dip procedure [37]. To select transgenic plants, T1 seeds were surface sterilized and germinated in MS agar medium containing 0.5% sucrose [38] and either 15 μg/ml hygromycin B (Roche, for pPCV812-Menchu–ATAF1) or 100 μg/ml kanamycin (Serva, for pPCV002-Gigi–ATAF1) under short day (8 h light/16 h dark cycle) at 20 °C, and then transferred into soil to obtain T2 offspring. Lines carrying single T-DNA insertion loci were identified by scoring for 3:1 segregation of antibiotics resistant versus sensitive progeny and used for analysis of phenotypes conferred by *ATAF1* expression. Root length measurements were performed by growing seedlings on vertical 0.5% sucrose MS agar plates.

2.3. Western blotting

For protein extraction, the plant material was homogenized in SDS-PAGE loading buffer (2% SDS, 6.5% glycerol, 62.5 mM Tris–HCl, 5% β-mercaptoethanol, 0.002% bromophenolblue; pH 6.8) and boiled for 5 min at 95 °C. The supernatants recovered after centrifugation (10 min, room temperature, 13,000×*g*) were separated by 12.5% SDS-PAGE according to Laemmli [39] and semidry-blotted to nitrocellulose membranes at 20 V, 400 mA for 40 min using 50 mM Tris, 50 mM boric acid and 10% (v/v) methanol transfer buffer. Blots were shaken for 1 h at room temperature in blocking buffer (TBST [137 mM NaCl, 0.1% (v/v) Tween 20, 20 mM Tris–HCl pH 7.6] containing 5% [w/v] non-fat dry milk powder), and then incubated overnight at 4 °C either with rat anti-HA or mouse anti-c-Myc monoclonal antibody (Roche) diluted in blocking buffer (1:2000 anti-HA; 1:1500 anti-c-Myc). After washing once for 15 min and four times for 5 min with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies (goat anti-mouse, Biotrend and goat anti-rat, Sigma) diluted 1:10,000 in blocking buffer for 1 h at room temperature. Washing steps were repeated as indicated above and detection was performed using the enhanced chemiluminescence method and exposure to X-ray films.

2.4. Yeast two-hybrid protein interaction assays

Yeast two-hybrid screens for AKIN-interacting factors were performed using an *Arabidopsis* cDNA library made from cell suspension in pACT2 as described [40]. The bait pAS2-1 (GBD, Trp^+) and prey pACT2 (GAD, Leu^+) plasmids were co-transformed into *Saccharomyces cerevisiae* strain Y190 (BD Biosciences/Clontech) using standard LiCl transformation protocol [41,42] and transformants were grown for 3–5 days at 30 °C on selective Trp^-/Leu^-/His^- SD medium [43] containing 50 mM 3-amino-triazole (3-AT). Protein interactions were monitored by performing LacZ filter lift assays with colonies grown on selective SD medium. Rescued prey plasmids were retransformed into yeast and interactions repeatedly assayed by mating of *S. cerevisiae* Matα strain Y187 (BD Biosciences/Clontech) carrying the pAS2-1 GBD-constructs with Mata strain Y190 harboring the pACT2 GAD-plasmid, according to Matchmaker system manual (BD Biosciences/Clontech).

2.5. In vitro protein binding assay

For *in vitro* transcription and translation using a TNT coupled reticulocyte lysate system (Promega), partial or full-length *ATAF1*

Table 1
Primer pairs for RT-PCR analysis of transcripts of ATAF subfamily, 35S::ATAF1 constructs and control β-actin 8.

Primer pair no.	Gene/transcript	Primer		Expected size PCR product (bp)	
		5′-End	3′-End	Genomic DNA	mRNA
1	35S::c-Myc-ATAF1	5′-CAAAAGTTGATTTCTGAGGAGGAT-3′[a]	5′-GAACGGACCGGTCAACGTCG-3′	–[b]	558
2	35S::HA–ATAF1	5′-TATCCATACGATGTTCCAGATTATG-3′[a]	5′-GAACGGACCGGTCAACGTCG-3′	–[b]	552
3	At1g01720, ANAC002, ATAF1	5′-GTTGACCGGTCCGTTCGC-3′	5′-TTCGGCGGCAGGAAAGGAA-3′[c]	585	498
4	At1g77450, ANAC032	5′-CGTTGACCGGTCGGTTCGT-3′	5′-CCACTTTCCACTAACTCTAATC-3′[c]	538	445
5	At5g08790, ANAC081, ATAF2	5′-TGTTGATAGATCAGCTTCTGTT-3′	5′-CACCTTTTTTGGTCTCTTCCG-3′[c]	569	478
6	At5g63790, ANAC102	5′-TCGATCGATCTGCTTCTACC-3′	5′-GCCGGTGATCGAAGACTCT-3′[c]	548	448
7	At1g49240, β-actin 8	5′-ATGAAGATTAAGGTCGTGGCA-3′	5′-ACCGGAAAGTTTCTCACATAGT-3′[c]	376	272

[a] Complementary only to spliced epitope sequence.
[b] No amplification product, only spliced transcripts would yield a product.
[c] Complementary to 3′untranslated region.

coding regions were PCR-amplified from the pACT2 constructs with a 5′-primer carrying a T7 promoter sequence (underlined): 5′-TAATACGACTCACTATAGGGAGACCACATGGAGGCCCCGGGGATCC-GAATT-3′ and 3′-primer 5′-ATGCACAGTTGAAGTGAACTT-3′. Glutahione-S-transferase (GST) fusion proteins of AKIN10 and AKIN11 were expressed in and purified from *Escherichia coli* according to Bhalerao et al. [15]. 5 μg GST–AKIN10, GST–AKIN11 and control GST proteins were coupled at 4 °C for 30 min in buffer B (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% (v/v) IGEPAL) to glutathione-Sepharose4B beads (GE Healthcare/Amersham Biosciences). After centrifugation (30 s, 2000×*g*), beads were resuspended in buffer BM (buffer B plus 5% [v/v] non-fat dry milk) and blocked for 30 min at 4 °C. Before addition of [^{35}S]-methinonine-labeled ATAF1 protein, the resin was washed with buffer BM followed by incubation of binding reactions for 60 min at 4 °C on a rotary shaker. Subsequently, the resin was pelleted by centrifugation and supernatant containing unbound [^{35}S]-ATAF1 was collected. After washing the resin four times with buffer B, bound proteins were eluted by SDS-PAGE loading buffer and denatured for 5 min at 95 °C. Aliquots of bound and unbound supernatant protein fractions were separated on SDS-PAGE as described above. After fixation by Coomassie blue staining, gels were soaked for 30 min in Amplify (GE Healthcare/Amersham Biosciences), dried under vacuum and the radioactively labeled ATAF1 proteins were detected by fluorography using X-ray films.

2.6. Northern RNA hybridization

Plant material was harvested from various organs of 8-week-old *Arabidopsis* grown in the greenhouse, whole seedlings (sterile culture) and cell suspension. Roots were obtained from plants cultured in liquid medium. Total RNA was isolated using the guanidinium-thiocyanate extraction protocol [41]. For northern blot analysis, 30 μg RNA samples were separated on formaldehyde agarose gel and transferred onto nylon membrane. The membrane was hybridized overnight at 42 °C in hybridization buffer (50% formamide, 6× SSPE, 5× Denhardt́s solution, 0.5% (w/v) SDS and 0.1 mg/ml (w/v) denatured salmon sperm DNA) with a ^{32}P-labeled cDNA probe, which was generated by random priming (Rediprime II; GE Healthcare/Amersham Biosciences) and carried 3′ *ATAF1* sequences encoding the non-conserved C-terminal region. After hybridization, the membrane was washed once with 2× SSC/0.5% SDS at 42 °C, 1× SSC/0.5% SDS at 50 °C and 0.5× SSC/0.5% SDS at 60 °C and subjected to autoradiography. As loading control the membrane was re-hybridized after stripping with α-actin *Act2* cDNA probe.

2.7. Reverse transcription-PCR (RT-PCR)

Total RNA from 100 mg *Arabidopsis* plantlets (six leaf-stage) of T2 lines either carrying a 35S::HA-GUS or the ATAF1 over-expression construct was extracted by TRI Reagent® according to the manufacturer's recommendations (Sigma). For RT-PCR, 2 μg RNA was applied for first-strand cDNA synthesis using $d(T)_{18}$-primers and reverse transcriptase (New England Biolabs). To ensure a specific amplification for each ATAF cDNA, primer pairs were used, from which one primer was complementary to the 3′untranslated region and the other to a subfamily member-specific part in the 3′-coding region (Table 1). PCR reactions with primer combinations 3–7 (Table 1) were performed using the following protocol: 5 min 95 °C, 40 cycles of 30 s 95 °C, 30 s 60 °C. 45 s 72 °C followed by 5 min 72 °C. For primers 1 and 2 (Table 1) the annealing temperature was 66 °C. The transcript of β-actin 8 (*At1g49240*) was used as RT-PCR loading control ([44], Table 1). A contamination of RNA samples with genomic DNA was excluded by an additional PCR reaction without preceding reverse transcription (data not shown).

3. Results

3.1. ATAF1 interacts with AKIN10 and AKIN11 catalytic subunits of Arabidopsis SNF1-related kinases

To identify interacting partners of SnRK1 catalytic α-subunits, two-hybrid screens were performed with pAS2-1 baits encoding fusions of AKIN10 (At3g01090) or AKIN11 (At3g29160) with the Gal4 DNA-binding domain (GBD) and a cDNA library made in the Gal4 activation domain (GAD) fusion vector pACT2 from dark-grown root derived *Arabidopsis* cell suspension as described [40]. A specific interaction of AKIN10 and AKIN11 with 13 classes of proteins was confirmed by monitoring histidine prototrophy and β-galactosidase activity [40]. Here we describe the characterization of ATAF1, one of the confirmed SnRK1-interacting factors (Fig. 1, Table 2). The pACT2 prey showing specific interaction with the pAS2-1–AKIN11 or pAS2-1–AKIN10 bait carried a cDNA sequence of 0.88 kb encoding an N-terminally truncated segment of 180 amino acids of NAC transcription factor ATAF1 (At1g01720, amino acid position 108–289; Fig. 1A) representing the non-conserved C-terminal domain [18]. Although the *Arabidopsis* NAC family comprises more than 100 members, many of which were represented in our pACT2-1 cDNA library, ATAF1 was the only NAC factor identified in the AKIN11 interaction screen, suggesting that unique C-terminal sequences of ATAF1 were responsible for binding of the kinase. To confirm that both SnRK1 catalytic α-subunits can also interact with the full-length ATAF1 protein, we cloned a cDNA carrying the complete coding domain in the GAD or GBD fusion vectors and repeated the two-hybrid assays with these constructs (Fig. 1B, Table 2). As controls, autonomous activation of the *lacZ* reporter gene and possible direct interactions of GAD–ATAF1 and GBD–AKIN11 with the GBD and GAD domains were also assayed (Fig. 1B, Table 2). Neither GBD–AKIN11 nor GAD–AKIN11 activated the *lacZ* reporter gene, and GBD–AKIN11 did not

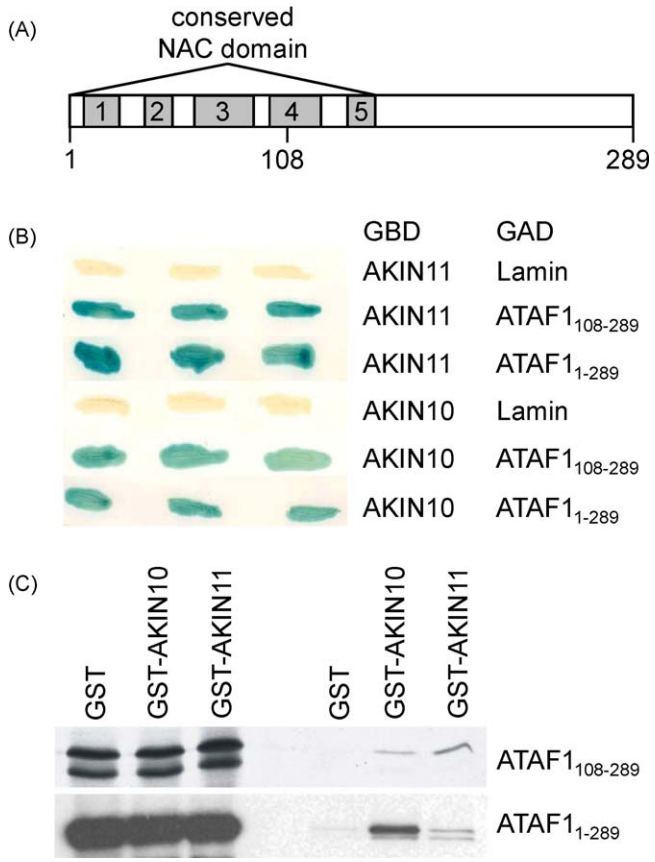

Fig. 1. ATAF1 interacts in the yeast two-hybrid system and *in vitro* with the SNF1-related kinases AKIN10 and AKIN11. (A) Schematic representation of ATAF1 protein domains. Five N-terminal motifs conserved in all members of NAC domain transcription factor family (numbered, black boxes) and amino acid positions of N-terminally truncated and full-length ATAF1 protein are indicated. (B) Test of two-hybrid protein interaction between either GAD–ATAF1$_{108-289}$ or GAD–ATAF1$_{1-289}$ and GBD–AKIN11 or AKIN11 using a qualitative β-galactosidase enzyme filter lift assay. As negative control GAD–Lamin was used. (C) Specific binding of ATAF1 to AKIN10 and AKIN11 *in vitro*. Equal amounts of [^{35}S]-methionine-labeled ATAF1$_{108-289}$ or ATAF1$_{1-289}$ were incubated with either GST, or GST–AKIN10 or GST–AKIN11 coupled to glutathione-Sepharose4B beads. The supernatant (left) and matrix-bound protein fractions (right) were separated by SDS-PAGE and the radioactively labeled ATAF1 was visualized by fluorography.

Table 2
Summary of two-hybrid interaction assays.

GBD vector	GAD vector	β-Galactosidase
AKIN11	ATAF1$_{108-289}$	+
AKIN10	ATAF1$_{108-289}$	+[c]
PRL1	ATAF1$_{108-289}$	–
VirD2	ATAF1$_{108-289}$	–
AKIN11	ATAF1$_{1-289}$	+
AKIN10	ATAF1$_{1-289}$	+[c]
PRL1	ATAF1$_{1-289}$	–
AKIN11	Lamin	–
AKIN10	Lamin	–[b]
AKIN11	–[a]	–
AKIN10	–[a]	–[b]
–[a]	AKIN11	–
ATAF1$_{108-289}$	–[a]	+
ATAF1$_{1-289}$	–[a]	+
–[a]	ATAF1$_{1-289}$	–
–[a]	ATAF1$_{1-289}$	–

GBD: Gal4 DNA-binding domain; GAD: Gal4 activation domain.
[a] Tests of single GDB bait and GAD prey vectors.
[b] Very weakly blue following incubation for more than 8 h.
[c] Strongly blue after 2 h.

exhibited an interaction with the control GAD–Lamin prey either. For GBD–AKIN10 the β-galactosidase activity-based filter lift assays revealed a very weak activation of the *lacZ* reporter gene following incubation for more than 8 h. Hence, GBD–AKIN10-specific interactions were monitored after 2 h incubation via direct comparison with control samples (Fig. 1B, Table 2). GAD-fusion of either full-length or N-terminally truncated ATAF1 showed no interaction with the control baits GBD–PRL1 and GBD–VirD2, and no auto-activation of the reporter genes. However, when targeted to the promoter of the *lacZ* reporter gene by the Gal4 DNA-binding domain, both N-terminally truncated GBD–ATAF1$_{108-289}$ and full-length GBD–ATAF1$_{1-289}$ constructs conferred autonomous activation of transcription. This confirmed the previous observations showing that ATAF1 carries a C-terminal transcription activation domain, analogously to other NAC domain proteins [31,45,46].

To demonstrate that the closely related SNRK1 α-subunits AKIN10 and AKIN11 can similarly recruit ATAF1, affinity-binding matrices were prepared by coupling glutathione S-transferase (GST)–AKIN10 or GST–AKIN11 fusion proteins to glutathione-Sepharose. These matrices were incubated with [^{35}S]-methionine-labeled ATAF1$_{1-289}$ or ATAF1$_{108-289}$ proteins prepared by coupled *in vitro* transcription and translation. As control, GST was similarly coupled to glutathione-Sepharose beads. The unbound and matrix-retained protein fractions were separated by SDS-PAGE and [^{35}S]-ATAF1 was detected by fluorography (Fig. 1C). [^{35}S]-ATAF1$_{108-289}$ carrying the C-terminal transcription activation domain exhibited specific binding to GST–AKIN10 and GST–AKIN11, and was not detectable in the control GST matrix-bound fraction. In contrast, the full-length ATAF1$_{1-289}$ protein revealed a very weak binding to the control GST matrix (Fig. 1C lower panel bound fraction on the right). Nevertheless, the [^{35}S]-ATAF1$_{1-289}$ displayed significantly stronger binding to both SNRK1 α-subunits in three independent experiments (example in Fig. 1C, lower panel and control supernatants on the left) confirming also a specific binding of the full-length protein to the GST-tagged AKIN10 and AKIN11.

3.2. Transcriptional co-regulation of members of ATAF subfamily of NAC factors

To characterize transcriptional regulation of *ATAF1*, northern hybridizations were performed. Because members of the *NAC* gene family show a high degree of sequence conservation in their 5′ coding regions [18], we used a divergent 3′-segment of *ATAF1* cDNA as specific probe in hybridizations with RNA samples prepared from cell suspension and different organs of flowering plants and seedlings (Fig. 2). Steady-state *ATAF1* mRNA levels were comparable in cells of dividing cell suspension culture, stems and

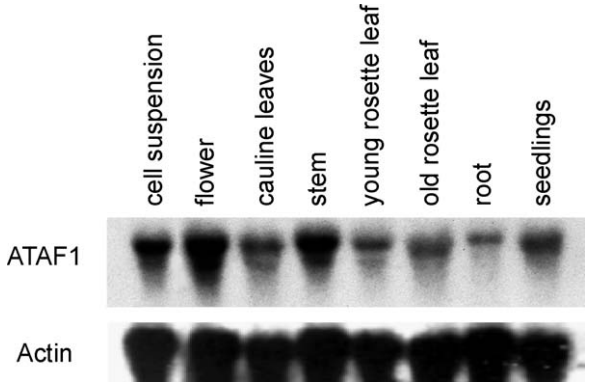

Fig. 2. Northern hybridization analysis of steady-state levels of *ATAF1* transcript in different *Arabidopsis* organs and cell suspension. The filter was hybridized with an *ATAF1* cDNA that carried 3′-sequences encoding the non-conserved C-terminus. An α-actin probe was used as loading control.

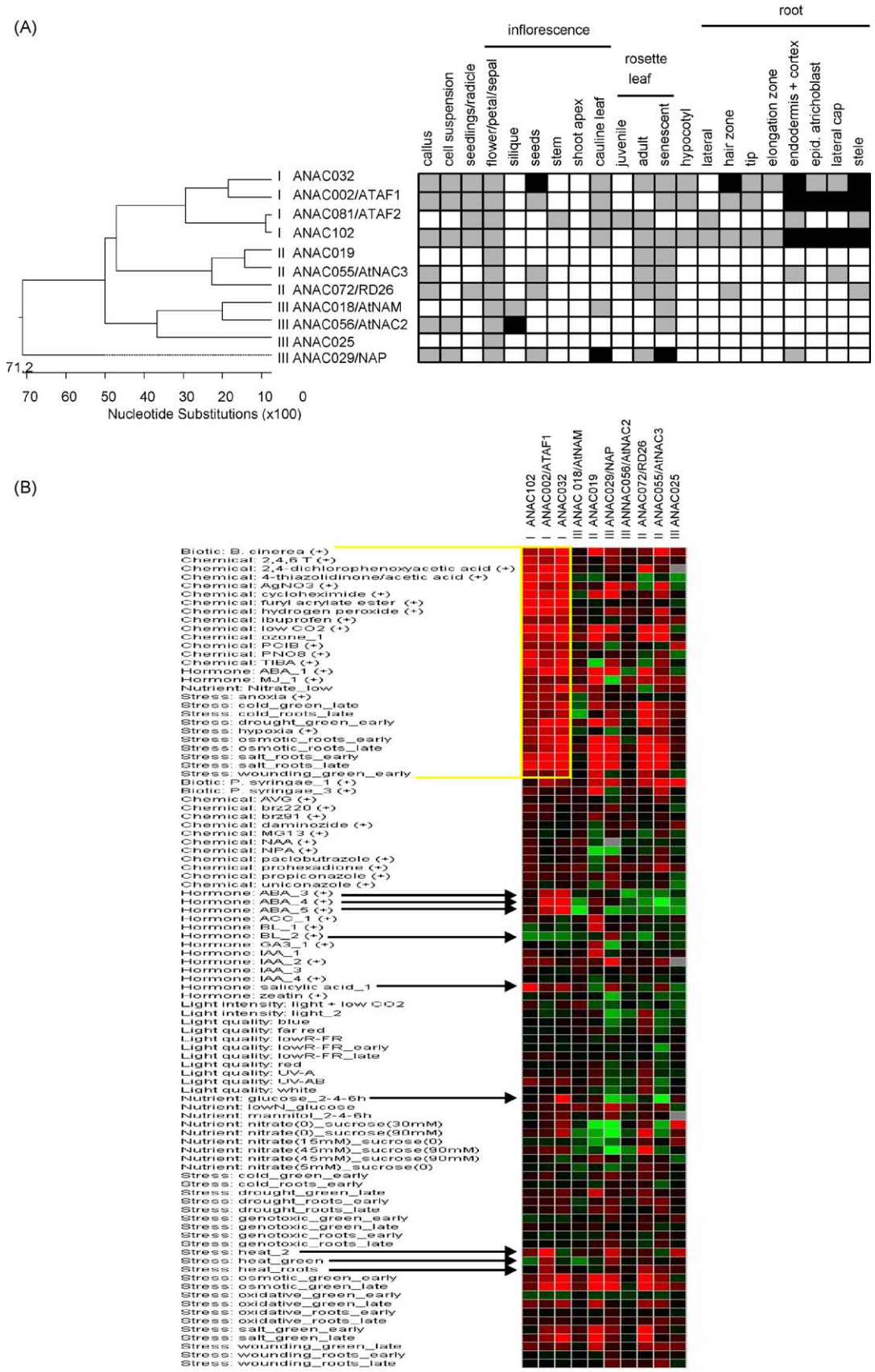

Fig. 3. Comparison of amino acid sequences and transcript profiles of NAC domain factors belonging to the ATAF, AtNAC3 and NAP subgroups. (A) Similarity between *Arabidopsis* NAC domain proteins predicted by ClustalW W phylogenetic tree analysis of amino acid sequences of ANAC032 (At1g77450), ANAC002/ATAF1 (At1g01720), ANAC081/ATAF2 (At5g08790), ANAC102 (At5g63790), ANAC019 (At1g52890), ANAC072/AtNAC3 (At4g27410), ANA0072/RD26 (At4g27410), ANAC018/AtNAM (At1g52880), ANAC056/AtNAC2 (At3g15510), ANAC025 (At1g61110) and ANAC029/NAP (At1g69490) is depicted on the left, and a comparison between transcript levels in different plant organs, callus and cell suspension is shown to the right. Presence of low to medium amounts of mRNA is represented by grey, medium to high by black and no detected mRNA

flower buds, whereas in other organs they were considerably lower. Similarly, microarray data available at the Genevestigator database [47] indicated transcription of *ATAF1* in callus, cell suspension, seedlings, seeds, cauline leaves, hypocotyls, rosette leaves, roots, flower petals and sepals (Fig. 3A). In contrast to our northern blot analysis, however, the microarray hybridizations revealed the highest *ATAF1* mRNA levels in root tissues and did not detect any transcript in stems. The anatomy microarray data sets of roots and stems where generated from plants in other developmental stages or growth conditions (7-day-old seedlings and 6-week-old plants grown in sand and liquid nutrition) than the ones used for our northern blot data; this might have caused the detected differences in transcript levels.

The ATAF subfamily of NACs includes three other members, ATAF2/ANAC081 (At5g08790), ANAC102 (At5g63790) and ANAC032 (At1g77450), and two closely related subclasses, AtNAC3 and NAP [20], as shown by phylogenetic tree and ClustalW analyses in Fig. 3A. Corresponding with the anatomy microarray data sets in Genevestigator [47], and with an independent analysis of *ATAF2* transcript levels and promoter–GUS fusions by Delessert et al. [48], the mRNA levels determined for *ATAF1* were comparable to those of *ATAF2*, *ANAC102* and *ANAC032* in callus, cell suspension, seedlings, inflorescences, and rosette leaves. All four genes showed the highest level of transcription in root tissues (Fig. 3A, I). In contrast, the microarray hybridizations detected mRNAs of the AtNAC3 (Fig. 3A, II) and NAP (Fig. 3A, III) subclasses only at very low level in root tissues (Genevestigator data base, [47]). The Genevestigator datasets as well as further previous studies indicated that transcription of *ATAF1* and *ATAF2* is induced by wounding, methyl jasmonate (MeJA), ABA, hydrogen peroxide (H_2O_2), pathogen infection, cold, drought, salt and osmotic stress (Fig. 3B, [18,20,46–51]). The ATAF family members *ANAC102*, *ATAF1*, and *ANAC032* group together in biclustering analysis illustrating a coordinate regulation of their transcription in response to a broad range of treatments (Fig. 3B, I, upper left, framed in yellow). Unlike the related AtNAC3 (Fig. 3B, II) and NAP (Fig. 3B, III) subclasses, members of the ATAF subfamily were similarly upregulated by *Botrytis cinerea* infection, H_2O_2, MeJA, wounding and drought at early time points in aerial organs, as well as by cold, osmotic and salt stress in roots (Fig. 3B, I, upper left, framed in yellow). Induction of *ATAF1* and *ANA032* transcription in shoots was significantly higher as compared to *ANAC102* upon osmotic treatment, whereas exposure to salt enhanced the expression of *ATAF1* and *ANAC032* only slightly in aerial organs. ABA treatment induced all members of the ATAF subfamily within 1 h (Fig. 3B, I, upper left, ABA_1), but upon 3 h of ABA exposure only *ATAF1* and *ANAC032* showed increased mRNA levels (Fig. 3B, arrow, ABA_3). The *ahg1-1* and *ahg3-1* mutations, causing ABA hypersensitivity, analogously enhanced the transcription of *ATAF1* and *ANAC032* (Fig. 3B; arrows, ABA_4/*ahg1-1*, ABA_5/*ahg3-1*). In addition, *ATAF1*, *ANAC102* and *ANAC032* were coordinately upregulated in response to low nitrate and CO_2 concentrations, anoxia, ozone exposure, auxin (2,4-dichlorophenoxyacetic acid), 4-thiazolidinone/acetic acid; furyl acrylate ester; and p-chlorophenoxyisobutyric acid [PCIB]; 2,3,5-triiodobenoicacid [TIBA]), as well as by inhibitors of photosystem II (N-octyl-3-nitro-2,4,6-trihydroxybenamide [PNO8]), protein synthesis (cycloheximide), ethylene signaling ($AgNO_3$), MeJA biosynthesis (ibuprofen), and auxin signaling and transport (2,4,6-trihydroxybenzamidine [2,4,6T] (Fig. 3B, I, upper left, framed in yellow). In contrast, members of the ATAF subfamily were down-regulated within 3 h by brassinosteroid (Fig. 3B, arrow, BL_2, brassinolid), but showed gene-specific transcriptional induction by heat stress (*ATAF1*), salicylic acid treatment (*ANAC102*) and low glucose concentration (*ANAC032*; Fig. 3B, arrows).

3.3. Transgenic Arabidopsis carrying an ATAF1 overexpression construct showed severe developmental defects and silencing of the ATAF subfamily

We identified and characterized T-DNA insertion mutations in all four genes encoding members of the ATAF subfamily [*ATAF1* (At1g01720), *ATAF2*/*ANAC081* (At5g08790), *ANAC032* (At1g77450) and *ANAC102* (At5g63790)] (for details see Supplementary Fig. 1 and Supplementary Table 1). Overall development, as well as growth responses of all *ataf* mutants to ABA, salt, glucose, sucrose, sorbitol and drought treatments were comparable to wild-type plants (data not shown), supporting the conclusion that members of the ATAF family perform redundant functions. To gain more information about possible biological functions of ATAF1, we generated 15 independent transgenic *Arabidopsis* lines overexpressing ATAF1 fusions with either N-terminal HA (35S::HA–ATAF1) or c-Myc (35S::c-Myc-ATAF1) epitope tags under the control of the CaMV 35S promoter. In the T2 generation, 21-day-old seedlings grown under selection for the T-DNA encoded antibiotics resistance marker were subjected to Western blot analysis to monitor expression of epitope-tagged ATAF1 (Fig. 4). Of 13 tested 35S::HA–ATAF1 lines six produced detectable levels of HA–ATAF1 (Fig. 4), and one of the two 35::c-Myc-ATAF1 lines displayed a considerable level of c-Myc-ATAF1 (line 5-3). From transgenic T2 progeny selected in sterile culture, plantlets of all lines were transferred into soil, and the seven-week-old plants were individually examined for the presence of epitope-tagged ATAF1 by Western blot analysis. Only one plant of T2 line 35::HA–ATAF1 3-8, which previously had revealed expression of HA–ATAF1 in the seedling stage, exhibited still a weak signal for HA–ATAF1 following immunodetection (data not shown).

Although all selected primary T1 transformants showed wild-type-like phenotypes or only mild dwarfism, in the T2 generation each transgenic line, including those that lacked detectable amounts of epitope-tagged ATAF1 protein, showed segregation of offspring with various grades of growth retardation (examples in Figs. 5 and 6). The T2 lines were classified according to the severity of developmental defects into 3 categories: class A with less than 10% of progeny showing dwarfism to a different degree (three lines); class B with 15–30% of dwarf progeny (five lines), and class C with 15–30% or a higher proportion of dwarf offspring displaying also other developmental defects such as early yellowing of leaves, sterility and reduced or absent flower initiation (seven lines; examples in Figs. 5 and 6).

Whereas classes A and B included those T2 lines in which no overproduction of ATAF1 protein could be detected in 21-day old seedlings by Western blotting, class C comprised lines producing detectable levels of ATAF1 in the seedling stage (examples in Figs. 5 and 6). A significant proportion (15–30%) of dwarf offspring in class C lines showed severely reduced root and shoot development, and seedling lethality within the first 14 days period after germination (Fig. 5C). The seedling lethal phenotype observed in the presence of antibiotics selection was not due to silencing of the T-DNA encoded selectable marker gene, since it was observed at exactly identical ratio upon germinating of the class C lines in the absence of

by white squares. (B) Accumulation of transcripts from the NAC genes indicated in (A) in response to different stimuli. Results from biclustering analysis of mRNA profiles are displayed as heat map (red up-regulation, green down-regulation). Clusters were boxed with a yellow line and other specific effects regarding the ATAF subclass transcript levels are highlighted by arrows. Data in (A) and (B) were extracted from the Genevestigator microarray database [47]. The *ATAF2* expression profile displayed in (A) is according to Delessert et al. [48]. Latin numbers indicate the subgroup classification: I for ATAF, II for AtNAC3 and III for NAP subfamily [20]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

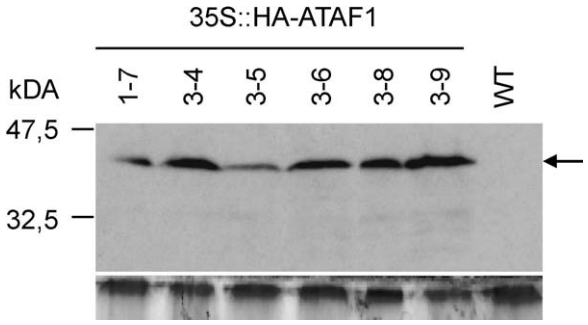

Fig. 4. Expression of epitope-tagged ATAF1 in different transgenic plant lines. Western immunoblot analysis of HA-tagged ATAF1 protein levels with a monoclonal anti-HA antibody using equal amounts of protein extracts from T2 seedlings carrying the 35S::HA–ATAF1 construct (upper panel). Protein extract from wild-type plants (WT) served as negative control. Positions of molecular mass markers are indicated at the right, and the position of HA–ATAF1 protein is marked by an arrow. Coomassie blue staining of the gel is shown as loading control (lower panel).

antibiotic selection (data not shown). Despite overproduction of ATAF1, which did not correlate with the number of independently segregating T-DNA loci in the examined transgenic lines (data not shown), each class C line yielded also relatively well developing transgenic T2 progeny, which in the T3 generation reproduced the phenotype of graded dwarfism combined with severe developmental defects (data not shown). In soil, a large proportion of class C progeny produced upward curling leaves, displayed early yellowing of older leaves and ultimate lethality before development of an inflorescence (example in Fig. 6D). As expected, control wild-type T2 lines lacking the ATAF1 overexpression construct produced 100% wild-type offspring in the next generation.

The fact that T2 lines lacking detectable levels of HA- or c-Myc-ATAF1 also segregated a dwarf progeny suggested that the observed phenotype might result from various degrees of silencing of *ATAF* gene family members by the modified epitope-tagged ATAF1 overexpression constructs. To test this idea, we monitored the transcript level of each ATAF subfamily member, as well as that of the corresponding epitope-labeled ATAF1 overexpression construct, in pools of T2 plants exhibiting dwarfism to a different extent by RT-PCR, respectively (Fig. 7, data not shown). However, from the 14 T2 lines tested, two (class C lines: 3-4 and 5-3) yielded sufficient amounts of plantlets showing growth arrest and other developmental defects allowing for an additional RT-PCR assay (Fig. 7, asterisk). In comparison to control plants of the same age carrying a 35S::HA-GUS construct, all tested samples from pooled transgenic lines exhibited a clear reduction in mRNA amounts of *ATAF1* and its closest homolog *ANAC032* (Fig. 7), the extent of which varied among the T2 lines. Similarly, a significant decrease in *ANAC102* and/or *ATAF2* transcript levels was detected in six (1-3, 1-7, 3-4, 3-8, 3-9 and 5-3) and eight (1-3, 1-4, 1-5, 1-7, 3-4, 3-5, 3-8 and 3-9) plant lines, respectively (Fig. 7). Remarkably, in the two samples representing exclusively plantlets with most pronounced developmental changes (Fig. 7, asterisk, lines 3-4 and 5-3), the *ATAF1* mRNA was not detectable at all, and the other three ATAF transcripts were even more strongly reduced than in the pooled samples. Thus, these two samples showed a clear correlation between the extent of transcript level reduction for members of the ATAF subfamily and the severity of phenotypic alteration. Only two transgenic lines displayed a detectable level of epitope-tagged *ATAF1* mRNA (data not shown, 35S::HA–ATAF1 1-6 and 3-3). On the whole, these results suggested a gradual built up of a silencing effect most likely stimulated by the ATAF1 overexpression, which ultimately led to silencing of the transgene, as well as to various levels of down-regulation of the endogenous ATAF1 gene transcription and of the three other closely related members of the ATAF subfamily.

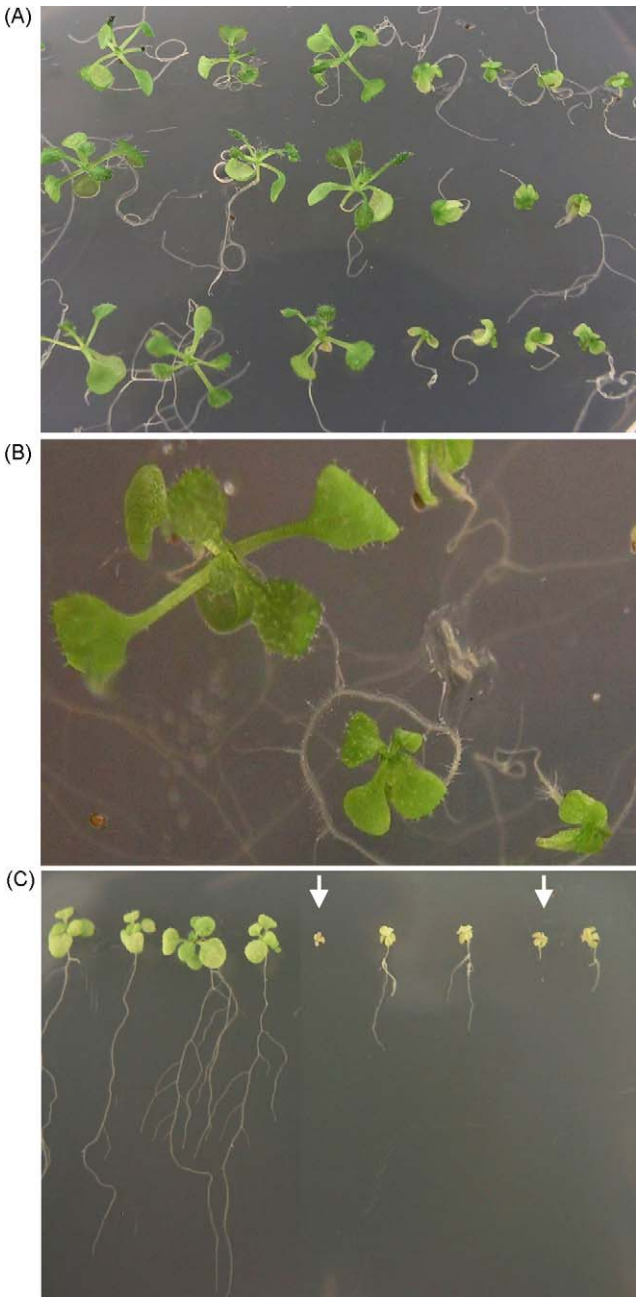

Fig. 5. *Arabidopsis* seedlings expressing HA–ATAF1 display severe developmental defects. (A–C) T2 seedlings were grown for 14 days in sterile culture on MS plates without selection. (A) Collection of wild-type-like (left) and dwarfed plantlets (right) from three independent lines carrying the CaMV 35S promoter-driven ATAF1 expression cassette (35S::HA–ATAF1 line 3-8 [upper panel] and line 1-7 [middle section]; and 35S::c-Myc-ATAF1 line 5-2 in the lower panel). (B) Close-up of 35S::HA–ATAF1 line 3-4 showing wild-type like (upper left), dwarfed (lower middle) and severely dwarfed plants with downward curled leaves (lower right). (C) Root length assay of wild-type (left) and 35S::HA–ATAF1 line 3-4 seedlings (right). Triangles point to seedlings with growth arrest.

4. Discussion

Our studies have identified for the first time a kinase as an interacting partner of a NAC domain transcription factor: *Arabidopsis* SnRK1 catalytic α-subunits AKIN10 and AKIN11 were found to bind directly to the ATAF1 NAC factor using yeast two-hybrid and *in vitro* protein interaction assays. In previous work, we had been able to show that AKIN10 and AKIN11 interacted as well

Fig. 6. The effect of ATAF1 overexpression on plant development. (A–C) T2 plantlets selected in sterile culture for the presence of transgene using either hygromycin (35::HA–ATAF1) or kanamycin (35S::c-Myc-ATAF1) selection were transferred into soil and grown parallel with control wild-type plants of identical age. Based on severity of developmental defects observed upon 7 weeks of growth, the transgenic plants were grouped into three phenotypic classes. (A) Example for class A: 35S::HA–ATAF1 line 3-5 (left) and wild-type (right). (B) Class B phenotype (35S::c-Myc-ATAF1 line 5-3 [left] and wild-type [right]) and close-up of the same plant in the upper left corner. (C) Class C plant from 35S::HA–ATAF1 line 3-4 (left) and a close-up of line 3-8 (upper left corner). A wild-type plant is depicted to the right. (D) As control, four transgenic lines were also directly grown in soil. Comparison of 11-week-old wild-type plants (left side) to a transgenic line (35S::HA–ATAF1 line 3-9, right side) of the same age displaying different grades of dwarfism. In the upper right corner a close-up of a dwarf plantlet with upward curled leaves is shown (marked below with an asterisks), whereas in the upper left corner a wild-type and two dwarf seedlings from the same transgenic line are displayed 14 days after germination. Note the early yellowing of older leaves in the transgenic line. A plantlet with growth arrest is marked by an arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

with the nuclear PRL1 WD-protein; a potential negative regulator of these kinases [15]. In the new study, two-hybrid assays have not revealed a PRL1 interaction with truncated and full-length ATAF1, denoting that these proteins occur in different SnRK1 complexes. Other studies detected for various members of the NAC protein family interactions with immunophilin, E3 ubiquitin ligase, N-acetylglucosamine transferase, various transcription factors and plant viral proteins [28–30,52–57].

Transcript profiling data available thus far from an experiment with transient expression of AKIN10 in leaf protoplasts indicated that this SnRK1 kinase controls the transcription of about 1200 genes that are known to be regulated by darkness, sugar and various stress stimuli [17]. The same study reported that transient overexpression of some bZIP transcription factors conferred similar regulatory effects as that of SnRK1α kinase AKIN10 on the transcription of certain target genes. Our novel evidence for an

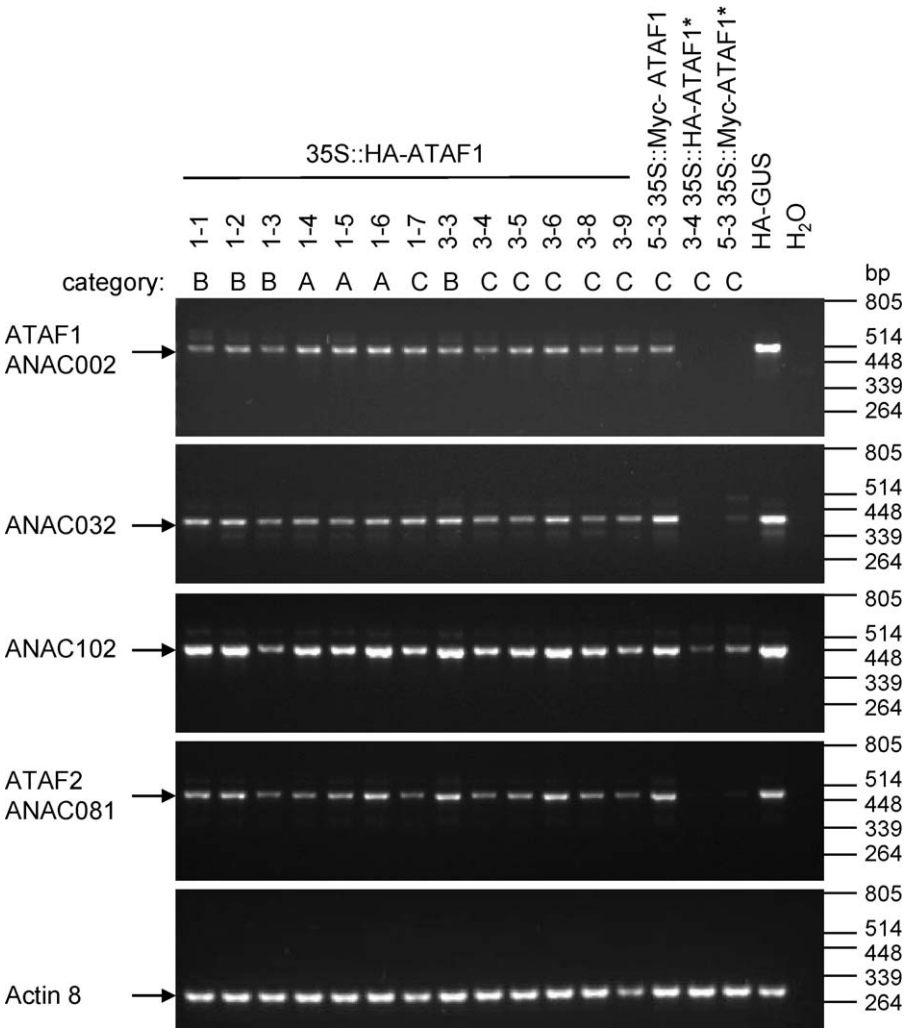

Fig. 7. Gene silencing effects in *Arabidopsis* plants carrying an epitope-tagged ATAF1 overexpression construct. Total RNA extracted from pooled dwarfed plantlets of class A (1-4, 1-5 and 1-6), class B (1-1, 1-2, 1-3 and 3-3), and class C T2 transgenic lines (1-7, 3-4, 3-5, 3-6, 3-8, 3-9 and 5-3), or a 35S::HA-GUS control line (HA-GUS) were assayed by RT-PCR for transcript levels of genes belonging to the ATAF subfamily. PCR products were separated in a 1.5% agarose gel along with a water control PCR reaction (H_2O) and a molecular weight marker (λ DNA, PstI-digested). PCR fragments corresponding to amplified specific cDNA sequences are labeled by arrows. Sizes of the expected genomic and cDNA fragments are listed in Table 1. A PCR product amplified from the β-actin 8 cDNA served as loading control. Samples derived exclusively from plantlets displaying growth arrest and other developmental defects are labeled by an asterisk.

interaction of ATAF1 with SnRK1α AKIN10 and AKIN11 in yeast and *in vitro* suggests that ATAF1 may function either as a possible downstream effector, tethering SnRK1 kinases to transcription regulatory complexes of target genes, or as a substrate of these protein kinases, or both. In this respect it is interesting that the SnRK1α-subunits have been identified as *in vivo* interacting partners of *Arabidopsis* SKP1/ASK1 protein, which is a common subunit of different SCF (SKP1-CULLIN1-F-box protein) E3 ubiquitin ligases [40]. The fact that a monomeric SnRK1 α-subunit has been shown to target SCF complexes to the PAD1/α7-subunit of proteasome 20S catalytic cylinder indicates that SnRK1 kinases may control the phosphorylation of SCF substrates and thereby regulate their ubiquitination and subsequent proteasomal degradation. Regulation of ubiquitination-mediated proteolysis by substrate phosphorylation represents a key signaling switch in many plant regulatory pathways, including some of those that converge on various NAC transcription [18,29,30,58,59]. Therefore we suppose that the observed SnRK1–ATAF1 interaction may target ATAF1 by SnRK1–SCF interaction to proteasomal degradation. Alternatively, a potential ATAF1 phosphorylation may modulate subcellular localization distribution, DNA-binding activity and protein interactions as described earlier for other transcription factors [60–64].

Consistent with the available transcript profiling data, our hybridization analysis of steady-state *ATAF1* mRNA levels showed that *ATAF1* is expressed in root tissues, but also in seeds, in actively dividing cultured cells and in most plant organs tested. These results are fully consistent with the expression pattern of *ATAF1* promoter–GUS fusions, which highlighted the vascular system throughout the entire plant and stomatal guard cells of mature leaf epidermis [46]. Spatial and temporal expression patterns of three other members of the ATAF subfamily (*ATAF2*, *ANAC102* and *ANAC032*) are very similar to that of *ATAF1*, but differed considerably from developmental expression patterns of less related members of AtNAC3 and NAP subfamilies (this study, [48]). Furthermore, clustering analyses of microarray transcript profiling data exhibited that *ATAF1*, *ANAC032* and *ANAC102* were analogously upregulated by wounding, H_2O_2, pathogen attack, MeJA, ABA, cold, drought, salt and osmotic stress [46,48–51]. Transcription of these ATAF subfamily members was also stimulated by inhibition of photosystem II, anoxia, and low CO_2 and nitrate concentrations, pointing at a role of these factors in metabolic and energy signaling cascades. These data support the conclusion that NAC transcription factors of the ATAF subgroup perform redundant functions and participate in the cross-talk between various

metabolic, hormonal and stress signaling pathways (this study, [46,48]).

Our analysis of T-DNA knock-out mutations in *ATAF1, ATAF2*, *ANAC032* and *ANAC102*, which caused no apparent alteration in any growth and developmental trait examined (Supplementary Fig. 1 and Supplementary Table 1) hint at a functional redundancy between members of the ATAF subfamily as well. In agreement with our findings, Delessert et al. [48] did not observe an obvious mutant phenotype in two *ATAF2* T-DNA insertion mutants (SALK_015750 and 136355). Analogously, also *ataf1-1* (SALK_067648) and *ataf1-2* (SALK_057618) mutations were reported to cause no evident changes in plant growth and development [45,46]. However, the *ataf1* insertion mutations were recently found to enhance the penetration rate of fungal pathogen *Blumeria graminis* f. sp. *hordei* into epidermal cells [45], and to increase the rate of recovery after drought stress along with a stimulation of the expression of some stress-inducible marker genes [46]. Based on these observations, ATAF1 has been proposed to play a role both in regulation of non-host resistance and drought stress responses. Notwithstanding, to our knowledge, a function in plant development has not been deduced from any previous experiments before.

With the aim to generate material suitable for the further characterization of ATAF1, we have produced a large set of transgenic lines expressing HA and c-Myc epitope-labeled ATAF1 proteins under the control of CaMV 35S promoter. Whereas nearly all T1 lines showed wild-type-like phenotype or only mild dwarfism, their T2 offspring exhibited yellowing and curling of leaves coupled to different severity of dwarfism that ultimately lead to growth arrest and seedling lethality. These traits also showed stable inheritance and manifestation in the T3 progeny accompanied by either complete lack, or by an only very low level of synthesis of epitope-tagged ATAF1 protein. Inspection of transcript levels of all four members of the ATAF subfamily, as well as those of the corresponding 35S promoter-driven *ATAF1* transgenes, indicated that the observed developmental alterations correlated with a silencing of ATAF family members and the 35S-ATAF1 construct. These data strongly imply that redundant functions of the transcription factors belonging to the ATAF subfamily are essential for normal plant growth and development.

The phenotypic alterations resulting from ATAF silencing resembled those obtained by siRNA-mediated silencing of SnRK1α genes *AKIN10* and *AKIN11* induced by 35S promoter-driven inverted repeat constructs [17]. SnRK1α-silenced *Arabidopsis* plants showed dramatic growth defects, such as small and curly leaves, and symptoms of early senescence, which in severe cases lead to senesced plants before flowering. Nonetheless, as transgene-mediated silencing often triggers a cascade of secondary siRNA production [65–67], it cannot be completely excluded that the observed leaf curving, dwarfism and seedling lethality phenotype involves amplification of additional silencing effects, rather than (only) of specific silencing of gene members in the ATAF subfamily.

Morphological changes induced by expression of *Brassica napus* NAC genes belonging to the ATAF subclass were similarly reported to cause severe developmental abnormalities. However, in this case it remained unclear whether these developmental alterations resulted from an increase or rather from silencing of ATAF expression [68]. In contrast, overexpression of ATAF2 was found to induce only mild developmental changes, such as yellowing and wrinkling of leaves accompanied by a decrease of chlorophyll and carotenoid content, and repression of pathogen- and wound-induced genes [48]. The severity grade of leaf yellowing was reported to correlate with the *ATAF2* mRNA levels; which were decreased in older leaves, apparently not due to a senescence response. Unlike in our *ATAF1* transgenic lines, the dominant *ATAF2* overexpression phenotype was shown to correlate with elevated *ATAF2* transcript levels [48], denoting a lack of gene silencing. A putatively different behavior of ATAF2 may also be inferred from experiments on rice, in which RT-PCR exhibited that increased transcript levels of the ATAF2 orthologue *OsNAC6* caused growth retardation, low reproductive yield, improved tolerance towards blast disease, dehydration and high-salt [69].

In summary, we report that: ATAF1 can recruit the SnRK1 kinase catalytic α-subunits AKIN10 and AKIN11 in protein interaction assays, suggesting that ATAF1 is a potential SnRK1 substrate; transcript levels of NAC factors belonging to the ATAF subfamily are co-regulated by a broad range of stimuli and show similar organ-specific expression patterns indicating functional redundancy between family members; down-regulation of transcript levels of endogenous *ATAF1* and related genes induced severe developmental alterations. Consequently, our data yield first evidence that upon activation by metabolic and phytohormone signaling or, alternatively, by biotic and abiotic stresses, the ATAF subfamily members might function as potent regulators of plant development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.plantsci.2009.06.011.

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