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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*

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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

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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*