Transcription Factor ATAF1 in Arabidopsis Promotes Senescence by Direct Regulation of Key Chloroplast Maintenance and Senescence Transcriptional Cascades

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Senescence represents a fundamental process of late leaf development. Transcription factors (TFs) play an important role for expression reprogramming during senescence; however, the gene regulatory networks through which they exert their functions, and their physiological integration, are still largely unknown. Here, we identify the Arabidopsis (Arabidopsis thaliana) abscisic acid (ABA)- and hydrogen peroxide-activated TF Arabidopsis thaliana ACTIVATING FACTOR1 (ATAF1) as a novel upstream regulator of senescence. ATAF1 executes its physiological role by affecting both key chloroplast maintenance and senescence-promoting TFs, namely GOLDEN2-LIKE1 (GLK1) and ORESARA1 (ARABIDOPSIS NAC092), respectively. Notably, while ATAF1 activates ORESARA1, it represses GLK1 expression by directly binding to their promoters, thereby generating a transcriptional output that shifts the physiological balance toward the progression of senescence. We furthermore demonstrate a key role of ATAF1 for ABA- and hydrogen peroxide-induced senescence, in accordance with a direct regulatory effect on ABA homeostasis genes, including NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3 involved in ABA biosynthesis and ABC TRANSPORTER G FAMILY MEMBER40, encoding an ABA transport protein. Thus, ATAF1 serves as a core transcriptional activator of senescence by coupling stress-related signaling with photosynthesis- and senescence-related transcriptional cascades.

Leaf senescence involves the degradation of cellular components and their remobilization to developing organs such as young and immature leaves during vegetative growth or seeds during the reproductive phase. It is assumed that the timely onset and progression of senescence confers an ecological fitness to the plant, as it contributes to optimizing the reuse of nitrogen and carbon during seed formation. Leaf senescence itself is an ordered physiological process, and although it is principally mediated by an underlying genetic program, environmental factors have a large impact on the onset and rate of progression of senescence. Abiotic stresses such as drought, high canopy temperature, soil salinity, and nutrient deficiencies hasten senescence and thus affect plant productivity.

Senescence is accompanied by transcriptional reprogramming of a large number of genes, including those encoding transcription factors (TFs), as shown by global transcriptome profiling in various plant species, including Arabidopsis (Arabidopsis thaliana; Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008; Breeze et al., 2011), wheat (Triticum aestivum; Gregersen and Holm, 2007; Cantu et al., 2011), aspen (Populus tremula; Andersson et al., 2004), maize (Zea mays; Zhang et al., 2014), and others. Recently, the role of several NAC (for NO APICAL MERISTEM/ARABIDOPSIS ACTIVATION FACTOR1 [ATAF1-2]/CUP-SHAPED COTYLEDON) TFs for senescence has been reported, and both positive and negative regulators were identified. In Arabidopsis, positive senescence regulators include ORESARA1 (ORE1; also called ARABIDOPSIS NAC092 [ANAC092] or AtNAC2; Kim et al., 2009; Balazadeh et al., 2010a; Rauf et al., 2013a), ORESARA1 SISTER1 (ORS1; ANAC059; Balazadeh et al., 2011), AtNAP (for Arabidopsis NAC-LIKE, ACTIVATED BY APETALA3/ PISTILLATA; ANAC029;
Guo and Gan, 2006), and ANAC016 (Kim et al., 2013), while negative regulators include JUNGBRUNNEN1 (JUB1; ANAC042; Wu et al., 2012) and VND-INTERACTING2 (ANAC083; Yang et al., 2011). Furthermore, downstream target genes of some of the senescence-regulated NAC TFs have been reported, including BIFUNCTIONAL NUCLEASE I (BFN1), RIBONUCLEASE3, SEVEN-IN-ABSENTIA1 (SINA1), and SENESCENCE-ASSOCIATED GENE29 (SAG29; SWEET15) for ORE1 (Balazadeh et al., 2010a; Matallana-Ramirez et al., 2013; Rauf et al., 2013a), DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2 for JUB1 (Wu et al., 2012), and SAG113 for AtNAP (Guo and Gan, 2006). These studies reveal first insights into the complex regulatory networks underlying plant senescence.

An important aspect of senescence is not only the up-regulation of the above-mentioned TFs and their target genes but also the reduced expression of photosynthesis-associated genes (PAGs), ultimately resulting in a decline of photosynthetic activity. The loss of chloroplast maintenance mechanisms and chlorophyll are important markers of senescence (Hensel et al., 1993). Two members of the GARP family of Myb TFs, namely GOLDEN2-LIKE1 (GLK1) and GLK2, are known to play key roles in chloroplast development and maintenance in Arabidopsis (Waters et al., 2008, 2009). Photosynthesis-related direct downstream targets of GLKs including light-harvesting chlorophyll a/b-binding (LHC) protein-encoding genes LHCb2.4, LHCb3, LHCA4, and LHCb4.2 have been identified (Waters et al., 2009). Recently, we reported that the positive senescence regulator ORE1 interacts at the protein level with the chloroplast maintenance TFs GLK1 and GLK2 (Rauf et al., 2013a). Our experimental data showed that GLK target genes are repressed when ORE1 expression is enhanced (most likely due to the formation of GLK-ORE1 heteromers), while ORE1 target genes are not affected. Based on our findings, we proposed the model that the onset of senescence is affected by the interaction of the two TFs (Rauf et al., 2013a).

Phytohormones play an important role in the modulation of the timing and progression of senescence (Guo and Gan, 2005; Lim et al., 2007; Jibran et al., 2013; Mueller-Roebel and Balazadeh, 2014). Abscisic acid (ABA) is one of the most effective plant hormones in terms of promoting leaf senescence apart from ethylene (Zacarias and Reid, 1990; Lee et al., 2011). ABA appears to initiate senescence, while ethylene appears to exert its effects at a later stage. An increase in endogenous ABA has been shown to coincide with the senescence of leaves, while genes involved in ABA signaling were induced earlier during senescence (Breeze et al., 2011). However, the molecular elements of the cellular regulatory networks that trigger the ABA-dependent control of senescence are largely unknown; however, recent experimental evidence indicates that AtNAP activates ABSICIC ALDEHYDE OXIDASE3, which encodes an enzyme responsible for the last step in ABA biosynthesis (Yang et al., 2014). ABA itself influences the redox as well as antioxidant status of the cell, as it involves hydrogen peroxide (H$_2$O$_2$) production via the activation of NADPH oxidases and the expression of antioxidant genes; thus, ABA helps balance both cellular protection activities and the progression of senescence (Torres and Dangl, 2005; Lim et al., 2007).

The NAC transcription factor ATAF1 in Arabidopsis negatively regulates the expression of stress-responsive genes under drought stress and ABA treatment, and functions in abiotic and biotic stress responses have been reported (Lu et al., 2007; Jensen et al., 2008; Wu et al., 2009).

Drought induction and/or recovery experiments revealed an enhanced drought tolerance in ataf1 knockout mutants (Lu et al., 2007; Jensen et al., 2008), indicating a negative regulatory role of ATAF1 in relation to drought tolerance. By contrast, in another report (Wu et al., 2009), ATAF1 overexpression led to increased tolerance to drought stress, demonstrating a positive correlation with drought tolerance. These authors also observed a marked increase of the expression of several stress-responsive marker genes in ATAF1 overexpressors at the later stress time points, in accordance with the enhanced drought tolerance observed. At the protein level, ATAF1 is known to interact with the catalytic subunits AKIN10 and AKIN11 of SUCROSE NONFERMENTING-RELATED SERINE/THRONEINE-PROTEIN KINASE1 (SnRK1; Kleinow et al., 2009). SnRK1 is a key integrator of transcription networks in response to stress and is implicated in sugar and ABA signaling pathways (Baena-González et al., 2007; Jossier et al., 2009). Recently, ATAF1 has been reported to directly regulate the expression of NINE-CIS-EPOXYCAROTENOÏD DIOXYGENASE3 (NCED3), which encodes a key enzyme of ABA biosynthesis in Arabidopsis (Jensen et al., 2013). Accordingly, overexpression of ATAF1 led to elevated ABA levels, and NCED3 transcript abundance was higher in ATAF1 overexpressors but lower in ataf1 knockout mutants compared with the wild type. The increased drought tolerance reported for ATAF1 overexpressors by Wu et al. (2009) is in accordance with this finding, but currently it cannot explain the contrasting observation by Jensen et al. (2008) that ataf1 mutant lines have a higher drought tolerance.

We previously observed that the expression of ATAF1 increases during developmental and salinity stress-induced leaf senescence (Balazadeh et al., 2008; Allu et al., 2014). Furthermore, ATAF1 is rapidly (within 1 h) induced in plants treated with H$_2$O$_2$ (Balazadeh et al., 2010b) and in seedlings treated with the catalase inhibitor 3-aminotriazole, which triggers an intracellular rise of H$_2$O$_2$ level (expression data are summarized in Supplemental Fig. S1, A–C). In addition, the expression of ATAF1 is enhanced in the catalase2-1 mutant (Gene-vestigator), which accumulates high levels of H$_2$O$_2$ under photorespiratory conditions (Hu et al., 2010). Notably, however, the expression of ATAF1 is not induced when senescence is induced by nitrogen starvation (Balazadeh et al., 2014); thus, ATAF1 appears to be preferentially involved in senescence-triggering stresses that involve H$_2$O$_2$ as a physiological signal or metabolite.
Here, we identify ATAF1 as a novel positive regulator of senescence in Arabidopsis. We demonstrate that ATAF1 regulates senescence by activating expression of the positive senescence regulator ORE1 and by repressing expression of the chloroplast maintenance TF GLK1. Thus, ATAF1 regulates senescence by oppositely acting on two downstream TF genes, which fully aligns with the previous finding that the interaction of ORE1 with GLKs at the protein level balances leaf senescence against maintenance by antagonizing the role of GLKs (Rauf et al., 2013a). Our data also reveal that ATAF1 affects the progression of senescence by regulating the expression of at least two ABA biosynthesis- and transport-related genes, namely NCED3 and ABC TRANSPORTER G FAMILY MEMBER40 (ABCG40). Furthermore, we show that \( \text{H}_2\text{O}_2\)-triggered expression of ORE1, and \( \text{H}_2\text{O}_2\)-triggered repression of GLKE1, require the presence of ATAF1. Thus, our data provide evidence that ATAF1 integrates ABA- and \( \text{H}_2\text{O}_2\)-dependent signaling with senescence through the direct regulation of key photosynthesis and senescence transcriptional cascades.

RESULTS

ATAF1 Positively Affects Senescence

As ATAF1 shows elevated expression during senescence (Fig. 1A; Balazadeh et al., 2008), we next tested its potential involvement in the regulation of this process. To this end, we compared Columbia-0 (Col-0) plants with transgenic plants overexpressing ATAF1 from the cauliflower mosaic virus (CaMV) 35S promoter and \( \text{ataf1} \) knockout mutants. We characterized three homozygous transfer DNA (T-DNA) insertion lines, namely \( \text{ataf1-2} \) (SALK-057618; Lu et al., 2007), \( \text{ataf1-3} \) (GK299B09), and \( \text{ataf1-4} \) (GK565H08; Supplemental Fig. S2A). The homozygosity of T-DNA insertions was confirmed by PCR-based genotyping, and the location of T-DNA insertion position was verified by PCR (Supplemental Fig. S2B) and sequencing. Absence of ATAF1 expression in all three \( \text{ataf1} \) mutants was demonstrated by end-point PCR and quantitative real-time (qRT)-PCR (Supplemental Fig. S2, C and D). In 35S:ATAF1 plants, elevated ATAF1 transcript levels were confirmed by qRT-PCR in three independent transformants (Supplemental Fig. S2E).

To test the effect of altered ATAF1 expression on senescence, we determined senescence-related parameters. Leaf yellowing due to chlorophyll loss became evident at approximately 25 d after sowing (DAS) in 35S:ATAF1 plants (OX-17 and OX-20), while loss of chlorophyll was delayed in leaves of all three \( \text{ataf1} \) knockout mutants compared with Col-0 (Fig. 1, B–E). Even at 45 DAS, chlorophyll content and number of leaves with 50% green blades were higher in the three \( \text{ataf1} \) null mutants than in 35S:ATAF1 and Col-0 plants (Fig. 1, D and E). Ion leakage as an indicator of membrane damage was higher in leaves of 35S:ATAF1 (OX-17) than wild-type plants, while \( \text{ataf1-2} \) and \( \text{ataf1-4} \) null mutants exhibited decreased ion leakage (Fig. 1F).

As a further indicator for leaf senescence, we measured the transcript abundance of early-senescence (SAG13) and late-senescence (SAG12) marker genes. At 45 DAS, SAG13 expression in leaf 9 was lower in the \( \text{ataf1-4} \) mutant than in the wild type, while SAG12 expression was not detected; on the contrary, SAG12 expression was highly elevated in 35S:ATAF1 plants, clearly indicating a senescence-promoting effect of ATAF1 (Fig. 1G). Notably, bolting time was not significantly different between the genotypes (days to inflorescence emergence, 26.6 ± 1, 25.9 ± 1.1, and 27.3 ± 0.9, and number of leaves at inflorescence emergence, 9.7 ± 1.2, 10.4 ± 1.6, and 9.5 ± 1.3 for Col-0, 35S:ATAF1, and \( \text{ataf1-4} \), respectively; \( n = 10 \) from two experiments).

We then expressed ATAF1 from an estradiol-inducible promoter (Zuo et al., 2000). ATAF1-IOE lines were sown on one-half-strength Murashige and Skoog (MS) medium containing 1% (w/v) Suc and 20 \( \mu \text{M} \) estradiol (or 0.2% [v/v] ethanol in control experiments; 16 h of light at 22°C/8 h of dark at 18°C). Similar to plants grown in soil, ATAF1-IOE plants grown in medium supplemented with estradiol showed precocious senescence compared with those grown in mock-supplemented medium (Fig. 2A). Accordingly, expression of PAGs such as \( \text{LHCB2.4}, \text{LHCB3}, \text{LHCa4}, \) and \( \text{LHCb4.2} \) was reduced in ATAF1-IOE seedlings (5 h after estradiol treatment) and in 27-d-old 35S:ATAF1 plants, while expression of the senescence marker SAG13 was elevated. On the contrary, expression of PAGs was enhanced in the 27-d-old \( \text{ataf1-4} \) mutant, while SAG13 expression was reduced (Fig. 2B; Supplemental Table S1). Collectively, these data reveal a role of ATAF1 in the control of developmental leaf senescence.

ATAF1 Is Involved in Dark-Induced Senescence

Although the molecular mechanisms underlying dark-induced senescence are not known in detail, some similarities with developmental senescence have been reported (van der Graaff et al., 2006). Microarray hybridization data obtained from Arabidopsis plants subjected to prolonged darkness for different time periods revealed a number of up- or down-regulated genes involved (Lin and Wu, 2004), including ATAF1, which was strongly up-regulated upon dark treatment (Fig. 3A). To test whether dark-induced senescence requires the presence of a functional ATAF1 gene, detached leaves from 3-week-old soil-grown Arabidopsis plants were incubated for 3 d in darkness on moist filter paper. Upon dark incubation, leaves of OX-17 turned yellowish faster than wild-type leaves, while leaf yellowing was slowed in \( \text{ataf1-4} \) (Fig. 3B). In accordance with this, we observed a more rapid decline in chlorophyll level in leaves of OX-17 and OX-20 plants than in Col-0 and a slowed decline in leaves of all three \( \text{ataf1} \) mutants (Fig. 3C). Furthermore, expression of SAG13 and ORE1 was enhanced, and expression of PAGs such as \( \text{LHCA4}, \text{LHCB3}, \text{LHCb4.2}, \) and \( \text{PROTCHLOROPHYLLIDE} \)
OXIDOREDUCTASE B was significantly reduced in dark-treated ATAF1 overexpression plants (Fig. 3D; Supplemental Table S1). Finally, we tested the expression of PPDK, which encodes pyruvate orthophosphate dikinase and was previously shown to be induced during dark-induced senescence; the enzyme is thought to play a role in generating Asn to function as a nitrogen carrier for nitrogen remobilization during senescence (Lin and Wu, 2004). PPDK transcript abundance was elevated in OX-17 leaves (Fig. 3E), in accordance with the more rapid senescence observed during dark incubation.

In contrast, expression of PPDK was reduced in the ataf1-4 mutant (Fig. 3E). These data suggest that, besides a role in developmental senescence, ATAF1 also constitutes an important regulatory element of the cellular networks controlling dark-induced senescence.

Identification of the Consensus Target Sequence of ATAF1

Identifying TF-binding sites represents an important step toward unraveling and predicting regulatory
ATAF1 directly regulated expression of \( \text{ORE1} \) and \( \text{GLK1} \) in vivo

The early-senescence phenotype observed in ATAF1-overexpressing plants and the delayed senescence in the \( \text{ataf1} \) mutant suggested a role for ATAF1 in regulating leaf senescence. To better understand the underlying mechanism of this regulation, we tested the expression of 15 PAGs and 158 senescence-associated genes (SAGs) by qRT-PCR in 45-d-old \( \text{OX-17} \), \( \text{ataf1-f} \), and Col-0 plants. This experiment revealed that expression of eight PAGs (including \( \text{GLK1} \) and \( \text{GLK2} \)) was down-regulated in ATAF1 overexpression plants compared with Col-0; in contrast, expression of the same genes was induced in the \( \text{ataf1-f} \) mutant (Supplemental Fig. S3A; Supplemental Table S3). With respect to SAGs, expression of 59 genes (37%) and 91 genes (58%) was down-regulated by 2- to 4-fold and by more than 4-fold, respectively, in \( \text{ataf1-f} \) compared with Col-0 (measured in leaf 9). In accordance with this, 38 genes (24%) and 24 genes (15%) of the SAGs were up-regulated by 2- to 4-fold and by more than 4-fold, respectively, in 35S:ATAF1 plants (Supplemental Fig. S3B; Supplemental Table S3). The fact that many PAGs and SAGs are affected at the expression level in ATAF1-modified plants suggested that some of them might be direct targets of the NAC TF.

To identify potential direct target genes of ATAF1, we analyzed the promoters of the affected genes for the presence of ATAF1-binding sites (Supplemental Table S2) and identified GLK1 [VDFHVnYRR(6N)YACGnMWSk(bS1) and RRWnnGCnTR(6-6n) DACTGnMWHd(bS2)] as ATAF1-binding sites (underlined nucleotides represent 100% conservation in the sequenced clones; Supplemental Table S2). Recently, Jensen et al. (2013) used protein-binding microarrays covering all possible 8-mer DNA sequences and reported the monopartite 6-mer motifs TT[A,C,G]CGT and T[A,C,G]CGT(A,G) as the binding sites of ATAF1. Due to technical constraints associated with protein-binding microarrays, longer (and here, bipartite) binding sites cannot be detected with high probability using this method. Still, the Jensen et al. (2013) binding site for ATAF1 is included in the spectrum of bipartite binding sites we detected here. The consensus binding site identified for ATAF1 bears similarity to the core binding motif (YACG or CGT) reported for a few other NAC factors: TCNNN\( \text{NNANACACAC} \) for the three highly homologous drought-responsive NAC factors ANAC019, ANAC055, and ANAC072 (Tran et al., 2004), (A/G)CGT(A/C)NNGNNN(C/T)ACGT(C/A)NACG for wheat TaNAC69 (Xue, 2005), (A/G)CGT(A/G)(4-5N) (A/G)(C/T)ACGCAA for ORE1 (Balazadeh et al., 2011), TGCCGT(7N)ACG and TGCCGT(7N)CCGC for JUB1 (Wu et al., 2012), and VMGTR(5-6N)YACR for ORE1 (Matallana-Ramirez et al., 2013).

Figure 2. Inducible ATAF1 expression triggers senescence. A, Induction of ATAF1 expression in ATAF1-IOE plants by estradiol (ESTR) treatment results in precocious senescence. Ten-day-old seedlings were transferred to agar plates containing one-half-strength MS medium supplemented with 20 \( \mu \text{M} \) estradiol and grown for another 8 d (16 h of light at 22˚C/8 h of dark at 18˚C). No leaf senescence is visible in estradiol-treated empty-vector (EV) control transformants or in mock-treated seedlings. B, Elevated expression of ATAF1 results in reduced expression of PAGs in ATAF1-IOE (5 h after estradiol induction) and \( \text{OX-17} \) plants, while knocking out ATAF1 in the \( \text{ataf1-f} \) mutant has the opposite effect (27 DAS). On the contrary, expression of the early-senescence marker gene \( \text{SAG13} \) is enhanced in ATAF1 overexpressors but reduced in \( \text{ataf1-f} \). The heat map indicates differences in the expression (log2 fold change) of genes in estradiol-induced versus mock-treated ATAF1-IOE plants, and in \( \text{OX-17} \) and \( \text{ataf1-f} \) plants, respectively, compared with Col-0 (corresponding data are given in Supplemental Table S1). Data represent means of three independent biological replicates, each determined in two technical replicates.
by qRT-PCR at different time points (1, 2, 5, 10, and 24 h) after estradiol (10 μM) treatment and compared with mock treatment. Interestingly, expression of ORE1 was rapidly (already within 1–2 h after estradiol treatment) up-regulated, while GLK1 was down-regulated in ATAF1-IOE plants (Fig. 4A; Supplemental Table S1).

To test the physical binding of recombinant ATAF1 transcription factor to the promoters of the two genes, we performed electrophoretic mobility shift assays (EMSAs). ATAF1 protein fused to CELD (see above) was incubated with 5′-DY682-labeled 40-bp double-stranded DNA fragments containing the binding sites of ATAF1. As shown in Figure 4B, ATAF1 binds to both the ORE1 and GLK1 promoter fragments. Binding of ATAF1 was significantly reduced when unlabeled promoter fragments were added in excess (competitor).

Next, we examined whether ATAF1 binds to the promoters of the two genes in vivo. To this end, we performed chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) using a transgenic line that expresses the ATAF1-GFP fusion protein under the control of the CaMV 35S promoter. We observed the presence of ATAF1-GFP protein in nuclei of transgenic plants (Fig. 4C) and elevated expression of ORE1 compared with Col-0, while expression of GLK1 was repressed (Fig. 4D), consistent with the model that ATAF1-GFP fusion protein faithfully functions as a transcription factor. Using these plants, we observed a significant
enrichment of the promoters of *ORE1* and *GLK1* (Fig. 4E), supporting the conclusion that both are direct downstream targets of ATAF1.

### ATAF1 Requires ORE1 for Full Control over Senescence

As *ORE1* (*ANAC092*) is a central positive regulator of senescence and a direct target of ATAF1, we hypothesized that ATAF1 executes its role in developmental senescence through *ORE1*. To test this model, we generated and analyzed plants overexpressing ATAF1 in the *anac092-1* mutant, which is null for *ORE1* (Balazadeh et al., 2010a). We determined *ATAF1* expression in 35S: *ATAF1*/*anac092-1* transformants and selected two independent lines (called L1 and L6; Fig. 5A) showing similar levels of *ATAF1* overexpression to the 35S: *ATAF1*/Col-0 plants (Supplemental Fig. S2E). As seen in Figure 5B, leaf senescence developed more rapidly in 35S: *ATAF1*/Col-0 (line OX-17) than in 35S: *ATAF1*/anac092-1 plants. We assessed the progression of leaf senescence by determining the number of leaves with at least 50% yellow blades at three different plant ages (35, 45, and 55 DAS) and membrane ion leakage at two time points (35 and 45 DAS). In accordance with previous findings (Balazadeh et al., 2010a), senescence was significantly retarded in the *anac092-1* mutant compared with the Col-0 wild type and was faster in 35S: *ATAF1*/Col-0 plants, while in 35S: *ATAF1*/anac092-1 plants (L1 and L6), senescence was only partly promoted (Fig. 5, C and D). Thus, although ATAF1 has the capacity to speed up senescence, it does require intact *ORE1* (*ANAC092*) to be able to fully execute its role.

In addition, to analyze senescence at the molecular level, we checked the expression of *SAG12* in leaf 9, which was approximately 4-fold less induced in 35-d-old 35S: *ATAF1*/anac092-1 than in *OX-17* plants (Fig. 5E).

- **Figure 4.** *ORE1* and *GLK1* are direct targets of ATAF1. A, Expression of *ORE1* and *GLK1* in 2-week-old *ATAF1-IOE* seedlings treated with estradiol (ESTR) for the indicated times. The heat map indicates expression compared with mock treatment (log₂ fold change). Data represent means of three independent biological replicates each. Note the rapid down- and up-regulation of *GLK1* and *ORE1*, respectively (for corresponding data, see Supplemental Table S1). B, EMSA. Purified ATAF1-CELD protein binds specifically to the ATAF1-binding site within the promoters of *ORE1* and *GLK1*. In vitro DNA-binding reactions were performed with 40-bp-long promoter fragments containing the respective ATAF1 motifs (5’-GTTCCTAACATCTACGCATCT-3’) and *GLK1* (5’-GACCAGTAGTCCTTTTACGAAAGT-3’). C, Nuclear localization of ATAF1-GFP fusion protein in transgenic Arabidopsis plants. D, Expression of *ORE1* and *GLK1* in 2-week-old 35S: *ATAF1-GFP* seedlings compared with expression in Col-0 plants. Numbers on the y axis indicate expression fold change (log₂ Fch). Data are means ± SD of three independent biological replicates, each determined in two technical replicates. E, ChIP-qPCR. Shoots of 2-week-old 35S: *ATAF1-GFP* seedlings were harvested for the ChIP experiment. qPCR was used to quantify the enrichment of the *ORE1* and *GLK1* promoter regions. As a negative control (NC), qPCR was performed on a promoter (*CLAVATA1*, *At1g75820*) lacking ATAF1-binding sites. Data represent means ± SD (two independent biological replicates, each with three technical replicates).
respectively, compared with the wild type, while in 35S:ATAF1/anac092-1 (line L6), only 24 genes (15%) and 18 genes (11%) were up-regulated by 2- to 4-fold and more than 4-fold, respectively (Fig. 5F; Supplemental Table S1). Notably, of the 82 SAGs repressed by at least 2-fold in the anac092-1 mutant (in accordance with the delay in senescence), 25 genes returned to normal level (log2 fold change difference from the wild type between 20.5 and 0.5) or were even induced as in OX-17. Importantly, a smaller fraction of the SAGs returned to normal level when ATAF1 was overexpressed in anac092-1 than in the Col-0 background (Fig. 5F; Supplemental Table S1). Collectively, our data clearly demonstrate that ORE1 is an important genetic determinant through which ATAF1 exerts its senescence regulatory function.

ORE1 has been shown previously to regulate the expression of several known SAGs, of which BFN1, SWEET15/SAG29, and SINA1 were shown to be direct targets (Balazadeh et al., 2010a, 2010b; Matallana-Ramirez et al., 2013). As ATAF1 regulates ORE1, we hypothesized that modifying ATAF1 expression affects ORE1 target genes. To test this model, we analyzed the expression of ORE1 targets in transgenic plants overexpressing ATAF1 in the Col-0 and anac092-1 backgrounds at 35 DAS. As shown in Figure 6A and Supplemental Table S1, expression of all tested ORE1 targets was

Figure 5. ATAF1 requires ORE1 for full control over senescence. A, Elevated expression of ATAF1 in anac092-1 mutant plants transformed with the 35S:ATAF1 construct at 25 DAS. Data are means ± so from three independent biological replications from T3 generation plants. Asterisks indicate significant differences from anac092-1 (Student’s t test, P < 0.05); dCT, Delta cycle threshold. B, Comparison of ATA {}{}F{}{} overexpressors with Col-0 and the anac092-1 mutant grown in long-day conditions. Note the earlier leaf yellowing in OX-17 compared with 35S:ATAF1/anac092-1 plants. C, Leaf yellowing. Values on the y axis indicate the percentage of leaves that had at least 50% yellow blades (inspected visually) at a given DAS (n = 12 for each plant line and time point). D, Higher ion leakage of plants overexpressing ATAF1 in the Col-0 and anac092-1 backgrounds compared with Col-0 and the anac092-1 mutant, respectively. Leaves 9 and 11 were combined and used for measuring ion leakage. Data in C and D are means ± so of three biological replicates (asterisks indicate significant differences from Col-0; Student’s t test, P < 0.05). E, Lower expression of the senescence marker gene SAG12 in 35S:ATAF1/anac092-1 than in 35S:ATAF1/Col-0 (OX-17) plants at 35 DAS indicates partial but not full recovery of the anac092-1 senescence phenotype. The expression levels of SAG12 in 35S:ATAF1/anac092-1 and 35S:ATAF1/Col-0 (OX-17) plants were normalized against ACTIN2 and compared with Col-0. Data are means ± so of three independent biological experiments. The asterisk indicates a significant difference from OX-17 (Student’s t test, P < 0.05). Fch, Fold change. F, Percentage of up- and down-regulated SAGs in leaf 9 of 35S:ATAF1/Col-0 (OX-17) and 35S:ATAF1/anac092-1 L6 plants as well as in the anac092-1 mutant compared with Col-0 (45 DAS). The corresponding gene expression data from three independent biological replications, each determined in two technical replicates, are given in Supplemental Table S1.
enhanced in ATAF1 overexpressors, while their expression, with the exception of SWEET15, remained low in 35S:ATAF1/anac092-1 plants, similar to the situation in the anac092-1 mutant itself. These results are in full accordance with the model that ORE1 is a direct target of ATAF1. The fact that the expression of SWEET15 returned to normal in plants overexpressing ATAF1 in the anac092-1 background may indicate that ATAF1 regulates SWEET15 through other transcription factors besides ORE1.

We next tested expression of the chloroplast maintenance TFs GLK1 and GLK2 and their target genes (Waters et al., 2009). As seen in Figure 6B and Supplemental Table S1, expression of the genes positively regulated by GLKs was significantly reduced in 35S:ATAF1/Col-0 (OX-17) plants, as expected (see above). Notably, expression of these genes was high in the anac092-1 mutant, consistent with the delayed senescence in this line and the fact that GLK targets require functional ORE1 protein for repression during senescence, as reported (Rauf et al., 2013a). The repression of GLKs and their target genes was largely restored when ATAF1 was overexpressed in the anac092-1 knockout mutant (Fig. 6B; Supplemental Table S1). Thus, collectively, our data are consistent with the model that ATAF1 regulates senescence by both inhibiting GLK1 expression and at the same time activating ORE1 expression. However, besides controlling senescence through the GLK/ORE1 transcriptional cascades, ATAF1 affects senescence through ABA signaling, as shown in the following.

Control of Senescence by ABA Is Mediated by ATAF1

ABA is one of the most effective plant hormones known to promote senescence (Zacarias and Reid, 1990; Lee et al., 2011; Finkelstein, 2013), and increases in endogenous ABA levels during senescence have been reported in several plant species, including oat (Avena sativa; Gepstein and Thimann, 1980), rice (Oryza sativa; Philosoph-Hadas et al., 1993), maize (He et al., 2005a), and Arabidopsis (Breeze et al., 2011). As expression of ATAF1 is well induced by ABA treatment (Lu et al., 2007; Wu et al., 2009), we tested whether ATAF1 plays a role in the hormonal control of senescence. To this end, we determined hormone levels in soil-grown plants. The levels of most hormones tested were not significantly altered in ataf1 null mutants compared with Col-0 at 35 DAS, with the exception of ABA, which was reduced significantly in ataf1 null mutants compared with Col-0 (Supplemental Table S4), similar to a previous report by Jensen et al. (2008). In addition, overexpression of ATAF1 was reported to lead to higher levels of ABA compared with wild-type plants (Jensen et al., 2013). Thus, the change in ABA levels (down in ataf1 knockout and up in ATAF1 overexpressors) well conforms to the change in senescence observed (delayed in ataf1 mutants and precocious in 35S:ATAF1 lines), suggesting that ABA is causally linked to senescence induction or progression.

Previous research has identified a role of ABA as a trigger of H$_2$O$_2$ accumulation under environmental stress (Jiang and Zhong, 2001; Kwak et al., 2003; Hu et al., 2005, 2006; Ye et al., 2011). In addition, H$_2$O$_2$ levels are known to increase in senescing leaves, and external application of H$_2$O$_2$ promotes leaf senescence, possibly through the activation of senescence-associated transcriptional cascades (Lesher, 1988; Navabpour et al., 2003; Mittler et al., 2004; Zimmermann and Zentgraf, 2005; Wu et al., 2012). Notably, ATAF1 is not...
only induced by ABA (Lu et al., 2007; Wu et al., 2009) but also by external treatment of plants with H$_2$O$_2$ (Balazadeh et al., 2010b) or intracellular elevation of H$_2$O$_2$ level by catalase inhibition (Supplemental Fig. S1C), indicating that it may be positioned at a cross-talk node connecting ABA and H$_2$O$_2$ signaling in plant senescence. Accordingly, we asked whether ATAF1 plays a role in ABA-induced leaf senescence. We tested the effect of exogenously applied ABA on the accumulation of H$_2$O$_2$ and the induction of senescence in ATAF1 transgenics (Supplemental Fig. S4). We selected nonsenescent leaves 4, 5, and 6 of 25-d-old plants for the experiment. After 3 d of ABA (20 $\mu$M) treatment under light, leaves of 35S:ATAF1/Col-0 and 35S:ATAF1/anac092-1 plants accumulated significantly higher H$_2$O$_2$ levels than Col-0; in contrast, leaves of the ataf1-4 mutant accumulated less H$_2$O$_2$ after ABA treatment, which was accompanied by a significantly better retention of chlorophyll compared with Col-0 (Supplemental Fig. S4, A–C).

Recently, Jia et al. (2013) reported that PHOSPHOLIPASE D6 (PLD6) plays an important role in ABA-promoted leaf senescence in Arabidopsis. The enzyme also functions in various environmental stresses, including the response to dehydration, salinity stress, and freezing, and plays a role in programmed cell death in response to H$_2$O$_2$, treatment. As shown by the authors, expression of PLD6 rises during ABA-stimulated senescence in detached leaves, and a pld6 knockout mutant exhibited delayed senescence in the presence of ABA, while no difference compared with the wild type was observed in the absence of the hormone. Here, we used qRT-PCR to test the expression of PLD6, along with genes involved in calcium signaling and chlorophyll degradation. We included calcium signaling genes in our analysis, as Ca$^{2+}$-mediated signal transduction plays a fundamental role in many abiotic stress responses, including those involving changes in ABA and reactive oxygen species (ROS; H$_2$O$_2$) levels (Pei et al., 2000; Tuteja and Mahajan, 2007; Wang and Song, 2008). We included chlorophyll degradation genes to monitor the transcriptional onset of pigment degradation as a proxy for senescence. As evident from the data shown in Supplemental Table S5, overexpression of ATAF1 significantly increased the expression of PLD6 and various calcium signaling-related genes, irrespective of the genetic background (Col-0 or anac092-1). Similarly, chlorophyll degradation genes were up-regulated upon ATAF1 overexpression (Supplemental Table S5). These results, together with the higher accumulation of H$_2$O$_2$ in ATAF1 overexpressors (see above), strongly support the model that ATAF1 affects senescence through ABA-mediated signaling and that this contributes to the partial restoration of the senescence syndrome observed in 35S:ATAF1/anac092-1 plants.

**ATAF1 Regulates ABA Homeostasis Genes**

ATAF1 modulates ABA biosynthesis by directly regulating the expression of the ABA biosynthetic gene NCED3 (Jensen et al., 2013). To test whether ATAF1 affects other genes involved in ABA homeostasis, GLK1 and ORE1, we examined the expression of 13 genes involved in ABA signaling, ABA metabolism/transport, and ABA responses in the ataf1-4 mutant as well as in 35S:ATAF1/Col-0 and 35S:ATAF1/anac092-1 plants (Fig. 7; Supplemental Table S1). The expression of BCH2 (involved in the synthesis of the epoxycarotenoid biosynthetic precursor zeaxanthin), NCED3 (involved in the cleavage of 9-cis-epoxycarotenoids to xanthoxin), and CYP707A1 (involved in ABA catabolism) was significantly down-regulated in the ataf1-4 mutant and up-regulated in 35S:ATAF1 plants. Also, the expression of genes involved in ABA signaling (such as protein phosphatase 2Cs, PYR1-LIKE6, and OPEN STOMATA1) and ABA import (ABCG40) as well as of ABA-responsive genes such as WRKY40 and WRKY63 was strongly down-regulated in the ataf1-4 mutant and up-regulated in ATAF1 overexpressors. Furthermore, expression of these genes was not significantly altered in the anac092-1 mutant compared with the wild type (Supplemental Table S1), while they were induced in 35S:ATAF1/anac092-1 plants, indicating that the ATAF1-dependent increase in the expression of the genes is independent of ORE1/ANAC092.

Next, we analyzed the promoters of the differentially expressed genes for potential ATAF1-binding sites and found full motifs [VDHVnnYRR(6-5n)YACGnMWSK] in the NCED3 (640 bp upstream the transcription start site) and ABCG40 (2,246 bp upstream) promoters, suggesting that both genes are direct downstream targets of ATAF1. As a further confirmation of this model,
we tested the expression of both genes in ATAF1-IOE lines after estradiol induction. Expression of ABCG40 was rapidly (within 1 h) induced after estradiol treatment, while expression of NCED3 was up-regulated at later time points (Fig. 8A; Supplemental Table S1). Using EMSA, we confirmed the in vitro binding of ATAF1 to 40-bp-long double-stranded oligonucleotides representing the respective promoter fragments, including the ATAF1 binding motifs (Fig. 8B). Finally, using ChIP-qPCR, we examined whether ATAF1 binds to the ABCG40 promoter in vivo. The expression of ABCG40 was induced in 35S:ATAF1-GFP plants, indicating faithful regulation of ABCG40 by the ATAF1-GFP fusion protein (Fig. 8C). Using these plants, we observed a significant enrichment of the ABCG40 promoter (Fig. 8D), supporting the conclusion that ABCG40 is a direct downstream target of ATAF1. Recently, Jensen et al. (2013) showed in vivo binding of ATAF1 to the promoter of NCED3.

ABA- and H2O2-Triggered Expression Changes Are Modified by ATAF1

In various plant cells or tissues, ABA can cause the generation of ROS such as H2O2 (Pei et al., 2000; Jiang and Zhang, 2001; Lin and Kao, 2001; Laloi et al., 2004; Hu et al., 2005), and increased levels of H2O2 result in an oxidative burst and ultimately cell death (Gechev and Hille, 2005). As the expression of ATAF1 is induced by treatment of plants with ABA (Lu et al., 2007; Wu et al., 2009) or H2O2 (Balazadeh et al., 2010b), we proposed an ABA- and H2O2-dependent regulation of its two photosynthesis- and senescence-related target genes (i.e. ÖRE1 and GLK1) through ATAF1. To test this hypothesis, we analyzed the expression of ÖRE1 and GLK1, as well as of their direct downstream targets, in 2-week-old Arabidopsis seedlings (Col-0, OX-17, and ataf1-4) after a 4-h treatment with ABA (10 μM) or H2O2 (10 mM). In both conditions, expression of GLK1 and its

Figure 8. NCED3 and ABCG40 are direct targets of ATAF1. A, Expression of NCED3 and ABCG40 in 2-week-old ATAF1-IOE seedlings after estradiol (ESTR) induction of ATAF1. The heat map indicates the difference in expression (log2 fold change) compared with mock-treated samples. Data represent means of three independent biological replicates, determined in two technical replicates each (corresponding data are given in Supplemental Table S1). B, EMSA. Purified ATAF1-CELD protein binds specifically to the ATAF1-binding site within the promoters of NCED3 and ABCG40. In vitro DNA-binding reactions were performed with 40-bp promoter fragments containing the respective ATAF1-binding motifs (NCED3, 5′-GACACTAATATAAACTACGTACC-3′; and ABCG40, 5′-AGATGCGTGGGAGGCTTGGGGAC-3′). C, Expression levels of ATAF1 and ABCG40 in 2-week-old 35S:ATAF1-GFP seedlings compared with Col-0; ATAF1 expression in 35S:ATAF1-GFP seedlings represents the combined expression of the endogenous ATAF1 gene and the ATAF1-GFP transgene. Numbers on the y axis indicate expression fold change (log2 Fch) of gene expression in 35S:ATAF1-GFP compared with Col-0. Data are means ± SD of three independent biological replicates, each determined in two technical replicates. D, ChIP-qPCR. Shoots of 2-week-old 35S:ATAF1-GFP seedlings were harvested for the ChIP experiment. qPCR was used to quantify enrichment of the ABCG40 promoter. As a negative control (NC), qPCR was performed on a promoter (CLAVATA1; At1g75820) lacking ATAF1-binding sites. Data represent means ± SD (two independent biological replicates, each with three technical replicates).
target genes was repressed in Col-0; notably, however, this repression was significantly stronger in OX-17 seedlings but less prominent in ataf1-4 seedlings (Supplemental Table S6). With respect to ORE1, the same stress treatments triggered a prominent increase of its expression in OX-17 seedlings, which was weaker in Col-0 and lowest in the ataf1-4 mutant; ORE1 targets showed the same clear response (Supplemental Table S6). Importantly, all three ABA-related genes we tested (i.e. NCED3, ABCG40, and CYP707A1) also showed enhanced expression after ABA or H₂O₂ treatment in Col-0 seedlings, a response that was stimulated in OX-17 but lowered in ataf1-4 lines (Supplemental Table S6). These data provide strong evidence for the model that ATAF1 regulates ABA- and H₂O₂-modulated senescence through GLK1 and ORE1. In addition, they reveal a tight cross talk between ABA- and H₂O₂-mediated signaling through the action of ATAF1.

Previously, delayed senescence in plants was observed to be associated with an increased tolerance to oxidative stress (Woo et al., 2004; Wu et al., 2012), and ATAF1 overexpressors were reported to be hypersensitive to oxidative stress imposed by treating seedlings with Rose Bengal or paraquat, which trigger the formation of singlet oxygen or superoxide free radical, respectively; however, no effect of these chemicals was observed in ataf1 knockout mutants (Wu et al., 2009). However, considering our above-reported results on the central role of ATAF1 in conveying the expresional effect of H₂O₂ through GLK1 and ORE1 on their downstream target genes, we wondered whether H₂O₂ treatment would differentially affect the phenotype of wild-type versus ATAF1-altered plants. To this end, 2-week-old ataf1 mutant seedlings were incubated for 2 d in liquid MS medium containing 10 mM H₂O₂ and the chlorophyll level was determined. The decrease in chlorophyll content after H₂O₂ treatment was less prominent in the three ataf1 mutants compared with the wild type (Fig. 9, A and B). A less severe H₂O₂-triggered chlorophyll loss was also observed in detached ataf1-2 (Fig. 9C) and ataf1-4 leaves (data not shown). In accordance with this finding, ATAF1 overexpressors accumulated more, and ataf1-2 mutants less, H₂O₂ than the Col-0 wild type, as shown by 3,3’-diaminobenzidine (DAB) staining (Fig. 9D). Similarly, overexpression of ATAF1 from an estradiol-inducible promoter (ATAF1-IOE line) stimulated chlorophyll loss in the presence of both estradiol and H₂O₂, confirming the important role of ATAF1 in this physiological response. Importantly also, estradiol treatment alone triggered chlorophyll loss (albeit to a lesser extent; Fig. 9, E and F), fully supporting the model of a close interaction between ABA- and H₂O₂-mediated signaling.

**DISCUSSION**

Senescence is a highly controlled process that requires the coordinated reprogramming of hundreds of genes. Previous work has identified a large number of NAC TFs being up-regulated during senescence, and SAGs are enriched for NAC-binding sites in their promoters, establishing the NAC family as the largest group of plant-specific senescence-related transcription factors (Balazadeh et al., 2008; Breeze et al., 2011). Several NAC TFs induced during senescence are also responsive to H₂O₂ treatment and are considered to play a role in ROS-mediated signaling networks that modulate plant longevity (Balazadeh et al., 2010b; Wu et al., 2012). Genes affected by senescence-associated NAC TFs have been reported for several members of the family, and in some cases direct target genes were identified, such as for ORE1 (Balazadeh et al., 2010a; Matallana-Ramirez et al., 2013; Rauf et al., 2013a), ATNAP (Zhang and Gan, 2012b), and JUB1 (Wu et al., 2012). Besides regulating senescence, NAC TFs play important roles in various other physiological processes, including the responses to abiotic and biotic stresses and in hormone signaling (Wu et al., 2012; De Clercq et al., 2013; Jensen et al., 2013; Rauf et al., 2013b; Xu et al., 2013; for review, see Nuruzzaman et al., 2013). Here, we demonstrate the senescence-associated role of another NAC TF, the ABA- and H₂O₂-induced factor ATAF1.

As shown, overexpression of ATAF1 promotes developmental as well as dark- and ABA-induced senescence, while knocking out the gene’s function delays senescence (Figs. 1 and 3; Supplemental Fig. S4), indicating that ATAF1 positively affects senescence. In line with this model, the expression of ATAF1 increases in leaves during developmental and dark-induced senescence (Lin and Wu, 2004; Balazadeh et al., 2008). Furthermore, a number of abiotic stresses, including salinity (Wu et al., 2009; Supplemental Fig. S1B), drought (Lu et al., 2007), and oxidative stress (Balazadeh et al., 2010a; Supplemental Fig. S1C), as well as pathogen attack (Jensen et al., 2008) trigger ATAF1 expression, in accordance with the widely known senescence-inducing effect these stresses have (Lim et al., 2007; Gepstein and Glick, 2013). An increase in ATAF1 expression is also observed after ABA treatment (Wu et al., 2009; Genevestigator).

To unravel the molecular mechanism through which ATAF1 regulates senescence, we performed qRT-PCR-based expression profiling of 15 PAGs and 158 SAGs in ATAF1 overexpressor and ataf1 mutant lines and compared the data with those obtained from the Col-0 wild type. Among the differentially expressed genes were GLK1 and ORE1, both of which contain bipartite ATAF1-binding sites in their 5’ upstream regulatory regions (promoters), revealing them as potential direct targets of ATAF1. Indeed, the transcript level of ORE1 increases rapidly, while that of GLK1 decreases, upon estradiol-mediated induction of ATAF1 in ATAF1-IOE plants. In addition, ATAF1 binds in vivo to the promoters of ORE1 and GLK1, as shown by ChIP, allowing the direct regulation of both genes in planta. However, it remains unknown at present how the same TF (i.e. ATAF1) can both repress (GLK1) and activate (ORE1) gene expression. One possible model is that
ATAF1 interacts with different other TFs to exert its different regulatory functions toward its target genes. A similar situation appears to exist for PHYTOCHROME-INTERACTING FACTOR4 (PIF4), which activates the chlorophyll degradation regulator NONYELLOWING1 but represses the expression of GLK2 by direct binding to their promoters, thereby triggering senescence (Song et al., 2014). As shown by several laboratories independently, ORE1 is a key positive regulator of senescence in Arabidopsis, limiting the longevity of leaves, cotyledons, and inflorescences (Kim et al., 2009; Balazadeh et al., 2010a; Trivellini et al., 2012). In accordance with this, several SAGs have been identified to be under direct or indirect control of ORE1 (Balazadeh et al., 2010a; Breeze et al., 2011; Matallana-Ramirez et al., 2013; Rauf et al., 2013a), highlighting this NAC as one of the core control elements of the transcriptome underlying the senescence syndrome. In contrast, GLK genes exist as a pair of functionally redundant homologous genes, GLK1 and GLK2, in Arabidopsis, and they have been shown to directly regulate the expression of photosynthesis apparatus-related genes during chloroplast development and maintenance (Waters et al., 2009).

We have shown previously that ORE1 and GLKs interact at the protein level to inhibit the transcriptional activation of GLK target genes, while the expression of ORE1 target genes is not affected (Rauf et al., 2013a). Thus, in developing and expanding leaves, when GLK1 and GLK2 expression is high and ORE1 expression is low, ORE1 cannot disrupt the chloroplast maintenance function of GLKs. However, this situation changes when leaves age and the expression of ORE1 increases and the formation of GLK-ORE1 heteromers becomes physically possible. Thus, ORE1 balances leaf senescence against maintenance by antagonizing GLK-mediated transcription (Rauf et al., 2013a). However, although the age-dependent expression change of GLKs (reduced during senescence) and ORE1 (induced during senescence) was known before, the molecular mechanism steering these opposing expression changes was not.

Here, we present convincing evidence that the NAC TF ATAF1 represents such an upstream regulatory element. In addition to ATAF1, several other TFs, namely PIF4 and PIF5, the basic Leu zippers ABSCISIC ACID INSENSITIVE5 and ENHANCED EM LEVEL involved in ABA signaling, and ETHYLENE-INSENSITIVE3 (EIN3), a key transcription factor in...
ethylene signaling, were found to bind to the ORE1 promoter to regulate its expression (Sakuraba et al., 2014). In addition, ORE1 is negatively regulated by microRNA164 (miR164), whose abundance is high in developing leaves but decreases in leaves undergoing senescence (Kim et al., 2009). Recently, EIN3 was shown to repress miR164 transcription by binding to its promoter, thereby up-regulating transcript levels of ORE1, leading to early senescence (Li et al., 2013). Thus, ethylene is an important trigger of ORE1 gene expression, as reported earlier (He et al., 2005b; Du et al., 2014).

ATAF1, shown here to function as a positive upstream regulator of ORE1, is induced by treatment with ABA or H$_2$O$_2$. Similarly, ORE1 expression is induced by both environmental signal mediators, while the expression of GLK1 shows the opposite response (i.e. repression by ABA or H$_2$O$_2$ treatment or conditions that lead to intracellular changes in H$_2$O$_2$ concentration; Supplemental Table S6; Supplemental Fig. S5, A and B), suggesting that the stress-dependent transcriptional response of ORE1 and GLK1 is conveyed by ATAF1. Indeed, as we show here, ABA-/H$_2$O$_2$-dependent expression changes are impaired in ataf1 mutants but stimulated in ATAF1 overexpressors (Supplemental Table S6). Thus, our data extend the existing molecular model of ORE1 regulation (Kim et al., 2009; Woo et al., 2013) by adding a further layer of control connecting it, via ATAF1, to ABA- and H$_2$O$_2$-dependent stress signaling (Fig. 9).

Lower and higher basal ABA levels in ataf1 mutants (Jensen et al., 2008; Supplemental Table S4) and ATAF1 overexpressors (Jensen et al., 2013), respectively, suggest a role of ATAF1 in ABA-induced physiological changes. Notably, exogenously applied ABA can act as an inducer of H$_2$O$_2$ generation in plant cells or tissues (Kwak et al., 2003; Hu et al., 2005; Xing et al., 2008; Ye et al., 2011), and increased levels of H$_2$O$_2$ trigger senescence through the activation of senescence-associated transcriptional cascades (Wu et al., 2012; Wang et al., 2013). As shown here, the ataf1-4 mutant exhibits a delayed progression of senescence, while ATAF1 overexpressors exhibit precocious senescence in response to exogenously applied ABA. Additionally, the expression of PLD6, which is an important mediator in ABA-promoted senescence (Jia et al., 2013), and genes involved in calcium signaling along with chlorophyll degradation is enhanced in ATAF1 overexpressors, highlighting the role of ATAF1 as a transcriptional regulator of ABA uptake and/or an amplifier of the signaling cascade that results in ABA-promoted senescence.

The synchronized expression of genes involved in ABA signaling as well as ABA metabolism and transport help to maintain ABA homeostasis in crops such as rice, wheat, and barley (Hordeum vulgare; Govind et al., 2011; Ji et al., 2011). The expression of genes involved in ABA signaling, ABA metabolism/import, and ABA responses was up-regulated in ATAF1 overexpressors. Considering the fact that ATAF1 overexpressors show higher sensitivity to ABA treatment and precocious senescence, we propose an overlap between ATAF1 regulatory pathways in response to altered ABA homeostasis and developmental senescence. Moreover, NCED3, which is an ABA biosynthetic gene, was found to be a direct target of ATAF1 (Jensen et al., 2013). Here, we confirmed the activation of NCED3 by ATAF1 and demonstrated in vitro binding of ATAF1 protein to its bipartite binding site within the NCED3 promoter (Fig. 8B), which, however, differed from the monopartite binding site identified by Jensen et al. (2013). Thus, ATAF1 might regulate NCED3 by binding to at least two 5'-upstream binding sites. We additionally found that ABCG40 (encoding an ABA importer) harbors the binding site in its promoter to which ATAF1 binds in vitro and in vivo (Fig. 8, B and D).

Taking the experimental data together, we conclude that ATAF1 regulates senescence through a gene regulatory network that involves various target genes that affect aging-induced cell death (ORE1), photosynthesis (GLK1), and ABA biosynthesis and import (NCED3 and ABCG40; Fig. 10). Our data thus provide a mechanistic explanation for the long-known stimulatory effect

![Figure 10. Model for the integration of ATAF1 function during developmental and stress-induced leaf senescence in Arabidopsis. ATAF1 activates ORE1 and represses GLK1 expression by directly binding to the promoters of both genes (ATAF1-binding sites are indicated by black boxes). Consequently, the expression of GLK target genes is impaired, resulting in an age-dependent decline in the expression of PAGs (GLK targets), while the expression of ORE1 target genes is enhanced, leading to the onset and subsequent progression of senescence. ATAF1 also activates the expression of genes involved in ABA biosynthesis and transport (NCED3 and ABCG40) and through this contributes to ABA-induced senescence. ATAF1 expression is regulated by unknown upstream TFs, allowing it to respond to ABA and H$_2$O$_2$ in a stress-related manner. Arrow-ending lines and T-ending lines indicate positive and negative interactions, respectively. The inhibitory effect of the ORE1 protein on GLK transcription factors was reported by Raul et al. (2013a).](https://www.plantphysiol.org/)

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**Figure 10.** Model for the integration of ATAF1 function during developmental and stress-induced leaf senescence in Arabidopsis. ATAF1 activates ORE1 and represses GLK1 expression by directly binding to the promoters of both genes (ATAF1-binding sites are indicated by black boxes). Consequently, the expression of GLK target genes is impaired, resulting in an age-dependent decline in the expression of PAGs (GLK targets), while the expression of ORE1 target genes is enhanced, leading to the onset and subsequent progression of senescence. ATAF1 also activates the expression of genes involved in ABA biosynthesis and transport (NCED3 and ABCG40) and through this contributes to ABA-induced senescence. ATAF1 expression is regulated by unknown upstream TFs, allowing it to respond to ABA and H$_2$O$_2$ in a stress-related manner. Arrow-ending lines and T-ending lines indicate positive and negative interactions, respectively. The inhibitory effect of the ORE1 protein on GLK transcription factors was reported by Raul et al. (2013a).
that ABA (and abiotic stresses promoting a rise in cellular ABA level, such as drought) has on chloroplast deterioration, the down-regulation of photosynthesis, and senescence (Noodén, 1988; Lim et al., 2007).

Another senescence regulatory NAC TF in Arabidopsis, namely AtNAP, has recently been shown to regulate ABA- and developmental senescence-dependent expression of SAG113 by directly binding to its promoter (Zhang and Gan, 2012a). SAG113 encodes a Golgi-localized protein phosphatase of the protein phosphatase 2C family; the protein is involved in the control of water loss from leaves (through stomata) during senescence (Zhang et al., 2012). In addition, recent data indicate that AtNAP also directly activates ABSCISIC ALDEHYDE OXIDASE3, which encodes an enzyme responsible for the last step in ABA biosynthesis (Yang et al., 2014). In rice, the functional ortholog of AtNAP (i.e. OsNAP) promotes ABA-induced senescence by activating SAGs, including those that encode chlorophyll degradation enzymes (Liang et al., 2014). In addition, a plasma membrane-anchored NAC TF, namely NAC WITH TRANSMEMBRANE MOTIF1-LIKE4 (ANAC053), has been reported to promote the production of ROS (H2O2 and superoxide radicals) during drought-stimulated senescence by activating the expression of RESPIRATORY BURST OXIDASE HOMOLOG genes leading to increased ROS formation; expression of NTL4 is induced by drought and ABA treatment as well as during developmental senescence (Lee et al., 2012). Thus, plants appear to have recruited various NAC factors to integrate different cellular activities into a coherent physiological process during stress-promoted leaf senescence. Further studies employing genetic and biochemical approaches will help to further unravel the intricate regulatory networks connecting developmental with stress-triggered senescence.

MATERIALS AND METHODS

General

Standard molecular techniques were performed as described (Sambrosk et al., 2001). Oligonucleotide sequences are given in Supplemental Table S7. Unless indicated otherwise, chemicals and reagents were obtained from Merck, Invitrogen, Sigma-Aldrich, and Fluuka. Molecular biological kits were obtained from Qiagen and Macherey-Nagel. qRT-PCR primers were designed using QuantPrime (www.quantprime.de). Genevestigator (www.genevestigator.com) and the eFP browser (www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) were used for gene expression pattern analysis.

Biological Material and Growth Conditions

All experiments were performed using Arabidopsis (Arabidopsis thaliana) accession Col-0 (Institut National de la Recherche Agronomique; http://dbgap.versailles.inra.fr/publiclines). Seeds were germinated in soil (Einheitserde CS90; Gruber Patzer) in a climate-controlled chamber with a 16-h daylength provided by fluorescent light at approximately 100 µmol m−2 s−1, day/night temperature of 20°C/16°C, and relative humidity of 60%/75%. After 2 weeks, seedlings were transferred to a growth chamber with a 16-h day (80 or 120 µmol m−2 s−1), day/night temperature of 22°C/16°C, and 60%/75% relative humidity. For growth on plates, Arabidopsis seeds were surface sterilized using 10% (v/v) sodium hypochlorite solution and sown on nutrient agar medium (1× MS medium and 1% [w/v] Suc, pH 5.8). The plates were stored for 2 days under vernalization conditions (16 h of light at 22°C and 8 h of dark at 4°C) and then transferred to long-day growth conditions (16 h of light at 22°C and 8 h of dark at 18°C). Seed stocks of AtAF1 T-DNA insertion lines (Supplemental Fig. S2) were obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). The anac092-1 T-DNA insertion mutant was reported previously (Balazadeh et al., 2010a).

DNA Constructs

 Constructs were generated employing PCR. Strategies for construct generation and primer sequences are listed in Supplemental Table S7. Amplicons generated by PCR were checked for correctness by DNA sequence analysis (Eurofins MWG Operon). Constructs were transformed into Arabidopsis via Agrobacterium tumefaciens GV3101 (pMP90)-mediated transformation.

Gene Expression Analysis

Total RNA extraction, synthesis of complementary DNA, and qRT-PCR using SYBR Green were performed as described (Caldana et al., 2007; Balazadeh et al., 2008). PCR was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). PCR experiments were generally performed on plants from two or three independent growth experiments, as indicated in the figure legends, and each sample was measured in two technical replicates. Expression levels were normalized against the expression level of ACTIN2. Statistical significance was estimated using Student’s t test.

DNA-Binding Site Selection

In vitro binding site selection was performed using the CELD system with the pTacATAFI-CELDOHIS construct, employing a biotin-labeled double-stranded oligonucleotide, which contained a 30-nucleotide random sequence (Xue, 2005). ATAFl-selected oligonucleotides were cloned and sequenced. The DNA-binding activity of ATAFl-CELER was measured using methylumbelleryl β-cellobiose as substrate (Xue, 2002). In control experiments, DNA-binding assays were performed with a biotin-labeled single-stranded oligonucleotide or a biotin-labeled double-stranded oligonucleotide lacking a target binding site.

EMSA

Recombinant ATAFl-CELER fusion protein was expressed in Escherichia coli strain BL21 (DE3) pLysS (Agilent Technologies) as described (Dortay et al., 2011). Protein was purified using Protino Ni-IDA 150 packed columns (Macherey-Nagel). EMSA was performed as described (Wu et al., 2012; Rauf et al., 2013b) using the Odyssey Infrared EMSA kit (Li-Cor). S-DY682-labeled DNA fragments were purchased from Eurofins MWG Operon.

ChIP-qPCR

ChIP-qPCR was performed with 2-week-old Arabidopsis seedlings expressing GFP-tagged ATAFl protein from the CaMV 35S promoter (35S: ATAFl-GFP). Col-0 plants served as negative controls. ChIP was performed as described (Kaufmann et al., 2010) employing anti-GFP antibody to immunoprecipitate protein-DNA complexes. The ChIP experiment was run in two biological replicates with three technical replications per assay. Primers used for qPCR flanked the ATAFl-binding sites within the promoter regions of ORE1, GLK1, and ABCG40. Primers annealing to promoter regions of an Arabidopsis gene (CLAVALATA1; At1g75820) lacking an ATAFl-binding site were employed in negative-control experiments. ChIP-qPCR data were analyzed as described (Wu et al., 2012).

Treatments

For estradiol induction, 12-d-old seedlings were incubated in liquid MS medium containing 10 µM estradiol (control treatment; 0.1% [v/v] ethanol). The seedlings were kept on a rotary shaker for 1, 2, 5, or 10 h, harvested, and immediately frozen in liquid nitrogen. Gene expression levels were determined by qRT-PCR. For estradiol induction on plates, MS medium was supplemented with 20 µM estradiol (control; 0.2% [v/v] ethanol). For ABA treatment, fully expanded green and nonsenescent leaves were detached from the rosettes of 25-d-old plants (Col-0, anac092-1, and ATAFl transgenics) grown on
soil at 22°C under a 16-h-light/8-h-dark photoperiod in 50% relative humidity and incubated for 3 d in water with or without ABA (20 μM) under white light at room temperature. For ABA or H2O2 treatment, 12-d-old seedlings were incubated for 4 h on a rotary shaker (90 rpm) in liquid MS medium containing 10 μM ABA or 10 mM H2O2, respectively.

**Determination of H2O2 Level**

Quantitative measurement of H2O2 level was performed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes) as described by Wu et al. (2012). For DAB staining, detached leaves were vacuum infiltrated in DAB solution (0.5 mg L−1) for 30 min and then incubated for 4 h at room temperature. Chlorophyll was removed by extraction with 80% (v/v) hot ethanol.

**Chlorophyll Measurement**

Chlorophyll content was determined using a SPAD analyzer (N-tester; Hydro Agt). Alternatively, frozen Arabidopsis leaves were ground in liquid nitrogen, resuspended in 5 mL of 80% (v/v) acetone in water, and homogenized at room temperature. Chlorophyll was removed by extraction with 80% (v/v) acetone in water, and homogenized for 1 min. Chlorophyll content was determined with a spectrophotometer at 663 and 646 nm as described by Arnon (1949).

**Electrolyte Leakage Measurements**

Ion leakage in pooled leaves 9 and 11 was determined by immersing them in 10 mL of deionized water and shaking at room temperature for 30 min. Initial electrical conductivity was measured at 25°C using a conductometer (Schott). Then, samples were boiled for 15 min and cooled down to 25°C, and total conductivity was measured again. Conductivity was expressed as the percentage of the initial conductivity versus the total conductivity.

**Other Methods**

Hormone levels were determined as reported by Müller and Munné-Bosch (2011). The presence of ATAF1-GFP fusion protein in transgenic plants was confirmed by fluorescence microscopy (Eclipse E600 microscope; Nikon). Student's t test embedded in Microsoft Excel was used for testing statistical significance; only the return of P ≤ 0.05 was regarded as statistically significant.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following codes: ABCG40 (At1g15520), ACTIN2 (At5g18780), ATAF1/ANA002 (At1g01720), GLK1 (At2g32570), ORE1/ANA002 (At1g39610), NACD3 (At5g14440), and PLD6 (At4g35790). Additional codes are given in Supplemental Tables S1, S3, S5, S6, and S7.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Senescence- and stress-dependent expression of ATAF1.

**Supplemental Figure S2.** T-DNA insertions and ATAF1 expression.

**Supplemental Figure S3.** Expression of PAGs and SAGs.

**Supplemental Figure S4.** ABA-induced senescence.

**Supplemental Figure S5.** Hydrogen peroxide effects on gene expression.

**Supplemental Table S1.** Data of figures.

**Supplemental Table S2.** DNA-binding site selection.

**Supplemental Table S3.** Data of supplemental figures.

**Supplemental Table S4.** Hormone concentrations.

**Supplemental Table S5.** Expression of genes involved in calcium signaling and chlorophyll degradation in leaves during ABA-promoted senescence.

**Supplemental Table S6.** Expression of ATAF1 target genes in H2O2- and ABA-treated seedlings.

**Supplemental Table S7.** Constructs and primer sequences.

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