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ORIGINAL ARTICLE

Dual function of *Arabidopsis ATAF1* in abiotic and biotic stress responses

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NAC family genes encode plant-specific transcription factors involved in diverse biological processes. In this study, the *Arabidopsis* NAC gene *ATAF1* was found to be induced by drought, high-salinity, abscisic acid (ABA), methyl jasmonate, mechanical wounding, and *Botrytis cinerea* infection. Significant induction of *ATAF1* was found in an ABA-deficient mutant *aba2* subjected to drought or high salinity, revealing an ABA-independent mechanism of expression. *Arabidopsis ATAF1*-overexpression lines displayed many altered phenotypes, including dwarfism and short primary roots. Furthermore, *in vivo* experiments indicate that *ATAF1* is a *bona fide* regulator modulating plant responses to many abiotic stresses and necrotrophic-pathogen infection. Overexpression of *ATAF1* in *Arabidopsis* increased plant sensitivity to ABA, salt, and oxidative stresses. Especially, *ATAF1* overexpression plants, but not mutant lines, showed remarkably enhanced plant tolerance to drought. Additionally, *ATAF1* overexpression enhanced plant susceptibility to the necrotrophic pathogen *B. cinerea*, but did not alter disease symptoms caused by avirulent or virulent strains of *P. syringae* pv *tomato* DC3000. Transgenic plants overexpressing *ATAF1* were hypersensitive to oxidative stress, suggesting that reactive oxygen intermediates may be related to *ATAF1*-mediated signaling in response to both pathogen and abiotic stresses.

***Keywords*:** abiotic stress, *Arabidopsis*, *ATAF1*, biotic stress

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Introduction

Plants are continuously exposed to various abiotic and biotic stresses in natural environment, and have evolved many adaptive mechanisms, which are dependent on a variety of signaling pathways, in order to react to altered conditions and combat pathogen attacks for survival. These stress-related signaling pathways are individually regulated by phytohormones including abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) [1-4], and can result in various physiological responses such as osmotic adjustment, stomatal movement, and oxygen bursts. The cellular processes of plant-stress responses are further complicated by web-type crosstalks among different signaling pathways. The identification and characterization of important signaling components have provided insights into these specific pathways; however, gaps still exist in our understanding of plant-stress response mechanisms. Transcriptome analyses have generated considerable data, which show extensive overlapping on gene expression between biotic and abiotic stresses. Many of these overlapped genes encode signaling components, including transcription factors and protein kinases [5-9].

Transcription factors are promising candidates of common players involved in crosstalk between abiotic and biotic stress signaling [4]. For instance, *AtMYC2/JIN1*, encoding the basic helix-loop-helix transcription factor, is not only involved in the ABA-mediated drought stress-

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signaling pathway, but also involved in JA-mediated wound response and JA/ET-mediated pathogen defense responses. Overexpression of *AtMYC2* resulted in improved osmotic stress tolerance. *AtMYC2* deficiency resulted in elevated expression of JA/ET-regulated defense genes and increased plant resistance to the necrotrophic fungal pathogen *Fusarium oxysporum* [10-12]. Additionally, BOS1, an R2R3MYB transcription factor, was shown to be required for biotic and abiotic stress responses in *Arabidopsis*. *bos1* mutant plants showed increased susceptibility to necrotrophic pathogens, and impaired tolerance to drought, salinity, and oxidative stress [13].

Many transcription factors involved in signaling crosstalk are multi-functional proteins. As a large multi-functional gene family, the *Arabidopsis* NAC family contains more than one hundred members. NAC proteins are plant-specific transcription factors characterized by a conserved N-terminal region known as the NAC domain. NAC proteins have crucial functions in plant developmental processes. Several NAC members were found to be determinative regulators in diverse plant developmental programs, including formation of apical shoots [14], lateral roots, and floral organs [15-17]; and leaf senescence [18]. Additionally, NAC proteins have been implicated in disease defense and abiotic stress signaling. Several NAC proteins have been shown to interact with virus proteins [19-21], or to play direct roles in regulating pathogen defense [22]. Some NAC proteins were recently identified as abiotic stress regulators. The NAC cDNAs *ANAC019*, *ANAC055,* and *ANAC072/RD26* were isolated as promoter-binding proteins of *ERD1* (*early-responsive to dehydration 1*). Their expression was induced by drought, high salinity, ABA, and methyl jasmonate (MeJA). Plants individually overexpressing these genes showed a significant increase in drought tolerance or salt tolerance [23-26]. The *anac019anac055* double mutant showed increased resistance to *B. cinerea* compared with the wild-type plants, and transgenic plants overexpressing *ANAC019* or *ANAC055* showed decreased resistance to this pathogen [27]. Moreover, NAC transcription factors such as ATAF2 and OsNAC6 have been shown to be involved in both biotic and abiotic stress responses. Overexpression of the *ATAF2* gene, which is a wound-, SA-, JA-, and salinity-responsive gene, resulted in increased susceptibility to fungal pathogens and decreased expression of pathogenesis-related genes [28]. Additionally, the rice gene *OsNAC6* was found to be an abiotic and biotic stress-related gene. *OsNAC6*-overexpression plants were tolerant to dehydration/high-salinity stresses and showed slightly increased tolerance to blast disease [29, 30].

ATAF1 (At1g01720), encoding a NAC protein, was previously reported to be a wounding-inducible gene [19]. The *ataf1-1* mutant line showed decreased resistance to a biotrophic fungus as a consequence of increased ABA level [22, 31]. ATAF1 was also reported to be a negative regulator in drought response based on analysis of *ataf1* mutants [32]. Here, we show that *ATAF1* plays important roles in both abiotic and biotic stress responses. We found that *ATAF1* overexpression lines, but not its null mutants, showed enhanced plant drought tolerance, revealing a positive function in plant drought response. Additionally, *ATAF1* overexpression lines were hypersensitive to high salinity, ABA, oxidative stress, and necrotrophic-pathogen infection. Our data indicate that *ATAF1* mediates *Arabidopsis* response to both abiotic and biotic stresses.

Results

Arabidopsis ATAF1 is a potential stress-related NAC gene

In order to systematically survey the function of *Arabidopsis* NAC members in stress signaling, we conducted an *in silico* analysis by mining public microarray data (Arabidopsis Biological Resource Center) for all NAC family genes under different stress treatments. As summarized in Supplementary information, Table S1, members of NAC family genes were found to be induced by multiple stresses, suggesting functional redundancy as well as specificities. We are in the process of applying forward and reverse genetic approaches to functionally study these stress-related NAC members. The following experimental results are based on our detailed investigation of one stress-related *NAC* representative, *ATAF1* (At1g01720, *ANAC002*).

Upregulation of ATAF1 in response to multiple abiotic stresses and Botrytis cinerea infection

To confirm the microarray data, detailed expression patterns of *ATAF1* were analyzed by RNA gel blot using an *ATAF1*-specific probe. *ATAF1* transcripts rapidly accumulated upon 250 mM NaCl, dehydration, or 50 μM ABA, and were maintained at a high level during the entire treatment period. Furthermore, *ATAF1* was found to be upregulated by mechanical wounding and 50 μM MeJA. In addition to induction of *ATAF1* by these abiotic stimuli or stress-related phytohormones, accumulation of *ATAF1* transcripts was also observed in seedlings infected with *B. cinerea* (*Botrytis cinerea*) (Figure 1). ABA is generally considered to be an important biomolecule regulating plant responses to drought and high salinity, and in particular it plays an essential role in some ABA-dependent stress response processes. To determine

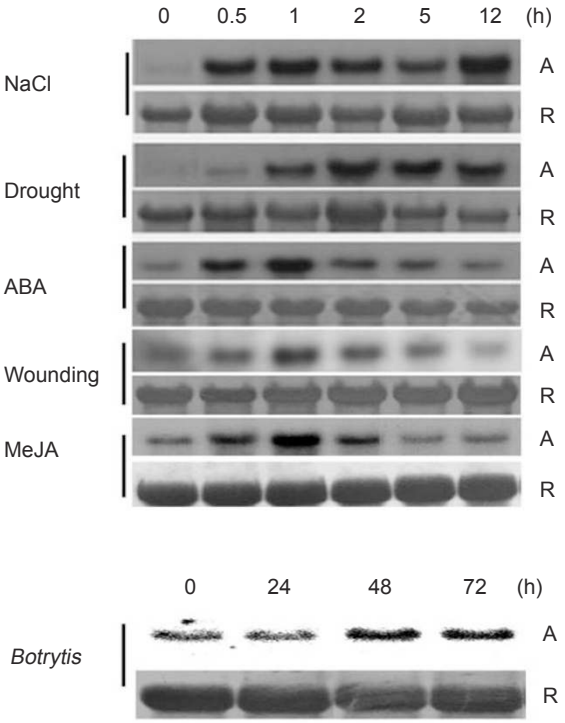

Figure 1 Northern blot analysis of *ATAF1* transcript. Expression of *ATAF1* in response to high salinity (250 mM NaCl), drought, ABA (50 μM), wound, MeJA (50 μM) treatments, and *B. cinerea* infection. A: *ATAF1* probe. R: rRNA: methylene blue-stained ribosome RNA used as loading controls.

whether *ATAF1* accumulation during drought and salinity stresses was dependent on ABA, *aba2* (an *Arabidopsis* ABA-deficient mutant) was used for analyzing *ATAF1* gene expression. The RNA gel blot revealed that *ATAF1* was still evidently induced in *aba2* plants subjected to drought or NaCl treatment (Supplementary information, Figure S1). This finding indicates that induction of *ATAF1* in response to drought or high salinity can be achieved through an ABA-independent pathway, although ABA alone is able to induce *ATAF1* expression.

Genetic analysis of ATAF1 gene

To investigate the functions of *ATAF1 in vivo*, we generated transgenic *Arabidopsis* plants overexpressing this gene. RNA gel blot revealed that all of the transgenic lines expressed *ATAF1* at higher levels compared with wild-type plants (data not shown). The highest level of *ATAF1* expression was found in three transgenic lines, designated as *ATAF1-O1*, *ATAF1-O2*, and *ATAF1-O3*. In western blot assays, these three lines exhibited high expression of the Myc-ATAF1 protein (Figure 2A). In addition, two T-DNA insertion lines with different target sites in *ATAF1* gene exons were obtained from the Salk mutant collection: Salk-067648 (*ataf1-1*) and Salk-057618 (*ataf1-2*). RT-PCR results showed that the expression of *ATAF1* was completely abolished in the *ataf1-1* and *ataf1-2* mutant lines, but was higher in the overexpressing lines (Figure 2B).

The *ATAF1*-transgenic lines showed altered phenotypes, such as relative dwarfism with small size, light green and round-shaped leaves, short petioles, sterility at the early flowering stage, and short siliques. Although *ataf1* mutants exhibited long primary roots (data not shown), their phenotypes bear a close resemblance to those of wild-type plants (Figure 2C).

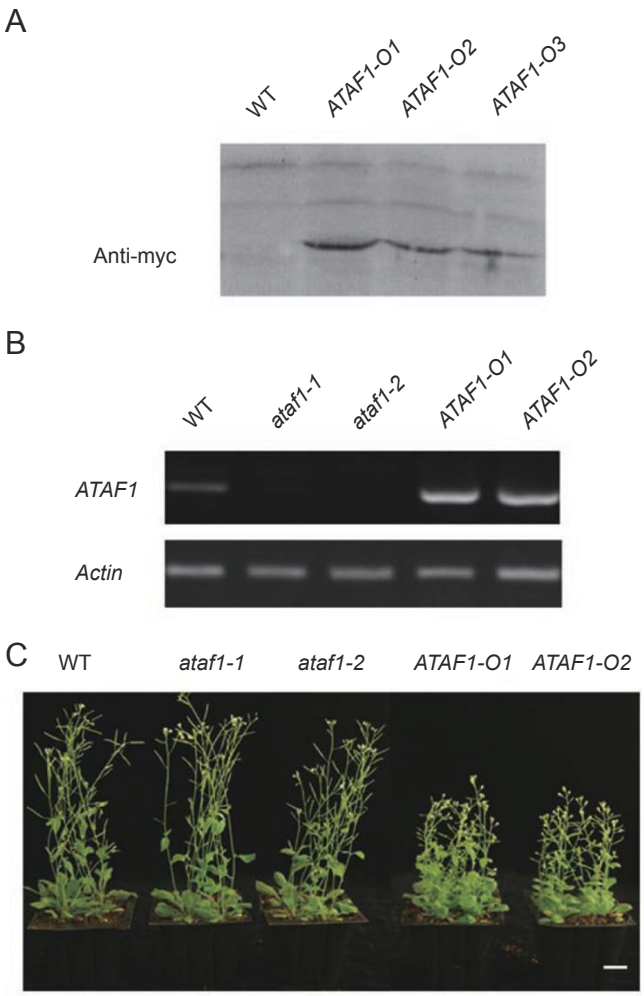

Figure 2 Genetic analysis of *ATAF1*. **(A)** *ATAF1* transcript and protein levels in *ATAF1*-overexpression lines. Western blot was performed with anti-Myc monoclonal antibody. **(B)** Comparison of *ATAF1* transcript levels in wild-type, *ataf1-1*, *ataf1-2*, overexpression plants *ATAF1-O1* and *ATAF1-O2*. **(C)** Growth phenotype of 35-day-old *ATAF1*-overexpression plants in comparison with *ataf1* mutants and wild-type plants. WT: wild-type plants. Bar = 1 cm.

Overexpression of ATAF1 confers drought tolerance in Arabidopsis

Since *ATAF1* is a drought-inducible gene, it likely regulates drought signaling. To characterize such a function of *ATAF1*, a whole-plant drought assay was performed in soil. Seedlings (10-day old) were transferred from MS plates to water-saturated soil for 7 days, and then water was withheld to cause a severe water deficit in soil. After 21 days, most of the control and mutant plants were wilted due to the extreme water deprivation (Figure 3A). After rewatering, 94% of overexpression plants continued to grow and successfully produced seeds, whereas most of the other plants did not recover and died from drought, showing a lower survival ratio (32% for wild-type, 28% for *ataf1-1*, and 30% for *ataf1-2*) (Figure 3A). This experiment was repeated several times with similar results. Our results indicate that high levels of *ATAF1* can enhance plant drought tolerance.

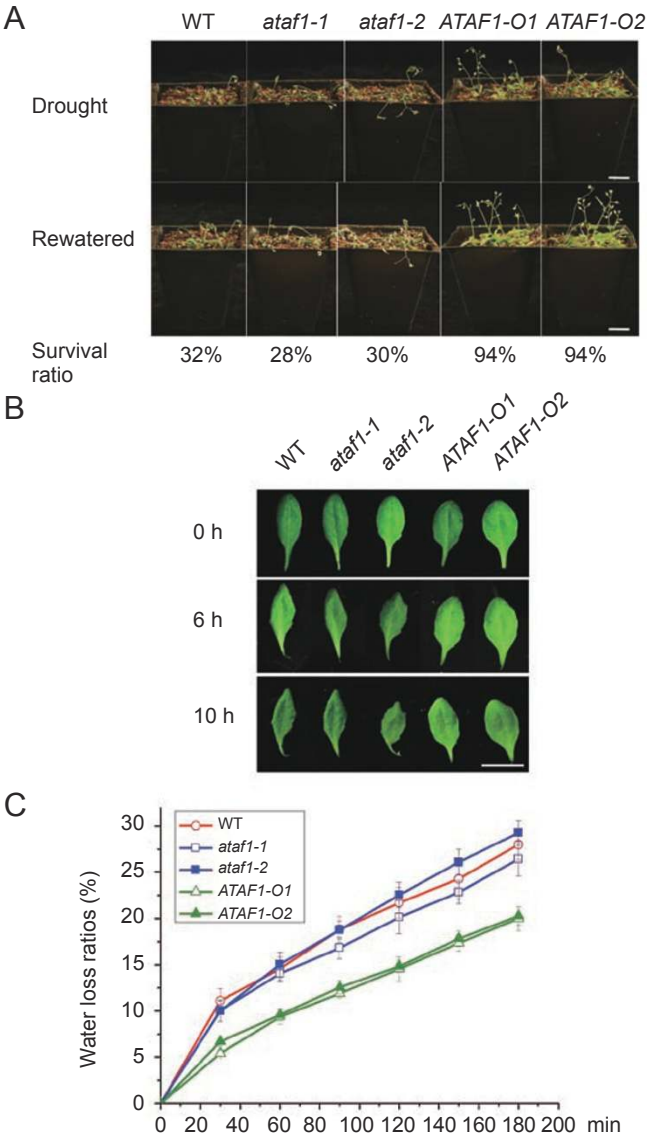

Figure 3 Drought-tolerance assays of wild-type, *ataf1-1*, *ataf1-2*, *ATAF1-O1*, and *ATAF1-O2* plants. **(A)** Photographs were taken when the drought-stressed phenotypes appeared and 6 h after being rewatered. Bar = 1 cm. **(B)** Comparison of phenotypes of excised leaves under the air-dry treatment for 0 h, 6 h, and 10 h. Bar = 1 cm. **(C)** Leaf water loss assay. Leaf weight was measured at the indicated time point (n = 18). Curves were drawn based on the data from three independent experiments, and bars indicate standard errors.

Regulation of transpiration plays a vital role in plant response to drought stress. To address whether the *ATAF1* function in enhancing plant drought tolerance was associated with transpiration, we compared the wilting phenotype of detached leaves from different plants that were subjected to air-dry treatment at room temperature with 65% humidity. After 6 h, obvious phenotypic changes were observed in these leaves (Figure 3B). At this time point, only the leaves from *ATAF1-O1* and *ATAF1-O2* plants retained normal shape and they only showed a slightly wilting phenotype, whereas the leaves of other plants were severely wilted. Similar phenomena were observed at 10 h of the treatment (Figure 3B). We further addressed the reasons for these phenotypic alterations by measuring water loss ratios in detached leaves from these plants (Figure 3C). In this assay, we found that the leaf weight of *ATAF1-O1* and *ATAF1-O2* plants decreased at the slowest rate of all analyzed plants, whereas other plants showed comparable water loss rates. Therefore, this quantitative result is consistent with the phenotypic observations in the leaf-wilting assay.

To reveal how *ATAF1* controls drought-responsive genes, we examined the expression of a group of marker genes in the drought-stressed plants (Supplementary information, Figure S2). *ATAF1-O1* plants produced dramatically high basal levels of *ADH1* (encoding an alcohol dehydrogenase) transcripts (0 h) and further increased its expression to reach the highest level compared with other plants. The expression patterns of *RD29A* and *COR47* were very similar. They showed low and comparable basal expression among different plants at 0 h, and then divergently increased in the assayed drought-stressed plants. After 2 h of treatment, transcripts of these two markers accumulated to a higher level in *ataf1-1* plants compared with wild-type or overexpression lines. However, transcript levels decreased in *ataf1-1* plants thereafter (10 vs 2 h). In contrast, *ATAF1-O1* plants induced small amounts of transcripts of the two marker genes at the 2-h time point, but accumulated the highest level of their transcripts at late stages of stress (10 h) in comparison with the other assayed plants. The drought-induction

of *RD22* was markedly repressed in *ATAF1-O1* plants, but slightly enhanced in the mutant plants. These results indicate that *ATAF1* may control different downstream ABA- and stress-responsive genes through specific manners that are different from other transcription factors.

Altered ATAF1 expression affects plant sensitivity to ABA

The transcript level of *ATAF1* was dramatically in-

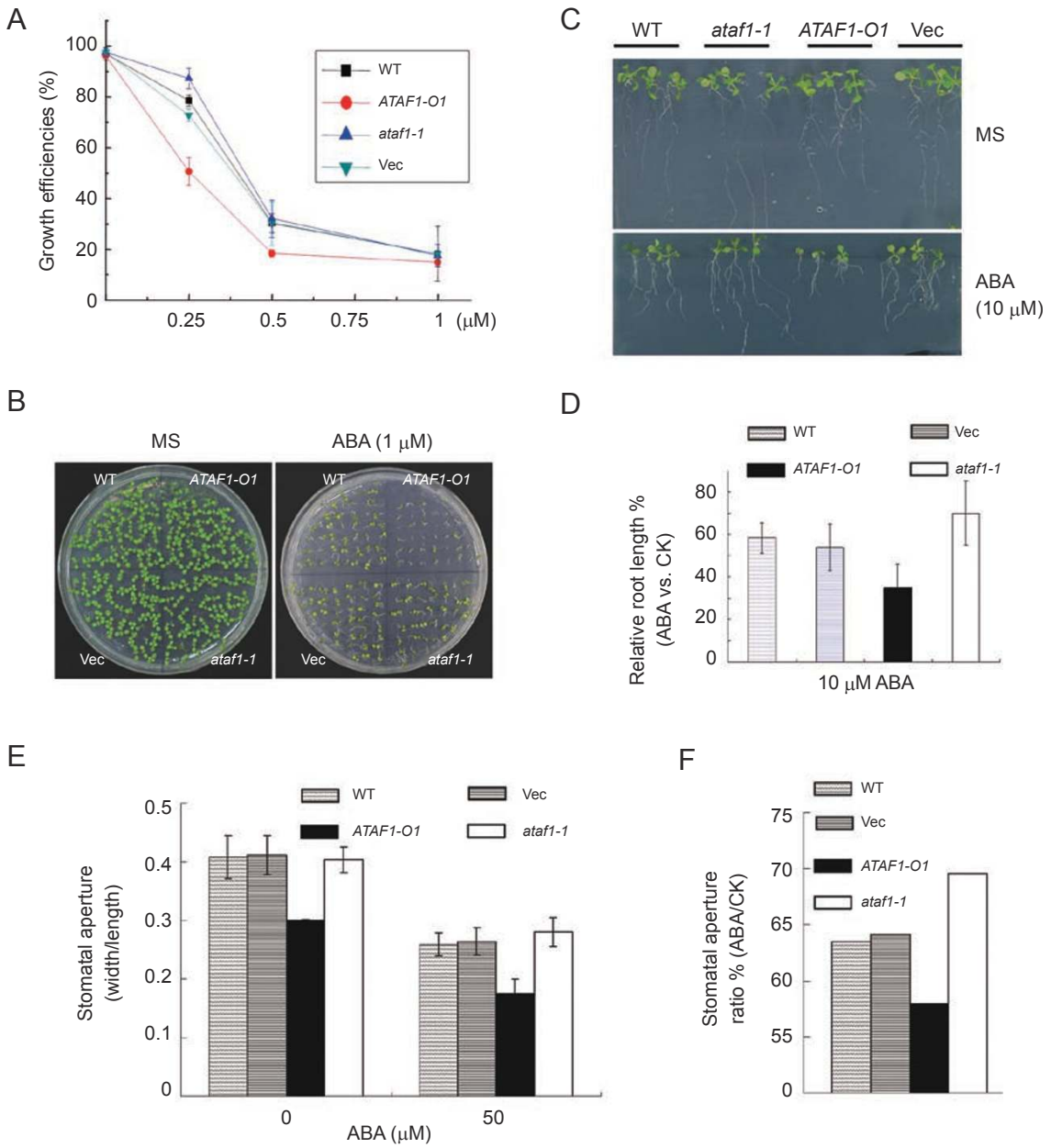

Figure 4 Sensitivity of *ATAF1* overexpression lines to ABA. **(A)** Effects of ABA on growth efficiencies. Growth efficiencies (green cotyledons) represented by the chart were based on three repeated experiments, in each of which an average of 90 seeds were counted. **(B)** Seedlings were grown on MS medium with or without 1 μM ABA and their phenotypes were scored on the 12th day after sowing. **(C)** Phenotypic comparison of root length in the presence of ABA. **(D)** Quantitative analysis of root growth. The experiment was performed in triplicate (n = 20) and repeated thrice. Bars indicate standard errors. **(E)** Effects of ABA on stomatal aperture of plants. Epidermal peels from plants were kept for 12 h in the dark, incubated under light in buffer for 3 h, and then treated with 0 and 50 μM ABA for 2 h before aperture measurements. Data are mean ratios of width to length ± SE of three independent experiments (n = 30 to 40). WT: wild type, Vec: vector control plants. **(F)** Stomatal aperture ratio, data are ratios of stomatal aperture of plants treated with 50 μM ABA to that of untreated plants.

duced by ABA in RNA gel blot assay (Figure 1), suggesting the potential function of *ATAF1* in ABA response. To elucidate the role of *ATAF1* in ABA signaling, *ATAF1-O1*, *ataf1-1* mutant, and control plants were germinated on MS plates with different amounts of ABA (0 , 0.25, 0.5, and 1 μM). No difference in the growth efficiencies (green cotyledons) and germination rates was observed among the untreated plants (Figure 4A). However, in the presence of 0.25 μM ABA for 3 days (the lowest concentration tested), the growth efficiencies decreased to 51.3% for *ATAF1-O1*, 78.5% for wild-type plants, 72.8% for vector plants, and 87.2% for *ataf1-1* plants. Similar inhibition effects were also observed for these plants grown on 0.5 μM ABA plates. *ATAF1-O1* plants exhibited the most severe growth inhibition by ABA among the assayed plants, indicating that high levels of *ATAF1* transcripts rendered plants more sensitive to ABA. The highest concentration of ABA (1 μM) used in this study led to extreme growth inhibition for all of the plants (Figure 4A); however, only *ATAF1-O1* plants displayed the most severely arrested growth under this condition, as reflected by retardation of cotyledon greening (Figure 4B). On the contrary, the germination rate of *ataf1-1* plants was the lowest and the germination rate of *ATAF1-O1* was the highest compared with that of the control plants (data not shown). To further correlate *ATAF1* function with plant sensitivity to ABA during the post-germination stage, root elongation inhibition was analyzed for these plants. Seedlings (4-day old) were transferred to MS medium with 10 μM ABA. After 7 days, root growth inhibition by ABA was the least severe for the *ataf1-1* mutant, but the most severe for *ATAF1-O1* plants among the assayed plants (Figures 4C, 4D). Taken together, these results indicate that *ATAF1* modulates plant ABA signaling and high *ATAF1* expression contributes to ABA hypersensitivity in *Arabidopsis*.

Stomata numbers on the leaf and aperture of stomata are major factors to determinate water evaporation. Under microscope, stomata numbers in identical area were counted for *ATAF1-O1*, *ataf1-1*, and control plants, and no stomata number changes were observed (data not shown). Overexpression of some ABA signaling components, such as *SDIR1*, *ABF3*, and *ABF4*, can reduce leaf water loss, because these genes have regulatory functions in controlling stomatal movement [33, 34]. To determine whether *ATAF1* can act on the adjustment of stomatal openings in a similar manner, we treated leaves of four genotypes with ABA to analyze stomatal aperture. Treating the leaf epidermis of *ATAF1-O1* plants with ABA caused nearly complete closure of stomata; the effect was not so pronounced in the control and *ataf1-1* plants (Figure 4E). Indeed, treating the leaf epidermis of *ATAF1-O1* plants with ABA caused the most severe closure of stomata (57.9% of untreated stomata aperture) compared with other plants (63.4% for wild-type plants, 64.2% for Vec control plants, and 69.5% for *ataf1-1* plants) (Figure 4F). Obviously, *ATAF1* overexpression elevated the sensitivity of stomatal closure mediated by ABA, whereas *ataf1-1* mutants showed slightly opposite phenotypes. These results indicate that *ATAF1* is involved in ABA-dependent stomatal closure.

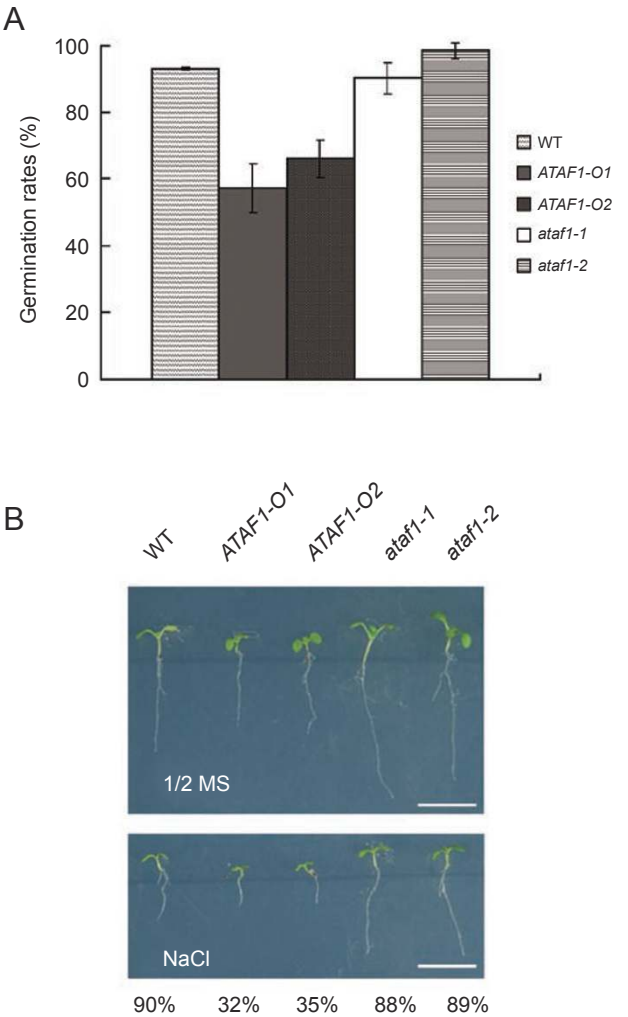

Figure 5 High-salinity assay of plants. **(A)** Germination rates (radical emergence) of wild type, *ataf1-1*, *ataf1-2*, *ATAF1-O1*, and *ATAF1-O2* plants on 1/2 MS medium containing 100 mM NaCl for 5 days. Percentages are means (n = 60 to 90 each) of three repeats ±SE. **(B)** Growth phenotype of transgenic and mutant plants on 1/2 MS medium (top panel) or containing 100 mM NaCl (bottom panel). Seeds were germinated and grown for 7 days. Numbers below the panel indicate the percentage of seedlings with green cotyledons in total seedlings grown on 100 mM NaCl medium (n = 60 to 90). Bar = 1 cm.

To further substantiate this finding, the expression of several ABA/stress-responsive marker genes was analyzed in *ATAF1-O1*, *ataf1-1*, and wild-type plants (Supplementary information, Figure S3). For *ADH1* gene, wild-type and *ataf1-1* plants showed similar expression patterns in response to ABA treatment. However, *ADH1* was highly expressed in *ATAF1-O1* plants at the 0 h time point and its transcripts were further increased by ABA treatment. Differently, *RD22* showed higher induction by ABA in the *ataf1-1* mutant than *ATAF1*-overexpression and wild-type plants (Supplementary information, Figure S3). The results suggest that *ATAF1* is involved in plant response to ABA by differentially regulating ABA/stress-responsive genes.

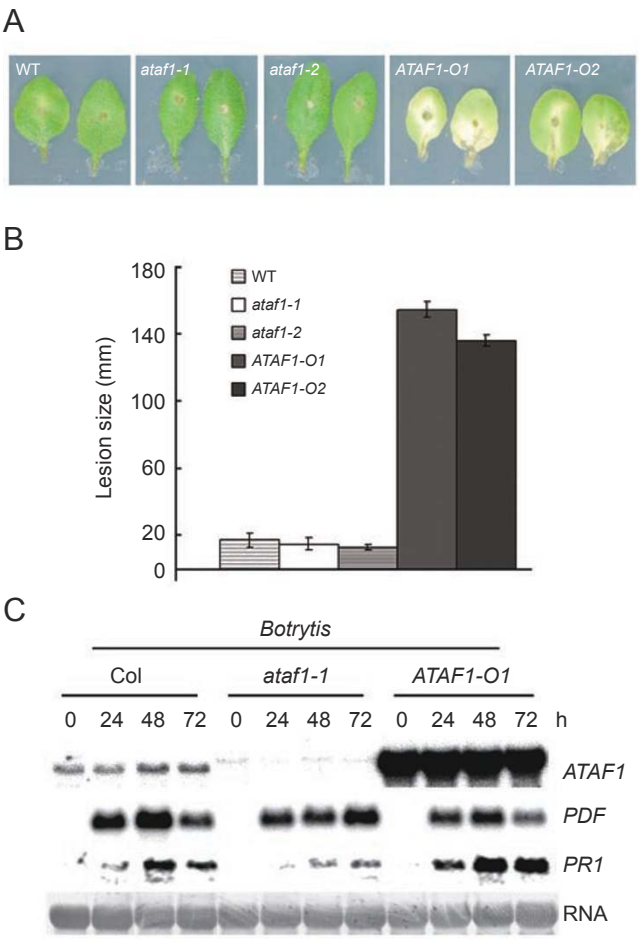

Figure 6 Responses of plants to *B. cinerea* infection. **(A)** Disease symptoms of detached leaves 5 days after inoculation with a 3-μl droplet of *B. cinerea* spores (10^5 spores/ml). **(B)** Lesion size was measured 5 days after inoculation. Date points represent average lesion size of measurements from 30 lesions. The experiments were repeated thrice. **(C)** Expression of *PR-1* and *PDF1.2* in response to *B. cinerea* infection. Each lane contains 15 μg total RNA.

Transgenic plants overexpressing ATAF1 are hypersensitive to high salinity

ATAF1 was found to be induced by salt; therefore, we asked whether this induction was correlated with plant-salinity response. As shown in Figure 5A, the germination rates (radicle emergence) of *ATAF1-O1* and *ATAF1-O2* were dramatically influenced by 100 mM NaCl compared with those of other plants. There was no difference of germination rates among all untreated plants (data not shown). Moreover, the greatest growth retardation and lesion were also found for *ATAF1-O1* and *ATAF1-O2* in the presence of 100 mM NaCl (Figure 5B). The percent of green seedlings was 32% and 35% for *ATAF1-O1* and *ATAF1-O2,* and nearly 90% for other plants (Figure 5B). Similar phenomena were also observed under a lower NaCl concentration (50 mM) (data not shown). These experiments indicate that high level of *ATAF1* transcripts sensitizes plants to high salinity.

To further address the regulatory function of *ATAF1* in salt response, expression patterns were checked for stress-responsive marker genes under NaCl treatment. Similar to ABA, NaCl induction led to high *ADH1* expression in *ATAF1-O1* plants, but did not notably change its expression in the *ataf1-1* mutant. Additionally, three downstream stress-signaling genes, *RD22*, *RD29A*, and *COR47*, showed similar expression patterns. Their expression, which was unaffected in the *ataf1-1* mutant, was reduced in *ATAF1-O1* plants at the early stage of salt stress (2 h). Interestingly, the transcripts of these three genes were slightly increased in *ATAF1-O1* plants at the late treatment stage (10 h). These results may suggest a feedback regulation of those genes affected by *ATAF1* under salt stress conditions (Supplementary information, Figure S4).

Altered ATAF1 expression levels affect plant susceptibility to the fungal pathogen B. cinerea

ATAF1 has been shown to be induced by JA, which is an important defense-related phytohormone implicated in the regulation of plant resistance to pathogens. In order to determine the function of *ATAF1* in biotic stress reactions, we checked the disease susceptibility of the different groups of plants inoculated with *B. cinerea,* a non-specific necrotrophic pathogen. Leaves from 4-week-old *ATAF1-O1* and *ATAF1-O2* plants, *ataf1-1* and *ataf1-2* mutants, and wild-type plants were drop-inoculated with the *B. cinerea* spore suspension, and disease progression was observed 7 days after inoculation. The examined disease symptoms included chlorosis, necrosis expansion, and tissue maceration surrounding the primary infection

sites. As shown in Figure 6A, *ATAF1-O* plants were more sensitive to *B. cinerea* infection in detached leaves than in *ataf1* mutants and wild-type plants, in which *B.cinerea* infection resulted in milder disease symptoms. Furthermore, we found that the leaf lesion size of *ATAF1-O* plants was larger than that of other plants (by 9- to 16-fold) (Figure 6B).

To define whether the role of *ATAF1* in biotic responses of plants was restricted in their reaction to a necrotrophic pathogen, we also tested its function by using the hemi-biotrophic pathogen *Pseudomonas syringae*. Both *ataf1-1* mutant and *ATAF1-O1* plants showed comparable disease symptoms in response to the avirulent bacterial strain (*P. syringae* pv *tomato* avirulent strain DC3000 carrying *avrRpm1*) and the virulent strain DC3000 in comparison with wild-type plants (Supplementary information, Figure S5).

We examined the expression of the defense-response markers *PDF1.2* (linked to JA and ET signalings), and *PR1* (linked to systemic acquired resistance (SAR)). As shown in Figure 6C, *PDF1.2* expression pattern was unaffected in the *ataf1-1* mutant and *ATAF1-O1* plants in response to *B. cinerea*, suggesting that the *ATAF1* gene functions via a resistance/response pathway that may be independent of *PDF1.2*. However, *ATAF1-O1* plants induced *PR1* expression more strongly than other plants, and the lowest expression of *PR1* was found in *ataf1-1* mutants (Figure 6C), revealing a negative correlation between *ATAF1* and plant disease resistance.

Transgenic plants overexpressing ATAF1 are hypersensitive to oxidative stress

Reactive oxygen intermediates (ROIs) have been implicated in signaling in response to both pathogens and abiotic stresses. In plant abiotic stresses, ROIs likely act as signals to induce reactive oxygen scavengers and other protective mechanisms, and as damaging agents that may contribute to stress injury in plants. Additionally, ROIs play important roles in plant-pathogen interactions such as cell wall strengthening and rapid host cell death. To test whether *ATAF1* was involved in ROI signaling, the sensitivity of plants to oxidative stress was assayed using rose bengal that generates ROIs in medium when exposed to light, and paraquat (methyl viologen) that increases the ROI formation by inhibiting electron transport in the reduction of NADP to NADPH during photosynthesis [13]. In the presence of 6 μM rose bengal, a complete block was found in the growth of *ATAF1-O1* and *ATAF1-O2* seedlings that were etiolated without any expanded green leaves (Figure 7). However, *ataf1-1*, *ataf1-2*, and control plants were minimally affected by this treatment, as these plants developed normally. Likewise, *ATAF1-O1* and *ATAF1-O2* seedlings showed more sensitivity than other seedlings when germinated on the 1/2 MS medium supplemented with 1 μM paraquat (Figure 7). These results suggest that *ATAF1*-mediated stress responses are likely related to ROI signaling pathways.

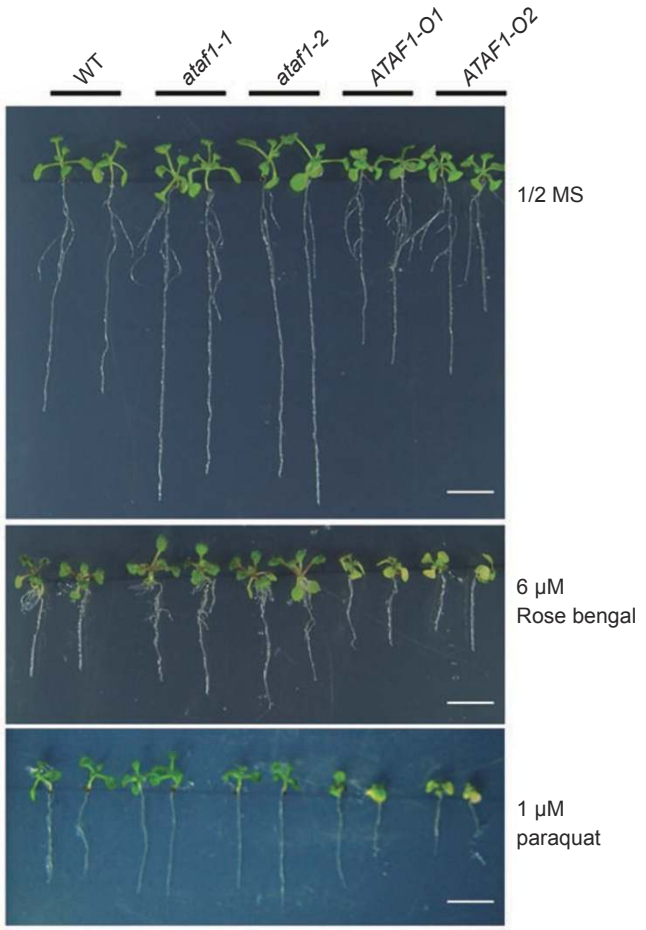

Figure 7 *ATAF1*-overexpressing plants are hypersensitive to oxidative stresses. Seedlings (5-day old) germinated on 1/2 MS medium were transferred to 1/2 MS medium with or without 6 μM rose bengal. Photographs were taken 5 days after treatment (middle panel). Young seedlings were compared between wild-type, *ataf1-1*, *ataf1-2*, *ATAF1-O1*, and *ATAF1-O2* plants in the presence of 1 μM paraquat (lower panel). The pictures were taken after 3 weeks. Bar = 1 cm.

Discussion

ATAF1 functions in ABA signaling and abiotic stress responses

ABA is an essential mediator in triggering plant responses to most of the common abiotic stresses, including drought, salinity, high temperature, oxidative stress, and cold [35, 36]. Nearly all ABA-deficient (*aba*) and

ABA-insensitive (*abi*) mutants exhibit salt insensitivity during germination and are susceptible to drought because of the impaired stomatal aperture regulation (e.g., *abi1* and *abi2*), while plants overexpressing the ABA-response pathway genes, such as *SDIR1*, *ABF3*, and *ABF4,* are tolerant to drought and hypersensitive to salt [33, 34, 37, 38]. *ATAF1* overexpression plants showed remarkably enhanced tolerance to drought but were hypersensitive to salt stress.

Stomatal closure is a key ABA-controlled process in dealing with water deficit conditions. Our results indicate that ATAF1 is involved in stomatal regulation. Its overexpression resulted in lower transpiration and enhanced drought tolerance. The stomatal openings of *ATAF1-O1* transgenic plants were smaller than those of wild-type plants under normal light growth conditions in soil. Furthermore, stomatal closure in the epidermis of *ATAF1-O1* plants was more sensitive than that of wild-type and *ataf1-1* plants after ABA treatment. *ATAF1-O1* plants also exhibited the most severe growth inhibition by ABA. Thus, our results suggest that ATAF1 may play a positive role in ABA-mediated guard cell control and regulation of drought response.

Recently, ATAF1 was described as a negative regulator in plant drought tolerance by phenotypically comparing wild-type plants and *ataf1* mutants [32]. However, here we found the opposite phenotypes of drought response. We have carefully read the previous paper and compared our experiments. We used the same Salk mutants to validate the documented function of ATAF1. Very surprisingly, we could not observe the same phenotype for these mutant lines as what was previously reported. In fact, we repeatedly observed that *ATAF1* overexpression lines, but not mutant plants, were more tolerant to soil-drought stress than controls. Such findings were also supported by other physiological experiments, including leaf water loss measurement and stomatal closure assay. Moreover, we found more rapid induction of some marker genes in the mutant plants compared with other assayed plants under drought stress, but the induction was dramatically diminished at late stage of the treatment. In contrast, *ATAF1-O1* plants had significant transcript accumulations of some stress-responsive markers at the late time point, demonstrating a positive correlation with the enhanced drought tolerance of these plants. Considering that the plant response to drought stress spans a relatively long time, high accumulation of stress-responsive genes at late stage of the stress may be a major contributor to plant drought tolerance.

ATAF1 functions in biotic stress

In this study, *ATAF1* enhanced susceptibility to infection by *B. cinerea*, but did not influence the plant response to *P. syringae* pv tomato. SAR is a mechanism of induced defense that confers long-lasting protection against a broad spectrum of microorganisms. Pathogen-triggered SAR process is dependent on the phytohormone SA. In contrast, plant pathogenesis caused by necrotrophic pathogens is closely related to JA and ET defense pathways [39]. *ATAF1* was found to rapidly respond to induction by MeJA, but not by SA (data not shown). To date, molecular and cellular mechanisms in plant-necrotrophic pathogen interactions remain largely elusive. However, ROIs are considered to be important molecules involved in plant disease reaction to necrophrophic pathogens such as *B. cinerea*. Elevated ROI levels can promote hypersensitive cell death. In one previous study, ROIs were reported to be induced by *B. cinerea* to accumulate to a high level, which may be toxic to host plants and consequently facilitate infection [40]. Conversely, reciprocal experiments indicated that chemical-mediated blockade of ROI generation decreased cell death in different plants challenged by pathogens [41, 42] and restricted necrosis development in *B. cinerea*-infected *Arabidopsis* [43]. The disease assay in our study showed that *ATAF1-O* plants displayed enhanced lesion formation after *B. cinerea* infection, indicating the negative role of *ATAF1* in plant defense to this necrotrophic fungus. Alternatively, *ATAF1* overexpression rendered plants hypersensitive to both the rose bengal and paraquat compounds, which promote the generation of reactive oxygen species. Considering the important roles of ROIs in necrotrophic disease processes, these findings suggest that *ATAF1* may sensitize the reaction of plant cells to ROIs and accordingly contribute to the enhancement of plant susceptibility after *B. cinerea* infection. In our study, more induction of *PR1* transcripts was found in *ATAF1-O1* plants infected with *B. cinerea*, implying that there was a *B. cinerea*-triggered hypersensitive response that was thought to positively correlate with *B. cinerea* growth [43]. In addition to pathogen challenge, abiotic stresses such as high salinity can elevate ROI levels in plants. The salt-hypersensitivity of *ATAF1-O* plants may also be a consequence of their enhanced sensitivity to ROIs.

Functional overlap between ATAF1 and other stress-regulatory NAC genes

In this study, mutant lines did not show distinct morphological variations as well as stress-related phenotypic changes when compared with control plants. Therefore, we speculate that there is likely functional redundancy among the *NAC* genes. Many *Arabidopsis* NAC members have been predicted to be stress-signaling related

(Table S1). Recent studies have demonstrated that several *NAC* genes were involved in plant abiotic stress responses, including *RD26* and its homologs *ANAC019* and *ANAC055*. These *NAC* genes are responsive to drought and high salinity, as well as plant hormones ABA and JA, with varied expression patterns. Overexpression of these *NAC* genes in plants increased the survival rate for plants grown in water-deficient soil [25]. In this study, *ATAF1* overexpression resulted in many stress-related physiological effects in plants. These effects were similar to the observations described for *RD26*, *ANAC019*, and *ANAC055*. *ATAF1* functionally resembles these characterized *NAC* genes, especially *RD26*. For example, both *ATAF1* and *RD26* were induced by exogenous ABA and by drought or high salinity in an ABA-independent manner, and their overexpression transgenic plants showed similar hypersensitivities to ABA. Obviously, functional overlap in stress responses exists among these *NAC* genes. The functional redundancy is likely responsible for the almost unchanged phenotype in the *ataf1* mutant lines.

In spite of the extensive functional similarities between *ATAF1* and other *NAC* homologs, the function of *ATAF1* is not completely identical to that of other *NAC* genes, including *RD26*. For example, transcript accumulation was observed for *RD26* in response to cold stress [44], but this was not found with *ATAF1* (data not shown). Recently, *OsNAC6,* a rice ortholog of *ATAF1*, was reported to be a positive contributor to disease resistance and salinity tolerance [29, 30]. This finding may indicate the functional discrepancies of *NAC* genes between monocotyledon and dicotyledon plants. In the biotic stress assay, we showed that *ATAF1* played a negative role in plant response to *B. cinerea,* resembling the function of *ATAF2* in plant pathogenesis. *ATAF2*, the closest *Arabidopsis* homolog of *ATAF1*, has been described as a repressor, which downregulated many pathogenesis-related genes, and consequently enhanced plant susceptibility to *Fusarium oxysporum* (a soil-borne fungal pathogen) in *ATAF2* overexpression lines. Both *ATAF1* and *ATAF2* can be induced by the wounding/pathogenesis-related phytohormone MeJA. Moreover, we repeatedly isolated ATAF2 as a protein partner of ATAF1 in yeast two-hybrid experiments (unpublished data). These findings suggest that these two proteins are functionally associated and redundant. Nevertheless, it seems that both *NAC* genes also maintain their unique features. For example, unlike *ATAF1, ATAF2* cannot be induced by ABA [28]. Taken together, many other *NAC* homologs could contribute to the plant abiotic and biotic stress responses. Plants have evolved multiple functionally related NAC proteins that may be beneficial for their survival, given the fact that they are frequently challenged by extreme circumstances.

ATAF1 is an important gene with diverse biological functions

Based on our experimental data, *ATAF1-O* transgenic lines showed many evident growth phenotypic changes such as delayed flowering time and sterility at early flowering stage. As a regulator in ABA signaling, ATAF1 can activate ABA-responsive gene expression and result in ABA sensitivity. Similarly, other ABA signaling-related transcription factors such as ABF4 and ABI3 were also described to produce the phenotype of delayed flowering time in their overexpression lines [34, 45]. Thus, we hypothesize that the ABA signaling pathway transduced by ATAF1 is likely involved in the flowering process. However, the intrinsic molecular link between ATAF1 and flowering control requires further elucidation.

In conclusion, ATAF1 appears to be a multiple-function transcription factor not only participating in abiotic and biotic stress responses, but also potentially regulating plant developmental processes.

Materials and Methods

Plant material and growth conditions

Arabidopsis accession Columbia (Col-0) was used for *ATAF1* cDNA cloning, gene transformation, and phenotypic control in this study. Salk067648 and Salk057618 (*ATAF1* T-DNA insertion mutants), as well as *aba2*, were obtained from the *Arabidopsis* Biological Resource Center. Plant growth conditions were as described previously [17].

Gene expression analysis

Seedlings (3-week old) were used for the following treatments. Dehydration treatment was carried out at room temperature with 75% humility for seedlings harvested from MS plates and placed on filter paper. Other treatments were carried out for seedlings grown on MS with 250 mM NaCl, 50 μM ABA, and 50 μM MeJA. For gene expression analysis, total RNA was isolated from nitrogen-frozen seedlings using the hot-phenol method [21]. Totally, 15 μg RNA was applied in each lane in RNA gel analysis. Hybridizations were performed with the α-^{32}p-labeled *ATAF1*-specific probe derived from its C-terminal region.

Constructs for plant transformation

To create the transgenic construct for overexpressing *ATAF1* in Arabidopsis, the cDNA was amplified by RT-PCR with the primers (5′-GGATCC TAA TGT CAG AAT TAT TAC AGT TG-3′ and 5′-ACTAGT CTA GTA AGG CTT CTG CAT G-3′), and cloned into the site of *Bam*HI and *Spe*I in the vector pBA002Myc. In this construct, N-terminal *ATAF1* was fused in frame to the C-terminal 6× *Myc* tag and the tagged protein was driven by the CaMV *35S* promoter. The transformants were selected by screening seedlings with resistance against Basta (8 μg/ml) on an MS plate. *Myc-ATAF1* expression level was checked by RT-PCR reac-

tion and western blot with an anti-Myc monoclonal antibody.

Abiotic stress assays

Seeds were sterilized and suspended in 0.15% agar and plated on MS medium (including vitamins, M0222.0050, Duchefa Biochemie. B.V., The Netherlands) supplemented with 1.5% sucrose, then stratified in darkness at 4 ºC for 3 days and transferred to growth chambers with the same environmental conditions described above. To conduct germination rate assay, seeds were plated on MS medium without or with ABA (0 μM, 0.25 μM, 0.5 μM, and 1 μM), NaCl (50 mM, 100 mM, and 150 mM), or 1 μM paraquat. To conduct root growth assay, seeds sown on MS plates were stratified for 3 days at 4 ºC and were vertically grown for 4 days under normal conditions, and then seedlings were transferred to vertical square MS plates with or without 10 μM ABA. For drought stress, 10-day-old seedlings were transferred from MS plates to water-saturated soil for 7 days, and then water was withheld until plants showed evident drought-stressed phenotypes. For leaf water loss assay, rosette leaves excised from plants grown in soil were put on filter paper for air-dry treatment. The leaf weight was measured at a series of time points. For rose bengal treatment, 5-day-old seedlings germinated on 1/2 MS medium were transferred to 1/2 medium with 6 μM rose bengal.

Effects of ABA on stomatal aperture of plants. Epidermal peels from 5-week-old plants were kept for 12 h in the dark, incubated under light in buffer (10 mM KCl, 7.5 mM potassium iminodiacetate, and 10 mM MES-KOH, pH 6.15) at 20 °C for 3 h, and then treated with 0 and 50 μM ABA for 2 h before aperture measurements (measured with a ZEISS microscope).

Fungal culture and plant inoculation

B. cinerea was grown on potato dextrose agar for 7-10 days at 24 °C with a 12-h photoperiod before spore collection. Spores were collected by flooding the dish and filtering the suspension with gauze. The conidia were resuspended with potato dextrose broth, and the concentration was adjusted to 10^5 spores/ml. Whole plants or rosette leaves were placed in dishes containing 0.8% agar and inoculations were performed with a 3 μl droplet of *B. cinerea* spores. After inoculation, plants were kept under a transparent cover to maintain high humidity and transferred to a growth chamber with 21 °C day and 18 °C night temperatures.

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