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The *Arabidopsis* homologs of CCR4-associated factor 1 show mRNA deadenylation activity and play a role in plant defence responses

Wenxing Liang[1, 2, *], Changbao Li[1, 3, *], Fang Liu[1, 2], Hongling Jiang[1], Shuyu Li[1, 2], Jiaqiang Sun[1], Xiaoyan Wu[1], Chuanyou Li[1]

[1]State Key Laboratory of Plant Genomics, National Centre for Plant Gene Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China; [2]Graduate School of Chinese Academy of Sciences, Beijing 100039, China; [3]The State Key Laboratory of Crop Biology, Agronomy College, Shandong Agricultural University, Taian 271018, China

Messenger RNA (mRNA) turnover in eukaryotic cells begins with shortening of the poly (A) tail at the 3′ end, a process called deadenylation. In yeast, the deadenylation reaction is predominantly mediated by CCR4 and CCR4-associated factor 1 (CAF1), two components of the well-characterised protein complex named CCR4-NOT. We report here that *AtCAF1a* and *AtCAF1b*, putative Arabidopsis homologs of the yeast *CAF1* gene, partially complement the growth defect of the yeast *caf1* mutant in the presence of caffeine or at high temperatures. The expression of *AtCAF1a* and *AtCAF1b* is induced by multiple stress-related hormones and stimuli. Both AtCAF1a and AtCAF1b show deadenylation activity *in vitro* and point mutations in the predicted active sites disrupt this activity. T-DNA insertion mutants disrupting the expression of *AtCAF1a* and/or *AtCAF1b* are defective in deadenylation of stress-related mRNAs, indicating that the two AtCAF1 proteins are involved in regulated mRNA deadenylation *in vivo*. Interestingly, the single and double mutants of *AtCAF1a* and *AtCAF1b* show reduced expression of pathogenesis-related (*PR*) genes *PR1* and *PR2* and are more susceptible to *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) infection, whereas transgenic plants over-expressing *AtCAF1a* show elevated expression of *PR1* and *PR2* and increased resistance to the same pathogen. Our results suggest roles of the AtCAF1 proteins in regulated mRNA deadenylation and defence responses to pathogen infections.

***Keywords*:** CCR4-associated factor 1 (CAF1), mRNA deadenylation, poly (A) tails, defence response, *Arabidopsis thaliana*
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Introduction

Proper regulation of gene expression is crucial for essentially all biological processes [1]. Although gene expression could be regulated at different steps, controlled RNA decay is an essential process that allows rapid changes in a cell's gene expression profile, especially in response to environmental signals. In both mammalian and yeast cells, messenger RNA (mRNA) degradation usually begins with the shortening of the poly (A) tail at the 3′ end of the mRNA (deadenylation) by a variety of deadenylases [2]. The deadenylated mRNA can then enter one of two decay pathways. In the first pathway, a complex consisting of the decapping enzymes DCP1 and DCP2 recognises the deadenylated mRNA and cleaves the 5′ cap, after which the XRN1 exoribonuclease hydrolyses the RNA body from its 5′ end. In the other pathway, deadenylated mRNAs can be degraded from the 3′ end by the cytoplasmic exosome complex [2]. In both cases, however, deadenylation is the initial and probably the rate-limiting step of mRNA turnover. Accumulating evidence indicates that, at least in yeast, deadenylation represents a central control point of mRNA abundance [3].

Three enzyme complexes, CCR4-NOT [4], PAN2-PAN3 [5, 6] and PARN [7-9], have been identified as mRNA deadenylases in eukaryotic cells. CCR4-associat-

*These two authors contributed equally to this work.
Correspondence: Chuanyou Li
Tel: +86-10-64865313; Fax: 86-10-64873428
E-mail: cyli@genetics.ac.cn
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ed factor 1 (CAF1) is a subunit of the CCR4-NOT complex, which is an evolutionarily conserved protein complex. The CCR4-NOT complex is involved in the control of diverse aspects of transcription and mRNA metabolism, including mRNA deadenylation and its subsequent degradation [4, 10]. In yeast, the complex consists of at least nine core subunits, including CCR4, CAF1, CAF40, CAF130, and five NOT proteins (NOT1-NOT5) [4, 11-13]. It has been shown that the CCR4 and CAF1 proteins are associated physically with each other and serve as the major cytoplasmic deadenylases in yeast cells [14-16]. However, the biochemical and physiological functions of CAF1 proteins are not clearly established. CAF1 proteins belong to the DEDDh subgroup of the DEDD family of nucleases, which requires three aspartates (D), a glutamate (E), and a nearby histidine for activity (DEDDh) [17, 18]. Although the yeast CAF1 shows deadenylase activity *in vitro* [17, 19], the role of this activity *in vivo* is unclear. Inactivation of the predicted key catalytic active sites of yeast CAF1 did not affect *in vivo* deadenylation function [20]. Although a *caf1* deletion reduces the rate of *in vivo* poly (A) shortening [14, 17], over-expression of *CCR4* can complement this defect [16]. These data support the notion that CCR4 is the principal deadenylase of the yeast CCR4-NOT complex, and a major role of CAF1 is to link CCR4 to the remainder of the CCR4-NOT complex [12]. However, a recent comprehensive structure-function analysis provided evidence showing that, in addition to its contact with CCR4, the yeast CAF1, like its animal counterparts, plays important roles in mRNA deadenylation [21].

The important role of CAF1 has also been exemplified by phenotypic analyses of loss-of-function mutants of CAF1 in yeast and animals. For example, yeast *caf1* mutants are hypersensitive to high temperature and caffeine [22, 23]. Mutant male mice that lack CAF1 function are sterile [24]. Loss-of-function of CAF1 in *Caenorhabditis elegans* causes early embryonic and larval lethality [25].

Relatively less is known about the biochemical and physiological roles of the *CAF1* genes in plants. One indication comes from the observation that over-expression of the pepper *CAF1* gene in tomato plants confers abnormal plant growth and altered pathogen resistance [26], suggesting roles for CAF1 in both plant development and defence responses. It was recently shown that an *Arabidopsis CAF1*-like gene is rapidly induced after mechanical wounding [27].

Here, we show that AtCAF1a and AtCAF1b, putative *Arabidopsis* homologs of the yeast CAF1 protein, exhibit deadenylation activity and act redundantly in regulated deadenylation of stress-responsive mRNAs. We also provide evidence showing that over-expression or reduced expression of *AtCAF1a* and *AtCAF1b* affects plant defence responses to pathogen infection.

Results

Hormone- and stress-induced expression of AtCAF1a and AtCAF1b

AtCAF1a (At3g44260) and *AtCAF1b* (At5g22250), which encode putative *Arabidopsis* homologs of the yeast CAF1 protein, were identified as jasmonic acid (JA)-inducible genes in our microarray analyses using the *Arabidopsis* whole genome chip (Affymetrix) [28]. Further RNA gel blot analysis indicated that, in addition to JA, the expression of the two genes was also strongly and transiently induced by abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene), salicylic acid (SA), mechanical wounding, and pathogen (*Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000)) infection. As shown in Figure 1, the transcript levels of *AtCAF1a* and *AtCAF1b* peaked 15 min after these treatments and decreased rapidly thereafter. The quick and transient induction of *AtCAF1a* and *AtCAF1b* expression by these stress-related hormones and stimuli suggested that these two genes might be associated with plant responses to biotic or abiotic stresses.

Reverse transcription (RT)-PCR analyses indicated that both *AtCAF1a* and *AtCAF1b* were expressed throughout the wild-type plant organs, including roots, stems, leaves, flowers and siliques (Figure 2A). The highest expression level of the two *AtCAF1* genes was found in leaves, followed by roots and siliques, with relatively low expression in stems and flowers (Figure 2A).

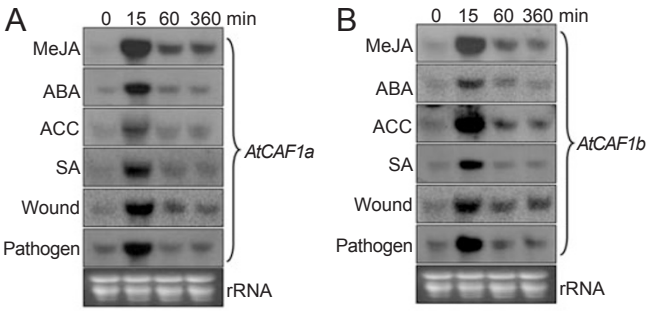

Figure 1 RNA gel blot analysis of *AtCAF1a* **(A)** and *AtCAF1b* **(B)** expression in response to different treatments. Two-week-old Col-0 plants were treated with 50 μM MeJA, 20 μM ABA, 50 μM ACC and 20 μM SA, wounded with a haemostat (Wound), or infected with *Pst* DC3000 (Pathogen). Leaves were harvested for RNA extraction at the indicated times after treatment. Each lane was loaded with 20 μg of total RNA. Ethidium bromide staining of rRNA served as a loading control.

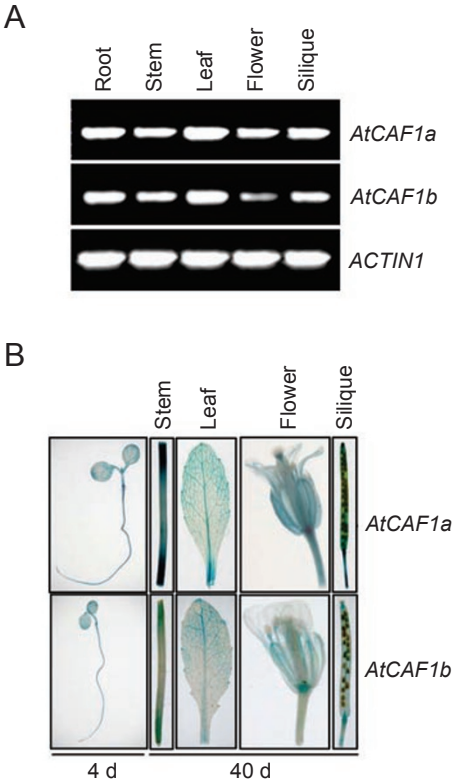

Figure 2 Spatial expression patterns of the *AtCAF1* genes. **(A)** Expression of the two *AtCAF1* genes in different organs revealed by RT-PCR analysis. Amplification of the *ACTIN1* gene served as a control. **(B)** Promoter-driven GUS expression patterns of the two *AtCAF1* genes. Shown are GUS staining of 4-d-old seedlings and organs from 40-d-old plants.

The tissue-specific expression patterns of these genes were also investigated with transgenic plants expressing the glucuronidase (GUS) reporter under the control of the *AtCAF1* promoters. GUS activities were detected in stems, leaves, floral parts and siliques (Figure 2B), confirming that the *AtCAF1* genes are ubiquitously expressed in different organs.

AtCAF1 genes partially complement the phenotypes of the yeast caf1 mutant

Two of the most prominent phenotypes of the yeast *caf1* mutants are their temperature and caffeine sensitivities [22, 23]. To examine the functional properties of the *AtCAF1* genes, the two genes were heterologously expressed in the yeast *caf1* mutant strain KY803-c1. As shown in Figure 3, the constructs pYES2-*AtCAF1a* and pYES2-*AtCAF1b* partially restored colony formation of the *caf1* mutant at 37 °C or on a YD plate containing 5 mM caffeine, but the vector control could not restore colony formation, indicating that AtCAF1a and AtCAF1b represent the *Arabidopsis* homologs of the yeast CAF1 protein.

AtCAF1 proteins exhibit 3′-5′ exonuclease activity in vitro

The high sequence similarity of AtCAF1 proteins to those from other eukaryotic organisms, and the conservation of the DEDDh amino acids required for deadenylation activity (Supplementary information, Figure S1), suggested that they may function as deadenylases. To test the role of AtCAF1a in deadenylation, a recombinant His-AtCAF1a fusion protein was expressed in *Escherichia coli*. The purified His-AtCAF1a fusion protein was incubated with poly (A) as described in Materials and Methods. Time-dependent shortening of the 5′-labelled poly (A) indicated that AtCAF1a is a functional 3′-5′ exonuclease

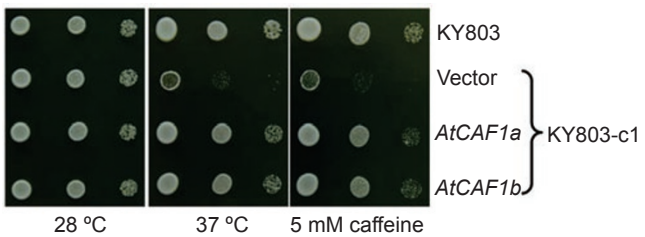

Figure 3 Effects of heterologous expression of *AtCAF1* genes on the growth of the yeast *caf1* mutant. Yeast strains KY803 and KY803-c1 with pYES2-*AtCAF1a*, pYES2-*AtCAF1b* or with pYES2 vector were grown on a YD plate at 28 °C, on a YD plate at 37 °C or on a YD plate with 5 mM caffeine. In each panel, from left to right, 20-fold fewer cells were plated in each column.

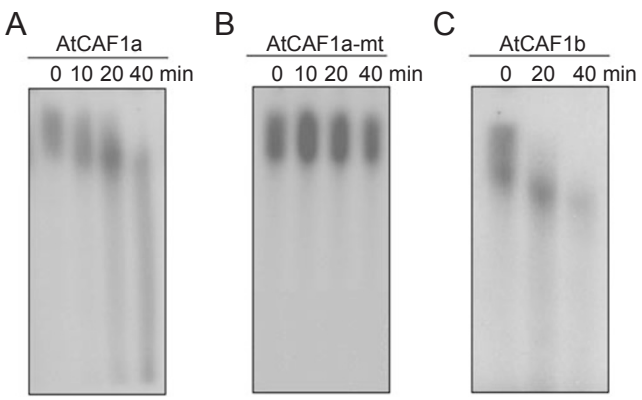

Figure 4 Nuclease activities of AtCAF1 proteins. **(A)** AtCAF1a shows 3′-5′ exonuclease activity *in vitro*. **(B)** AtCAF1a-mt does not show exonuclease activity. **(C)** AtCAF1b shows exonuclease activity. For all panels, purified His-AtCAF1 fusion proteins were incubated with 5′-labelled poly (A) for the indicated time periods and the reaction mixtures were resolved on polyacrylamide gels.

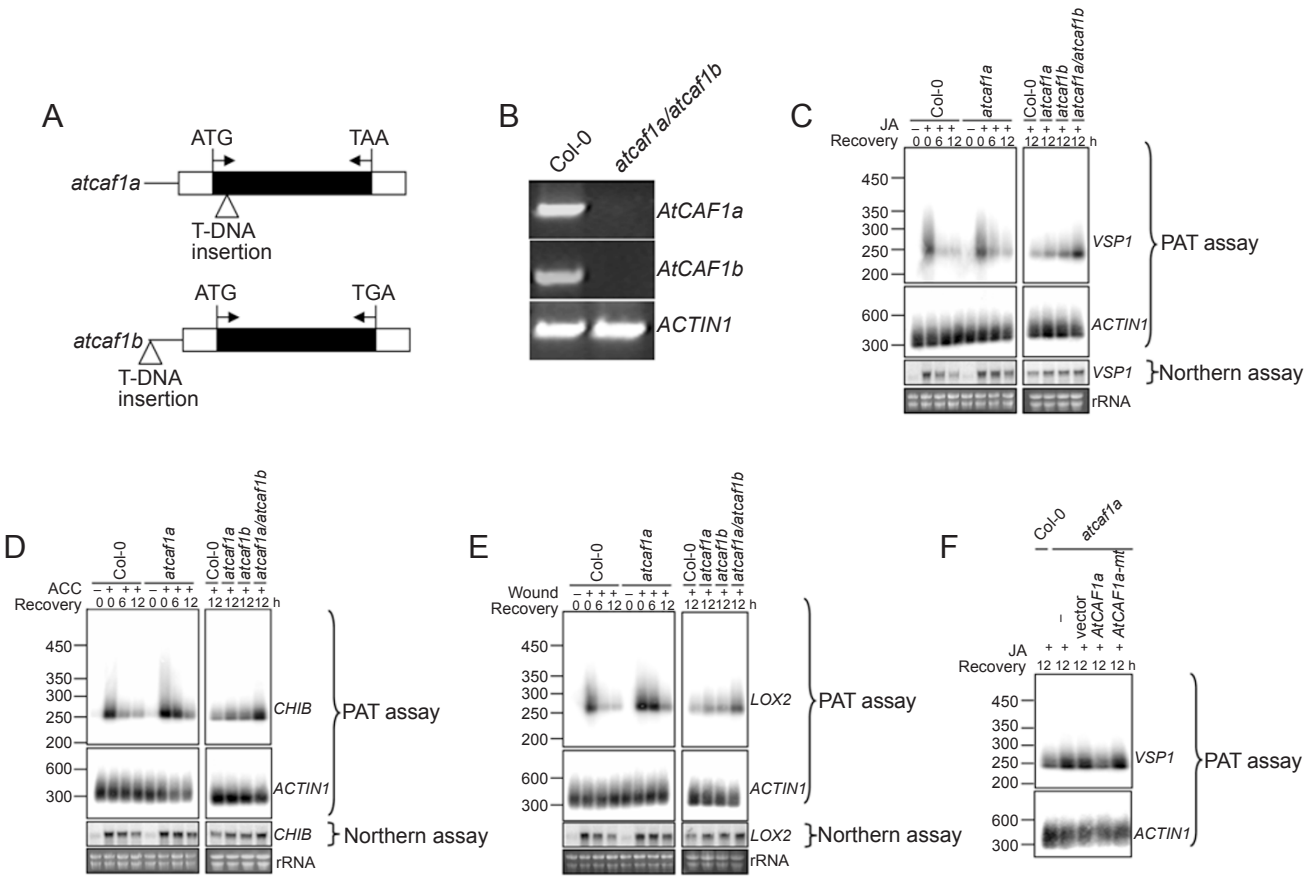

Figure 5 Loss-of-function of *AtCAF1a* or *AtCAF1b* leads to defective mRNA deadenylation. **(A)** Schemes of T-DNA insertion lines of each locus. Gray boxes indicate open reading frames (ORFs), and white boxes indicate untranslated regions (UTRs). Arrows indicate the relative positions of the primers used for RT-PCR analysis to check the expression of *AtCAF1a* or *AtCAF1b* in T-DNA insertion lines. Locus numbers, open reading frames, and T-DNA insertion sites are shown. **(B)** *AtCAF1a* and *AtCAF1b* expression in wild type and *atcaf1a/atcaf1b* revealed by RT-PCR analysis. Total RNAs from 2-week-old seedlings of the indicated genotypes were used as a template. Amplification of the *ACTIN1* gene serves as a control. **(C)** PAT assays measured the deadenylation of *VSP1* mRNA. Two-week-old wild-type and *atcaf1a* seedlings were not treated (–) or were treated with 50 μM MeJA for 6 h (+). Plant tissues were then collected for RNA extraction at the indicated times (h) after MeJA treatment. *atcaf1b* and *atcaf1a*/*atcaf1b* mutants were only harvested 12 h after JA treatment. RNA gel blot analysis was performed to show the actual expression levels of *VSP1*. **(D)** PAT assays measured the deadenylation of *CHIB* mRNA. Two-week-old wild-type and *atcaf1a* seedlings were not treated (–) or were treated with ACC for 24 h (+). Plant tissues were then collected for RNA extraction at the indicated times (h) after ACC treatment. *atcaf1b* and *atcaf1a*/*atcaf1b* mutants were only harvested 12 h after ACC treatment. RNA gel blot analysis was performed to show the actual expression levels of *CHIB*. **(E)** PAT assays measured the deadenylation of *LOX2* mRNA. Two-week-old wild-type and *atcaf1a* seedlings were not wounded (–) or were wounded with a haemostat and incubated for 3 h (+) in a growth chamber. Plant tissues were then collected for RNA extraction at the indicated times (h). *atcaf1b* and *atcaf1a*/*atcaf1b* mutants were only harvested after 12 h. RNA gel blot analysis was performed to show the actual expression levels of *LOX2*. **(F)** Deadenylation of *VSP1* mRNA *in vivo* is dependent on the deadenylase activity of AtCAF1a. Empty plasmid vector, wild-type *AtCAF1* or *AtCAF1a-mt* was expressed in *atcaf1a* plants. Plants were harvested 12 h after a 6-h treatment with MeJA treatment. The deadenylation of the *Arabidopsis ACTIN1* gene, which is not induced by stress treatments, was used as a control in all of the PAT assays. The positions of DNA size markers (in bp) are indicated on the left.

(Figure 4A). AtCAF1a-mt, which contains point mutations in the conserved putative catalytic residues D47/A and E49/A, completely abolished the exonuclease activity (Figure 4B). These results showed that AtCAF1a is a functional deadenylase *in vitro* and that its conserved DEDDh domain is required for its enzymatic activity. Similar exonuclease activity was also observed with AtCAF1b (Figure 4C).

AtCAF1a and AtCAF1b are required for regulated mRNA deadenylation

To examine whether *AtCAF1a* plays a role in mRNA deadenylation *in vivo*, we determined whether *atcaf1a*, a T-DNA insertion mutant that disrupts the expression of *AtCAF1a* (Figure 5A and 5B), shows defective deadenylation activity. The finding that the expression of *AtCAF1a* was transiently induced by JA prompted us to investigate whether the *atcaf1a* mutation affects the decay of the *vegetative storage protein 1 (VSP1)* mRNA, which is widely used as a JA-inducible marker gene in *Arabidopsis* [29]. For this experiment, wild-type and *atcaf1a* plants were treated with methyl jasmonate (MeJA) for 6 h and then transferred to MS media for the indicated time periods (Figure 5C). Thus, a pool of newly transcribed mRNAs was produced and a transcriptional pulse-chase experiment to access the time course of deadenylation was possible. Poly (A) tail length (PAT) assays indicated that, compared with that in wild type, the deadenylation rate of *VSP1* mRNA was reduced in the *atcaf1a* mutant (Figure 5C). Similarly, the deadenylation rate of *VSP1* mRNA was also decreased in *atcaf1b*, a T-DNA insertion mutant that affects the expression of *AtCAF1b* (Figure 5A-5C). Furthermore, the impaired deadenylation rate of *VSP1* mRNA was more severe in the *atcaf1a/atcaf1b* double mutant than those in the single mutants or in wild type (Figure 5B and 5C). Consistent with their defective deadenylation activities, our RNA gel blot analysis indicated that the actual accumulation levels of *VSP1* mRNA in the single and double mutants were higher than those in wild type (Figure 5C). These results suggested that *AtCAF1a* and *AtCAF1b* act redundantly in *VSP1* mRNA deadenylation.

The single mutants, *atcaf1a* and *atcaf1b*, and the double mutant were also compared with wild type for their deadenylation rates of the stress-related genes *CHITINASE B* (*CHIB*) and *LIPOXYGENASE2* (*LOX2*). The ethylene- and JA-responsive *CHIB* encodes a basic chitinase with antimicrobial properties [30, 31]. *LOX2* encodes a key enzyme in the octadecanoid pathway leading to JA biosynthesis [32] and is extensively used as a marker for wound responses. Results from the PAT assays showed that the mutants also showed substantially reduced deadenylation rates of *CHIB* (Figure 5D) and *LOX2* (Figure 5E) mRNAs. In addition, RNA gel blot analyses indicated that the defective deadenylation capacities in these mutants led to increased mRNA levels of *CHIB* and *LOX2* (Figure 5D and 5E). These observations indicated that *AtCAF1a* and *AtCAF1b* are required for regulated deadenylation of a broad spectrum of stress-responsive mRNAs. In contrast, our parallel experiments indicated that *AtCAF1a* and *AtCAF1b* have little effect on the deadenylation of the *Arabidopsis ACTIN1* gene (Figure 5C-5E), which is usually not induced by stresses.

Deadenylase activity of AtCAF1a is crucial for regulated mRNA deadenylation in vivo

To determine whether the exonuclease activity of AtCAF1a is required for deadenylation of *VSP1* mRNA, we introduced *AtCAF1a* or *AtCAF1a-mt*, a mutant version of *AtCAF1a*, into the *atcaf1a* mutant (Figure 6F and 6G). As described above, *AtCAF1a-mt* contains two missense substitutions that inactivate the deadenylase activity of AtCAF1a (Figure 4B). PAT assays indicated that *AtCAF1a* rescued the *atcaf1a* defect in *VSP1* mRNA deadenylation, but the empty vector and *AtCAF1a-mt* did not rescue the defect (Figure 5F). Similarly, *AtCAF1a* also rescued the *atcaf1a* defect in mRNA deadenylation of *CHIB* and *LOX2* (data not shown). These results provided evidence that the enzymatic activity of AtCAF1a is essential for regulated deadenylation of mRNAs *in vivo*.

Over- and under-expression of AtCAF1a or AtCAF1b affect plant responses to pathogen infection

The expression of *AtCAF1a* and *AtCAF1b* was upregulated after infection by *Pst* DC3000 (Figure 1). In addition, the expression levels of *AtCAF1a* or *AtCAF1b* affected the deadenylation of several stress-responsive mRNAs. These two findings prompted us to investigate whether over- or under-expression of these genes result in altered responses of plants to pathogen infection. To this end, we generated transgenic plants showing increased expression of *AtCAF1a* under the control of the CaMV 35S promoter (Figure 6A and 6B). The *AtCAF1a* over-expression line (all *AtCAF1a* over-expression lines showed similar results; thus, only data obtained with one line are shown below), together with the single and double mutants of *AtCAF1a* and *AtCAF1b*, were compared with wild type for their responses to *Pst* DC3000 infection. Quantification of pathogen growth indicated that, while the *AtCAF1a* over-expression line showed significantly increased resistance to *Pst* DC3000, the single and double mutants were more susceptible to this pathogen (Figure 6C).

In agreement with the performances of the different genotypes to pathogen infection, our RT-PCR and quantitative real-time PCR analyses indicated that, compared with wild-type plants, the *AtCAF1a* over-expression plants showed constitutively higher expression of the pathogenesis-related (*PR*) genes *PR1* and *PR2*. In contrast, the single and double mutants of *AtCAF1a* and *AtCAF1b* showed reduced expression levels of *PR1* and *PR2* (Figure 6A and 6B). Given that both AtCAF1a and AtCAF1b show deadenylation activity, we tested whether

the two proteins act directly on the poly (A) tails of *PR1* and *PR2* transcripts. Our PAT assays indicated that the poly (A) tail levels of *PR1* and *PR2* in *AtCAF1a* overexpression lines were significantly higher than those in wild-type plants. In the double-mutant plants, however, the poly (A) tail levels of *PR1* and *PR2* mRNAs were much lower than those in wild-type and single-mutant plants (Figure 6D and 6E). These results are the opposite of what would be predicted if AtCAF1 proteins act directly on the deadenylation of *PR1* and *PR2*. Together, our data suggest that, even though AtCAF1a and AtCAF1b are functional deadenylases, they do not act directly on the poly (A) tails of *PR1* and *PR2*.

Deadenylase activity of AtCAF1a is essential for the elevated expression of PR1 and PR2

To test whether the AtCAF1a enzymatic activity is required for *PR1* and *PR2* expression, we analysed the expression levels of *PR1* and *PR2* in *atcaf1a* mutants over-expressing *AtCAF1a* or the above-described *AtCAF1a-mt*. Both the wild type and the mutant version of *AtCAF1a* were expressed at nearly the same level in the *atcaf1a* mutant background (Figure 6F and 6G). Overexpression of wild-type *AtCAF1a* increased the expres-

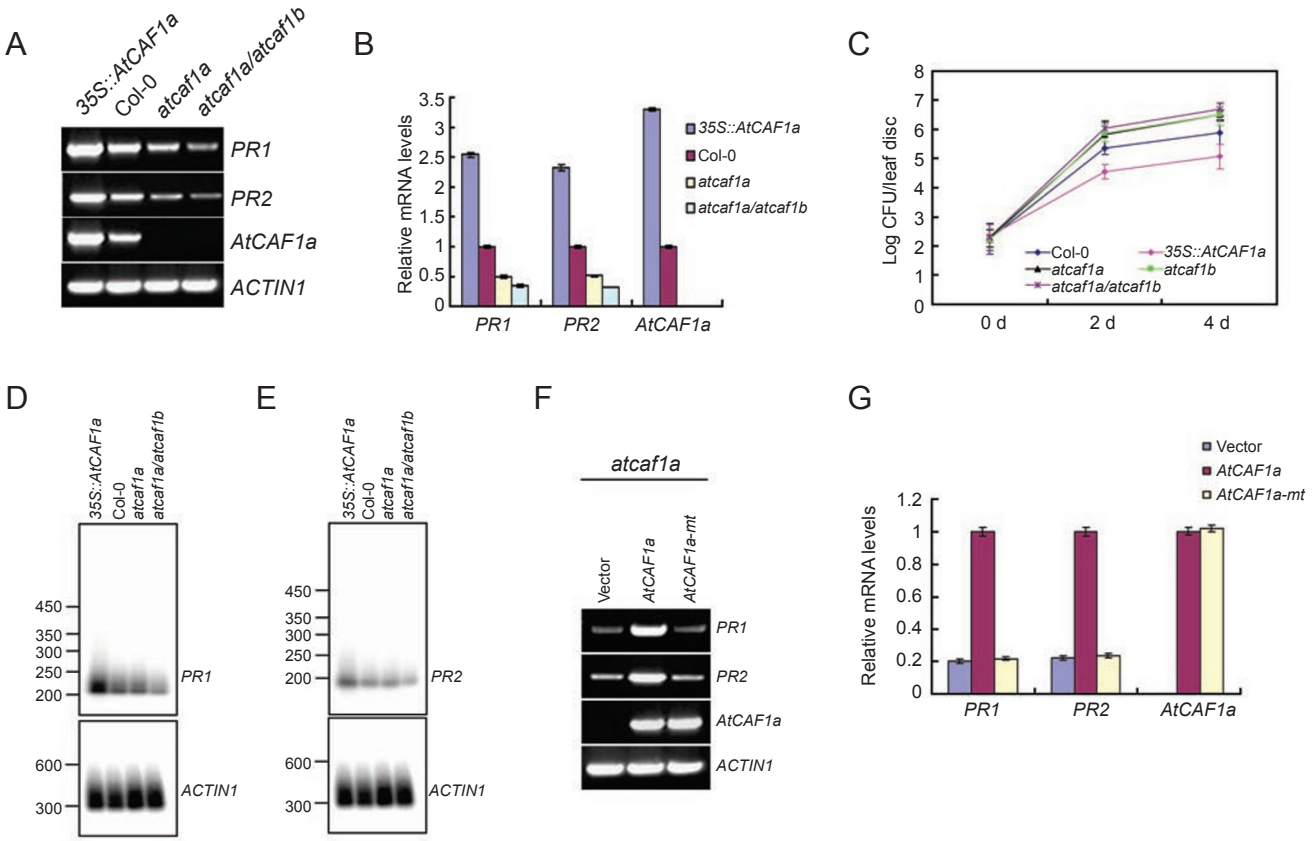

Figure 6 AtCAF1a and AtCAF1b affect plant response to *Pst* DC3000. **(A)** Steady-state expression levels of *PR1* and *PR2* in the indicated genotypes revealed by RT-PCR assays. Amplification of the *ACTIN1* gene serves as a control. **(B)** Steady-state expression levels of *PR1* and *PR2* in the indicated genotypes measured by qRT-PCR assays. Transcript levels of *PR1* and *PR2* were normalised to the expression of the *ACTIN1* gene measured in the same RNA samples. Data are mean ± SD of three independent experiments. **(C)** Bacterial growth in wild-type, *35S::AtCAF1a, atcaf1a*, *atcaf1b*, and *atcaf1a*/*atcaf1b* plants. The number of colony-forming units (CFU) per leaf disk was determined 0, 2, and 4 days after infiltration. Data are mean ± SD of three replicates. **(D)** Deadenylation of *PR1* mRNA in the indicated genotypes as revealed by PAT assay. **(E)** Deadenylation of *PR2* mRNA in the indicated genotypes as revealed by PAT assay. For (D) and (E), 2-week-old seedlings grown under normal conditions were used for RNA extraction. Deadenylation of the *ACTIN1* gene in the same RNA samples served as a control. **(F)** Expression levels of *PR1* and *PR2* under normal conditions in the indicated genotypes revealed by RT-PCR assays. Amplification of the *ACTIN1* gene served as a control. **(G)** Expression levels of *PR1* and *PR2* under normal conditions in the indicated genotypes as measured in qRT-PCR assays. Transcript levels of *PR1* and *PR2* were normalised to the expression of the *ACTIN1* gene measured in the same RNA samples. Data are mean ± SD of three independent experiments.

sion levels of *PR1* and *PR2*, but over-expression of the mutant did not (Figure 6F and 6G). These results indicate that the enzymatic activity of AtCAF1a is important for the elevated expression of *PR1* and *PR2*.

Discussion

In yeast (*Saccharomyces cerevisiae*), the CAF1 protein functions as one of the nine components of the CCR4-NOT complex [4, 10]. Our sequence analysis and yeast two-hybrid assays revealed that the genome of *Arabidopsis* contains homolog(s) for all of the major components of the yeast CCR4-NOT complex except CAF130 (data not shown). We demonstrate herein that several stress-related hormones and stress stimuli (mechanical wounding and pathogen infection) induce the expression of *AtCAF1a* and *AtCAF1b*, putative *Arabidopsis* homologs of the yeast *CAF1* gene (Figure 1). Both AtCAF1a and AtCAF1b partially complement the phenotypes of the yeast *caf1* mutant and exhibit 3′-5′ exonuclease activity *in vitro* (Figure 4), suggesting that they may act as functional deadenylases *in vivo*. We further explored the physiological significance of the deadenylation activities of the AtCAF1 proteins in mRNA degradation *in vivo*. Stress-related hormones and environmental stresses induce the expression of many defence-related mRNAs, and fulfilment of these signal transduction pathways requires the removal of earlier products of gene expression. Although some of these events occur at the protein level, including the rapid proteolysis of the AUX/IAA proteins following auxin binding to the TIR1 F-box protein [33, 34], some occur at the RNA level. It is well known that JA treatment leads to increased production and stabilisation of *VSP1* mRNA [29]; recovery from JA treatment, however, results in deadenylation and decay of *VSP1* mRNA. Our data indicate that AtCAF1a and AtCAF1b redundantly function in regulating deadenylation of *VSP1* and other stress-responsive mRNAs. Importantly, although the RNase activity is not required for the *in vivo* function of the yeast CAF1 protein [20], mutation in the putative catalytic residues of AtCAF1a, which abolishes its exonuclease activity *in vitro*, impairs the ability of mRNA deadenylation. These data support the hypothesis that the exonuclease activity of AtCAF1a is crucial for *in vivo* deadenylation of mRNAs.

Of interest is our findings that the expression of the *AtCAF1* genes was induced by ABA, ACC, SA, mechanical wounding, and pathogen infection, in addition to their induction by MeJA (Figure 1). Induction of *AtCAF1* genes by versatile stress-related hormones and stimuli suggests that *AtCAF1a* and *AtCAF1b* may play a general role in the deadenylation of a wide range of mRNAs, rather than specifically acting on a particular stress-response pathway. It is reasonable to speculate that, whenever there is a sudden increase in mRNA production, *AtCAF1* genes are induced at the transcriptional level and then perform their deadenylation role on mRNAs that need to be degraded.

Emerging evidence suggested important roles for plant *CAF1* genes in defence responses against abiotic or biotic stresses. For example, over-expression of the pepper *CAF1* gene (*CaCAF1*) in tomato plants resulted in enhanced resistance against the oomycete pathogen, *Phytophthora infestans*. In addition, multiple defence-related genes, including *PR1* and *PR6*, are constitutively up-regulated in these transgenic plants [26]. One of the *Arabidopsis* homologs of *CAF1*, named *CAF1-like* (*AtCAF1a* in this study), was recently shown to be wound- and biotic stress-inducible *in vivo* using stable transgenic lines expressing transcriptional luciferase fusions [27]

In line with these observations, our results indicated that transgenic *Arabidopsis* plants over-expressing *AtCAF1a* constitutively express higher levels of *PR1* and *PR2* and are more resistant than wild type to the infection of *Pst* DC3000. On the contrary, mutants of *AtCAF1* genes show reduced expression of *PR1* and *PR2* and are more susceptible to the same pathogen. These results raised the interesting question of how AtCAF1a affects the expression levels of *PR1* and *PR2*. Significantly, our PAT assays (Figure 6D and 6E) and complementation experiments (Figure 6F and 6G) indicate that, even though AtCAF1a does not act directly on the poly (A) tails of *PR1* and *PR2* mRNAs, its deadenylation activity is essential for the maintenance of the elevated expression levels of *PR1* and *PR2* in transgenic plants over-expressing *AtCAF1a*. It is likely that AtCAF1a acts indirectly in controlling *PR1* and *PR2* expression levels. We speculate that AtCAF1 proteins are required for degradation of a particular mRNA species specifying a repressor of *PR1* and *PR2* transcription. Wild-type plants, in which AtCAF1 proteins partially degrade the repressor, exhibit an intermediate level of *PR1* and *PR2* expression and disease resistance. In plants that over-express the *AtCAF1* genes, the repressor is largely or completely degraded, conferring an enhanced defence response by production of higher levels of *PR1* and *PR2*. In mutants that lack the deadenylase activity of AtCAF1 proteins, the repressor is more stable and interferes with *PR1* and *PR2* expression, consequently reducing pathogen resistance. Support of this hypothesis comes from the characterisation of the *Arabidopsis* CER7 (WAX-DEFICIENT ECERIFERUM 7) protein, which is a putative 3′-5′ exoribonuclease homologous to yeast Ribonuclease PH45 (RRP45p), a core subunit of the RNA processing and degrading exosome

[35]. CER7 regulates cuticular wax biosynthesis, probably by degrading a specific mRNA species that encodes a negative regulator of the transcription of *CER3/WAX2/YRE,* a key wax biosynthetic gene [35]. Further studies are required to elucidate the detailed mechanisms of how AtCAF1 proteins affect the expression of *PR1/2* and perhaps other defence-related genes.

Disruption of genes encoding AtCAF1a or/and AtCAF1b causes no apparent developmental phenotype. This is likely because of a redundancy in mRNA deadenylation systems in *Arabidopsis*. AtPARN, homolog of the important deadenylase PARN in mammalian cells, has deadenylation activity on some embryo-specific transcripts [8]. Furthermore, the genome of *Arabidopsis* also encodes a putative homolog of yeast PAN2 protein. These two deadenylases might compensate for the AtCAF1a and AtCAF1b defects of the mutants.

Materials and Methods

Oligonucleotides used in this study

All oligonucleotides used in this study are listed in Supplementary information, Table S1. Restriction enzyme sites and point mutation sequences are underlined.

Plant growth conditions

All *Arabidopsis* lines used were in the Columbia (Col-0) background. *Arabidopsis* seeds were surface sterilised with 10% (v/v) bleach for 10 min and washed five times with sterile water. Sterilised seeds were then suspended in 0.1% agarose and plated on Murashige and Skoog media. Plants were vernalised in darkness for 3 d at 4 °C and then transferred to a phytotrone set at 22 °C with a 16-h light/8-h dark cycle. After 2-3 weeks, seedlings were also potted in soil and placed in a growth room at 22 °C with a 16-h light/8-h dark cycle.

Mutant identification and generation of transgenic plants

Arabidopsis mutants *atcaf1a* (SALK_070336) and *atcaf1b* (SALK_092761) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Homozygous T-DNA insertion lines for *atcaf1a* and *atcaf1b* were identified with diagnostic PCR using gene-specific primers and T-DNA primers (Supplementary information, Table S1). Disruption of target gene expression in these mutants was verified by RT-PCR (Figure 5B). Mutant *atcaf1a* and *atcaf1b* lines were crossed, and an *atcaf1a/atcaf1b* double-mutant line was identified from the resulting F2 population by PCR-based genotyping.

The coding sequence of *AtCAF1a* was amplified by PCR using the primer pairs as indicated in Supplementary information, Table S1. The resulting fragments were cloned into the *Bam*HI and *Sac*I sites of the binary vector pCanG-HA under the control of the 35S promoter to generate the *35S::AtCAF1a* construct. Similarly, we also generated a *35S::AtCAF1a-mt* construct. Plant transformation was done with the vacuum infiltration method [36].

Plant treatments and bacterial infection

All hormones were purchased from Sigma (St Louis, MO). For hormone treatments, 2-week-old seedlings grown on MS media were treated with 50 μM MeJA, 20 μM ABA, 50 μM ACC, or 20 μM SA. For the wounding treatment, leaves of 2-week-old plants were crushed two times across the apical lamina with a haemostat. Plants were incubated for various periods, after which tissues were harvested for RNA extraction.

Soil-grown plants that were 4-week old were infected with *Pst* DC3000. Bacteria were applied in a density of 10^4 CFU/ml with a needleless syringe in the middle of a leaf, and leaf discs were cut 0, 2, and 4 d after infection. Leaf discs were cut with a cork borer and immediately homogenised with sterile water. Appropriate dilutions were plated on Kings B plates with 50 μg/ml rifampicin and incubated for 48 h at 28 °C.

Gene expression analyses

Total RNA was isolated from 14-d-old seedlings grown on MS media using a guanidine thiocyanate extraction method. Total RNA (20 μg) was separated by electrophoresis on a 1% MOPS-agarose gel containing 5.8% formaldehyde, and RNA gel blot analysis was performed as described previously [28]. For RT-PCR, 5 μg of total RNA was used for first-strand cDNA synthesis by M-MLV (Promega). PCR conditions were as follows: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C; 30 s at 55 °C; and 1 min at 72 °C. Quantitative real-time RT-PCR (qRT-PCR) analyses were performed using the QuantiTec SYBR green system (Qiagen) and the Opticon PCR machine (MJ Research, Waltham, MA). Data were treated using the Opticon Monitor 3 software provided by the manufacturer. Gene-specific primers used for RT-PCR and qRT-PCR assays are listed in Supplementary information, Table S1.

To express GUS under the control of *Arabidopsis* native promoters, 2628-bp and 1456-bp regions of 5′ upstream sequences of *AtCAF1a* and *AtCAF1b*, respectively, were amplified from the relevant genomic regions. These promoter fragments were fused to a GUS coding sequence and transformed into wild-type *Arabidopsis* plants. Histochemical staining for GUS activity in transgenic plants was performed as described previously [37].

Site-directed mutagenesis of AtCAF1a

Point mutations of *AtCAF1a* were done with the MutantBEST Kit (TaKaRa) according to the manufacturer's instructions.

Yeast complementation

Yeast strains KY803 (*MATa leu2-PET56 trp1-Δ1 ura3-52 gal2 gcn4-Δ1*) and KY803-c1 (*MATa leu2-PET56 trp1-Δ1 ura3-52 gal2 gcn4-Δ1 caf1::LEU2*) were kindly provided by Clyde L. Denis. Yeast strains were grown on YEP medium (1% yeast extract, 2% Bacto peptone) supplemented with 2% glucose. YD plates consisted of YEP media supplemented with 2% glucose and 2% agar. The open reading frames (ORFs) of *AtCAF1* genes were cloned into pYES2, and yeast complementation studies were done according to Ohn *et al*. [21].

Nuclease assay

For the expression of AtCAF1 proteins as fusions with the His tag, the open reading frames of *AtCAF1* genes were cloned into pET-28a (+) (Novagen). Expression of His-AtCAF1 fusions was carried out in BL21 (DE3) host strains, and purification was done with Ni-NPA according to the manufacturer's instructions (Novagen). *In vitro* RNase assays were performed in 20 mM Tris/Cl (pH

7.0), 150 mM NaCl, 2 mM $MgCl_2$, 5 U RNasin (Promega), 1 mM poly (A) substrate (Amersham) labelled with ^{32}P at the 5′ end, and 0.1 μg of purified His-AtCAF1 fusion proteins. A volume of 10 μl of the reaction mixture was incubated at 25 °C for the indicated time. Reactions were stopped by the addition of formamide/EDTA buffer and then loaded onto 7 M urea/10% acrylamide (19:1) gels [19].

PAT assay

For PAT assays, 2-week-old plants were treated with 50 μM MeJA for 6 h, 50 μM ACC for 24 h, or wounded with a haemostat and incubated for 3 h, after which plant tissues were harvested at various times for RNA extraction. PAT assays were carried out according to the method of Sallés *et al*. [38] with minor modifications. Briefly, 2 μg of total RNA was used for RT with an anchoring nucleotide-fused oligo $(dT)_{15}$ primer. PCR was performed with the anchor primer and a sense primer VSP1-mF (CHIB-mF/LOX2-mF) targeting a specific sequence in the cDNA of interest. PCR cycles were as follows: 3 min at 94 °C, followed by 25 cycles of 30 s at 94 °C; 30 s at 60 °C; 1 min at 72 °C. The PCR products were resolved on 2% agarose gels and subjected to Southern blot analysis.

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)