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Research Article

Overexpression of *AtCAF1*, CCR4-associated factor 1 homologue in *Arabidopsis thaliana*, negatively regulates wounding-mediated disease resistance

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Abstract The CCR4-CAF1-NOT complex-mediated degradation of mRNA is a fundamental aspect of gene regulation in eukaryotes. We herein examined the role of *AtCAF1* in the innate immune and wound responses of plants. Our results showed that overexpression of *AtCAF1* significantly down-regulated the transcript level of *EFR* but not *FLS2* and *BRI1*, as well as abolished up-regulated expression pattern of *EFR* in response to wounding. Consistently, *Agrobacterium*-mediated transient expression of GUS was highly enhanced in the transgenic plants overexpressing *AtCAF*. Furthermore, JA responsive genes were down-regulated by overexpression of *AtCAF*, causing the transgenic plants overexpressing *AtCAF* more susceptible to necrotrophic fungal pathogen, *Botrytis cinerea*. These results suggest that The CCR4-CAF1-NOT complex-mediated degradation of mRNA negatively regulates wounding-mediated disease resistance in *Arabidopsis thaliana*.

Keywords CCR4-CAF1-NOT complex, CCR4 associated factor 1, plant innate immunity, wound response

Introduction

The mRNA degradation is a fundamental aspect of gene regulation in response to various physiologic and environmental signals in eukaryotes, which is preceded by deadenylation of the poly (A) tail and followed by decapping of the 5' cap structure and rapid 5' to 3' exonucleolytic digestion of mRNA body (Tucker et al. 2004). In particular, deadenylation is a rate-limiting step in the control of the mRNA degradation rate, which is mainly mediated by the major cytoplasmic deadenylase complex, the CCR4-CAF1-NOT complex (Collart and Timmers 2004). The CCR4-CAF1-NOT complex is composed of at least seven core subunits, including the carbon catabolite repressor factor 4 (CCR4), CCR4-associated factor 1 (CAF1), and five negative regulator of transcription (NOT1-5). This complex is a highly conserved regulator of mRNA metabolism (Ohn et al. 2007).

Recently, it has been proposed that deadenylation-mediated mRNA degradation is an important mechanism of gene regulation to deploy the proper defense responses against invading pathogens in animals and plants. *CCR4* knockout mice exhibited increased transcript levels of various *Toll-Like Receptor* (*TLR*) genes encoding the pattern recognition receptors that recognize various pathogen-associated molecular patterns (PAMP) from invading microbes. Consequently, *CCR4* knockout mice enhanced recognition of the PAMPs of invading bacteria, resulting in a faster activation of innate immune responses and a significant resistance to bacterial infection (Ness et al. 2006). In addition, the pepper (*Capsicum annuum*) and *Arabidopsis thaliana CAF1* homologues, *CaCAF1*and *AtCAF1*, were reported to positively regulate the salicylic acid (SA)-mediated defense response (Sarowar et al. 2007; Liang et al. 2009).

On the basis of these reports, it is tempting to speculate that plant innate immune responses triggered with recognition of various PAMPs by cognate receptor-like kinases can also be regulated by mRNA degradation mediated by the CCR4-CAF1-NOT complex. In this study, we examined the role of *AtCAF1* gene in the innate immune responses of plants mediated by pattern-recognition receptor kinases EFR (Zipfel et al. 2006) and FLS2 (Gómez-Gómez L and Boller T 2000) recognizing the bacterial pathogen-associated

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molecular patterns EF-Tu and flagellin, respectively. We found that overexpression of *AtCAF1* negatively regulates the transcript level of *EFR* but not *FLS2,* as well as attenuates JA signaling that regulates plant defense during necrotrophic pathogens infection. These results provide new insight on the regulatory role of mRNA degradation by CCR4-CAF1-NOT complex in determining JA/wounding-mediated disease resistance in plants.

Materials and Methods

Plant lines and growth conditions

All the plants used in this work were *Arabidopsis thaliana* (Col-0) background. Wild-type, mutant, and transgenic plants were grown for 3- to 4-weeks in soil in a growth chamber at 22℃ with an 8/16 h light/dark cycle. Seeds of the SALK T-DNA knockout line of the *AtCAF1* gene, Salk_050279, was ordered from the Arabidopsis Biological Resource Center at the Ohio State University.

Mutant identification and generation of transgenic plants

Total gemonic DNA was prepared from 3- to 4-week-old individual plants. The genotype was confirmed using PCR with genomic DNA from individual plants. The primer sets AtCAF1 For/ pROK2 T-DNA LB and AtCAF1 For / AtCAF1 Rev (Table 1) were used to confirm the presence of the T-DNA insertion in the middle of *AtCAF1* gene. Full-length *AtCAF1* was amplified by PCR using the primer set AtCAF1 For-1/AtCAF1 Rev-1 (Table 1). The purified PCR product was cloned into pBluescript and sequenced. Then, an *Xba*I fragment containing full-length *AtCAF1* was inserted into pCAMBIA1300-35S binary vector for expression of the *AtCAF1* gene under the control of the CaMV 35S promoter. Transgenic plants were generated by vacuum infiltrating *A. tumerifaciens* (GV3101) carrying recombinant binary vector into flowering *Arabidopsis* as described by Clough and Bent (1998).

Wound treatment and gene expression analyses

The plants were wounded by punctuation of the leaves with a custom made pin-tool consisting of 16 needles (2 needles/1cm^2). Three times of consecutive application pierced on average three or four distinct wholes per leaf. Leaf samples were harvested at 1 and 2 h after start experiment.

Table 1 List of oligonucleotide primers used in this study

Primer	Sequence(5’-3’)
AtCAF1 For	CCGAGATCTCAAACCGGACG
AtCAF1 Rev	AGCATGTTTCTCCGGCCCAT
pROK2 T-DNA LB	GCGTGGACCGCTTGCTGCAACT
AtCAF1 For-1	CATCTAGAATGGCGATCATTAAACCAAAC
AtCAF1 Rev-1	GATCTAGATTAAAAAACCTCAAGCCCATA
PR1 For	CCACAAGATTATCTAAGGGTTC
PR1 Rev	GGCTTCTCGTTCACATAATTCC
PR2 For	GATACCTTGCCAAGTCCATCGGACGTTG
PR2 Rev	TCTCTATAGCTTTCCCTGGCCTTCTCGG
PR5 For	GCTGTTATGGCCACAGACTTCAC
PR5 Rev	GTTAGCTCCGGTACAAGTGAAGG
PDF1.2 For	AAGTTTGCTTCCATCATCACC
PDF1.2 Rev	ATACACACGATTTAGCACCAA
AtVSP For	ACGTCCAGTCTTCGGCATCC
AtVSP Rev	GAGCTTAAAAACCCTTCCAG
Thi2.1 For	GTGATCAAACAAGTAAACCAT
Thi2.1 Rev	AACAAACCTTCTACGACACAT
EFR For	CCGGTTGGTAAGCCTAAAGA
EFR Rev	GGTCATGACAATGAACGTGC
FLS2 For	GCGATTTTGGAACTGCTCGG
FLS2 Rev	ATCAGGTCGATCTTCAGGTC
ACT2 For	AGTGTGTCTTGTCTTATCTGGTTCG
ACT2 Rev	AATAGCTGCATTGTCACCCGATACT

Total RNA was extracted from plant tissue using Trizol reagent (Invitrogen). Using the SuperScript Ⅲ One-Step RT-PCR kit, semi-quantitative RT-PCRs were performed according to the manufacturer’s instructions (Invitrogen). Gene-specific primer sets (Table 1) of the *PR1*, *PDF1.2*, *AtVSP*, *EFR*, *FLS2*and *ACT2* genes were used to investigate the transcript levels of specific genes from various RNAs by RT-PCR. The amount of template cDNA and the number of PCR cycles necessary were determined in preliminary experiments to ensure that amplification occurred in the linear range. Northern blot analysis was performed as described previously (Seo et al. 2008)

Bacterial and fungal growths and plant inoculations

We used *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) containing the empty vector pVSP61 and *Pst* DC3000 (*avrRpt2*) as controls of compatible interactions and incompatible interactions, respectively. All the bacterial strains with resistance to rifampicin and kanamycin were grown overnight in King's B medium containing 100 mg/l rifampicin

and 10 mg/l kanamycin, washed twice, resuspended, and diluted to a final density of 10^5 cfu/ml in 10 mM $MgCl_2$. For measurements of bacterial growth, 4-week-old *Arabidopsis* plant leaves were infiltrated with a bacterial suspension using a 1 ml syringe without a needle. For each sample, four leaf discs were pooled, three times per data point. Leaf discs were bored from the infiltrated area, ground in 10 mM $MgCl_2$, serially diluted, and plated on Pseudomonas Agar F medium (Difco) to determine the number bacteria. *Botrytis cinerea* was cultured on 20 g/l malt extract, 10 g/l peptone (Difco), and 15 g/l agar for 7 ~ 10 days at 24℃ with a 12 h photoperiod before collection of spores. Pathogenicity test was performed as described in Ferrari et al. (2003). Briefly, rosette leaves from 4-week-old soil-grown *Arabidopsis* plants were placed in Petri dishes containing 0.8% agar, with the petiole embedded in the medium. Inoculation was performed by placing 5 μl of a suspension of 5×10^5 conidiospores/ml in 24 g/l potato dextrose broth (PDB; Difco) on the upper middle vein. The plates were incubated at 22℃ with a 12 h photoperiod. High humidity was maintained by covering the plates with a clear plastic lid. Under these experimental conditions, most inoculations resulted in rapidly expanding water-soaked lesions of comparable diameter. Lesion size was determined by measuring the diameter of the necrotic area.

Agrobacterium-mediated transient transformation assays

Transient transformations of *Arabidopsis* leaf and root segments were performed as described in Zipel et al. (2006) and Nam et al. (1997), respectively, with *Agrobacterium* GV301 carrying GUS-intron transgene driven by super-promoter (pBISN1). Bacterial suspension in 0.9% NaCl (OD_{600} = 0.4) was directly injected into leaves of 3- to 4-week-old plants or cocultivated with root segments on the B5 medium. The efficiency of transient transformation was determined with GUS activity in the infected tissues.

Results

AtCAF1 is one member of a small gene family containing a ribonuclease domain

Analysis of *Arabidopsis* genomic sequence revealed six additional genes with significant homology to *AtCAF1* (At3g44260). A pairwise analysis of the members of this gene family revealed an amino acid similarity between 51 and 78%. In particular, *AtCAF1b*, At5g22250, and a potential ortholog of *AtCAF1* found in *Capsicum annuum*, *CaCAF1* (ABG66307), shared a high degree of sequence similarity with each other. As indicated in a phylogenetic diagram analysis (Fig. 1A), these genes were clustered together as a clade, which may have a similar functional role *in vivo*, connecting CCR4 and NOT to form the major cytoplasmic deadenylase complex, CCR4-CAF1-NOT.

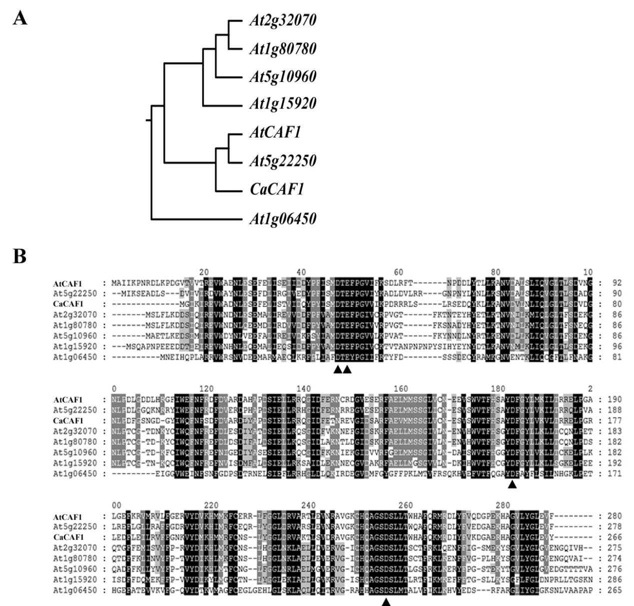

Fig. 1 Phylogenetic analysis and sequence alignment of AtCAF1 family proteins and CaCAF1 protein. (A) A phylogenetic tree was constructed from the seven *Arabidopsis* CAF1 homologues and the CaCAF1 protein. (B) The protein sequence of AtCAF1 was aligned with homologues from *Arabidopsis thaliana* and *Capsicum annuum* using the ClustalW program. The gray and black boxes represent homologous and identical amino acids, respectively. DEDD catalytic site residues are indicated with arrowheads

CAF1 is related to the ribonuclease D family which belongs to the DEDD superfamily. The DEDD superfamily is composed of ribonuclease along with deoxyribonuclease containing four conserved acidic residues, DEDD, that are responsible for binding the two metal ions involved in catalysis (Thore et al., 2003). Plant CAF1 proteins also have conserved and potentially fully functional active sites for deadenylation (Fig. 1B). Although CCR4 is the active deadenylase in the yeast CCR4-CAF1-NOT complex, mammalian and plant CAF1 is also a bona fide adenylase that regulates mRNA decay (Liang et al., 2009).

Constitutive high expression of *AtCAF1* down-regulates *EFR* transcript level

Because CAF1 regulates the deadenylase activity of CCR4 that is reported as a key regulator for innate immunity in animal by forming the CCR4-CAF1-NOT complex (Ness et al. 2006), we hypothesized that AtCAF1 might be a key

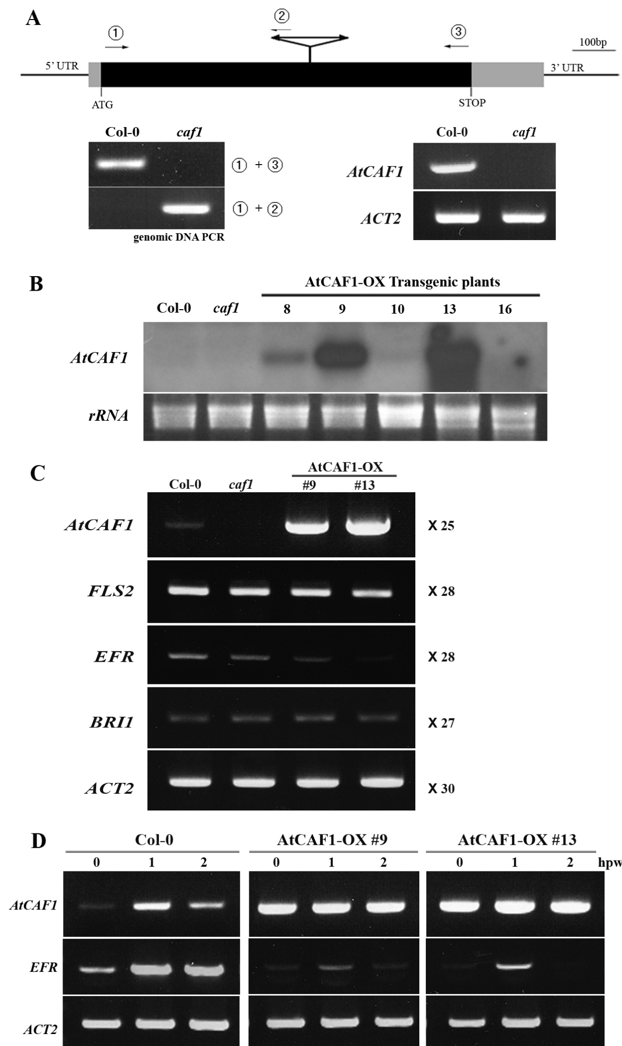

Fig. 2 Effect of *AtCAF1* transcript levels on expression of receptor-like kinase genes. (A) Structure of the *AtCAF1* gene is shown with the insertion site of T-DNA in the knockout line. Using specific primers indicated by small black arrows and numbers, genomic PCR and RT-PCR confirmed the presence of T-DNA insertion in the coding region and a loss of mRNA of the *AtCAF1* gene in *AtCAF1*-KO line, respectively. Amplification of *ACT2* from the same cDNA preparation was used as a positive control. (B) The putative transgenic plants overexpressing *AtCAF1* were confirmed with RNA blot analysis. The transgenic #9 and line #13 were selected for further experiments. (C) Expression of receptor-like kinase genes in wild-type, *AtCAF1*-KO, and *AtCAF1*-OX plants. The number of cycles for detecting specific gene transcript levels with RT-PCR is indicated as x and number. (D) Kinetics of EFR expression after wounding in wild-type, *AtCAF1*-KO, and *AtCAF1*-OX plants. Hpw stands for hour post wounding

modulator of innate immunity in plants. To confirm our hypothesis, we first identified an *Arabidopsis* T-DNA insertion knockout line of the *AtCAF1* gene (AtCAF-KO), and used RT-PCR to confirm that *AtCAF* mRNA was not present in the mutant (Fig. 2A). We also generated transgenic *Arabidopsis* plants that overexpressed the *AtCAF1* gene under the control of the CaMV 35S promoter (AtCAF1-OX lines). *AtCAF1* expression levels were confirmed with RNA blot analysis (Fig. 2B). There was no significant difference in morphology that correlated with the various expression levels of *AtCAF1* (data not shown).

Depending on expression levels of *AtCAF1,* we investigated the transcript levels of *FLS2*, *EFR,* and *BRI1* encoding receptor kinases that are structurally similar LRR-type. Interestingly, only *EFR* transcript was significantly decreased in the AtCAF1-OX lines compared to wild-type plants (Fig. 2C). Furthermore, the kinetics of *EFR* expression in response to wound was highly attenuated by the constitutively enhanced expression of *AtCAF1* (Fig. 2D). These results suggest that CCR4-CAF1-NOT complex may have preferential targets for properly responding to endogenous development programs and diverse environmental cues.

EFR-mediated disease resistance is attenuated in the AtCAF1-OX lines

To investigate whether the expression levels of *AtCAF1* affected on the innate immunity, we first tested the growth of nonpathogenic *hrc* derivative of *Pst* DC3000, which is very sensitive to the status of innate immunity in host plants because of the lack of suppression of PAMP-mediated innate immune responses by the effectors (Kim et al. 2005). The growth of *Pst* DC3000 *hrcC* was not affected by expression levels of *AtCAF1* (data not shown), resulting from constant expression of *FLS2* responding to flagellin of *Pst* DC3000 *hrcC* regardless of the expression levels of *AtCAF1.*

To further test the effect of *AtCAF1* expression levels on the EF-Tu perception by EFR and activation of basal resistance without activation of FLS2-mediated innate immunity, we also performed *Agrobacterium*-mediated transient plant transformation with leaves and roots of wild-type, AtCAF1-KO, and AtCAF1-OX plants as described in materials and methods. The leaves and root segments of AtCAF1-OX plants infected with *Agrobacterium* carrying GUS-intron construct driven by the super promoter consistently exhibited intense GUS staining, compared to those of wild-type and AtCAF1-KO plants (Fig. 3). The staining intensity was increased in proportion to *AtCAF1* expression levels in the AtCAF1-OX plants, but not significant different between wild-type and AtCAF1-KO plants. These results indicate that the ability of EF-Tu recognition by EFR in the AtCAF1-OX plants might be attenuated by the decreased transcript level of *ERF* and consequently,

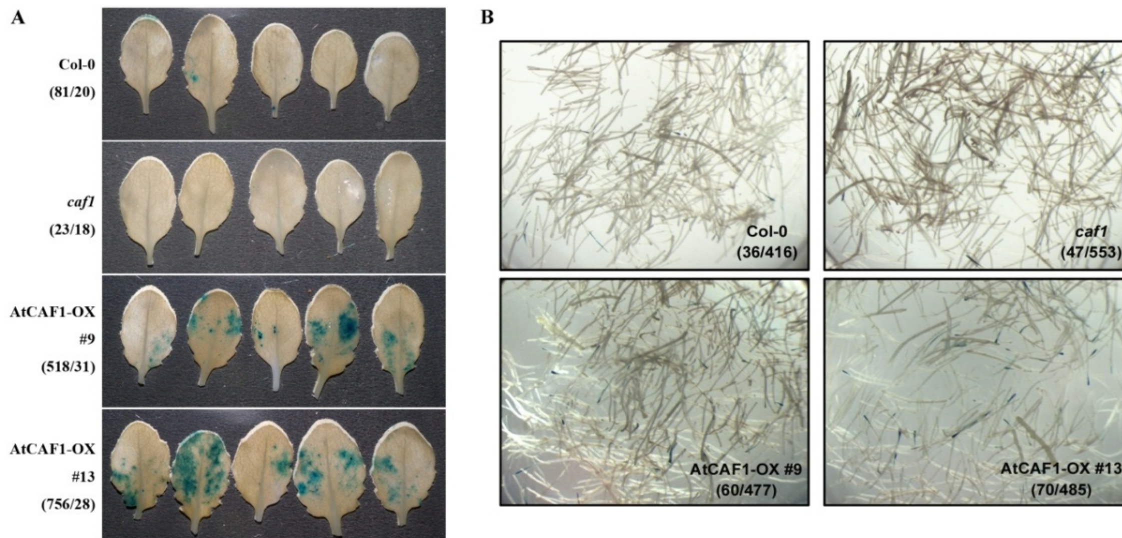

Fig. 3 Constitutive high expression of *AtCAF1* enhances the efficiency of *Agrobacterium*-mediated transient transformation. Staining for GUS activity in leaves (A) and root segments (B) of wild-type, AtCAF1-KO, and AtCAF1-OX plants after infiltration or cocultivation of *Agrobacterium* GV301 carrying GUS-intron transgene driven by superpromoter (pBISN1). Values in parentheses indicate total number of spots with GUS staining per total leaves or root segments infected with *Agrobacterium*

innate immune responses against invading *Agrobacterium* might be compromised, resulting in high frequency of *Agrobacterium*-mediated transformation events in the AtCAF1-OX plants.

Overexpression of AtCAF1 leads to enhanced disease susceptibility to necrotrophic fungus *Botrytis cinerea*

Based on the transient expression of *AtCAF1* in response to wounding (Fig. 2D), we speculated on the possible role of AtCAF1 in the JA-mediated disease resistance responses. We first analyzed the expression levels of several JA-responsive genes including transcription factors, *MYC2* and *EFR1*, and downstream marker genes, *PDF1.2* and *AtVSP*, in different plants carrying the various expression levels of *AtCAF1*. The expression levels of these JA-response genes were highly decreased in the AtCAF1-OX plants, but SA-response gene PR1 expression was not changed regardless of AtCAF1 expression level (Fig. 4A). Furthermore, constitutive high expression of *AtCAF1* significantly attenuated the response kinetics of *PDF1.2* and *AtVSP* after wounding (Fig. 4B). These results indicate that enhanced AtCAF1 might specifically down-regulate JA-mediated wound responses.

Because JA positively regulates resistance to necrotrophic fungal pathogens and insect attacks (Dong 1998), we also tested whether decreased JA responses of AtCAF1-OX plants could result in the enhanced disease susceptibility. Notably, AtCAF1-OX was more susceptible to the virulent necrotrophic fungus *Botrytis cinerea* (Fig. 5A, B), but not

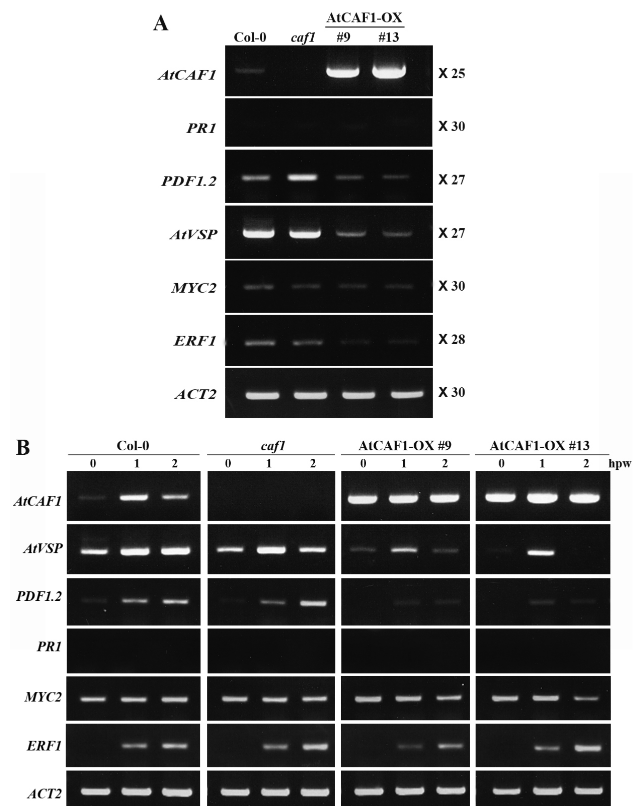

Fig. 4 Effect of *AtCAF1* transcript levels on expression of JA-response genes and their kinetics after wounding. (A) Expression levels of jasmonic acid responsive genes in AtCAF1-KO, AtCAF1-OX, and Col-0 plants (B) Kinetic analysis of JA-response gene expressions after wounding. Transcript levels of each gene were determined with RT-PCR. The number of cycles for detecting specific gene transcript levels with RT-PCR is indicated as x and number. As an internal control, expression of the *ACT2* gene was measured under the same conditions. Hpw stands for hour post wounding

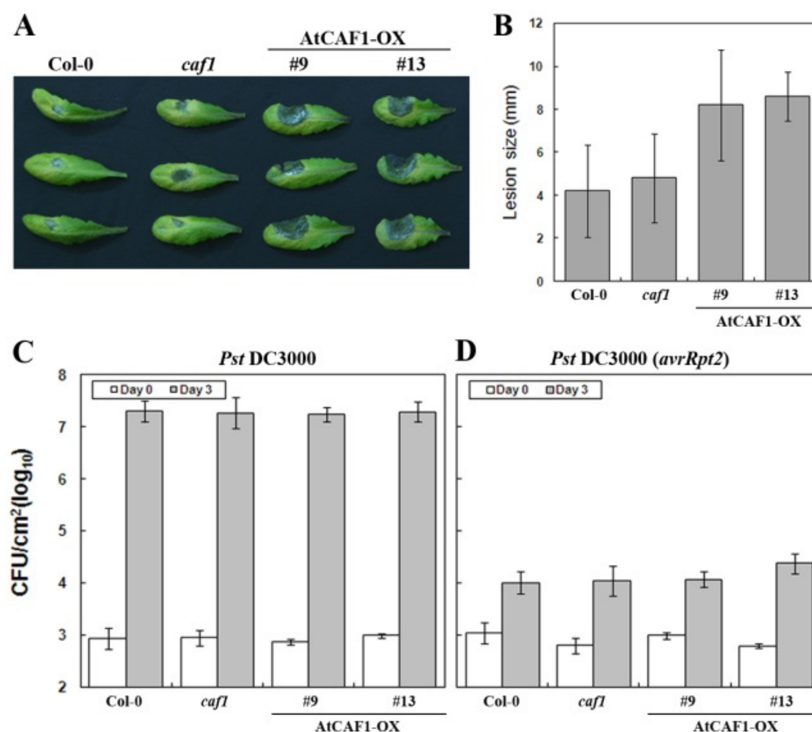

Fig. 5 Transgenic plants overexpressing *AtCAF1* are more susceptible to necrotrophic fungal pathogen but not to biotrophic bacterial pathogen. Susceptibility of wild-type, AtCAF1-KO, and AtCAF1-OX plants to *Botrytis cinerea* was investigated by macroscopic symptoms and disease rating (A) and lesion diameter (B) at 5 days post-inoculation as described in materials and methods. Results are average±SD (n=10) and asterisks indicate $P<0.05$ by t-test. Susceptibility of wild-type, AtCAF1-KO, and AtCAF1-OX plants to compatible pathogen *Pst DC3000* (C) and the incompatible pathogen *Pst DC3000 avrRpt2* (D) was determined by bacterial numbers measured at 0 and 3 days post-inoculation as described in materials and methods. Results are average±SD (n=3) and two sample *t*-tests were performed; these showed that there were no significant differences among wild-type, AtCAF1-KO, and AtCAF1-OX

to the boitrophic bacterial pathogens *Pseudomonas syringae* of which infection is highly dependent SA-mediated resistance in the compatible and incompatible interactions (Fig. 5C, D)

Discussion

Innate immunity in plant is the receptor-mediated surveillance system that detects the presence of pathogens through recognition of their specific PAMPs and activates diverse defense genes as the front line of host defense against infection. Recently, it has been reported that biogenesis and functional activities of FLS2 and EFR are dynamically regulated through post translational processes such as protein complex formation and endoplasmic reticulum quality control (Chinchilla et al. 2007). However, the molecular mechanism for their transcriptional regulation remains elucidated. In this report, we demonstrate that deadenylase complex containing AtCAF1 regulates transcript levels of *EFR* and several wound-response genes involving in disease resistance in *Arabidopsis*.

Transcriptional gene regulation is a dominant mechanism for wound-activated responses directing to healing of damaged tissue and to activation of defense mechanism to prevent further damage and pathogen infections (Leon et al. 2001). Interestingly, *AtCAF1* expression in response to wound and stress-related plant hormones is transiently up-regulated in 30 min after treatments and then quickly down-regulated to the basal level, followed by high induction of major wound-response genes including *ERF* (Fig. 1D and 3B). These results suggest that there is a strong correlation between the expression profile of *AtCAF1* and its role as an important component of CCR4-CAF1-Not1 deadenylase complex that is a major regulator of mRNA turnovers. Therefore, we hypothesize that deadenylase complex containing AtCAF1 might target the mRNAs of positive regulators of wound-response genes while *AtCAF1* is transiently up-regulated, leading to delay the burst activation of wound-mediated defense response. EFR highly accumulated during late wounding response recognizes *Agrobacterium*-derived EF-Tu as a PAMS and triggers ROS-mediated innate immune response to restrict *Agrobacterium* infection (Zipfel et al. 2006). Therefore, high susceptibility to *Agrobacterium*-

mediated transformation in AtCAF1-OX plants might result from the long-lasting high expression of *AtCAF1* under the CaMV 35S promoter, which might enhance the activity of deadenylase complex and decrease the ability to restrict *Agrobacterium* infection due to lack of EFR (Fig. 2D and Fig. 3). However, direct deadenylation of EFR mRNA by deadenylase complex need to be determined empirically. The persisting high deadenylase activity decreased not only *EFR* expression but also other wound-response genes such as *VSP1* and *PDF1.2* of which expressions are strongly dependent on JA-mediated signal pathway. Consistently, AtCAF1-OX plants are more susceptible to necrotrophic fungal pathogen *Botrytis cinerea*, but not to biotrophic bacterial pathogen *Pseudomonas syringae* (Fig. 4 and Fig. 5). In line with these results, recently, Liang *et al* (2009) demonstrated that AtCAF1 is required for the direct mRNA deadenylation of several wound/JA-response genes including *AtVSP1*, but not for *PR* with in *vitro* poly (A) tail length (PAT) assay. However, they also showed that *PR1* gene expression and resistance to *Pst* DC3000 have a direct correlation with AtCAF1 gene expression level, which is in conflict with our results. This discrepancy may result from the differences in AtCAF1 gene expression in transgenic plants and experiment conditions used in each research group. Of interest here is the fact that the tight regulation of AtCAF1 expression in spatial and temporal might be prerequisite for controlling the activity of general CCR4-CAF1-Not1deadenylase complex specifically which is directly responsible for biotic and abiotic stress responses.

Acknowledgements

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