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Note

Cytokinin Response Factor 2 positively regulates salicylic acid-mediated plant immunity in *Arabidopsis thaliana*

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Abstract The phytohormone cytokinin affects various growth and developmental processes and plant immune responses to various pathogens. However, understanding of the molecular mechanism by which cytokinin regulates plant immunity is at the early stage compared to that of growth and development. Therefore, the role of *Arabidopsis thaliana CYTOKININ RESPONSE FACTOR2* (*AtCRF2*) in plant immunity were investigated. AtCRF2 is a plant specific APETALA2 (AP2)/ethylene-responsive factor (ERF) type transcription factor that is transiently upregulated by cytokinin in a type-B *Arabidopsis* response regulator (ARR) dependent manner. *Arabidopsis* transgenic plants overexpressing *AtCRF2* showed an accelerated leaf senescence phenotype, but extreme *AtCRF2* overexpression was lethal at an early stage of growth with lesion-mimic phenotypes in leaves. Moreover, growth of the compatible hemibiotrophic bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000, was strongly compromised in transgenic than in wild-type plants. Furthermore, there was a strong correlation between the expressions of *AtCRF2* and pathogenesis related (*PR*) genes. However, the phenotypes of transgenic plants overexpressing *AtCRF2* were completely suppressed by expressing bacterial salicylic hydroxylase, *NahG*. These results suggest that AtCRF2 may enhance salicylic acid (SA) biosynthesis, leading to autoimmune responses and leaf senescence.

Key words: AP2/ERF transcription factor, cytokinin response factor, plant immunity.

Plant hormones regulate various growth and developmental processes and are fundamental for adaptation to environmental changes. They interact in complex circuitries to balance the response to developmental and environmental cues, and limit the fitness costs associated with adaptation to the environment (Denancé et al. 2013). The molecular mechanisms that govern these hormonal networks are beginning to be revealed. In particular, cytokinin has been studied as an essential growth regulating hormone that promotes cell division, shoot formation, nutrient mobilization, and leaf longevity (Hwang et al. 2012; Mok and Mok 2001). Cytokinin signaling is carried out by a two-component system similar to the phosphorelay signaling mechanism from yeast and bacteria (Hwang and Sheen 2001). The signaling cascade is initiated by binding of cytokinin to the *Arabidopsis* sensor histidine kinases (AHKs), resulting in autophosphorylation at a conserved histidine. The phosphate group is then transferred to the *Arabidopsis* histidine-containing phosphotransfer proteins (AHPs), which are then translocated into the nucleus where they activate the *Arabidopsis* type-B response regulators (ARRs) by transferring the phosphate. The activated type-B ARRs promote the transcriptional response to cytokinin, including the induction the type-A ARRs that are negative regulators of the primary signal transduction pathway (Argyros et al. 2008; Kiba et al. 2003; To et al. 2004). Recently, cytokinin and SA have been found to synergistically modulate the plant immunity. Cytokinins promote plant immunity by modulating salicylic acid signaling through the interaction between ARR2 and the salicylic acid response factor TGA3, which binds to the *PATHENOGENESIS-RELATED 1* (*PR1*) promoter in *Arabidopsis* (Choi et al. 2010). In addition, high cytokinin concentrations lead to increased SA-mediated defense responses against a virulent oomycete pathogen infection (Argueso et al. 2012).

Cytokinin Response Factors (CRFs) that regulate the transcriptional response to cytokinin are a subfamily of APETALA2/Ethylene Response Factor (AP2/ERF) transcription factor genes found in terrestrial plants (Rashotte et al. 2006; Rashotte and Goertzen, 2010). These CRFs contain a single AP2/ERF domain for DNA-binding and regulate gene expression of primary cytokinin response genes that is dependent on the presence of type-B ARRs (Rashotte et al. 2006). They are also able to form homo- and heterodimers and specifically interact with the AHPs of the cytokinin-signaling pathway (Cutcliffe et al. 2011). However, their biological functions remain elusive. Particularly, *AtCRF2* and *AtCRF5* genes among the *AtCRF* gene

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family in *A. thaliana* are rapidly induced by cytokinin in a type-B ARRs-dependent manner. *AtCRF2* expression is transient, but *AtCRF5* is sustainable (Rashotte et al. 2006). In addition, *Arabidopsis* transgenic plants overexpressing *AtCRF5* enhance resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Liang et al. 2010). Therefore, to investigate the function of *AtCRF2* in cytokinin-mediated plant immune responses, transgenic plants overexpressing *AtCRF2* under the control of the CaMV 35S promoter (*CRF2-OX* lines) in *A. thaliana* were generated using *Agrobacterium tumefaciens* carrying the recombinant binary vector pCAMBIA1300-35S::*AtCRF2* that contained *AtCRF2* cDNA amplified by PCR using the primer set AtCRF2 For XbaI/AtCRF2 Rev XbaI (Supplementary Table S1). Particularly, *AtCRF2-OX* transgenic line 1 that showed a strong senescence phenotype compared to other lines, which was correlated with expression level of *AtCRF2*, was used for further experiments (Figure 1A and 1B). *AtCRF2-OX* plants showed accelerated senescence in rosette leaves, compared with that of the wild-type Col-0 plants (Figure 1C). Consistently, trypan blue staining assay indicated that age-dependent cell death was enhanced in *AtCRF2-OX* leaves, and the senescence-associated genes, *SAG12* (Gan and Amasino, 1995) and *SAG113* (Zhang et al. 2012), were highly expressed in *AtCRF2-OX* plants (Figure 1D and 1E). Furthermore, when *AtCRF2* gene was highly overexpressed, *AtCRF2-OX* plants were small with a high mortality rate at the early stage of growth with lesion-mimic phenotypes in leaves (Figure 2A). Consistent with these results, *AtCRF-DEX* transgenic seedlings expressing *AtCRF2* with a dexamethasone-inducible conditional expression system (Aoyama and Chua 1997) developed severe necrotic cell death when treated with 20 μM dexamethasone (DEX) (Figure 2B). To test effect of expression of *AtCRF2* gene in mature plants, 4-week old *AtCRF-DEX* plants were treated with DEX. DEX treated *AtCRF-DEX* plants showed precocious leaf senescence and cell death phenotypes, similar to *AtCRF2-OX* plants (Figure 2C). This result was confirmed with trypan

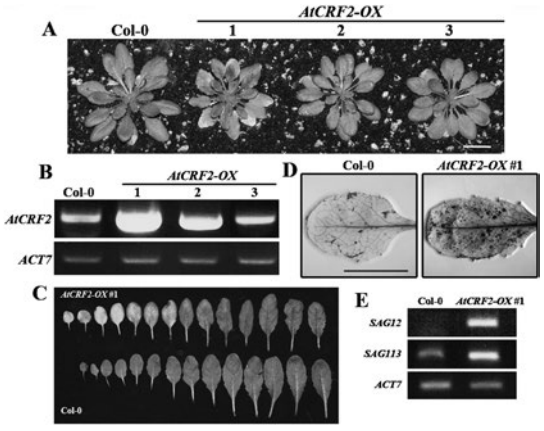

Figure 1. Age-dependent leaf senescence phenotypes of transgenic plants overexpressing *AtCRF2*. (A) Four-week old wild-type and three independent *AtCRF2-OX* transgenic lines. All plants were grown in soil in a growth chamber at 23°C with an 8/16 h light/dark cycle. (B) RT-PCR analysis of *AtCRF2* gene expression in wild-type and transgenic lines. The housekeeping gene, *actin*, was used as an endogenous control gene. (C) Rosstte leaves of 4-week old wild-type and *AtCRF2-OX* (line 1) plants. (D) Trypan blue staining of the fifth rosette leaves of wild-type and *AtCRF2-OX* plants. Dark blue indicates cell death. Scale bar = 1 cm. (E) RT-PCR analyses of transcript levels of senescence-associated genes in wild-type and *AtCRF2-OX* plants.

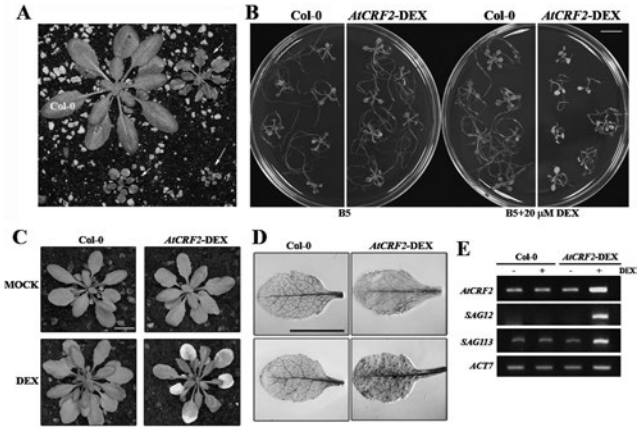

Figure 2. Developmental phenotypes of transgenic plants extremely overexpressing *AtCRF2* and expressing dexamethasone-inducible *AtCRF2*. (A) Growth phenotypes of wild-type and transgenic plants extremely overexpressing *AtCRF2*. Arrows indicated the transgenic plants extremely overexpressing *AtCRF2*. (B) Effect of dexamethasone (DEX) treatment on seedlings of wild-type and transgenic plants expressing dexamethasone-inducible *AtCRF2*. One-week old seedlings cultured on Gamborg medium (B5) under sterile conditions were transferred onto B5 and B5 containing DEX (20 μM) and cultured for 7 days. (C) Visible phenotypes of wild-type and transgenic plants carrying DEX-inducible *AtCRF2* after treatment with mock or DEX. Pictures of representative plants were taken 7 days after DEX treatment. (D) AtCRF2-induced lesions showing cell-death characteristics. Trypan blue stained leaves after DEX treatment for 7 days. Scale bar = 1 cm. (E) Expression of *AtCRF2* and *SAG* genes was observed 7 days after DEX treatment.

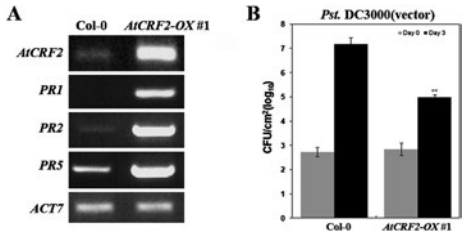

Figure 3. Transgenic plants overexpressing *AtCRF2* enhanced immune responses. (A) Expression of *AtCRF2* and SA-response genes in wild-type and *AtCRF2-OX* plants. (B) Resistance of wild-type and *AtCRF2-OX* plants to virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Bacterial growth was determined by counting bacterial numbers at 0 and 3 days post-inoculation with 1×10^5 cfu/ml bacterial suspension in 10 mM $MgCl_2$. For measurements of bacterial growth, 4-week old *Arabidopsis* leaves were infiltrated with a bacterial suspension using a 1 ml syringe without a needle. Leaf discs were bored from the infiltrated area, ground in 10 mM $MgCl_2$, serially diluted, and plated on King's B medium and incubated at 29°C for 2 days. Asterisks indicate a significant difference from wild-type plants using a *t*-test (** $p<0.01$). Similar results were obtained in three independent experiments.

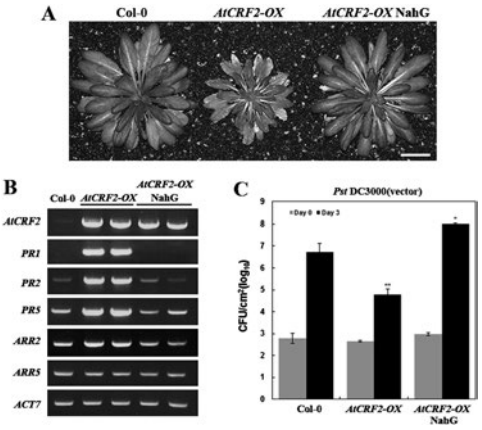

Figure 4. Suppression of autoimmune responses of *AtCRF2-OX* plants by *NahG* expression. (A) Five-week old wild-type, *AtCRF2-OX*, and *AtCRF2-OX* expressing *NahG* plants. Scale bar = 1 cm. (B) Expression of SA- and cytokinin-response genes in *AtCRF2-OX* and *AtCRF2-OX* expressing *NahG* plants. Semi-quantitative RT-PCRs were performed with gene specific primer sets described in Supplementary Table S1. (C) Bacterial growth of wild-type, *AtCRF2-OX*, and *AtCRF2-OX* expressing *NahG* plants at 0 and 3 days post-inoculation with 1×10^5 cfu/ml *Pst* DC3000 bacterial suspension in 10 mM $MgCl_2$. Asterisks indicate a significant difference from wild-type plants using a *t*-test (* $p<0.05$, ** $p<0.01$). Similar results were obtained in three independent experiments.

blue staining assay that showed markedly enhanced cell death, but not in mock-treated transgenic or wild-type plants (Figure 2D). The expression of *SAG12* and *SAG113* genes also increased in the DEX-treated leaves (Figure 2E). These results suggest that AtCRF2 positively regulates age-dependent leaf senescence and cell death in plants. Many lesion-mimic mutants show autoimmune responses that express SA-mediated defense response genes highly without pathogen infection (Bowling et al. 1997; Greenberg and Ausubel 1993). Consistently, the SA responsive *PR* genes were highly expressed in *AtCRF2-OX* plants and the growth of the virulent bacterial pathogen, *Pst* DC3000 was significantly compromised in *AtCRF2-OX* than wild-type plants (Figure 3). The importance of the SA in leaf senescence further supported that AtCRF2 plays a pivotal role in regulating senescence and the basal defense responses against invading biotrophic pathogens via the activation of SA-mediated signal pathway (Rivas-San Vicente and Plasencia, 2011).

The expression of the bacterial *NahG* transgene, which encodes salicylate hydroxylase and inhibits salicylic acid accumulation, suppresses spontaneous lesion formation and autoimmune response in some lesion-mimic mutants (Brodersen et al. 2005; Pilloff et al. 2002). Thus, the *NahG* gene driven by the CaMV 35S promoter was introduced into *AtCRF2-OX* plants. As shown in Figure 4A, the accelerated senescence in rosette leaves that was characteristic of *AtCRF2-OX* plants was suppressed in *AtCRF2-OX* plants expressing *NahG*. Furthermore, the expression levels of several SA-responsive *PR* genes that were highly expressed in *AtCRF2-OX* plants were decreased by *NahG* expression regardless of *AtCRF2* expression (Figure 4B). Accordingly, the bacterial growth of *Pst* DC3000 in *AtCRF2-OX* plants expressing *NahG* was completely suppressed to that of wild-type plants (Figure 4C). These results suggest that the accelerated leaf senescence and autoimmune response of the *AtCRF2-OX* plants may result from elevated SA production, leading to the constitutive activation of SA response genes. These findings expand the current model that cytokinin promotes plant immunity by modulating SA-mediated defense gene expression (Choi et al. 2010). As a result, cytokinin signaling phosphorelay circuitry activated type B ARRs, specially, ARR2, which could upregulate *AtCRF2* expression to enhance SA biosynthesis that is a prerequisite for cytokinin-mediated defense responses. Furthermore, increased *ARR2* expression in *AtCRF2-OX* plants was decreased, but not *ARR5* by *NahG* expression (Figure 4B). This result suggests that there is a positive feedback loop that stimulates *ARR2* expression by SA to enhance SA production, similar to the SA-associated positive feedback loop that potentiated plant defense by enhancing *EDS1* and *PAD4* expression operating upstream of pathogen-induced SA accumulation (Rustérucci et al. 2001; Shirasu et al. 1997). SA has also been reported to negatively regulate the cytokinin-signaling pathway to fine-tune the cytokinin responses (Argueso et al. 2012). This fine-tuning mechanism may result in the transient expression of *AtCRF2* by cytokinin and prevent SA overproduction and autoimmune response, leading to balanced developmental processes and biotic environmental responses.

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Supplementary Table S1. List of oligonucleotide primers used in this study

Primer	Sequence (5'-3')
AtCRF2 For XbaI	AATCTAGAATGGAAGCGGAGAAGAAA
AtCRF2 Rev XbaI	AATCTAGATTAAACAGCTAAAAGAGG
SAG12 For	TGAGGATGTCCCGGTTAATG
SAG12 Rev	TTCTCCCCATTTTGTTCCCC
SAG113 For	GGAGTACTTGCAATGTCACG
SAG113 Rev	TTTCCCTTTCCGGTGATGAT
PR1 For	CCACAAGATTATCTAAGGGTTC
PR1 Rev	GGCTTCTCGTTCACATAATTCC
PR2 For	GATACCTTGCCAAGTCCATCGGACGTTG
PR2 Rev	TCTCTATAGCTTTCCCTGGCCTTCTCGG
PR5 For	GCTGTTATGGCCACAGACTTCAC
PR5 Rev	GTTAGCTCCGGTACAAGTGAAGG
ARR2 For	CATTTTCCACTTCGGAAGCA
ARR2 Rev	GATGGGATGCCTTCCTGTTT
ARR5 For	TTCAGAGAACATCTTGCCTCGT
ARR5 Rev	CGCGTTTTAGCTGCGAGTAG
ACT7 For	AGTGTGTCTTGTCTTATCTGGTTCG
ACT7 Rev	AATAGCTGCATTGTCACCCGATACT