Functional analysis of hot pepper ethylene responsive factor 1A in plant defense

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ABSTRACT
Ethylene-responsive factors play important roles in the biotic and abiotic stresses. Only some ERF genes from Capsicum annuum have been characterized. In the study, the CaERF1A gene is characterized in response to biotic stress. CaERF1A transcripts were induced by various plant defense-related hormone treatments. Knockdown of CaERF1A in hot pepper plants are negatively affected Tobacco mosaic virus-P0-mediated hypersensitive response cell death, resulting in reduced gene expression of pathogenesis-related genes and ethylene and jasmonic acid synthesis-related gene. Overexpressing CaERF1A transgenic plants show enhanced resistance to fungal pathogen via regulating ethylene and jasmonic acid synthesis-related gene expression. Thus, CaERF1A is a positive regulator of plant defense by modulating ethylene and jasmonic acid synthesis-related gene expressions.

Introduction
Plants undergo many physiological changes to cope with various biotic challenges. The survival of plants mainly depends on their ability to evolve complicated defense system against different pathogen attacks through signaling networks. In plants, transcription factors (TFs) play important roles in gene expression regulating response to biotic stress. Among the TF families, the apetala 2/ethylene-responsive factor (AP2/ERF) transcription factors are unique to plants and can be subdivided into four major subfamilies, AP2, related to abscisic acid insensitive 3/viviparous 1 (RAV), ethylene-responsive factor (ERF), and dehydration-responsive element-binding protein (DREB). AP2/ERF family is one of the largest TFs in the plant kingdom such as 122 Arabidopsis, 139 rice, 148 soybean, 121 barley, and 175 hot pepper genes. AP/ERF contains essential 68 amino acid repeat motifs that are designated the AP2 domain and contains DNA binding activity. ERF can activate or inhibit the transcription of genes that have GCC-box (AGCCGCCC) motif in target gene promoters. Additionally, some ERFs also can bind to a CRT/DRE (A/GCCGAC) motif to regulate the expression of genes in response to biotic or abiotic stresses.

Notably, ERFs are integrators of the hormonal pathways and directly responsible for the transcriptional regulation of several jasmonic acid (JA)/ethylene (ET)-responsive defense genes. Basically, constitutive expression of ERF family members known as positive activators of transcription is sufficient to activate the expression of JA/ET-dependent defense genes and to trigger resistance against necrotrophic pathogens. Arabidopsis ERF1 functions in the ET signaling pathway by activating the expression of PDF1.2 and PR3. Constitutive expression of ATERFI, AtERF6, and ORA59 was shown to enhance resistance against necrotrophic pathogens. Additionally, the ethylene-insensitive 3 (EIN3) and ethylene-insensitive 3 like-1 (EIL1) also play in the JA/ET signaling pathway and EIN3 directly bind to the ERF1 promoter to activate transcriptional activity. Expression of ERF96 transcripts are highly upregulated after methyl jasmonate (MeJA) or the ET precursor 1-amino-2-cyclopropane-1-carboxylic acid (ACC) treatment. ERF96 overexpressing transgenic Arabidopsis plants show resistance phenotype to necrotrophic pathogens via increased up-regulation of JA/ET-dependent PR genes such as PDF1.2, PR3, and PR4. NtERF5 overexpressing plants show a strong resistance to TMV infection. The role of ERFs in plant immunity is unlimited. Here, CaERF1A is a positive regulator in biotic stress response against TMV and necrotrophic fungal pathogens. CaERF1A is required for full L-gene-mediated TMV resistance via regulation of ET/JA-synthesis-related gene expression and defense-related genes. This enhanced resistance is consistent with A. alternata infection in CaERF1A overexpressing Arabidopsis plants.

Materials and methods

Plant materials and growth conditions
Hot pepper (Capsicum annuum L.) cultivars Bugang and Nokkwang were grown in a growth room at 25° C with a 16 h light and 8 h dark photoperiod cycle. Arabidopsis thaliana Col-0 wild-type and transgenic plants were grown in a 16 h light/8 h dark photoperiod at 23° C in soil. For the constitutive expression of CaERF1A and CaERF1A mutant, a Gateway vector was used, and the CaERF1A open reading frame (ORF) was amplified using Pfu-polymerase and cloned. CaERF1A construct was generated by deletion of putative nuclear localization signal (NLS) ‘KRRKK’ in the C-terminal using PCR.
**Virus-induced gene silencing (VIGS)**

The 214 bp of 3′ untranslated region (UTR) and partial open reading frame (ORF) fragment of CaERF1A were cloned into the pTRV2 vector containing a part of the Tobacco rattle virus (TRV) genome using BamHI sites. The pTRV1 and pTRV2-GFP or pTRV2-CaERF1A were introduced into Agrobacterium strain GV3101. The Agro-cells are infiltrated using infiltration buffer (20 mM citric acid, 2% sucrose, and 200 μM acetosyringone, pH 5.2), adjusted to an OD_{600} = 0.2.

**Gene expression analysis**

Six-week-old plants were used for virus inoculation and chemical treatments. Virus-containing sap TMV-P₀ (avirulent) or PMMoV-P₁, 2, 3 (virulent) strains were inoculated by rubbed fully expanded leaves with carborundum. For fugal pathogen inoculation, *Alternaria alternata* FBCC-F68 strain was grown and used for inoculation as described. In brief, *A. alternata* was grown on PDA plates for 6–8 d with 100% humidity at 25°C with a 16-h light/8-h dark cycle. *A. alternata* spore was inoculated by spray in the *Arabidopsis* leaves.

For chemical treatments, 6-week-old plants were sprayed with a solution of 10 mM salicylic acid (SA), 100 μM methyl jasmonate (MeJA), and 10 mM ethephon (ET). Total RNA was prepared with the samples using Trizol RNA extraction method. Quantitative RT-PCR was performed with SYBR Green according to the manufacturer’s instructions (KapaBiosystems). Primers used in these experiments are listed in Table S1.

**Enzyme-linked immunosorbent assay (ELISA) and Western blot**

ELISA was performed as described previously. Total proteins were extracted from TMV-P₀-inoculated plants. The polyclonal TMV-CP antibody (diluted 1:5000) was used, and plates were incubated for 1 h at 37°C. TBS buffer containing alkaline phosphatase-conjugated goat anti-rabbit IgG was added and the plates were incubated further. p-Nitrophenyl phosphate was used as substrate and color values were measured at a wavelength of 405 nm by an ELISA reader. Western blot was performed with mCherry-specific antibody.

**Subcellular localization analysis of CaERF1A**

*Araabidopsis* transgenic carrying CaERF1A-mCherry or CaERF1A^nl5-mCherry plant leaves were used for confocal microscopy analysis. The mCherry signals were detected using an LSM 510 Meta confocal microscope (Carl Zeiss).

**Electrolyte leakage assay**

The electrolyte leakage assay was conducted with leaves of hot pepper plants inoculated with TMV-P₀. Briefly, five leaf discs were taken from one leaf and transferred to a six-well dish containing 5 ml water. After 1 hr of washing with gentle agitation, the leaf discs were transferred to new glass tubes containing 10 ml water. The conductivity of the samples was determined using portable conductivity meter (Thermo Orion). This experiment was repeated three times.

**Measurement of ethylene**

The seedlings of *Araabidopsis* CaERF1A-OE transgenic and WT were grown in 500 mL uncapped air-tight plastic bottles (SPL) for 2 weeks at growth chamber. The *Araabidopsis* plants were inoculated with *A. alternata* FBCC-F68. Water was used as negative controls. After the bottles were sealed for the 24 hr, 1 mL of headspace of each bottle was measured for ethylene contents using gas chromatography with a flame ionization detector (Shimadzu).

**Results**

*CaERF1A, a member of hot pepper AP/ERF family, is strongly induced by plant defense-related hormones and avirulent TMV-P₀ inoculation*

Previously, we performed microarray analysis of TMV-P₀-mediated cell death condition to better understand the mechanism underlying L-gene-mediated cell death upon TMV infection. And we focused on the ERF genes that were up-regulated during TMV-P₀ infection (Figure 1a). Among them, highly induced *CaERF1A* (LOC107868079) gene was selected. *CaERF1A* contains conserved AP/ERF DNA binding domain and has a putative ‘KKRRKK’ nucleus localization signal peptide in the C-terminal region (Fig S1a). From the phylogenetic tress analysis, *CaERF1A* is close to group V *Araabidopsis* ERF1A and ERF2 but more closely related to tobacco ERF2 members (Fig S1). To confirm the microarray data, we performed qRT-PCR analysis with TMV-P₀ (avirulent strain) and PMMoV-P₁,2,3 (virulent strain)-inoculated hot pepper leaves. Expression of *CaERF1A* is significantly increased in TMV-P₀-inoculated plants but not PMMoV-P₁,2,3, suggesting that *CaERF1A* is specifically involved in TMV-P₀-mediated hypersensitive response but not basal defense (Figures 1b and 1c).

To further characterize the function of *CaERF1A* in plant defense, we analyzed the about 18 2 kb promoter region of *CaERF1A* using PlantCARE tool. In the analysis of the *CaERF1A* promoter, we found ERF1A function might be related to ethylene (ET)/jasmonic acid (JA)-mediated signaling (Figure 1d). Indeed, expression patterns of *CaERF1A* in hot pepper plants treated with wounding, methyl jasmonic acid (MeJA), ET, and salicylic acid (SA) were significantly increased (Figure 1e). These results indicate that *CaERF1A* function in the TMV-P₀-mediated hypersensitive response cell death might regulate ET/JA-mediated signaling. Similarly, activation of AtERF1 requires 19 both ET and JA. AtERF1 expression in JA-insensitive mutant is inactivated.

To examine whether *CaERF1A* functions in the TMV-P₀-mediated hypersensitive response cell death, we generated a *CaERF1A*-silencing construct with *Tobacco rattle virus* (TRV)-based VIGS system (Figure 2a). Gene silencing efficiency of *CaERF1A* showed about 65% (Figure 2b). To test the effect of TMV-P₀-mediated cell death in TRV2-CaERF1A
and TRV2-GFP, electrolyte leakage was measured. The conductivity of TRV2-CaERF1A plants was dramatically reduced by about 52% compared with TRV2-GFP plants (Figure 2c). In addition, accumulations of TMV virus coat protein are significantly reduced local and upper leaves in TRV2-CaERF1A plants when compared with control TRV2-GFP plants (Figures 2d and 2e). Thus, CaERF1A could be involved in TMV-P0-mediated cell death.
Figure 2. CaERF1A is involved in L-mediated resistance upon TMV-P0 infection. (a) A schematic illustration of VIGS region of CaERF1A. Partial 3' UTR and ORF fragments of CaERF1A cDNA were used for VIGS. (b) Silencing efficiency of CaERF1A in TRV2-CaERF1A-silenced plants using qRT-PCR analysis. Error bars indicate standard deviations (n = 3). Student’s t-test. ***P < 0.0001. (c) For quantification of TMV-P0-mediated HR cell death, conductivity was measured by electrolyte leakage assay upon TMV-P0-inoculated or mock-treated TRV2-CaERF1A and TRV2-GFP plants at 3 dpi. The error bars show the mean of the standard deviation (SD) of the replicate samples. (d) For quantification of TMV-P0 accumulation, ELISA analysis was performed in TMV-P0-inoculated upper and local leaves at 3 dpi. (e) ELISA was performed in non-TMV-P0-inoculated upper leaves. The error bars show the mean value of the standard deviation (SD) of the replicate samples. The student’s t-test is used to check whether two sets of data differ significantly (*p < 0.01, **p < 0.001, ***p < 0.0001). (f and g) qRT-PCR analysis for basal defense-related genes (CaPR1, CaPR2, and CaPR10) and HR cell death-related genes (CaHIN1, CaAlaAT1, and CaHSR203J) in TMV-P0-inoculated CaERF1A-silenced plants at 2 dpi. The error bars show the mean value of the standard deviation (SD) of the replicate samples. The statistical significance of the difference was confirmed by Student’s t-test (*p < 0.01, **p < 0.001, ***p < 0.0001).
**Knockdown of CaERF1A affects TMV-P0-mediated HR cell death and defense-related gene expression**

To investigate the role of CaERF1A as a transcriptional factor, we also checked expression patterns of pathogenesis-related genes (PRs) and HR cell death-related genes in TRV2-CaERF1A plants upon TMV-P0 inoculation. As expected, gene expression levels of CaPR1, CaPR2, and CaPR10 were significantly down-regulated in TMV-P0-inoculated TRV2-CaERF1A plants when compared with TRV2-GFP control (Figure 2f). Furthermore, CaHIN1, CaAlaAT1, and CaHSR203L transcript levels also reduced in TMV-P0-inoculated TRV2-CaERF1A plants when compared with TRV2-GFP plants (Figure 2g). Thus, our data indicated that CaERF1A has a transcriptional reprogramming function in the TMV-P0-mediated cell death.

**CaERF1A regulates ethylene and jasmonic synthesis-related gene expression upon TMV-P0-mediated HR response**

JA, ET, and SA have shown that key elements for HR cell death and defense signaling control. To explore whether expression of ET/JA synthesis genes is changed in TMV-P0-mediated cell death, we analyzed expression patterns of ET/JA hormone synthesis gene in the microarray data. As expected, expression of some ET synthesis-related genes such as 1-aminocyclopropane-1-carboxylic acid (ACC), ACC synthase (ACS), and ACC oxidase (ACO) was induced in TMV-P0-mediated cell death (Figure 3a). We also found that upregulation of JA synthesis-related genes such as lipoxigenase (LOX), allene oxide cyclase (AOC), allene oxide synthase (AOS), and 12-oxophytodienoate reductase (OPR) (Figure 3a), suggesting that TMV-P0-mediated cell death is required for ET/JA synthesis as well as SA. To confirm CaERF1A is involved in regulation of ET/JA synthesis-related gene expression in TMV-P0-mediated cell death, we performed qRT-PCR using TRV2-CaERF1A silencing and TRV2-GFP control plants upon TMV-P0 inoculation. Most of the ET/JA synthesis-related genes were reduced in TMV-P0-inoculated TRV2-CaERF1A silencing plants compared with TRV2-GFP plants (Figures 3b and 3c).

**CaERF1A overexpression in Arabidopsis enhances resistance to necrotrophic pathogen via ET and JA synthesis-related gene regulation**

To investigate gain of function of CaERF1A, we generated 35S::CaERF1A-mCherry and 35S::CaERF1A<sup>3A</sup>-mCherry transgenic Arabidopsis plants (Figure 4a). CaERF1A<sup>3A</sup> construct was

![Figure 3. Suppression of ET/JA synthesis-related gene expression in TMV-P0-inoculated CaERF1A-silenced plants. (a) Gene expression analysis of ET/JA synthesis-related gene expression in TMV-P0-inoculated hot pepper plants using microarray data. (b and c) For monitoring of ET/JA synthesis-related genes expression patterns in TMV-P0-silenced CaERF1A-silenced plants at 2 dpi, qRT-PCR analysis was performed using gene specific primers of ET synthesis-related genes (CaACS1, CaACS2, CaACO2, and CaACO3-like) and JA synthesis-related genes (CaLOX1, CaAO3, and CaOPR3). The statistical significance of the difference was confirmed by Student's t-test (*P < 0.01, ***P < 0.0001).](image-url)
generated by deletion of putative nucleus localization signal (NLS) ‘KRRKK’ in the C-terminal end (Fig. S1). As expected, CaERF1A localizes in the nucleus and signal of CaERF1A<sup>ΔmCherry</sup> is mainly observed in the cytosol region but not nucleus (Figure 4b). We also confirmed protein expression by Western blot in the Arabidopsis transgenic plants (Figure 4c).

We next tested whether CaERF1A might be required for ET/JA signaling-mediated resistance upon necrotrophic fungus, Alternaria alternata strain FBCC-F68. As shown Figure 4d, overexpression CaERF1A plants show more less symptoms when compared with control plants. Ethylene production was measured A. <i>alternata</i>-inoculated and non-inoculated Arabidopsis plants using gas chromatography. It was confirmed that ET produced more significantly in the CaERF1A transgenic plants compared with WT plant upon A. <i>alternata</i> inoculation (Figure 4e). We also checked accumulation of A. <i>alternata</i> with A. <i>alternata</i>-specific Tubulin gene primers. In A. <i>alternata</i>-inoculated 35S:CaERF1A-mCherry transgenic plants, accumulations of <i>AaTubulin</i> gene are highly reduced when compared with control plants (Figure 4f). These results are consistent with reduced gene expression levels of AtACS1 and AtLOX1 in A. <i>alternata</i>-inoculated 35S:CaERF1A-mCherry transgenic plants (Figures 4g and 4h). Thus, CaERF1A could enhance to JA/ET synthesis-related gene expression and ET production against fungal pathogen infection.

**Discussion**

<i> Capsicum annuum</i> is an important horticultural crop that is susceptible to various pathogens, especially virus and necrotrophic fungal pathogens. Many transcription factors have been characterized for their defense role in hot pepper immunity to pathogens, but less information is known regarding the ERF family in hot pepper.

Ethylene-responsive factors (ERF) perform multifarious functions in plant resistance/tolerance to biotic and biotic stresses. The DNA-binding domain of AP2/ERF transcription factors, which consists of a sheet with three β-strands followed by α-helix, recognizes cis-regulatory elements, including the GCC- and GCC-boxes. Purified ethylene-responsive factor like protein 1 (CaERFLP1) protein was able to make a specific complex with both the GCC box and DRE/CRT motif.

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**Figure 4.** Overexpression of CaERF1A confers resistance to necrotrophic fungus in Arabidopsis. (a) Arabidopsis transgenic carrying 35:CaERF1A-mCherry and NLS deletion mutant 35:CaERF1A<sup>ΔmCherry</sup> plants shows no developmental phenotype. (b) CaERF1A localized to the nucleus but not CaERF1A<sup>ΔmCherry</sup> mutant in Arabidopsis transgenic plants. (c) Western blot analysis of CaERF1A-mCherry and CaERF1A<sup>ΔmCherry</sup> transgenic plants using mCherry specific antibody. (d) Alternaria alternata inoculation symptoms at 3 dpi. (e) The Arabidopsis transgenic carrying 35:CaERF1A-mCherry plants showed increased ethylene production in response to A. alternata. After 1 dpi upon A. alternata, ethylene contents from seedlings of 35:CaERF1A-mCherry transgenic and WT plants were measured using gas chromatography. The error bars show the mean value of the standard deviation of the replicate samples. The statistical significance of the difference was confirmed by Student’s t-test (**P < 0.0001). (f) A. alternata<sup>Tubulin</sup> gene expression analysis in the A. alternata-inoculated 35:CaERF1A-mCherry transgenic and WT plants. The samples were harvested at 2 dpi. The error bars show the mean value of the standard deviation (SD) of the replicate samples. The statistical significance of the difference was confirmed by Student’s t-test (**P < 0.0001). (g) and (h) Gene expression analysis of AtACS1 and AtLOX1. The 35:CaERF1A-mCherry transgenic and WT plants were inoculated by A. alternata and the samples were harvested at 2 dpi. The error bars show the mean value of the standard deviation (SD) of the replicate samples. The statistical significance of the difference was confirmed by Student’s t-test (**P < 0.0001).
Overexpression of CaERF1A in transgenic tobacco plants showed improved resistance against the bacterial pathogen *Pseudomonas syringae*.\(^3^0\) Consistently, various defense-related genes, including GCC box-containing PR genes were constitutively expressed in 35S:CaERF1P1 tobacco plants.\(^3^0\) Identified ERFs regulated the expression of ET/JA-responsive genes by targeting GCC box present in their promoters.\(^3^1\) Similarly, CaERF1A also might bind to the GCC box of ACS1 and LOX1 promoters to regulate ET/JA synthesis against TMV and necrotic fungal pathogen infections.

The hot pepper plant contains CC-NB-LRR type L-gene resistance proteins, which confer resistance to *tobacco mosaic virus* (TMV) by restricting virus spread at the primary infection site.\(^3^2\) The resistance proteins are immune receptors that possess nucleotide-binding (NB) domain and leucine-rich repeat-containing (LRR) domains. They are part of a broad family conserved between plants and animals known as Nod-like receptors (NLR).\(^3^3\) NLRs mediate elicitor recognition and activate downstream signaling responses leading to programmed cell death termed the hypersensitive response.\(^3^4\) NLRs detect pathogen-derived effectors either directly or indirectly and then activate immune responses, including the accumulation of the defense hormones salicylic acid (SA) or jasmonic acid (JA), ethylene (ET), reactive oxygen species (ROS) and PR proteins.\(^3^5\) In L-gene-mediated resistance to TMV, we showed that expression of ET/JA biosynthesis-related genes are highly induced and ET production is induced upon fungal inoculation in *Arabidopsis 35S:CaERF1A* transgenic plants (Figure 4e).

SA is an important plant defense hormone, which positively regulates many ERFs.\(^3^6\) These data imply that JA/ET cross-talks with SA to fine-tune plants’ defense signaling pathway. Indeed, the transcript level of CaERF1A was strongly increased under JA/ET treatments. Thus, CaERF1A function might be associated with ET/SA signaling pathway in TMV-P0-mediated cell death. In *Arabidopsis*, ethylene signaling is required for the acceleration of cell death by activation mutant AtMEK5.\(^3^7\)\(^3^8\) Moreover, ethylene production is increased in TMV-inoculated NN tobacco plants and it is correlated with overexpression of ACSs and ACOs.\(^3^9\)\(^4^0\) Thus, CaERF1A is a positively regulator of ET/JA synthesis-related gene to enhance TMV-P0-mediated cell death and protect fungal pathogen infection.

**Conclusion**

CaERF1A is identified with microarray analysis in TMV-P0-mediated cell death. AP/ERF transcription factors function as a positive or negative regulator in plant defense as well as environmental stresses. CaERF1A is a transcriptional positive regulator in the nucleus. CaERF1A is required for TMV-P0-mediated cell via regulating ET/JA synthesis-related gene expression. Furthermore, overexpression of CaERF1A confers a strong resistance against necrotrophic fungal pathogen infection. CaERF1A might be a useful genetic trait for engineering crop plant in the future.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by the Next-Generation BioGreen 21 Program (Project No. PJ01365301) by Rural Development Administration.

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