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The *CaAP2/ERF064* Regulates Dual Functions in Pepper: Plant Cell Death and Resistance to *Phytophthora capsici*

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Abstract: *Phytophthora* blight is one of the most destructive diseases of pepper (*Capsicum annuum* L.) globally. The APETALA2/Ethylene Responsive Factors (*AP2/ERF*) genes play a crucial role in plant response to biotic stresses but, to date, have not been studied in the context of *Phytophthora* resistance in pepper. Here, we documented potential roles for the pepper *CaAP2/ERF064* gene in inducing cell death and conferring resistance to *Phytophthora capsici* (*P. capsici*) infection. Results revealed that the N-terminal, AP2 domain, and C-terminal of *CaAP2/ERF064* protein is responsible for triggering cell death in *Nicotiana benthamiana* (*N. benthamiana*). Moreover, the transcription of *CaAP2/ERF064* in plant is synergistically regulated by the Methyl-Jasmonate (MeJA) and ethephon (ET) signaling pathway. *CaAP2/ERF064* was found to regulate the expression of *CaBPR1*, which is a pathogenesis-related (*PR*) gene of pepper. Furthermore, the silencing of *CaAP2/ERF064* compromised the pepper plant resistance to *P. capsici* by reducing the transcript level of defense-related genes *CaBPR1*, *CaPO2*, and *CaSAR82*, while the ectopic expression of *CaAP2/ERF064* in *N. benthamiana* plant elevated the expression level of *NbPR1b* and enhanced resistance to *P. capsici*. These results suggest that *CaAP2/ERF064* could positively regulate the defense response against *P. capsici* by modulating the transcription of *PR* genes in the plant.

Keywords: pepper; *AP2/ERF*; *P. capsici*; cell death; *PR* gene

1. Introduction

Plants are continuously exposed to various pathogens and have evolved complex mechanisms to regulate defense response [1,2]. Members of the APETALA2/Ethylene Responsive Factor (*AP2/ERF*) gene family play an important role in biotic and abiotic defense responses in plants [3,4]. In a previous study in tobacco, *ERF* genes were identified through binding to the GCC-Box cis-element of the pathogenesis-related (*PR*) genes [5]. In *Arabidopsis*, *AtERF96* promotes the expression levels of several defense genes (*PDF1.2*, *PR-3*, and *PR-4*), by directly binding to GCC-Box elements of their promoters, and enhances the plant resistance to necrotrophic pathogens [6]. Furthermore, it was found that the expression of the *ERF* gene *RAP2.2* was upregulated upon the infection of *Botrytis cinerea* in *Arabidopsis*, while this upregulation was disrupted in *ethylene insensitive 2* (*ein2*) and *ein3* mutants. Moreover, the over-expression of *RAP2.2* in *ein2* and *ein3* mutants restored the *Arabidopsis* plant resistance to *Botrytis cinerea* [7]. It has also been shown that the *Arabidopsis* *ERF5* gene promotes plant defense responses to multiple pathogens through coordinating chitin pathways [8].

A subset of *AP2/ERF* factors contains an *ERF*-associated amphiphilic repression (*EAR*) domain and have been shown to function as a transcriptional repressors of defense-related genes [9,10].

For example, *AtERF4* was found to negatively regulate plant response to *Fusarium oxysporum* infection in *Arabidopsis* [9], while *StERF3* negatively regulates plant resistance to *Phytophthora infestans* and salt stress [11], whereas the over-expression of *GmERF5* increases the transcription level of *PR* genes and also enhances plant resistance to *Phytophthora sojae* infection [12]. Recently, it was found that *AtERF8* gene positively regulates plant resistance to *Pseudomonas syringae* [13,14].

In previous studies, several ERF genes were identified in the pepper plant. *CaERFLP1* was transcriptionally activated during the infection of pathogens in pepper [15]. The over-expression of *CaPF1* in *Arabidopsis* and tobacco plants increased resistance to *Pseudomonas syringae* [16], whereas the *CaPT11* gene has been shown to play a crucial role in resistance to *Phytophthora capsici* (*P. capsici*) infection [17]. Recently, the *CaAIEF1* gene was found to regulate the response to drought stress in plants [18].

Oomycetes include a large number of pathogens. *P. capsici* causes root and fruit rot on pepper and other vegetables (tomato, cucurbits, and lima beans) plant. *P. capsici* is a highly destructive pathogen and is considered to be one of the most significant oomycete pathogens limiting production of these vegetables [19,20]. Members of the AP2/ERF gene family have been reported to play a key role in plant response to different pathogens. However, little is known about the exact role of *CaAP2/ERF* genes in pepper plant response to *P. capsici*.

In the previous study, the AP2/ERF family genes were identified from the latest pepper genome database in response to *P. capsici* infection and various phytohormones treatments. Moreover, one of these genes, *CaAP2/ERF064*, was found to respond to the infection of *P. capsici*, and *CaAP2/ERF064* protein was located in the nucleus [21]. In this study, the expression pattern of *CaAP2/ERF064* in response to various combinations of phytohormones was investigated with qRT-PCR. Moreover, its function in plant defense response to *P. capsici* was elucidated by the virus-induced gene silencing (VIGS) technique and ectopic transformation in *Nicotiana benthamiana* (*N. benthamiana*). Additionally, the transcription regulation relationship between *CaAP2/ERF064* and the *CaBPR1* gene was also identified with a yeast one-hybrid assay. The cell's death, which induced by the over-expression of *CaAP2/ERF064* and RNA silencing suppressor *P19*, was investigated with a transient expression assay in *N. benthamiana*.

2. Materials and Methods

2.1. Plant Materials and Phytohormones Treatments

The *Phytophthora capsici*-resistant pepper (*Capsicum annuum* L.) lines Y5 was obtained from Vegetable Plant Biotechnology and Germplasm Innovation lab, Northwest A&F University-China. Pepper seedlings were grown under 16 h light and 8 h dark photoperiods, with 25 and 18 °C day and night temperatures, respectively, and 65% relative humidity. *Nicotiana benthamiana* plants were grown under 16 h light and 8 h dark photoperiods, with 22 and 20 °C day and night temperatures, respectively, and 65% relative humidity.

For phytohormones treatments, the Y5 plants, at 6–8 leaves stage, were sprayed with 1mM salicylic acid (SA), 100 µM Methyl-Jasmonate (MeJA) or 100 µM ethephon (ET). Control plants were sprayed with mock solution. Leaf samples were collected at 0, 3, 6, and 12 h post-treatment (hpt).

2.2. Pathogen Preparation and Inoculation

P. capsici strain HX-9 was obtained from our laboratory, and zoospores of *P. capsici* were prepared as previously described [21,22]. Briefly, *P. capsici* on potato dextrose agar (PDA) medium was divided into pieces and covered with liquid carrot broth (200 g/L) in a Petri dish for three days at 28 °C. Then, the cultures were washed with sterile water and covered with Petri broth for five days in the dark. To release zoospores, cultures with sporangia were covered with cold sterile water and placed at 4 °C for 30 min. Finally, the concentration of zoospores was adjusted to 1×10^6 zoospores/mL with sterile water.

For the detached leaf assay, 10 μ L zoospores suspension was applied on the dorsal side of each leaf, and the leaves were then incubated on 22–25 °C [23], while for the inoculation of tobacco plants, 5 mL zoospores suspension was applied to each plant by a foliar and root drench, and the plants were then incubated at 25 °C [24]. The leave samples were collected at 0, 3, and 7 days post-inoculation (dpi). The disease index of tobacco plant was determined at 0, 7, and 14 dpi, as described in our previous paper [24], while symptoms on tobacco plants are characterized into different levels (L₀–L₆) which are: Level₀: no symptoms; Level₁: stem shrink < 1 cm (from the base); Level₂: >1–2 cm and leaves wilting; Level₃: > 2–3 cm and leaves become yellow; Level₄: > 3–4 cm and leaves become yellow; Level₅: > 4–5 cm and the leaves become yellow and withered; and Level₆: > 5 cm or whole plant died.

2.3. Transient Expression Assay in Tobacco

To construct the transient expression vector, the coding sequence (CDS) and deletion fragments of *CaAP2/ERF064* gene and its homologs (*StERF1B-l*, *SITSRF1*, and *NbERF1B-l*) (excluding stop-codon) were cloned into a pCAMBIA3301-green fluorescent protein (GFP) vector, while the *PcINF1* and *NbCD1* were used as positive controls (Table S1). The recombinant constructs were transformed into *Agrobacterium tumefaciens* (GV3101) with the freeze-thaw method.

The transient expression assay was performed as described in our previous study [21]. Briefly, *Agrobacterium* strains were grown in Luria-Bertani (LB) liquid medium at 28 °C for 12–16 h, then collected by centrifugation and suspended with the infiltration medium (10 mM MgCl₂, 10 mM MES, pH 5.5, 200 μ M acetosyringone) to an OD₆₀₀ of 0.8. The suspension cells were mixed in a 1:1 ratio with *P19* (RNA-silencing suppressor), and then the mixture was injected in *N. benthamiana* leaves through a needleless syringe. Cell death was monitored at 4–7 days post infiltration. For the transient expression of *CaAP2/ERF064* and derived deletion mutants in Pro_{CaBPR1}:*CaBPR1* transgenic tobacco plants, infiltration was done identically, and samples were collected 3 days after infiltration.

While for the subcellular localization of different homologs and deletion mutants of *CaAP2/ERF064* gene, the GFP signal in infiltrated tobacco leaves was detected with OLYMPUS BX63 (OLYMPUS, Tokyo, Japan) fluorescence microscope at 2–3 days post-infiltration.

2.4. Transcriptional Activation and Yeast One-Hybrid Assay

To evaluate transcriptional activation, the *CaAP2/ERF064* coding sequence and derived deletion fragments were cloned into the pGBKT7 vector. The recombination constructs were transformed into yeast strain (Y 2-H gold) according to the manufacturer's protocol (Clontech, CA, U.S.). The resulting strains were grown on SD/-Trp, SD/-Trp-His-Ade, and SD/-Trp-His-Ade + X- α -Gal at 30 °C for 2–3 days.

For yeast one-hybrid assay, the CDS of *CaAP2/ERF064* and the promoter fragment of *CaBPR1* were cloned into the pGADT7 and pHIS2 vector, respectively. Then, the resulting strains (Y187), which contained these constructs, were grown on SD/-Leu-Trp and SD/-Leu-Trp-His medium contained 90 mM 3-AT (the minimal inhibitory concentration of the promoter activation of *CaBPR1*) at 30 °C for three days.

2.5. Virus-Induced Gene Silencing in Pepper

For VIGS assays, a 267-bp specific fragment of *CaAP2/ERF064* was cloned into pTRV2 to generate the TRV2:CaAP2/ERF064 vector. VIGS was performed as in our previous study [25]. Briefly, *Agrobacterium* strains (TRV2:CaAP2/ERF064, TRV2:GFP, and TRV2:CaPDS), as well as pTRV1, were grown in induction medium at 28 °C for 12–18 h; then cells were collected by centrifugation and suspended with the infiltration medium (10 mM MgCl₂, 10 mM MES, pH 5.5, 200 μ M acetosyringone). The suspension was mixed in a 1:1 ratio with suspension of pTRV1 and used for infiltration of two-weeks-old pepper seedlings. Finally, the pepper plants were incubated in the chamber at 22 °C.

2.6. Tobacco Transformation

The CDS of *CaAP2/ERF064* was cloned into pCAMBIA2300-RC to generate the pCaMV35S:CaAP2/ERF064 vector, while the promoter (2016-bp) and CDS fragment of *CaBPR1* gene (excluding stop-codon) was cloned into the pBI121 vector. Both of these constructs were transformed into *Agrobacterium tumefaciens* (GV3101) and then used for the tobacco transformation. The pCaMV35S:CaAP2/ERF064 and Pro_{CaBPR1}:CaBPR1 transgenic tobacco plants were generated with the *Agrobacterium*-mediated leaf disc transformation assay [26]. All the transgenic tobacco plants were confirmed with qRT-PCR and T₂ generation plants were used for further analysis.

2.7. Histochemical Staining

3,3-diaminobenzidine (DAB) and trypan-blue staining were conducted as described by Choi, et al. [27]. For DAB staining, the tobacco leaves were incubated in DAB solution (1 mg mL⁻¹, pH 3.8) for 8–10 h. Then, the leaves were cleared with 75% ethanol, while for trypan-blue staining, pepper and tobacco leaves were boiled in trypan-blue solution (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue, dissolved in 10 mL distilled water) for 2–5 min. Then, leaves were de-stained with chloral hydrate (2.5 g mL⁻¹).

2.8. qRT-PCR Analysis

For gene expression analysis by qRT-PCR, total RNA was isolated with the Trizol method as previously described [24], and cDNA was synthesized with reverse transcript kit according to the manufacturer's instructions (Vazyme, China). The qRT-PCR assay was performed as previously described [21,24]. The β -glucuronidase (GUS) gene primers were used to detect the expression level of *CaBPR1* in Pro_{CaBPR1}:CaBPR1 transgenic tobacco plants. *CaUBI3* and *NbEF1 α* were used as reference genes in pepper or tobacco, respectively. The relative expression levels of each gene were calculated by the 2^{- $\Delta\Delta$ Ct} method [28]. All the primers used for qRT-PCR are listed in Table S1.

2.9. Statistical Analysis

Data Processing System software was used for statistical analysis with least significant difference (LSD) at $p < 0.05$ levels. All experiments were performed and analyzed independently with at least three biological replicates.

3. Results

3.1. Expression Analysis of *CaAP2/ERF064* in Response to Combinations of Phytohormones

Our previous study showed that *CaAP2/ERF064* gene exhibited a high response to the treatment of SA, MeJA, and ET [21]. To investigate potential for cross-talk signaling in the regulation of *CaAP2/ERF064*, we treated pepper plants with combinations of these phytohormones. As shown in Figure 1, with ET treatment, the highest (19.65-fold) and lowest (5.08-fold) expression level of *CaAP2/ERF064* were detected at 3 and 12 h post-treatment (hpt), respectively, while the expression level of *CaAP2/ERF064* in treatment with ET + SA, ET + MeJA, SA + MeJA, and ET + SA + JA peaked at 3 hpt, which is 19.91, 32.29, 14.73, and 27.67-fold, respectively. The expression level of *CaAP2/ERF064* in treatment of ET + MeJA (32.29-fold) and ET + SA + MeJA (27.67-fold) was higher than that in treatment of ET (19.65-fold) and MeJA (2.64-fold) [21] at 3 hpt, while in the SA + MeJA treatment, the response was lower than that in the ET treatment. These results indicated that MeJA augments the effect of ET on the transcription of *CaAP2/ERF064* gene in the pepper.

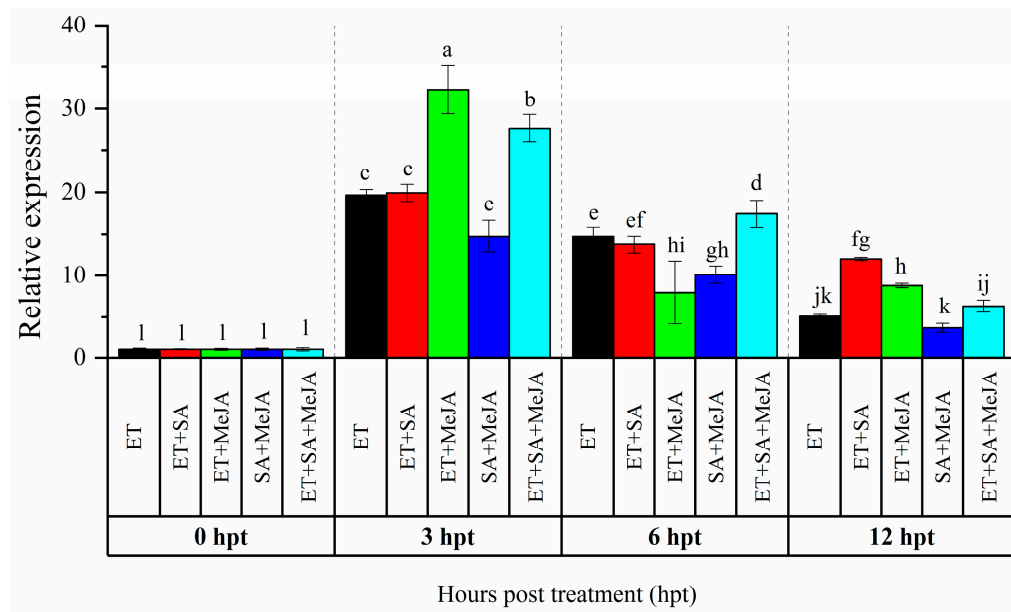


Figure 1. Expression analysis of *CaAP2/ERF064* in response to various combinations of phytohormones. Values are means \pm SD. Small letters indicate a significant difference (least significant difference (LSD), $p < 0.05$).

3.2. Transient Over-Expression of *CaAP2/ERF064* Induces Cell Death in *N. benthamiana*

We found that the tobacco leaves co-infiltrated with *CaAP2/ERF064* and *P19* (suppressor of RNA silencing) [29,30] exhibited cell death phenotypes in the infiltrated area (Figure 2). The cell death area was confirmed by using trypan-blue staining (Figure 2B). Furthermore, to identify the domains of *CaAP2/ERF064* responsible for the induction of cell death, different deletion mutants of *CaAP2/ERF064* were tested for their potency in cell death upon transient over-expression by agro-infiltration. Results showed that its deletion mutant N2 induced the collapse of the epidermis but not cell death in tobacco leaves (Figure 2). The other deletion mutants and empty vector (EV) control did not induce any obvious symptoms in the infiltration area. In the absence of *P19*, both *CaAP2/ERF064* and its N2-mutant could induce the collapse of the epidermis in leaves. The positive control, *NbCD1*, also caused cell death when over-expressed in tobacco. However, the death symptom caused by *NbCD1* was weaker when *P19* was absent. In contrast, the elicitor of *P. capsici*, *PcINF1*, induced strong hypersensitive cell death in the leaves at four days post-infiltration.

Furthermore, the protein localization of different mutants of *CaAP2/ERF064* was detected with a fluorescence microscope. Results showed that *CaAP2/ERF064* and its N2 mutant proteins have a strong fluorescence signal in the nucleus (Figure S1) [21], whereas the deletion mutant C1 protein was located in the cytoplasm, and the N1, C2, and C3 mutant proteins were detected in both the cytoplasm and nucleus. The homologs (*StERF1B-l*, *SITSRF1*, and *NbERF1B-l*) of *CaAP2/ERF064* also caused cell death when co-expressed with *P19* in tobacco leaves (Figure 3A,B), and all showed localization in the nucleus (Figure 3C). Moreover, other ERF genes (*CaAP2/ERF049* and *CaAP2/ERF109*) did not induce the cell death when co-overexpressed with *P19* in tobacco plants (Figure S2). These results suggested that transient over-expression of *CaAP2/ERF064* induces cell death, and its N-terminal, AP2 domain, and C-terminal are necessary to trigger cell death in *N. benthamiana*.

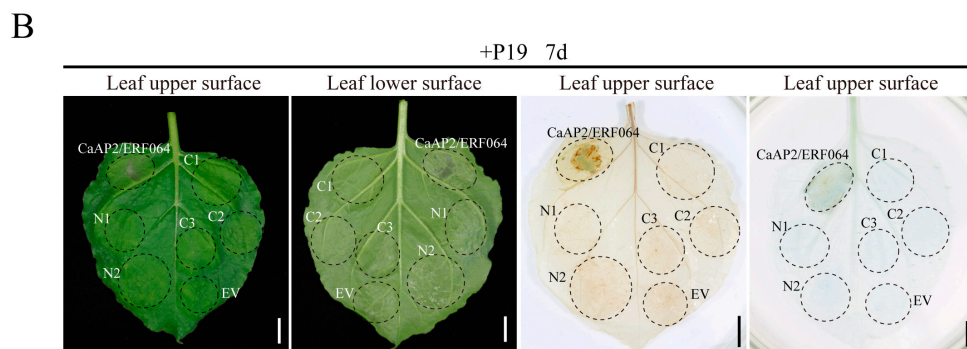
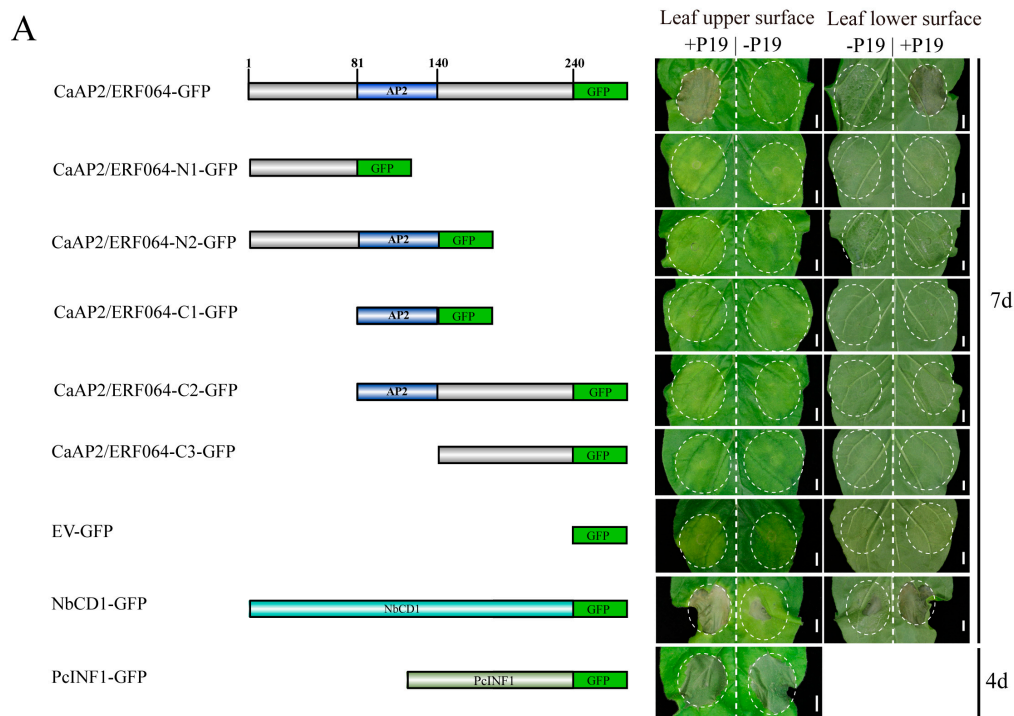


Figure 2. Transient over-expression of *CaAP2/ERF064* triggers cell death in *Nicotiana benthamiana* (*N. benthamiana*). (A) *CaAP2/ERF064* and its deletion mutants in *N. benthamiana*. Scale bar represents 0.5 cm. (B) 3,3-diaminobenzidine (DAB) and trypan-blue staining of tobacco leaves. Scale bar represents 1 cm.

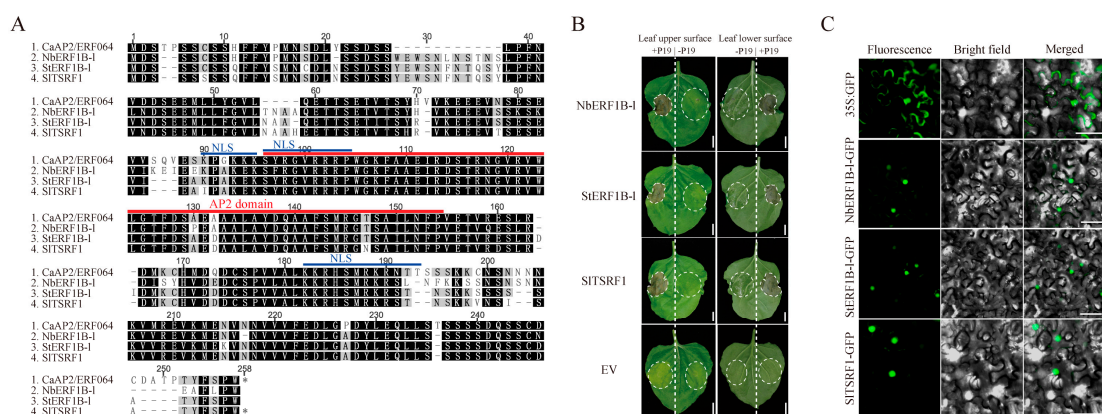


Figure 3. Induction of cell death by *CaAP2/ERF064* homologues in *N. benthamiana*. (A) Sequence alignment of *CaAP2/ERF064* and its homology proteins. (B) Transient over-expression of *StERF1B-1*, *NbERF1B-1*, and *SITSRF1* in *N. benthamiana*. EV was short for empty vector. Scale bar represents 1 cm. The photograph was taken at seven days post-infiltration. (C) Localization of *StERF1B-1*, *NbERF1B-1*, *SITSRF1* proteins in *N. benthamiana* epidermal cells. The scale bar represents 50 μ m.

3.3. CaAP2/ERF064 Regulates the Expression of CaBPR1

To determine if CaAP2/ERF064 protein might function as a transcription factor in pepper, its transcriptional activity was evaluated in yeast. As shown in Figure 4A, the yeast strain containing BD-CaAP2/ERF064 protein showed activation of reporter gene LacZ and grew well in the medium of SD/-Trp-His-Ade and SD/-Trp-His-Ade + X- α -Gal. The deletion mutant C3 of CaAP2/ERF064 also exhibited strong transcription activity, while the deletion mutants N1, N2, and C2 showed weak activity, and the C2 mutant showed no activity in yeast. These results indicated that the CaAP2/ERF064 protein can act as a transcriptional activator.

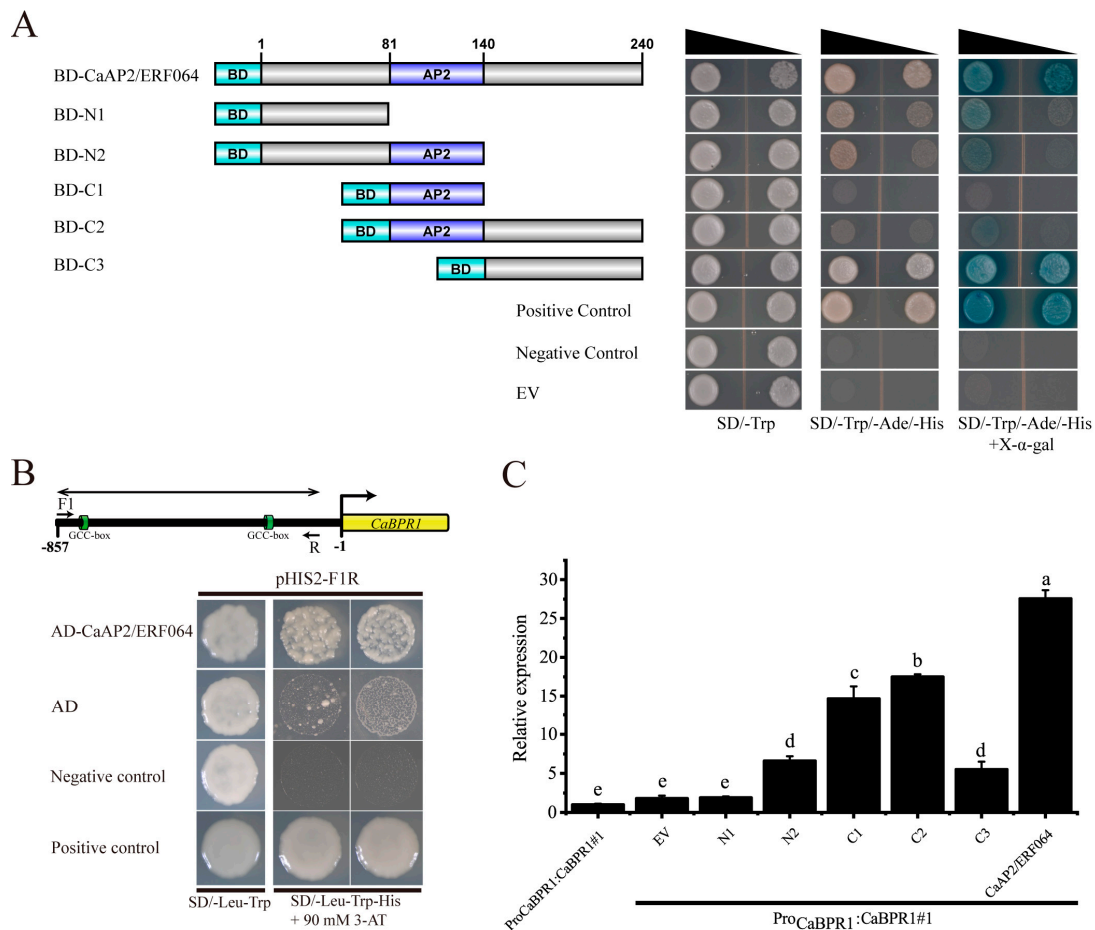


Figure 4. CaAP2/ERF064 promotes the transcription of CaBPR1. (A) Transcriptional activation of CaAP2/ERF064 in yeast. (B) Interaction of CaAP2/ERF064 and the promoter fragments (F1R) of CaBPR1 in yeast. (C) Expression analysis of CaBPR1 in ProCaBPR1:CaBPR1 transgenic tobacco plants after infiltration of CaAP2/ERF064 and its deletion mutants. Values are means \pm SD. Small letters indicate a significant difference (LSD, $p < 0.05$).

Furthermore, ERF transcription factors can bind to the GCC-box element of target genes, and the CaBPR1 (PR1 gene) contains two GCC-box elements in the promoter region [31]. Then, the yeast one-hybrid experiment was performed to detect whether CaAP2/ERF064 protein could bind to the promoter fragment (F1R) of the CaBPR1 gene. The results showed that the yeast strain containing AD-CaAP2/ERF064 protein and pHIS2-F1R grew well in the SD/-Leu-Trp-His medium (90 mM 3-AT), indicating that CaAP2/ERF064 protein could bind to this segment (F1R) of the CaBPR1 gene (Figure 4B).

For further confirmation, the Pro_{CaBPR1}:CaBPR1 transgenic tobacco plants (Figure S3) were subjected to infiltration with expression constructs of CaAP2/ERF064 and its deletion mutants. As shown in Figure 4C, the expression level of CaBPR1 under the treatment of CaAP2/ERF064 was 27.57-fold compared to control at three days post-infiltration, while the expression level of CaBPR1 under the treatment of N2, C1, C2, and C3 mutants were 6.61, 14.66, 17.50, and 5.51-fold, respectively. Both CaAP2/ERF064 and its C2 mutant significantly induced the transcription level of CaBPR1 in Pro_{CaBPR1}:CaBPR1 transgenic tobacco plants. These results demonstrated that CaAP2/ERF064 protein can activate the expression of the CaBPR1 gene in plants.

3.4. Silencing of CaAP2/ERF064 in Pepper Enhances the Susceptibility to *P. capsici*

To evaluate a potential function for CaAP2/ERF064 in resistance to *P. capsici*, the VIGS technique was used to silence CaAP2/ERF064 in the pepper cultivar Y5, which is highly resistance to *P. capsici*. To visually verify the success of CaAP2/ERF064-silencing, a TRV2:CaPDS vector (positive control) was used for the knock-down of the CaPDS gene, which produced a typical white color in the leaves, as a mark of photo-bleaching phenotype. Additionally, the TRV2:GFP vector was used as a negative control. After six weeks of infiltration, the CaPDS-silenced plants showed photo-bleaching phenotypes in the leaves, demonstrating the success of the VIGS (Figure S4). At the same time, the silencing efficiency of CaAP2/ERF064 was examined using qRT-PCR analysis, which revealed that the CaAP2/ERF064 gene in TRV2:CaAP2/ERF064 (CaAP2/ERF064-silenced) plants was 67% lower than TRV2:GFP (negative control) plants (Figure 5A). To evaluate the resistance of CaAP2/ERF064-silenced Y5 plants to *Phytophthora* blight disease, the detached leaf assay was used. After four days of inoculation, the *Phytophthora* blight lesions were found on the leaves of both CaAP2/ERF064-silenced and control plants, but the infected area of the silenced plants was significantly larger than the control plants (Figure 5B,C). Phenotypically, trypan-blue staining showed more extensive cell death in the detached leaves of CaAP2/ERF064-silenced plant (Figure 5B).

Furthermore, the qRT-PCR analysis was used to investigate whether the silencing of CaAP2/ERF064 regulated the defense-related genes (CaBPR1 [32], CaPO2 [33], and CaSAR82 [34]) in pepper plants. So, a substantial decrease was observed in the expression level of the CaAP2/ERF064 gene in the silenced plant compared to the control after *P. capsici* infection (Figure 5D). However, it was found that the expression level of CaBPR1 in CaAP2/ERF064-silenced plants was significantly lower (46.39-fold) than the control (76.85-fold) at seven days post-inoculation (dpi) (Figure 5D). Moreover, the expression level of CaSAR82 in CaAP2/ERF064-silenced plants was also lower (2.51-fold) than that in the control (3.71-fold) at 7 dpi, while the CaPO2 gene only exhibited a lower (7.50-fold) expression level in CaAP2/ERF064-silenced plants when compared with that in the control (17.70-fold) at 3 dpi. These findings indicated that silencing of CaAP2/ERF064 gene might alter the expression of defense-related genes and enhance the plants more prone to *P. capsici* infection.

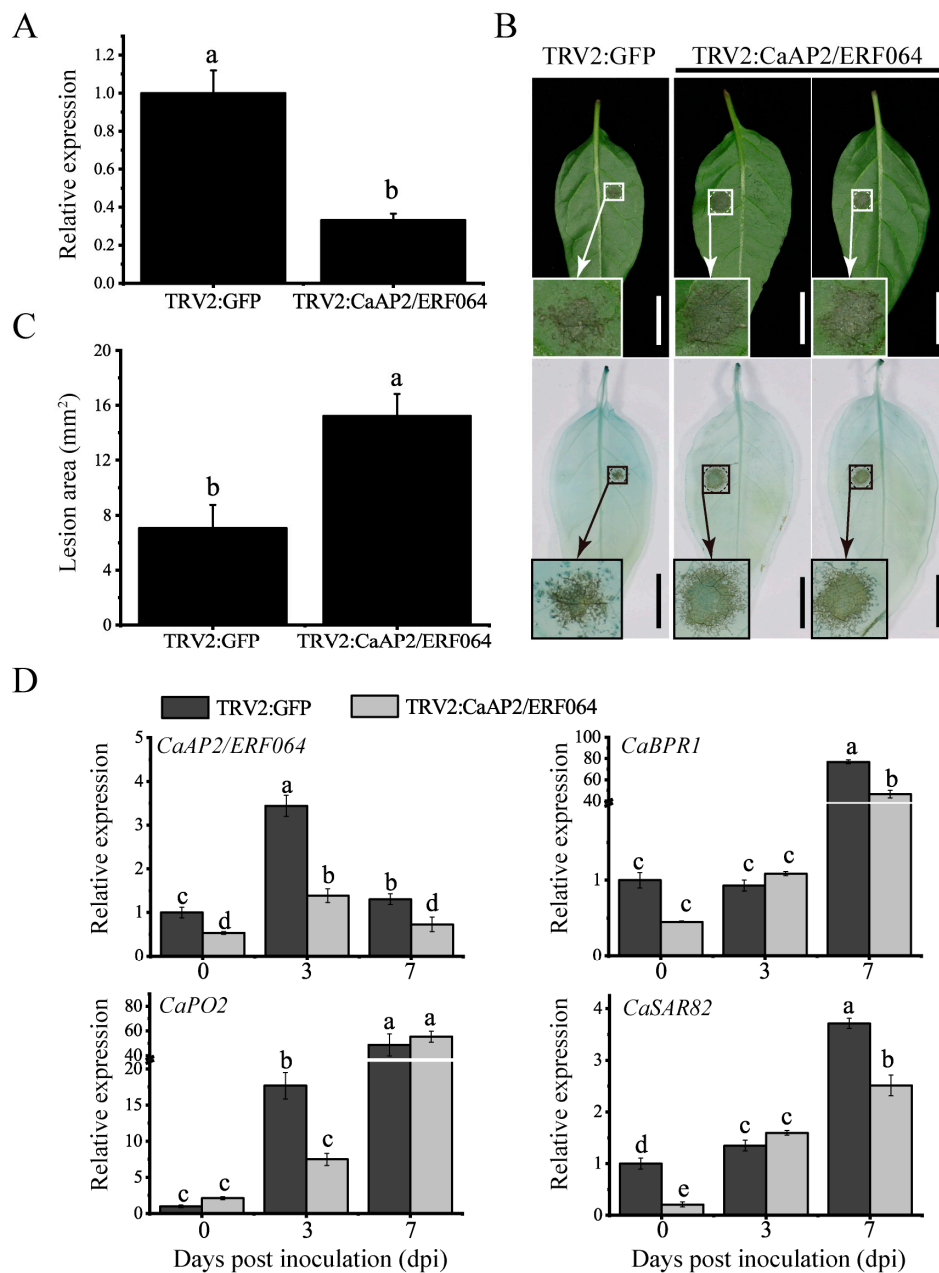


Figure 5. Virus-induced gene silencing (VIGS) of *CaAP2/ERF064* in pepper enhanced susceptibility to *P. capsici* infection. (A) Silencing efficiency of *CaAP2/ERF064* in silenced and control plants. (B) Disease symptom on pepper leaves at 4 dpi. (C) Analysis of leaf lesion and quantified using ImageJ software. (D) Expression analysis of defense-related genes in *CaAP2/ERF064*-silenced and control plants. Values are means \pm SD. Small letters indicate a significant difference (LSD, $p < 0.05$).

3.5. Ectopic Expression of *CaAP2/ERF064* Enhances Tobacco Resistance to *P. capsici*

To further assess the potential role of *CaAP2/ERF064* in plant defense response to *P. capsici* infection, the pCaMV35S:*CaAP2/ERF064*-transgenic tobacco plants were generated and used for the subsequent experiment. As shown in Figure 6A,B, the *CaAP2/ERF064*-OE plants exhibited a higher expression level of *CaAP2/ERF064*, but the plant height is slightly lower than the wild-type (WT) plants. Furthermore, the results of the detached leaf assay showed that the disease lesions on the leaves of *CaAP2/ERF064*-OE plants were significantly smaller (55.12%) than the control after the infection of *P. capsici* (Figure 6C,D), and the cell death was also less extensive in the *CaAP2/ERF064*-OE plants (Figure 6C).

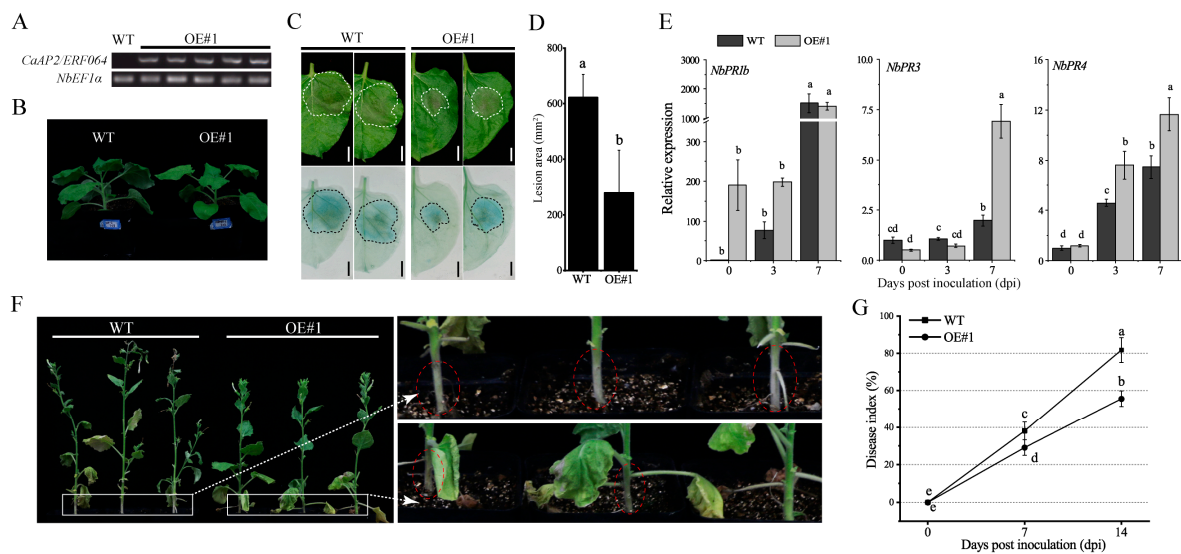


Figure 6. *CaAP2/ERF064*-OE tobacco plants show enhanced resistance to *P. capsici*. (A) Expression of *CaAP2/ERF064* in OE and wild-type (WT) plants. (B) Phenotype of OE and WT plants. (C, D) Disease symptoms and analysis of lesion area at 3 dpi. (E) Expression analysis of defense-related genes after *P. capsici* infection. (F) Phenotypes of OE and WT plants after *P. capsici* inoculation at 7 dpi. Circles indicate the shrinkage of the stem. (G) Disease index percentage. Values are means \pm SD. Small letters indicate a significant difference (LSD, $p < 0.05$).

To further confirm the role of *CaAP2/ERF064* in disease resistance and to elucidate its possible molecular mode of action, the transcriptional responses of defense-related genes in *CaAP2/ERF064*-OE and WT plants were investigated by qRT-PCR. The defense marker gene *NbPR1b* showed a higher expression level in *CaAP2/ERF064*-OE plants as compared to WT, which is 198.23 and 76.17-fold, respectively, at 3 dpi (Figure 6E). Moreover, the chitinase gene *NbPR3* showed higher (6.92-fold) induction in *CaAP2/ERF064*-OE plants when compared with control (1.98-fold) at 7 dpi. The expression level of *NbPR4* in *CaAP2/ERF064*-OE plants was 7.61 and 11.63-fold, which was also higher than the control (4.60 and 7.44-fold) at 3 and 7 dpi, respectively. Furthermore, severe disease symptoms (wilting, yellowing of leaves, and stem shrinkage) were observed in WT plants at 7 dpi, whereas *CaAP2/ERF064*-OE plants were shorter but less affected (Figure 6F). As is evident by a calculated disease index, *CaAP2/ERF064*-OE plants showed lower infection than the control at both 7 and 14 dpi (Figure 6G). These results demonstrated that over-expression of *CaAP2/ERF064* in tobacco plants might directly participate in the defense system, at least partly through promoting expression of PR genes.

4. Discussion

Phytohormones (SA, JA, and ET) are essential signaling molecules and play a crucial role in the regulation of plant immune response to pathogens [35–37]. ERF genes are the downstream components of the ET signaling pathway and are recognized to integrate various phytohormone signaling pathways in plants [3]. The cross-talk between ET and other phytohormones signaling pathways resulted in a differentiated disease resistance response in plants [35]. Previously, we found that *CaAP2/ERF064* could respond to the treatment of MeJA (2.64-fold at 3 hpt) [21]. In this study, we found that the expression level of *CaAP2/ERF064* in treatment of ET + MeJA (32.29-fold) was significantly higher than that in treatment of ET (19.65-fold) and MeJA (2.64-fold) at 3 hpt. These results indicated that the transcription of *CaAP2/ERF064* is synergistically regulated by MeJA and ET signaling pathway. This is consistent with a previous study of *Arabidopsis ERF1*, which reported that this gene could be induced by ethylene and JA, as well as by both phytohormones synergistically [38].

Previous studies suggested that some ERF genes which contained the EAR domain could induce cell death when transient over-expressed in tobacco plant. For example, the *NbCD1* gene induced

hypersensitive cell death when transient over-expressed in *N. benthamiana*, while the NbCD1 protein contained the EAR domain and its EAR domain in C-terminal was indispensable for triggering cell death [39]. Likewise, *NtERF3* could also cause hypersensitive cell death when over-expressed in *Nicotiana tabacum* (*N. tabacum*). Loss of the EAR domain in *NtERF3* protein did not influence its localization to the nucleus but did abrogate the cell death response [40]. Recently, *AtERF8* was found to trigger cell death when co-overexpressed with CaMV35S:HC-Pro (gene silencing suppressor) in *N. benthamiana*, and its intact EAR domain was necessary for this activity [13]. In contrast with previous studies, we found that CaAP2/ERF064 caused cell death when expressed in *N. benthamiana*, even though the protein does not contain an obvious EAR domain. The homologs of CaAP2/ERF064, such as StERF1B-1, NbERF1B-1, and SITSRF1 proteins, could also induce cell death in *N. benthamiana*. In other studies, it was found that the homologs of *NtERF3* in rice and tobacco could trigger cell death response when over-expressed in *N. tabacum* [40,41]. However, the other *CaAP2/ERF* genes (*CaAP2/ERF049* and *CaAP2/ERF109*) could not induce the cell death in *N. benthamiana*. All of these results indicated the distinctive role of *CaAP2/ERF064* gene in cell death response.

Additionally, the CaAP2/ERF064 protein and its N2 mutant, as well as its homologs (StERF1B-1, NbERF1B-1, and SITSRF1), proteins were located in the nucleus. Furthermore, *CaAP2/ERF064* and its homology genes belong to ERF transcription factors family and function in the nucleus. These results speculated on whether the nucleus location of CaAP2/ERF064 proteins was the inducement of cell death response. We attempted to explore this further by adding a nuclear export-signal (NES) [42] to the C-terminal of CaAP2/ERF064-GFP fusion protein but found that the fusion protein was still localized in the nucleus (Figure S5). Disruption of the nuclear localization signals (NLS) in CaAP2/ERF064 may be an effective, alternative way to approach this, while the RPW8.2 protein confers broad-spectrum resistance against powdery mildew, and the cytoplasmic localization of RPW8.2 could trigger cell death. In contrast, the RPW8.2 protein localization in the nucleus could result in plant resistance to powdery mildew [43,44].

Current research suggests that that CaAP2/ERF064 protein had the transcriptional activity and its active domain (AD) may be located in C-terminal. A previous study of the *CaAIEF1* gene also demonstrated that an AD domain is found in its C-terminal [18]. In contrast, the AD domain of the SITSRF1 protein is located in the N-terminal [45]. In this study, we found that the CaAP2/ERF064 protein could bind to the promoter fragment (F1R) of the *CaBPR1* gene and that the transient over-expression of CaAP2/ERF064 or its deletion mutant C2 significantly increased the transcription level of *CaBPR1* in Pro_{CaBPR1}:CaBPR1 transgenic tobacco plants, while the conserved AP2 domain of ERF proteins has the ability of DNA-binding [5,46]. Therefore, the AD domain of CaAP2/ERF064 protein may be located in the C-terminal, as our research showed that the C3 mutant, which contains the AD domain, exhibited strong transcriptional activity in yeast. Moreover, the C2 mutant containing both the AP2 (DNA-binding domain) and AD domains binds to the promoter and activates expression of the *CaBPR1* gene in Pro_{CaBPR1}:CaBPR1 transgenic plants. These results suggested that *CaAP2/ERF064* is functioning as a transcriptional activator and could promote the expression of the *CaBPR1* gene in plants.

The loss of function or silencing of *CaAP2/ERF064* also reduced the expression of defense-related genes *CaBPR1*, *CaPO2*, and *CaSAR82* and increased the susceptibility of pepper plants to *P. capsici*. Similarly, silencing of the *CaWRKY22* gene altered the transcription level of *CaPO2* and *CaBPR1* in plants after inoculation with *Ralstonia solanacearum* [47]. Previous studies found that over-expression of *CaBPR1* enhanced resistance to *Phytophthora nicotianae*, *Ralstonia solanacearum*, and *Pseudomonas syringae* pv. *tabaci* in *N. tabacum* [48]. The decrease in the expression level of *CaBPR1* may be due to the knock-down of *CaAP2/ERF064* in TRV2:CaAP2/ERF064 plants, while *CaAP2/ERF064* is likely only one of several or many transcription factors that regulate *CaBPR1*; further research is needed to elucidate the exact role of *CaAP2/ERF064* in transcription regulation of *CaBPR1*, as well as in plant defense response to *P. capsici*.

The over-expression of the *CaAP2/ERF064* gene in tobacco plants increased the transcription of *NbPR1b*, *NbPR3*, and *NbPR4* that enhanced resistance to *P. capsici*. Additionally, the over-expression

of *CaWRKY40* in *N. tabacum* also enhanced the plant defense response to *R. solanacearum* infection by upregulating the expression of *NtPR1b* and *NtPR3* [49]. *SpWRKY6* transgenic tomato plants showed enhanced resistance to *Phytophthora infestans* infection and increased the expression of *PR1*, *PR2*, *PR3*, and *PR5* [50]. However, the over-expression of *GmERF5* in soybean significantly enhanced plant resistance to *P. sojae* by increasing the expression level of *PR* (*PR10*, *PR1-1*, and *PR10-1*) genes [13]. Moreover, the transcript level of *PDF1.2* in *ERF96-RNAi Arabidopsis* plants was lower than WT, which suggests that *ERF96* plays a positive role in plant resistance to *Botrytis cinerea* [6], while *Arabidopsis* plants expressing *SlERF84* exhibited compromised immunity resistance against *Pseudomonas syringae* pv. *tomato* DC3000, and this was associated with diminished expression of *AtPR1* and *AtPR3* [51]. Recently, it was found that the over-expression of *ERF19* could repress plant pattern-triggered immunity (PTI) response and increase plant susceptibility to *Botrytis cinerea* and *Pseudomonas syringae* [52]. Furthermore, *CaAP2/ERF064-OE* tobacco plants are slightly shorter than WT plants, similar to the stunted phenotype reported for *Arabidopsis* plants overexpressing *AtERF14*. Similar to our observations, those *Arabidopsis* plants also showed enhanced expression of the defense-related genes *PR1*, *ChiB*, and *PDF1.2* [53]. Inappropriate activation of immune response may reduce growth of the plant [54,55]. We found that the expression level of *NbPR1b* in *CaAP2/ERF064-OE* plants was much higher than the control before inoculation (Figure 6E). Taken together, all these results indicate a vital role for *CaAP2/ERF064* in the defense mechanism against *P. capsici*.

In conclusion, the transient over-expression of *CaAP2/ERF064* with *P19* induced cell death in *N. benthamiana* leaves. Additionally, the transcription of *CaAP2/ERF064* is synergistically regulated by the MeJA and ET signaling pathway. *CaAP2/ERF064* works as a transcription activator and promotes the expression of the *CaBPR1* gene in plants. Moreover, it also positively regulates plant defense responses against *P. capsici* by modulating the transcription of *PR* genes. To elucidate the mechanisms associated with this activity, further studies are needed to identify additional target genes of *CaAP2/ERF064* and factors that participate in its specific role in cell death.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4425/10/7/541/s1>, Table S1: Primers used in this study, Figure S1: Localization of deletion mutants of *CaAP2/ERF064* protein in *N. benthamiana* epidermal cells. Scale bar represent 100 μ m, Figure S2: Transient over-expression of *CaAP2/ERF049* and *CaAP2/ERF109* in *N. benthamiana*. EV was short for empty vector. The photograph was taken at 7 days post infiltration. Scale bar represent 1 cm, Figure S3: Detection of Pro_{CaBPR1}:*CaBPR1* transgenic tobacco plants. (A) Expression analysis of *CaBPR1* in Pro_{CaBPR1}:*CaBPR1* transgenic tobacco plants. (B) Phenotypes of WT and Pro_{CaBPR1}:*CaBPR1* transgenic plants, Figure S4: Phenotype of *CaPDS*-silenced pepper plants at 6 weeks post infiltration, Figure S5: Localization of *CaAP2/ERF064* proteins in *N. benthamiana* epidermal cells. (A) and (B) Localization of *CaAP2/ERF064*-GFP-NLS and *CaAP2/ERF064*-GFP-NES proteins in tobacco. The Scale bar represents 100 μ m.

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