

Ethylene-Inducible AP2/ERF Transcription Factor Promotes Capsaicinoid Biosynthesis in *Capsicum*

minghua deng (✉ dengminghua2013@sina.com)

Yunnan Agriculture University <https://orcid.org/0000-0001-8293-9035>

kai zhao

Yunnan Agriculture University

junheng lv

Yunnan Agriculture University

xiang zhang

Yunnan Agriculture University

zuosen li

Yunnan Agriculture University

hong zhang

Yunnan Agriculture University

jinlong huo

Yunnan Agriculture University

hongjian wan

Zhejiang Academy of Agricultural Sciences

ziran wang

Yunnan Agriculture University

haishan zhu

Yunnan Agriculture University

xuexiao zou

Hunan Agricultural University

jinfen wen

Kunming University of Science and Technology

Research Article

Keywords: CcERF2, Capsaicinoids, Ethylene, VIGS

Posted Date: November 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1080827/v1>

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Abstract

Ethylene is very important in the process of plant development and regulates the biosynthesis of a lot of secondary metabolites. In these regulatory mechanisms, transcription factors that mediate ethylene signals play a very important role. Capsaicinoids (CAPs) only synthesized and accumulated in *Capsicum* species, causing their fruit to have a special pungent taste, which can protect against attack from herbivores and pathogens. In this study, we identified the transcription factor *CcERF2*, which is induced by ethylene, and demonstrated its regulatory effect on CAPs biosynthesis. Transcriptome sequencing analysis revealed the expression patterns of *CcERF2* and multiple genes associated to CAPs biosynthesis were basically the same. The spatiotemporal expression results showed *CcERF2* was preferentially expressed in the placenta of the spicy fruit. Ethylene can induce the expression of *CcERF2* and CAPs biosynthesis genes (CBGs). *CcERF2* gene silenced, 1-MCP and PZA treatments caused a decrease in expression of CBGs and a sharp decrease in content of CAPs. The results indicated *CcERF2* was associated with the CAPs biosynthesis.

Background

Peppers (*Capsicum* spp.) are important vegetables worldwide. Pepper fruits are diverse in color, rich in nutrients, have a special pungency and aroma, and so are widely used as food additives (Ou et al, 2018). The pungency of pepper fruit is derived from capsaicinoids (CAPs), which are only biosynthesized in *Capsicum* plants (Iwai et al, 1979; Liu et al, 2019). The CAPs have the effect of curbing attack by herbivores and microorganisms. At the same time, CAPs are also widely used in many fields such as food, medicine, cosmetics, and agricultural pest control; CAPs are also widely used in riot prevention, personal defense, and in the military and national defense (Lejeune et al, 2007; Tewksbury et al, 2008; Ludy et al, 2012). The CAPs are synthesized and accumulated in fruit placental tissues (Iwai et al, 1979). So far, more than 22 CAPs have been identified in peppers. The most important components of CAPs are capsaicin (CaP) and dihydrocapsaicin (DhCaP). Approximately 90% of the total content of CAPs are them. The accumulation of CAPs is affected by a lot of factors, including endogenous (for example, variety and developmental stage of the fruit) and exogenous factors (for example, light, temperature, water conditions and biological stress) (Garruña-Hernández et al, 2013; Phimchan and Techawongstien 2012). The content of CAPs may vary significantly between and within pepper genotype. Genotype is the most important determinant of CAPs content (Bosland et al, 2012; Liu et al, 2017); among the five domesticated species of *Capsicum*, *C. chinense* and *C. frutescens* accumulated significantly higher content of CAPs (Deng et al, 2009; Bosland et al, 2012). At the same time, the content of CAPs has high plasticity, which can be affected by many complex ecological-physiological reactions and environment. It is reported that factors such as plant growth regulators, chemicals, temperature, light, and drought stress can change the content of CAPs (Gurung et al, 2011).

The CAPs are synthesized by the fusion of phenylpropane and branched-chain fatty acid pathways in the placenta (Aluru et al, 2003; Blum et al, 2003; Mazourek et al, 2009). The CAPs biosynthesis genes (CBGs) that have been determined to be involved in biosynthesis of CAPs include *PAL*, *Ca4H*, *4CL*, *BCAT*, *Kas*,

FatA, *ACL*, *ACS*, *CoMT*, *pAMT* and *CS* (Abraham-Juárez et al, 2008; Liu et al, 2013). The biosynthetic mechanism of CAPs has been widely elucidated using bioinformatics analysis and multi-omics (Qin et al, 2014). As CAPs have considerable application value and commercial use, a lot of effort have been performed to enhance their content. However, their biosynthesis changed largely in space/time, and expression level of CBGs is precisely regulated at the transcriptional level. Manipulating the expression level of crucial CBGs seems to be determined the increasing content of CAPs (Abraham-Juárez et al, 2008; Sun et al, 2019; Zhu et al, 2019). In highly spicy peppers, the transcription level of CBGs (such as *pAMT*, *Kas*, and *CS*) is always higher than that of less spicy varieties (Abraham-Juárez et al, 2008). It is very important that manipulating the expression levels of some transcription factors (TFs) usually changes the transcription levels of all genes in the metabolic pathway, thereby affecting the content of the compound at last.

For example, the TFs *CaMYB31*, *CaMYB108*, *CaMYB48*, *ERF* and *Jerf* are associated with regulating the content of CAPs (Keyhaninejad et al, 2014; Phimchan et al, 2014; Arce-Rodríguez et al, 2017; Sun et al, 2019; Zhu et al, 2019). It was found that the Solanaceae-specific TF *MYB31* affects the accumulation of CAPs by directly targeting CBGs resulting changing the expression level of CBGs (Zhu et al, 2019). TF *CaMYB108* activates the CBG promoters, especially those of *COMT*, *pAMT*, and *KasI*, and promotes enhanced expression of CBGs, thereby promoting the biosynthesis of CAPs (Sun et al, 2019). By directly binding and regulating the expression of CBGs, TF *CaMYB48* participates in the biosynthesis of CAPs, but the transcriptional regulation of CAPs biosynthesis has not been fully clarified (Sun et al, 2020). Therefore, it is necessary to identify TF associated with the biosynthesis of CAPs.

Ethylene plays a significantly role in plant life process (Hu et al, 2020). After synthesis of ethylene, it binds to the receptor ETR and transmits the signal to the nucleus through MAPKK and EIN2 (Gray 2004). The EIN2 binds to EIN3/EIL1 Andthe EIN3/EIL1 bind to the *ERF1* promoter. ERF1 binds to the GCC-box-containing genes in the downstream promoter region specifically and their expression can promote secondary metabolism product synthesis (Fujimoto et al, 2000; Brown et al, 2003; Paul et al, 2019). For example, in tobacco, ERF189 and ERF163 can bind to the GCC-box in the promoter region of the tobacco nicotine synthesis-related gene *PMT2* specifically, and directly promote synthesis of tobacco alkaloids (Shoji et al, 2010). The B3 subfamily proteins ORCA3 and ORA59 of the ERF family are two typical TFs associated with the regulation of secondary metabolites. The ORCA3 can upregulate the expression level of indole alkaloid synthesis-related genes in terpenoids, and promote the terpenoid indole biosynthesis (van der Fits and Memelink 2000, 2001). *Lithospermum erythrorhizum* *LeERF-1* affects the secondary metabolites of shikonin positively through a mechanism similar to that of ORCA3 in affecting secondary metabolites (Zhang et al, 2011). In *Catharanthus roseus*, *CrERF5* upregulates the biosynthesis and accumulation of bisindole alkaloids (Pan et al, 2019).

The TFs and key enzymes in ethylene synthesis and its signal transduction can promote the biosynthesis of secondary metabolites. In many cases (plants, cells, and hairy roots), the addition of ethylene, addition of 1-aminocyclopropane 1-carboxylic acid (ACC), and overexpression of related TFS can increase the biosynthesis of secondary metabolites (Gantet and Memelink 2002). For example, adding 0.018 mL/L of

ethylene to yew cell culture can greatly increase production of taxane (Pan et al, 2000); adding ACC to *Arabidopsis* can promote synthesis of flavonoids (Buer et al, 2006); adding ethylene or treating cypress cells (*Platycladns orientalis*) with ACC can promote the biosynthesis of β -rubiin (Zhao et al, 2004); and overexpression of *ORC1/ERF221* can upregulate the synthesis of pyridine alkaloids (De Boer et al, 2011).

In this paper, the ethylene-induced AP2/ERF TFs *CcERF2*, which was particularly expressed in the placenta was identified. We confirmed that *CcERF2* was associated with the production of CAPs biosynthesis.

1. Materials And Methods

1.1 Experimental materials

The *C. chinense* inbred line SL08 has a high CAPs content and was derived from Shuan La, the hottest pepper genotype in China (Deng et al, 2009). The *C. annuum* inbred line H19 has a low CAPs content, and was derived from Xiangtan Chi Ban Jiao. Pepper seeds germinated in the soil of cell plastic flats in complete darkness at 28°C. The seedlings were grown in a greenhouse of Yunnan Agricultural University on campus under normal conditions.

1.2 Extraction and detection of CaP and DhCaP

The CaP and DhCaP were extracted and detected according to our method (Deng et al, 2009). The total CAPs content was calculated as $(CaP + DhCaP)/0.91$. Fruits in different development stages of SL08 and H19 were used as material for extraction and detection of CaP and DhCaP.

1.3 RNA-sep and analysis

RNA-sep was performed according to our method (Liu et al, 2012). Fruits of SL08 at 4, 14, 24, 34, 44, 54 days after pollination (DAP) were used to isolate total RNA.

1.4 Exogenous substance treatments

Fruit at 24 DAP was used to investigate the effect of different exogenous substances on expression level of *CcERF2* and CBGs after 0.5, 1.0, 1.5, 3.5, 5.5, 8.5, and 11.5 h. Treated with exogenous substances, the 24-DAP fruits of SL08 were soaked in sterile water with 0.01 g/L 6-benzylaminopurine (6-BA), 20 g/L PEG6000, 30% H₂O₂, 0.17 g/L gibberellic acid (GA₃), 0.14 g/L salicylic acid (SA), 17.53 g/L NaCl, 90 g/L glutamate (Glu), or 0.1 mmol/L ethephon (with sterile water as control) under normal conditions. After treatment, all fruits were frozen in liquid nitrogen and stored at -80°C immediately for gene expression analysis.

1.5 *CcERF2* cloning and bioinformatics analysis

The open reading frame of *CcERF2* was cloned according to Deng et al (2012). The primers used in the experiment are shown in Table S1. The BLAST-protein-nucleic acid (BLASTP) analysis was performed on *CcERF2* through a database (www.ncbi.nlm.nih.gov); PSORT Prediction was performed for subcellular location prediction; and MEME was used to analyze the amino acid sequence motif. The Portparam tool was used to predict the physical and chemical properties of the protein. SignalP-5.0, TMHMM, and Nepos were used for signal peptide, transmembrane structure, and phosphorylation site of the protein prediction, respectively; SOPMA was used to predict its secondary structure; and ClustalX was used for sequence alignment and phylogenetic analysis. The MEGA 6.0 software was performed to construct a phylogenetic tree based on the amino acid sequence through the neighbor joining method, and the bootstrap method was used to evaluate the reliability of each node in the tree, repeated 1,000 times.

1.6 Subcellular localization and transcriptional activation analysis

Subcellular localization and transcriptional activation analysis were performed according to Sun et al (2020) and Zhu et al (2019). The full-length coding sequences of *CcERF2* was cloned into the pAN580 (green fluorescent protein, GFP) vector and fused to the N-terminus of GFP under the control of the CaMV 35S promoter. The constructs were separately introduced into tobacco protoplasts for transient expression, and the GFP fluorescence signals were detected using a Zeiss lsm710 confocal laser scanning microscope (Carl Zeiss, Inc, Jena, Germany)

1.7 Gene expression analysis

The RNAiso Plus (Takara, Dalian, P R China) was used to extract total RNA from pepper fruit. The synthesis of cDNA first strand refers to the instructions of the High Fidelity PrimeScript[®] RT-PCR Kit (Takara). According to the sequence of related genes revealed by transcriptome sequencing data, specific primers were respectively designed, and the internal reference was the *β-ACTIN* gene. Quantitative real-time PCR (qRT-PCR) was performed according to the method provided by the SYBR[®]Premix Ex Taq[™] II (Tli RNaseH Plus) kit (Takara). The primers used in this study are shown in Table S1.

1.8 VIGS analysis

A fragment of the *CcERF2* coding sequence (CDS) with low similarity to other genes was cloned into pTRV2 and generated the silencing vector pTRV2-*CcERF2*. The VIGS were carried as reported (Zhu et al, 2019). In short, the pTRV2-*CcERF2* and pTVR1 vectors were co-injected into the cotyledon stage seedlings of line SL08. The empty vectors pTRV2 and pTVR1 were co-infiltrated as a control, and pTRV2-PDS and pTVR1 were co-infiltrated as a technical control. The RNA isolated from 24-DAP fruits was used for expression analysis, and 34-DAP fruits were used for CaP and DhCaP measurement. The primer information used in this study is shown in Table S1.

1.9 Effects of 1-methylcyclopropene (1-MCP) and Pyrazinamide (PZA) on the expression of CBGs and the content of CAPs

The SL08 pepper fruit of 24DAP was used to study the effects of 1-MCP and PZA on the expression of CBGs and the content of CAPs. 1-MCP treatment: 1 $\mu\text{L L}^{-1}$ 1-MCP fumigated for 12 hours and played in a dark environment with a temperature of $20\pm 2^\circ\text{C}$, a humidity of $85\pm 5\%$. PZA treatment: Spray 100 mM PZA on the surface of the fruit until there are droplets, and played in a dark environment with a temperature of $20\pm 2^\circ\text{C}$, a humidity of $85\pm 5\%$. The fruits after 5 days of treatment were stored in the liquid nitrogen used for subsequent analysis. All experiments were repeated 3 times.

1.10 Ethylene release rate determination

Ethylene release rate was investigated according to the method of Xu et al (2018).

1.11 Statistical analyses

Each experiment contained three biological replicates and three technical replicates. The error bars mean the standard errors. One-way ANOVA was performed to identify significant differences. The relative gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak 2001). Without induction at the 0 h time point was used as control expression.

2. Results And Analysis

2.1 Accumulation of CAPs

Accumulation of CaP and DhCaP was detected in both lines, but the content of CaP was much higher (about 3-4 times) than that of DhCaP. The CaP and DhCaP contents were much higher in the placenta of SL08 than in H19 (Figure S1). Both CaP and DhCaP began to accumulate at 9 DAP in the placenta of SL08, reached a peak at 44 DAP, and then began to decline. Both CaP and DhCaP began to accumulate at 11 DAP in the placenta of H19, and began to decrease at 49 DAP. The CaP, DhCaP, and total CAPs contents in the placenta of SL08 were 44.4, 63.1, and 48.0 times those in H19, respectively.

2.2 Expression of *CcERF2* and CBGs is consistent with CAPs biosynthesis

Expression patterns of CBGs during development stages of the placenta were analyzed using transcriptome sequencing data of the placenta and pericarp of line SL08 during the early stage of the laboratory study (Figure 1). Expressions of *CcPAL*, *CcCa4H*, *CcACL*, *CcCoMT*, *CcpAMT*, *CcCS*, *CcBCAT*, *CcKAS*, *CcACL*, *CcFAT*, and *CcACS* genes were positively associated with accumulation of CAPs in SL08. The expression of these genes (except *CcACL*) showed a low expression level in fruit at 4 DAP, increased, and then decreased. Gene *CcCS* (Capana05g000531) was not expressed in 4-DAP fruit, and reached a

maximum in 24-DAP fruit. Expression of *CcCoMT* (Capana03g001811), *CATCcBCAT* (Capana04g000751), *CcKAS* (Capana01g000111), and *CcFATA* (Capana06g000197) reached their highest values in 14-DAP fruit. Expression of *CcCa4H* (Capana06g000272) and *CcpAMT* (Capana10g001341) genes reached their maximum in 34-DAP fruit. Expression of *CcPAL* (Capana09g002199) reached its highest value in 44-DAP fruit.

Based on the transcriptome sequencing data of lines SL08 and H19 at different developmental stages of placenta completed in the laboratory, combined with the changes in the CaP and DhCaP contents of the placenta of both lines (Figure S1), the AP2/ERF TF *CcERF2* (Capana01g000677) was identified. Its expression pattern was similar to that of CBGs (Figure 1). Expression of *CcERF2* was almost undetectable in the early stage of placenta (4 DAP), then rose rapidly, reached a maximum at 34 DAP, and then decreased.

We measured the expression level of *CcERF2* in some tissues of line SL08 and found that it was mainly expressed in the placenta (Figure 2A). It also showed considerable level of expression in seeds and pericarp, meaning that it has a role in these tissues. We also investigated *CcERF2* expression in the placenta of both lines, and found significantly higher expression in SL08 than in H19 (Figure 2B). Expression of *CcERF2* in the placenta has the same pattern with the content of CaP and DhCaP. Thus, our results indicated that *CcERF2* may play a vital role in of CAPs biosynthesis.

2.3 *CcERF2* gene cloning and bioinformatics analysis

Based on our transcriptome sequencing data of SL08 and the pepper genome data in the public database, specific primers were designed to clone the full-length CDS of *CcERF2* from SL08. Nucleotide sequence analysis showed that *CcERF2* length was 795 bp. The molecular formula of *CcERF2* is $C_{1328}H_{2050}N_{376}O_{409}S_9$, molecular weight is 30,115.70 D, and theoretical isoelectric point is 5.74. It is a fat-soluble, hydrophilic, and unstable protein. *CcERF2* had no signal peptide and no transmembrane structure, and is located in the nucleus (88.8% probability).

The protein domain prediction results showed that *CcERF2* belonged to the AP2 superfamily, and the conservative amino acid sequence position was 74-131 (LYRGIRQRPWGKWAAEIRDPRKGVVRVWLGTFTNTAEAAAYDKEARKIRGEKAKVNFP) (Figure 3C). This domain specifically bound to the 11-bp GCC box of the ethylene response element and was essential for the ethylene response. Based on BLAST, 48 amino acid sequences including *CcERF2* were obtained, and 43 motifs were obtained after motif significance test and analysis. On the whole, the predicted motifs differed within the same family, but the conserved motifs in the same subgroup were almost the same; that is, the closer the related species, the more similar were the motifs. For peppers, they all contained 13 conserved motifs: 1-8, 11, 12, 17, 19, and 22. Of these, motifs 1, 3, and 5 covered the AP2 conserved structure domain (Figure 3B). The secondary structure of *CcERF2* protein contained 73 alpha helices (27.56%), 22 extension chains (8.42%), and 9 beta turns (3.41%). There were 160 random coils (60.61%). The evolutionary relationship of amino acid sequences showed that the selected ERF sequences were

grouped together in different families. This meant that they were conserved in the evolutionary relationship. In genetic relationship, CcERF2 protein was the closest to Solanaceae. In the Solanaceae, it was closest to *Capsicum* and *Solanum*, and most distant to *Petunia* (Figure 3A). The phosphorylation sites of CcERF2 protein were predicted, and 19 amino acid residue types were screened: six types of T, three types of Y, and 10 types of S. CcERF2 was localized to the cell nucleus (Figure 4A) and the CcERF2 protein has strong activation activity (Figure 4B).

2.4 *CcERF2* is a TF induced by ethylene

. Exogenous substances were applied to the 24-DAP fruit of SL08 to test the regulation model. Expression of *CcERF2* in placental tissues was analyzed using qRT-PCR (Figure 5).

The fruits were treated with ethephon, which significantly induced *CcERF2* expression. Compared with the control, *CcERF2* expression increased by 64 times within 11.5 h of applying ethephon. At the same time, the effects of exogenous 6-BA, PEG, H₂O₂, GA₃, SA, NaCl, and Glu on *CcERF2* expression were also tested. Of these, 6-BA, H₂O₂, GA₃, SA, and NaCl significantly induced *CcERF2* expression. Compared with the control, these exogenous substances increased *CcERF2* expression; however, the modes of induced expression were not the same. In the test time range, under the 6-BA, H₂O₂, and NaCl treatments, the *CcERF2* expression showed a trend of initial increase and then decrease; however, under GA₃ and SA treatment, *CcERF2* expression showed an increasing trend. Treatment with Glu had no significant effect on *CcERF2* expression and PEG treatment was inhibitory.

2.5 Effect of ethephon on expression of CBGs

Ethephon treatment significantly increased expression of CBGs, but the induction effect was inconsistent. Compared to controls, within 11.5 h of applying ethephon, expression levels of *CcPAL*, *CcCoMT*, and *CcFAT* were 2.3–5.0 times; of *CcCa4H*, *CcpAmt*, *CcBCAT*, *CcACL*, and *CcACS* were 5.8–8.3 times; of *CcKAS* gene was 11.1 times; and of *CcCS* was more than 100 times. Considering that ethephon significantly induces the expression of *CcERF2*, our results showed that *CcERF2* was a key factor in ethylene-mediated biosynthesis of CAPs (Figure 6).

2.6 The effect of *CcERF2*-silenced on CAPs content and metabolic pathways

The VIGS vector pTRV2–*CcERF2* was constructed using a vector derived from tobacco rattle virus (TRV), and a *CcERF2*-silenced experiment was performed. The 24-DAP fruits of SL08 were used to study. It was demonstrated that *Agrobacterium* infection with an empty pTRV2 vector resulted in indistinctive changes in the expression level of CBGs in pepper fruits (Abraham-Juárez et al, 2008). Compared with empty vector plants, the fruits infected with the pTRV2–*CcERF2* construct resulted in a distinctive reduction in *CcERF2*

expression (22.4% of the empty vector) (Figure 7A). Consistent with the expression level of *CcERF2*, CBGs in silent plants were also significantly downregulated. In the *CcERF2*-silent placenta, the expression of genes derived from fatty acid metabolism pathways (such as *CcKAS*, *CcACL*, *CcFAT* and *CcACS*) only slightly changed. However, expression of genes derived from the phenylpropane pathway (such as *CcPAL*, *CcCa4H*, *Cc4CL*, *CcComt*, and *CcpAmt*) and *CcCS* underwent significant changes, and the genes whose expression was altered were consistent with the transcription level of *CcERF2* (Figure 7A).

The CAPs contents of the pepper fruits infected with the empty pTRV2 vector were similar to those of uninfected plants (Abraham-Juárez et al, 2008). The 34-DAP fruits were used to study the effect of *CcERF2* silencing on the CaP and DhCaP contents. Comparing with fruits infected with empty pTRV2, the CaP and DhCaP contents in fruits infected with pTRV2–*CcERF2* were significantly reduced by 74.2% and 73.0%, respectively (Figure 7B). The above results strongly supported that *CcERF2* regulated certain CBGs to control CAPs biosynthesis.

2.7 Effects of 1-MCP on CBGs expression and CAPs contents

The effect of blocking ethylene signal transmission by 1-MCP on CBGs expression and CAPs contents had been studied. The results showed that 1-MCP treatment could remarkably reduce the expression of *PAL*, *C4H*, *COMT*, *pAMT* and *CS* genes (Figure 8A). 1-MCP treatment significantly reduced the content of CAPs (Figure 8B). The results indicated that the ethylene signal transduction was involved in the regulation of capsaicinoids biosynthesis.

2.8 Effects of PZA on CBGs expression and CAPs contents

The results indicated that after ethylene biosynthesis inhibitor PZA treated pepper fruits for 12 h, the expression of *ACC oxidase-2 (ACO2)* gene was extremely reduced (Figure 8C); The endogenous ethylene release rate was conspicuously reduced (Figure 8E); The expression of CBGs such as *PAL*, *C4H*, *COMT*, *pAMT* and *CS* was inhibited (Figure 8C); The CAPs contents were also obviously reduced (Figure 8D). It indicated that the content of endogenous ethylene significantly affects the biosynthesis of capsaicinoids.

3. Discussion

Plant secondary metabolism comprises the life activity, formed in the long-term evolutionary process. The secondary metabolites are not only associated with the regulation of plant growth, but also allow plants to effectively deal with all kinds of stresses, such as fungi, pests, and herbivores (Dangl and Jones 2001). The distribution of secondary metabolites is usually specific to species, organs, and tissues; CAPs are unique synthesized in the fruit placenta of *Capsicum* and have obvious specificity (Mazourek et al, 2009). A variety of stress conditions (such as high temperature, hydropenia, and herbivore invasion) can induce the production of secondary metabolites, and this process is affected by a lot of plant endogenous hormones (such as ethylene). Studies have found that low temperature promotes the accumulation of CAPs in pepper fruits (Keyhaninejad et al, 2014; Arce-Rodríguez et al, 2017); drought induces biosynthesis

of CAPs(Phimchan et al, 2014); and injury significantly increases the CAPs content of pepper fruits(Arce-Rodríguez et al, 2017). Plants under stress (such as high temperature, hydropenia, and herbivore attack) usually show an increase in ethylene content, which can increase the formation of secondary metabolites. Ethylene is plays a vital factor in response to all kinds of stresses (Fang et al, 2014; Hu et al, 2020). However, the effect of CAPs biosynthesis induced by ethylene needs further study.

We investigated the mechanism of ethylene on the expression of *CcERF2* and CBGs. After fruits of line SL08 were treated with ethephon, expressions of *CcERF2* and CBGs were significantly increased, equivalent to 2.1-110.3 times that of the control. However, the response pattern of each gene induced by ethephon slightly differed (Figure 5). A lot of studies have indicated that ethephon can upregulate the expression of multiple genes in the phenylpropane metabolic pathway, thereby promoting biosynthesis of flavonoids, anthocyanins, rutin, lignin, and procyanidins (El-Kereamy et al, 2003; Buer et al, 2006; Hossain et al, 2009; Pan et al, 2019; Paul et al, 2019; Hu et al, 2020; Zhao et al, 2020). Our experimental results are similar to those reports.

Most AP2/ERF family proteins have conserved AP2 domains, which endow them with the ability to bind to specific DNA, and have been shown to be used as regulatory factors for regulating different types of special metabolites, such as phenylpropane, betaine, glucosinolates, and analogs of carotene (Pan et al, 2019; Paul et al, 2019; Hu et al, 2020; Zhao et al, 2020). These AP2/ERF factors seem to play a similar role in regulation metabolic genes (Hu et al, 2020). In this paper, the AP2/ERF TF *CcERF2*, which can be used as a transcription activator to regulate CAPs biosynthesis was identified. There were 43 motifs obtained by analyzing the motif significance of the *CcERF2* protein. On the whole, the predicted motifs differed within the same family, but the conserved motifs in the same subgroups were almost the same. That is, the closer the related species, the more similar were the motifs. The peppers all contained 13 conserved motifs (1–8, 11, 12, 17, 19, and 22), of which motifs 1, 3, and 5 covered the conserved AP2 structural domain. It was reported that this type of TF can regulate the synthesis of plant secondary metabolites (Paul et al, 2019). Our results showed that the function of *CcERF2* was convergent and divergent among different plant species.

Various genes related to CAPs biosynthesis have been studied (Aluru et al, 2003). However, very few TFs associated to CAPs biosynthesis have been isolated and characterized, except for some MYB TFs (Arce-Rodríguez et al, 2017; Sun et al, 2019; 2020; Zhu et al, 2019). We need to identify some TFs, especially AP2/ERF family TFs, which can be used as positive regulators to promote accumulation of CAPs. In the current study, based on transcription data, the AP2/ERF TF *CcERF2* was selected for further analysis, because *CcERF2* had a similar expression pattern to the CBGs in the transcriptome data. Silencing *CcERF2* downregulated the expression of CBGs, especially the expression level of genes associated with the phenylpropane pathway, and therefore reduced its CAPs content. The results showed that *CcERF2* was involved in regulating this metabolic process.

1-MCP is an ethylene receptor inhibitor (Sisler and Serek 1997; Huber 2008). It can effectively inhibit ethylene signal transduction (Du et al, 2021). A large number of studies have shown that 1-MCP can

affect the maturation, senescence and secondary metabolism of non-atmospheric fruits by blocking ethylene signal transduction (Gómez-Lobato et al, 2012; Li et al, 2016; Zhang et al, 2018). Our research results showed that: 1-MCP significantly reduces the expression of some capsaicin biosynthesis genes, and obviously reduces the content of capsaicinoids in fruits.

PZA is a new type of ethylene biosynthesis inhibitor by inhibiting the expression of *ACO2* (Sun et al, 2017). Our research found that PZA could obviously reduce the expression of *ACO2* in pepper fruits and remarkably reduce the production of ethylene. At the same time, it was found that PZA treatment could extremely reduce the expression of some genes related to capsaicin biosynthesis, thereby reducing the content of capsaicinoids.

Declarations

Author contributions

D.M. and Z.K. conceived and designed the study; L.J., Z.X., L.Z., Z.H., H.J., and W.H. performed the research; W.Z., Z.H., Z.X., and W.J. analyzed the data; and D.M., Z.X., and W.J. prepared the paper.

Conflict of interest

The authors declare that they have no conflicts of interest

Acknowledgements

This study was supported by the Natural Science Foundation of China (Grant No 31560556, 31160394) and the Major Science and Technology Projects in Yunnan Province (2018BB020).

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Figures

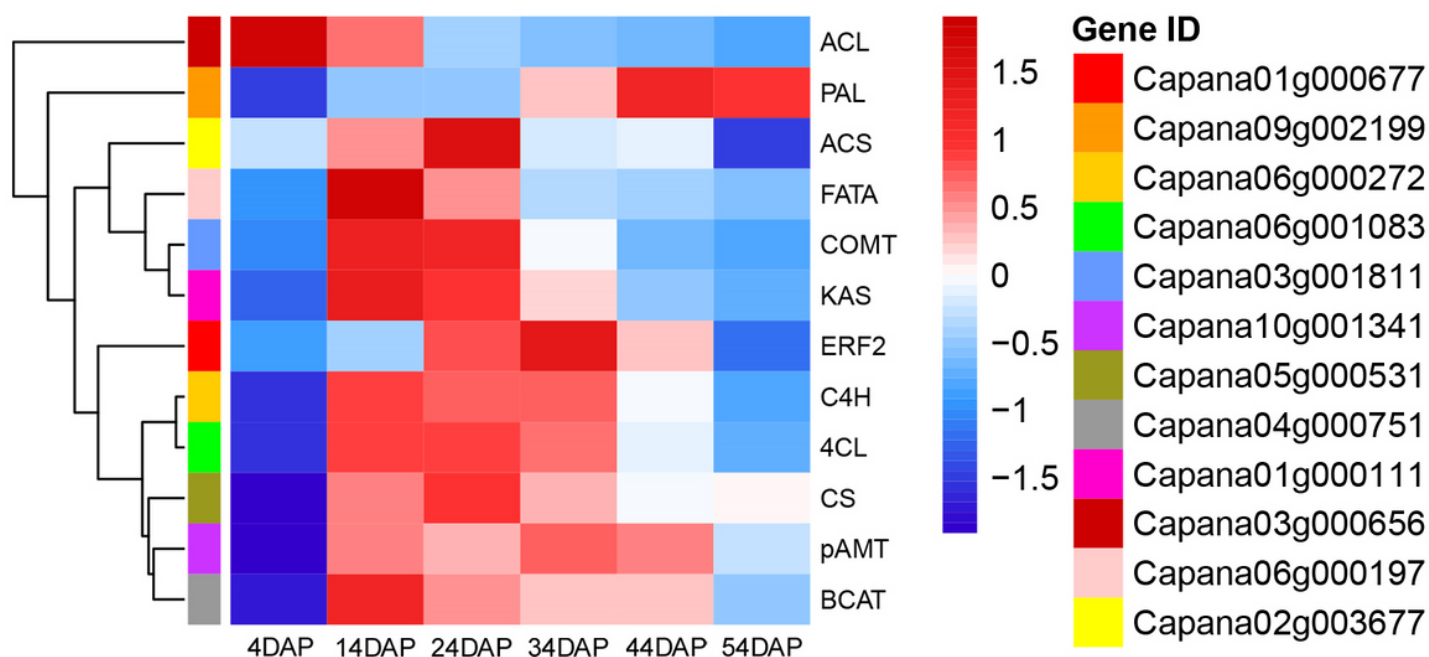


Figure 1

Heat map indicating the CBGs and CcERF2 expression patterns in fruit of inbred line SL08. The heatmap was generated by transcriptome sequencing data with R software packages. The data were normalized in

each row, and the numbers on the right side of the figure indicate the gene expression level corresponding to color.

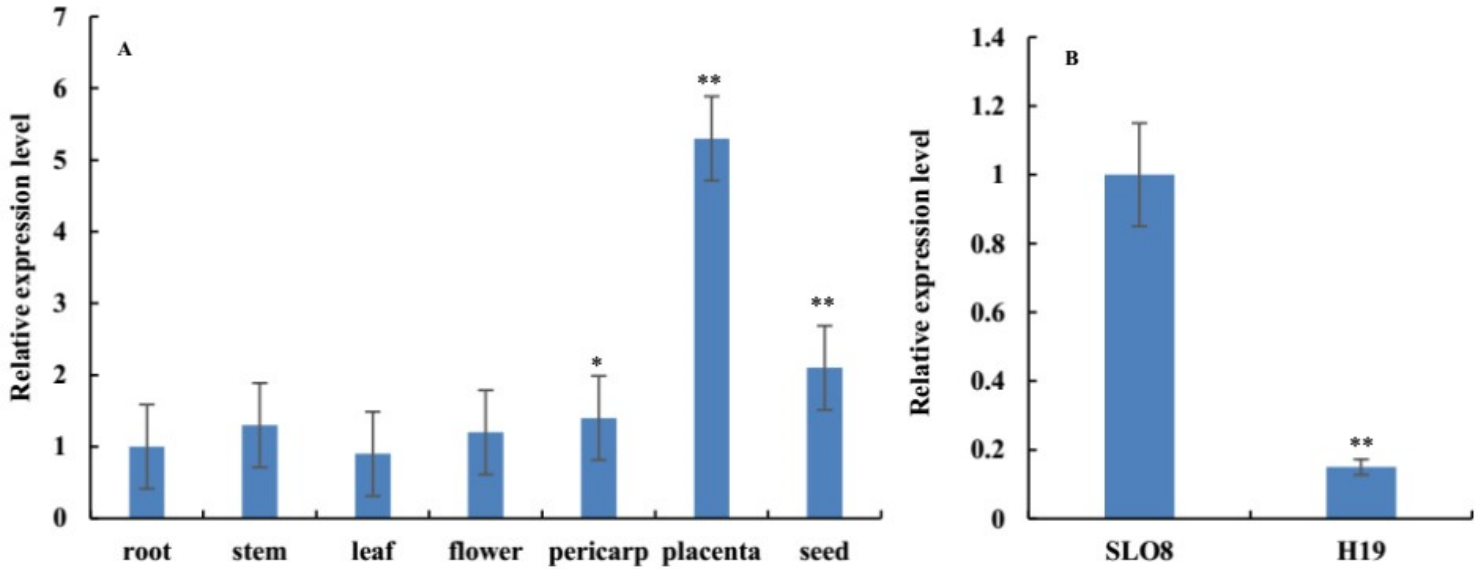


Figure 2

Differential expression assays of CcERF2 (A) Differential expression assays of CcERF2 in different tissues of pepper inbred line SL08; Roots, stems, leaves and flowers were collected from 30-day-old seedlings. Fruits at 24 DAP was collected and was divided into pericarps, placentas and seeds. The relative expression of the root was set to 1, and that of all the other tissues was measured relative to that of the root. (B) Differential expression assays of CcERF2 in pepper inbred line SL08 and H19 fruits. Fruits at 24 DAP was collected to investigate the CcERF2 expression. The relative expression of the SL08 was set to 1, and that of H19 was measured relative to that of the SL08. The experiments were replicated three biological times and three technical times. Data are expressed as the mean \pm SD (n = 9). Student's t test was used to identify significant differences compared to the control (*P < 0.05, **P < 0.01).

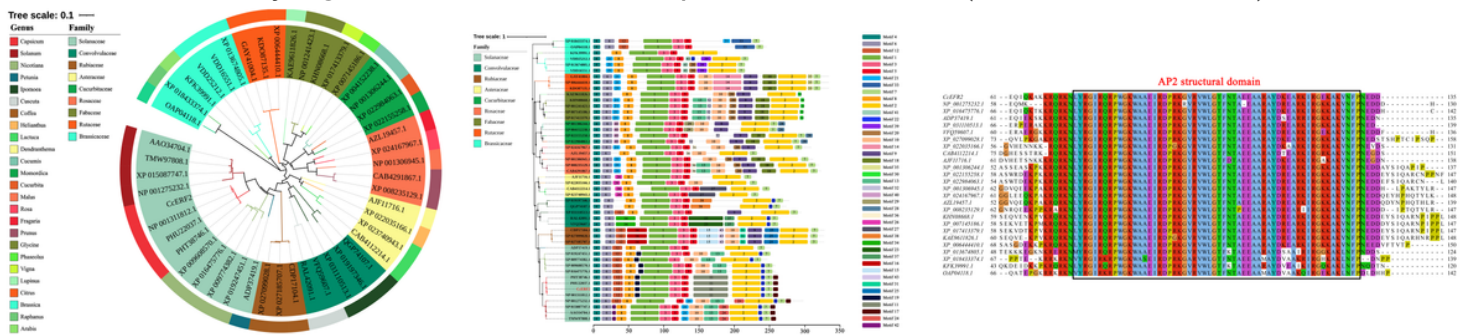


Figure 3

Phylogenetic tree of ERF TFs, analysis of promoter cis-acting elements and multiple amino acid sequence alignment of CcERF2 TFs. (A) Phylogenetic tree comprising CcERF2 in *C. chinense* and 47 ERF2 TFs in plant. (B) Promoter cis-acting elements comprising CcERF2 in *C. chinense* and 47 ERF2 domain promoters in plant. (C) Multiple amino acid sequence alignment of ERF2 TFs.

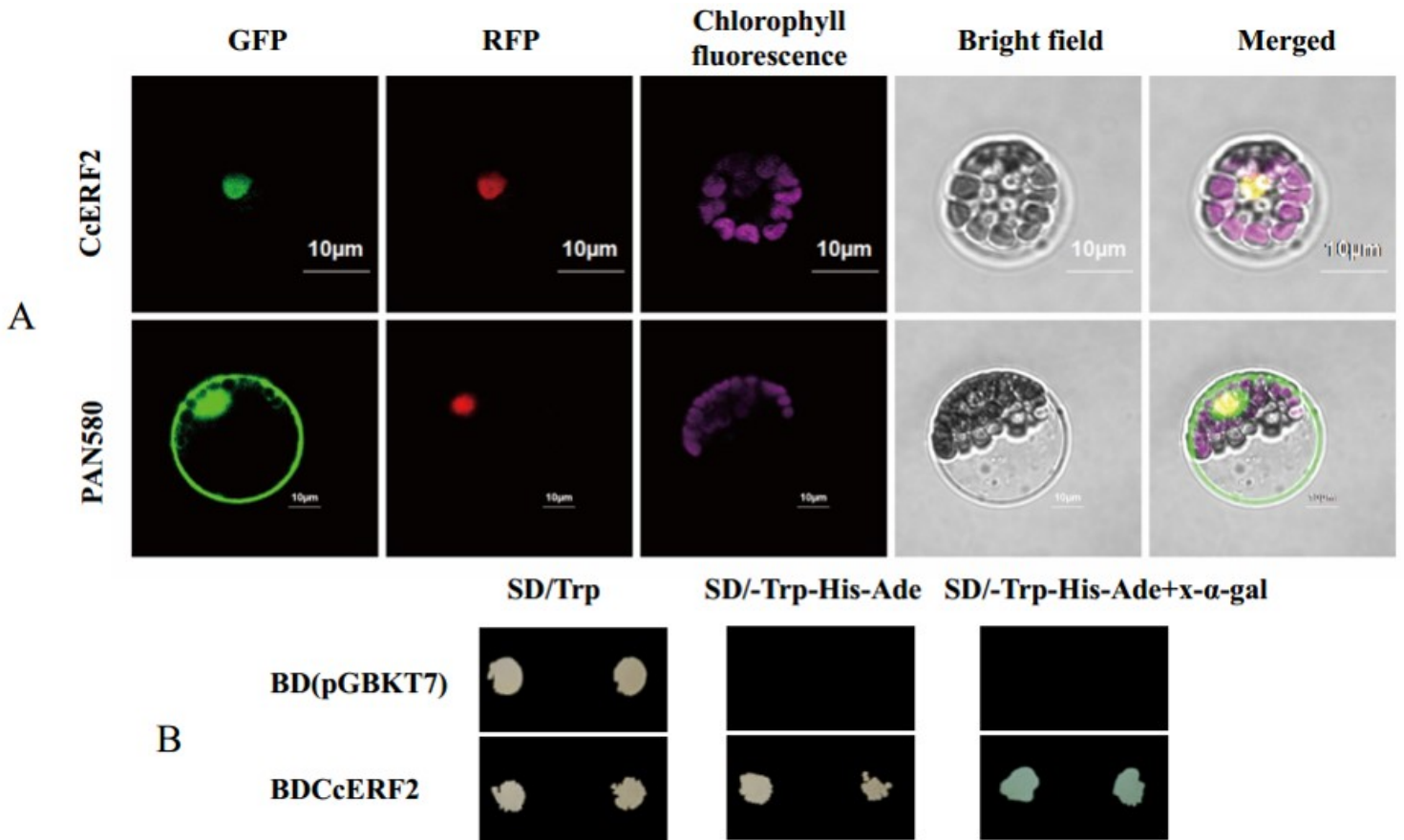


Figure 4

Subcellular localization and transcriptional activation analysis (A) GFP signals indicated that CcERF2 was localized to nucleus. GFP fluorescence signals were detected using a Zeiss lsm710 confocal laser scanning microscope (Carl Zeiss, Inc., Jena, Germany). Scale bars: 10 µm. (B) Auxotroph plates of SD/-Leu-His-Ade (middle) and SD/-Leu-His-Ade-x-α-gal (right) showing transcriptional activation of protein. SD/Trp, medium lacking tryptophan; SD/-Trp-His-Ade, medium lacking tryptophan, histidine and adenine.

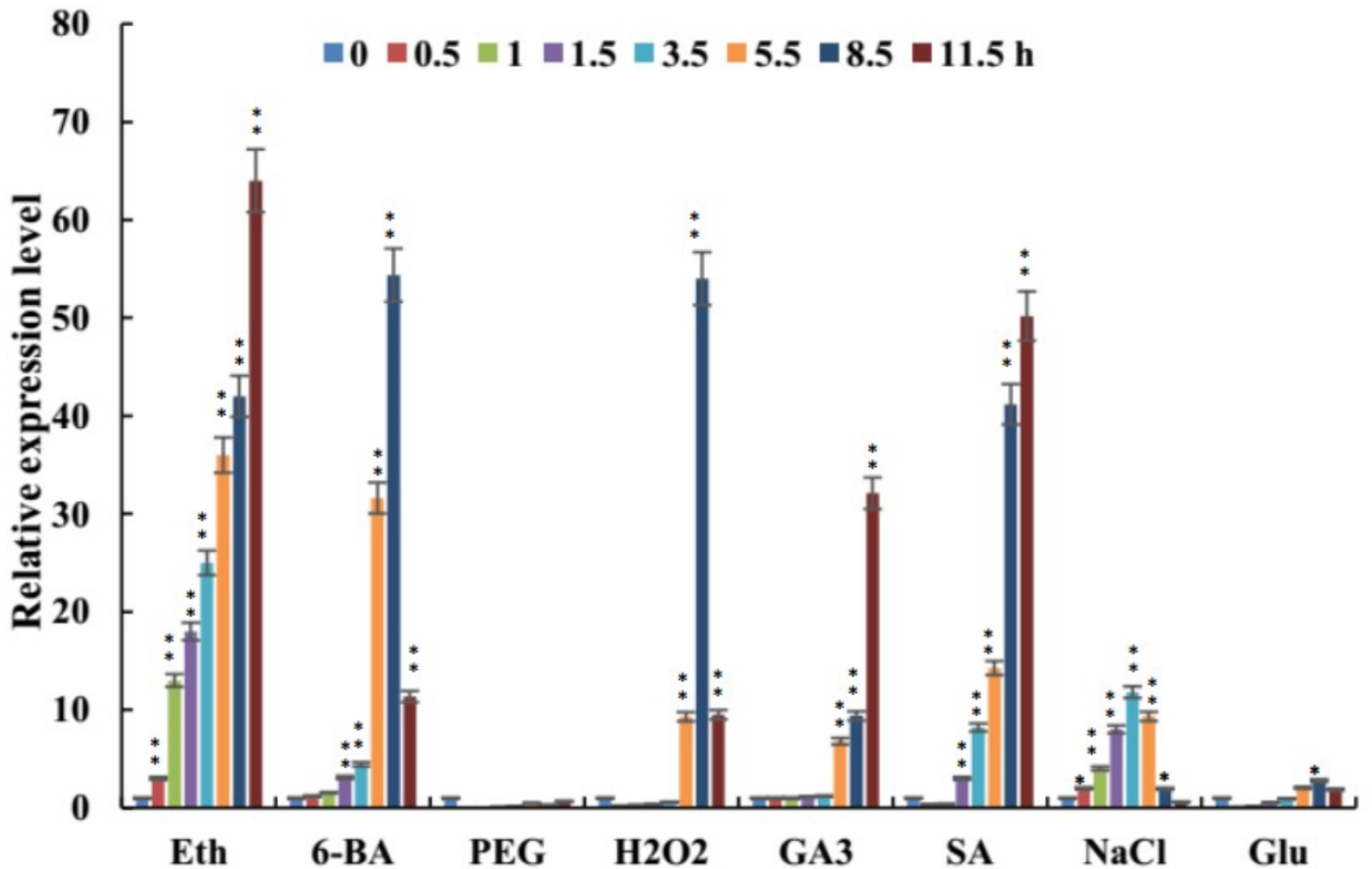


Figure 5

Effect of elicitors (Eth, 6-BA, PEG, H2O2, GA3, SA, NaCl and Glu) on CcERF2 expression. The 24 DAP pepper fruits were treated with elicitors (Eth, 6-BA, PEG, H2O2, GA3, SA, NaCl or Glu), and the fruit were sampled after elicitation for 0.5, 1.0, 1.5, 3.5, 5.5, 8.5, and 11.5 h. Without induction at the 0 h time point was used as control expression. The experiments were replicated three biological times and three technical times. Data are expressed as the mean \pm SD (n = 9). Student's t test was used to identify significant differences compared to the control (*P < 0.05, **P < 0.01).

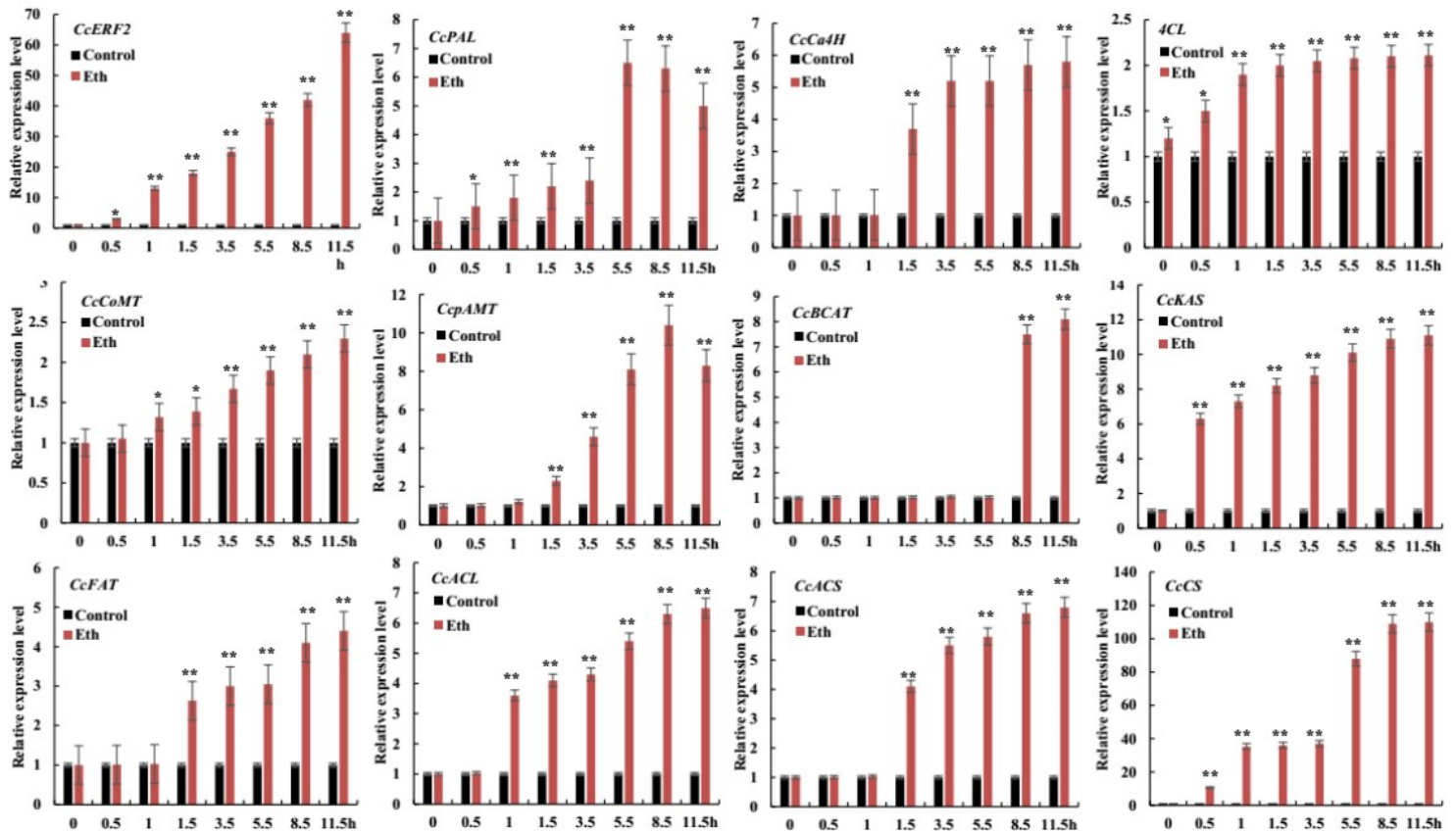


Figure 6

Expression profiles of CBGs after treatment with Eth. The 24 DAP pepper fruits were treated with Eth, and the fruit were sampled after elicitation for 0.5, 1.0, 1.5, 3.5, 5.5, 8.5, and 11.5 h. Without induction at the 0 h time point was used as control expression. The experiments were replicated three biological times and three technical times. Data are expressed as the mean \pm SD (n = 9). Student's t test was used to identify significant differences compared to the control (*P < 0.05, **P < 0.01).

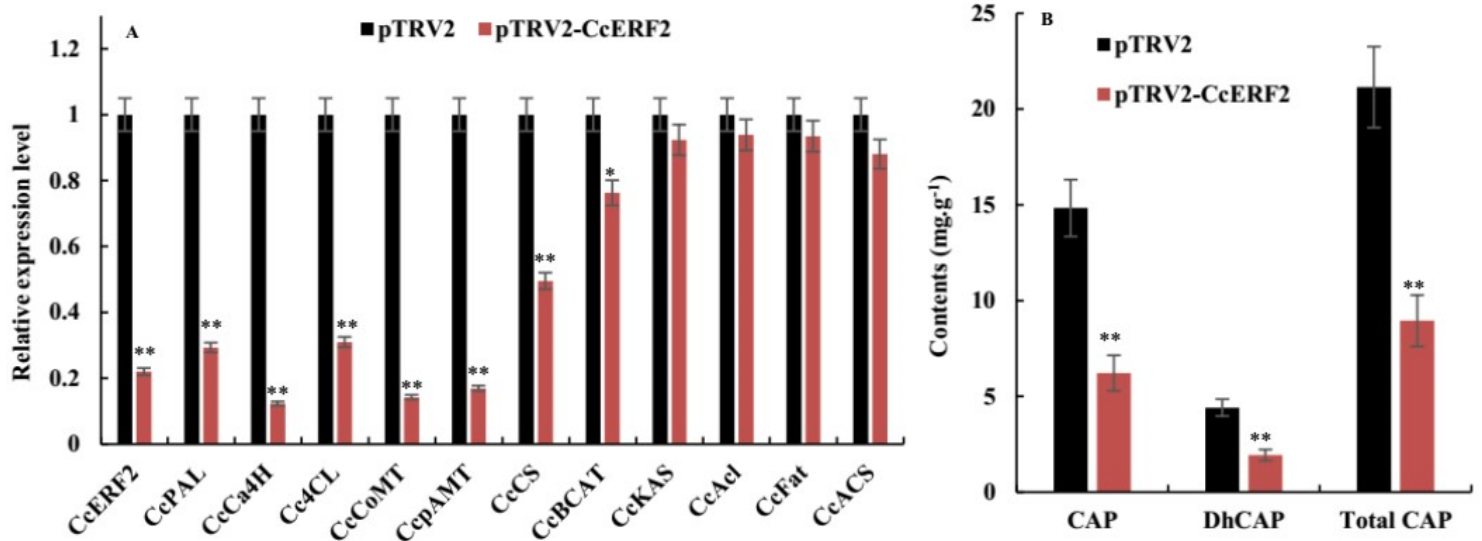


Figure 7

Expression level of CBGs and CcERF2, relative contents of CaP, DhCaP and CAPs in the control (empty vector) and CcERF2-silenced fruit. (A) Expression level of CBGs and CcERF2 in the control (empty vector) and CcERF2-silenced fruit. Fruits at 24 DAP was sampled to perform the expression level of CBGs and CcERF2. The relative expression level of the CBGs and CcERF2 in the control were set to 1, and that of in the CcERF2-silenced fruit were measured relative to that in the control. (B) Relative contents of CaP, DhCaP and CAPs in the control (empty vector) and CcERF2-silenced fruit at 34 DAP. The experiments were replicated three biological times and three technical times. Data are expressed as the mean \pm SD (n = 9). Student's t test was used to identify significant differences compared to the control (*P < 0.05, **P < 0.01).

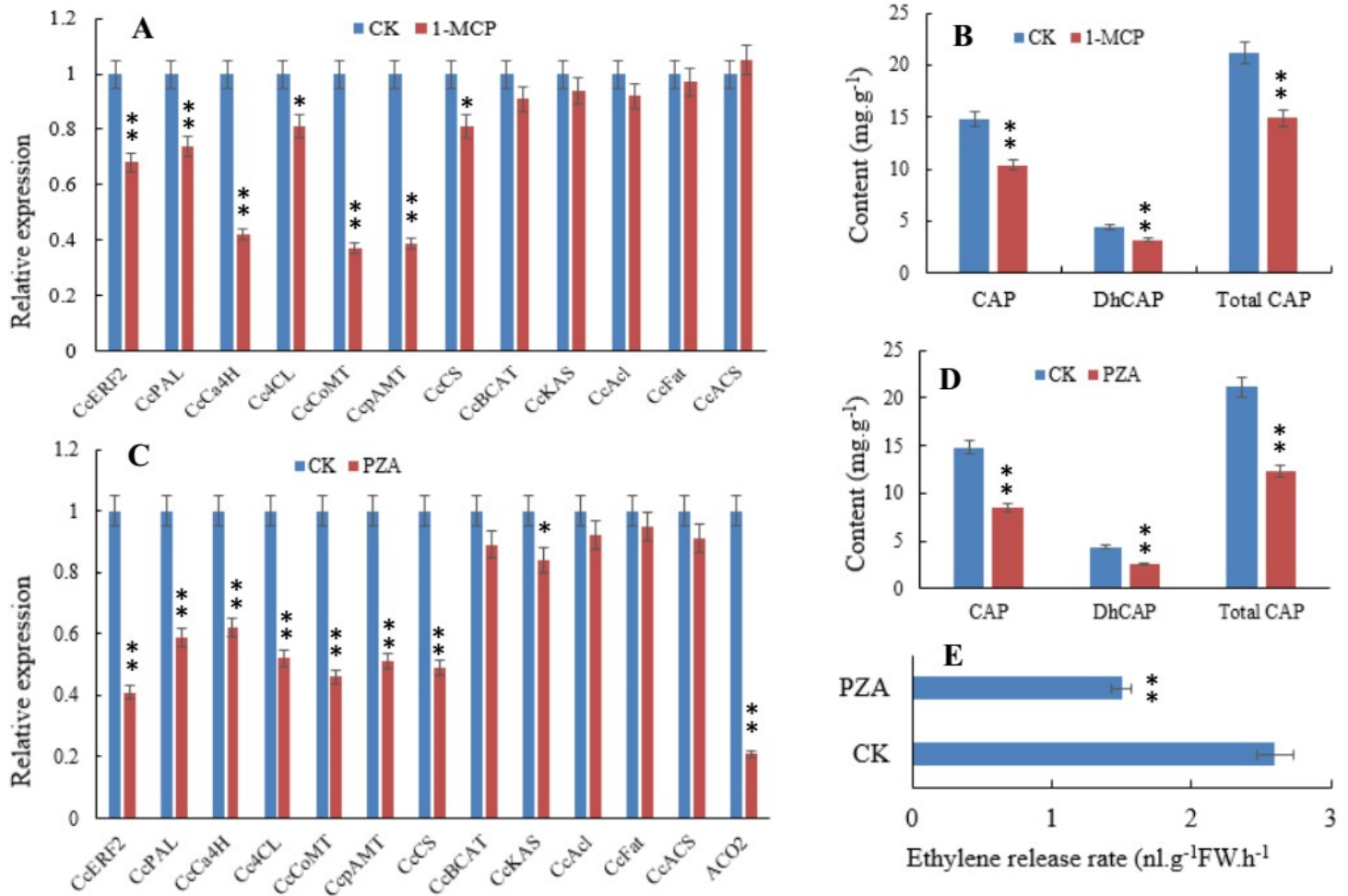


Figure 8

Expression level of CBGs and CcERF2, relative contents of CaP, DhCaP and CAPs in 1-MCP and PZA treatment fruit. (A) Expression level of CBGs and CcERF2 in the 1-MCP treatment fruit. Fruits at 24 DAP was sampled to perform the expression level of CBGs and CcERF2. The relative expression level of the CBGs and CcERF2 in the control were set to 1, and that of in 1-MCP treatment fruit were measured relative to that in the control. (B) Relative contents of CaP, DhCaP and CAPs in the 1-MCP treatment fruit at 29 DAP. (C) Expression level of CBGs, CcERF2 and ACO2 in the PZA treatment fruit. Fruits at 24 DAP was sampled to perform the expression level of CBGs and CcERF2. The relative expression level of the CBGs and CcERF2 in the control were set to 1, and that of in PZA treatment fruit were measured relative to that

in the control. (D) Relative contents of CaP, DhCaP and CAPs in the PZA treatment fruit at 29 DAP. (E) Ethylene release rate in the PZA treatment fruit at 29 DAP. The experiments were replicated three biological times and three technical times. Data are expressed as the mean \pm SD (n = 9). Student's t test was used to identify significant differences compared to the control (*P < 0.05, **P < 0.01).

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