

# Ethylene accelerates grape ripening *via* increasing VvERF75-induced ethylene synthesis and chlorophyll degradation

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## Abstract

Ethylene plays an important role in grape ripening, acting through ethylene response factors (ERFs) that are downstream of ethylene signaling pathways, yet little else is known about these molecular mechanisms. In our study, we demonstrate that ethylene treatment promotes grape ripening and that the transcription factor VvERF75 shows increased expression during grape ripening. VvERF75 was found to contain a conserved AP2/ERF domain, to be a member of the ERF IXa subfamily, and to localize to the nucleus and to have transactivation activity in yeast. Our *in vitro* assays showed that VvERF75 can positively regulate expression of *pheophorbide a oxygenase* (VvPAO1) and *1-aminocyclopropane-1-carboxylic acid synthase* (VvACS5) by binding to DRE motifs in their promoters. Overexpression of VvERF75 in tomato increased ethylene production and resulted in dwarfed plants, early flowering and fruit ripening, increased SIPAO and SIACS transcript levels, and decreased chlorophyll content. In general, our study revealed that VvERF75 may regulate fruit ripening by promoting ethylene biosynthesis and chlorophyll degradation, a discovery that lays a foundation for deciphering the molecular mechanisms of grape ripening.

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## INTRODUCTION

Grape (*Vitis vinifera* L.) is an important economic fruit planted worldwide. Fruit ripening is crucial to the quality of a cluster of grapes, which affects both consumer selection and storability of the fruit. Fruit ripening is a complicated procedure that involves numerous physiological and biochemical reactions that result in color and textural changes, soluble sugar accumulation, and aroma formation in the fruit. Therefore, to improve fruit appearance, quality and storability, one must understand the regulatory mechanisms underlying fruit ripening and how these mechanisms are similar and different between fruit types.

Fleshy fruits are traditionally classified into climacteric and non-climacteric, according to whether there is a peak of respiration and ethylene release at the beginning of ripening. It has been widely studied how ethylene accelerates ripening of climacteric fruits<sup>[1–3]</sup>. Grape is a non-climacteric fruit, however, previous research implies that ethylene is required for grape ripening<sup>[4,5]</sup>, that exogenous ethylene can affect coloration and ripening in non-climacteric fruit<sup>[6–8]</sup>, and that ethylene treatment of grapes one week before veraison can enhance fruit color and anthocyanin content together with the expression of genes related to anthocyanin biosynthesis<sup>[8–10]</sup>. Less is known about the mechanism through which ethylene regulates grape berry ripening.

Grape berry ripening is firstly accompanied by changes in the color that result from chlorophyll degradation and anthocyanin accumulation<sup>[8–10]</sup>. Chl degradation has been widely investigated during leaf senescence and fruit ripening, resulting in the degreening of leaves or fruits<sup>[11–14]</sup>, with the genes encoding

the enzymes needed for the catalysis of Chl degradation identified, including NON-YELLOW COLOURING (NYC), PAO, chlorophyllase (CLH), pheophorbide hydrolase (PPH), Red chlorophyll catabolite reductase (RCCR) and STAYGREEN/STSYGREEN-LIKE (SGR/SGRL)<sup>[11,15–18]</sup>. Degradation of Chl during apple storage proved to be regulated by ethylene, which activates the expression of Chl catabolic genes (CCGs) that will result in accelerated Chl degradation<sup>[3,4,19]</sup>.

Ethylene-induced Chl degradation is regulated transcriptionally<sup>[4,5,19]</sup> through activation of a downstream Ethylene Response Factor (ERF). ERFs belong to the AP2/ERF transcription factor superfamily<sup>[20]</sup> and regulate expression of CCGs during fruit ripening<sup>[4,5]</sup>, leaf senescence, and iron deficiency<sup>[21,22]</sup>, among other processes. Moreover, ERF transcription factors can modulate ethylene production by regulating expression of ACS and ACC oxidase (ACO), which encode key enzymes in ethylene biosynthesis<sup>[2,3,23,24]</sup>. Genome-wide identification of AP2/ERF genes in grape has been carried out, and 122 ERF TFs were identified, which can be classified into 12 subfamilies<sup>[25]</sup>. VvERF17 has been shown to control chlorophyll degradation in berry skin during fruit development<sup>[26]</sup>, while VvERF95 was proven to regulate chlorophyll degradation during rachis degreening during postharvest storage. However, relatively few functional analyses of grape ERF genes have been reported, and it remains unknown if ERF transcription factors are involved in ethylene biosynthesis and Chl degradation during grape berry coloration.

In this study, VvERF75 is shown to have a higher expression level after ethylene treatment and a positive correlation with

ethylene production, but a significantly negative correlation with chlorophyll content. We further studied the VvERF75-controlled regulation of CCGs and ethylene biosynthesis genes and demonstrated the functions of VvERF75 in Chl degradation and ethylene biosynthesis. The results of this study advance our understanding of the mechanisms through which ethylene influences grape ripening.

## MATERIALS AND METHODS

### Plant material

Vines of the cultivar 'Summer Black' (*Vitis vinifera* × *Vitis labrusca*) were planted in Henan Agricultural University (China) and maintained under normal growth conditions. Ethylene (500 mg/L) was sprayed on berries one week before veraison, with berries sprayed with distilled water used as control. Nine grapevine clusters were sampled randomly at 0, 5, 10 and 15 days (d) after treatment. At each timepoint, 30 berries were randomly selected for measurement of longitudinal diameter, transverse diameter, single berry weight (mm), soluble solids content (%), and titratable acidity (%). Eighty grapevine berries were deseeded, immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until assayed.

### Gene isolation, phylogenetic tree construction, multiple sequence alignment and motif analysis of promoters

The full-length coding sequence (CDS) of VvERF75 was amplified using primers listed in [Supplemental Table S1](#). Protein sequences of ERF genes used in phylogenetic tree construction and multiple sequence alignment were downloaded from the TAIR database and the NCBI database; accession numbers for each gene are listed in [Supplemental Table S2](#) and [S3](#). MEGA 7.0 software was used for sequence alignments and phylogenetic trees. The DRE motif (A/TCCGAC)<sup>[4,5]</sup>, REV motif (CAACA)<sup>[27]</sup> and CE-box motif (CCACC)<sup>[28]</sup> were manually searched. Mutations in the promoters of VvPAO1 and VvACS5 was conducted using QuikChange Lightning Site-Directed Mutagenesis Kit to generate the VvPAO<sub>m</sub> and VvACS5<sub>m</sub> constructs.

### Subcellular localization and transcriptional activation analysis of VvERF75

Subcellular localization and transcriptional activation assays were conducted as described<sup>[29]</sup>. For these experiments, the CDS of VvERF75 without its stop codon was introduced into the pSAK277-GFP and pGBKT7 vectors, respectively. The empty vectors of pSAK277-GFP and pGBKT7 was used as controls.

### Yeast one-hybrid analysis

The CDS sequence of VvERF75 was introduced into the pB42AD vector, while the promoters of various CCG genes were introduced into the placZi vector. Yeast one-hybrid experiments were performed as previous reported<sup>[4,30]</sup>. To accurately identify the DNA regions/motifs than can interact with VvERF75, the target genes identified in the first round of screening were further divided into 100-bp and 50-bp segments to conduct second- and third-round screens.

### Dual-luciferase assays

For dual-luciferase assays, the promoter sequences of VvPAO1 and VvACS5 were inserted into pGreen 0800-LUC to yield reporter vectors. The CDS of VvERF75 was inserted into pSAK277 to yield effector vector. The reporter vectors and

effector vectors were co-transformed into *Nicotiana benthamiana* leaves, with the empty pSAK277 vector used as a negative control. The experiment was carried out according to previous reports<sup>[31,32]</sup>.

### EMSA assays

For EMSA assays, the CDS of VvERF75 was introduced upstream of the GST-tag in the pCold plasmid. Recombinant GST-VvERF75 protein was expressed in *Escherichia coli* strain BL21 and purified. The probe and mutated probe sequences were synthesized in Sangon Biotechnology (Shanghai, China). Each probe was biotin-labeled at the 5' end. The reported method was used for EMSA assays<sup>[31]</sup>.

### Real-time quantitative PCR analysis

The plant RNA kit (Omega) was used for RNA extraction from frozen grape berry samples. qRT-PCR was carried out according to a previous report<sup>[32]</sup> with GAPDH (CB973647) serving as an internal control using the primers listed in [Supplemental Table S1](#).

### Genetic transformation and confirmation of VvERF75

The CDS sequence of VvERF75 was inserted into the binary vector pSAK277 to generate the 35Spro-VvERF75-GFP construct. Tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were used for VvERF75 overexpression. Transgenic plants were generated and cultured as described<sup>[30]</sup>. DNA and RNA were extracted from leaves of candidate transgenic plants to identify VvERF75 overexpressing (VvERF75-OE) plants by qRT-PCR and PCR. The expression of SIPAO, SIACS2 and SIACS4 in tomato were detected by qRT-PCR using primers in [Supplemental Table S1](#).

### Measurement of ethylene production, chlorophyll and lycopene content in fruit and rachis of tomato

Fruits of VvERF75-OE transgenic and WT tomato plants were detached at the breaker, breaker+3d, breaker+5d, and breaker+7d stages of fruit ripening. Ethylene production, and chlorophyll level content were measured as described previously<sup>[33–35]</sup>.

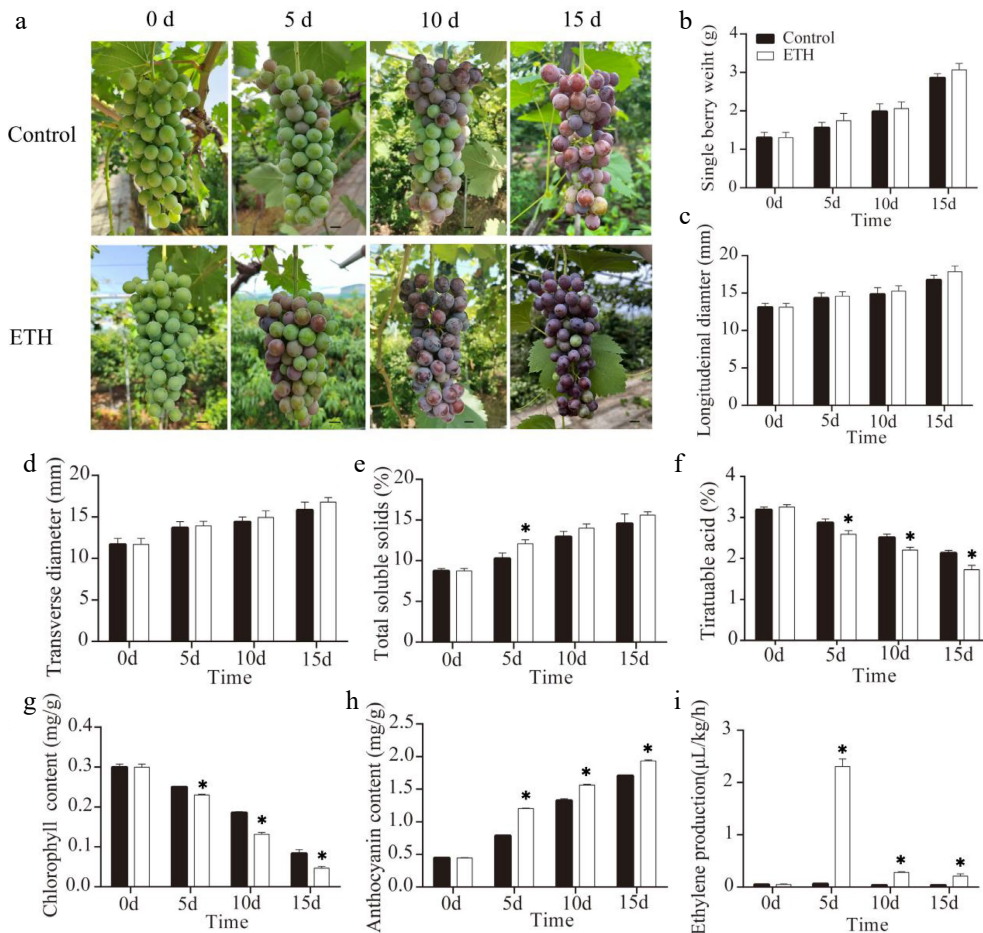
### Statistical analysis

Experiments in this study were conducted in three replicates. Statistics were analyzed using DPS software. Error bars represent standard deviation. \* indicate significant difference ( $P < 0.05$ ) as obtained by one-way analysis of variance and the least significant difference test.

## RESULTS

### Expression of VvERF75 is closely associated with chlorophyll content and is induced by ethylene

Ethylene treatment of maturing grape clusters was carried out one week before veraison, and clusters were detached 5, 10 and 15 d after treatment (DAT). Berry coloration was advanced by about 5 d in ethylene-treated clusters compared with controls ([Fig. 1a](#)). Single berry weight ([Fig. 1b](#)), longitudinal diameter ([Fig. 1c](#)) and transverse diameter ([Fig. 1d](#)) showed no significant variation after treatment, while total soluble solids were higher at 5 d after treatment but similar to the controls afterwards ([Fig. 1e](#)). Titratable acid ([Fig. 1f](#)) and chlorophyll content ([Fig. 1g](#)) were decreased, while anthocyanin content ([Fig. 1h](#)) and ethylene production ([Fig. 1i](#)) were increased after



**Fig. 1** The effects of ethylene treatment on fruit ripening in 'Summer Black' grape. (a) Ripening phenotype in response to ethylene treatment (on day 0), Bar = 0.5 cm. (b) Single berry weight. (c) Longitudinal diameter. (d) Transverse diameter. (e) Total soluble solids. (f) Titratable acid. (g) Anthocyanin content. (h) Chlorophyll content. (i) Ethylene production.

ethylene treatment (Fig. 1a). This indicated that ethylene treatment can promote coloration of 'Summer Black' grape.

### VvERF75 belongs to the ERF IXa subfamily and has a AP2/ERF domain

ERF transcription factors take part in fruit ripening by modulating expression of genes related to ethylene production or chlorophyll degradation<sup>[2,3,36]</sup>. The ERF transcription factor family can be classified into 12 subfamilies<sup>[20]</sup>. We identified VvERF75 in our previous genome analysis of genes responsive to 1-MCP treatment<sup>[34]</sup>. Here, VvERF75 expression was negatively correlated with chlorophyll content, with a Pearson correlation coefficient of 0.799\* (Supplemental Fig. S1). A phylogenetic tree of VvERF75 and some of the ERF proteins from Arabidopsis revealed that VvERF75 belongs to subfamily IX (Supplemental Fig. S2a). Group IX can be further divided into Subgroups IXa, IXb and IXc, with VvERF75 belonging to group IXa (Supplemental Fig. S2b). The Chl degradation and ethylene biosynthetic roles of most genes in group IXa are largely unclear. Alignment of protein sequences of VvERF75 and its homologs from *Ziziphus jujube* (*ZjPIT5-like*), *Mangifera indica* (*MiPIT5-like*), *Quercus suber* (*QsPIT5-like*), *Citrus sinensis* (*CsPIT5*) and *Medicago truncatula* (*MtPIT5*) showed that VvERF75 contains a conserved AP2/ERF domain ( $\beta$ -1  $\beta$ -2,  $\beta$ -3, and  $\alpha$ -helix) (Supplemental Fig. S2c).

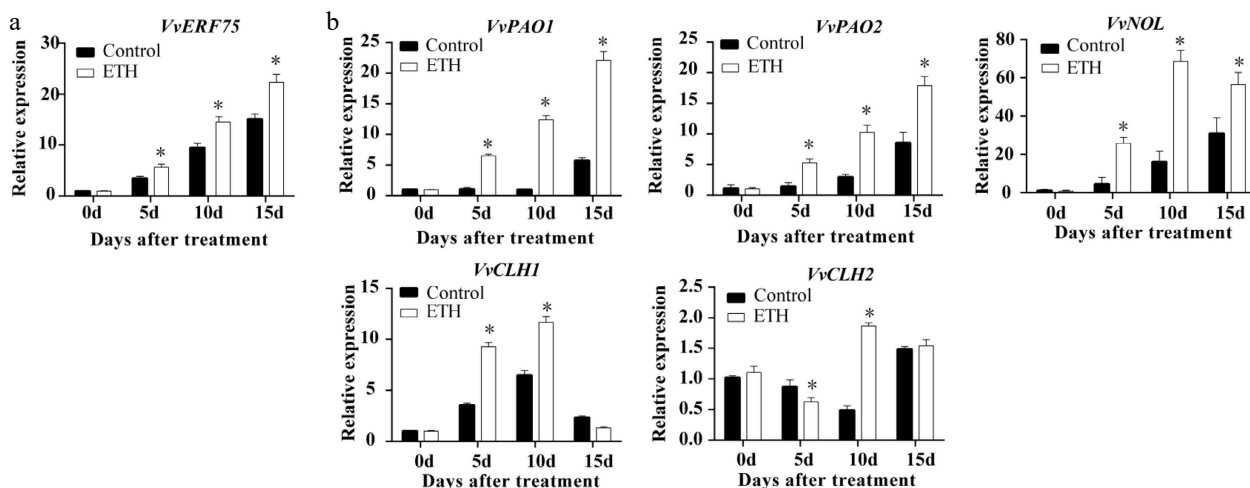
The expression of the transcription factor VvERF75 and several potential downstream CCGs (*VvPAO1*, *VvPAO2*, *VvNYC1*,

*VvNOL*, *VvCLH1* and *VvRCCR*) were examined after veraison. Expression of VvERF75 and VvPAO1, VvPAO2, VvCLH1 and VvNOL increased in response to ethylene treatment, while expression of VvCLH2 showed more variation, decreasing at 5 DAT and increasing at 10 DAT (Fig. 2). Expression of VvERF75 showed a similar pattern to ethylene production (Fig. 1i) and expression of the chlorophyll degradation genes VvPAO1, VvPAO2, VvCLH1, VvNOL and VvCLH2 (Fig. 2), but the opposite pattern to chlorophyll content in grape after ethylene treatment (Fig. 1g). These correlations indicated that VvERF75 may have a potential role in regulation of ethylene biosynthesis and chlorophyll degradation.

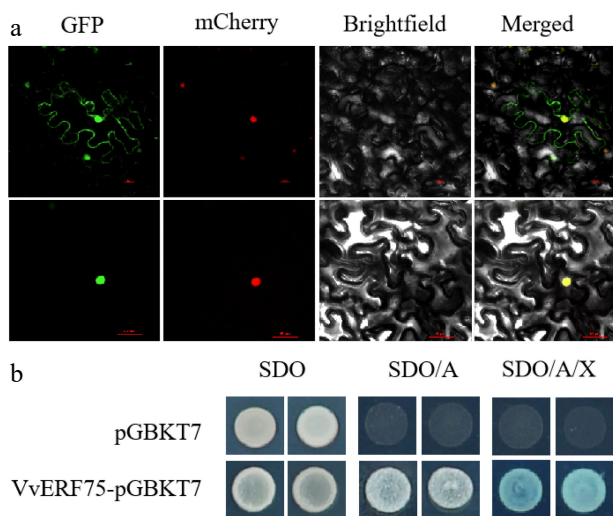
### VvERF75 is localized in the nucleus and has transcriptional activity

The GFP-VvERF75 construct was transiently transformed into tobacco leaves to verify the subcellular localization of VvERF75. The fluorescence of GFP alone was observed in both the nucleus and the cytoplasm. The fluorescence of the GFP-VvERF75 protein was observed only in the nucleus, as verified with NLS-mCherry (a nuclear-localized marker) (Fig. 3a), suggesting that VvERF75 is localized in the nucleus.

A transcription activation assay was conducted in yeast to identify if VvERF75 has transcriptional activity. VvERF75-pGBKT7 and the empty pGBKT7 vector were separately transformed into yeast strain Y2H gold. All transformants survived on



**Fig. 2** Expression in 'Summer Black' grape of (a) *VvERF75* and (b) chlorophyll degradation related genes in response to ethylene treatment.



**Fig. 3** Subcellular localization in tobacco and transcriptional activation in yeast of *VvERF75*. (a) Subcellular localization of *VvERF75* in *Nicotiana benthamiana* leaves. *VvERF75*-GFP was transformed into *Nicotiana benthamiana*. *VvERF75*-GFP, GFP signal; Brightfield, white light; Merged, combined GFP and brightfield signals. NLS mCherry was used as a nuclear-localized marker. Bar = 50  $\mu$ m. (b) *VvERF75* trans-activation activity assay. Yeast cells were dotted on plates containing either SDO, synthetic dropout media; SDO/A, synthetic dropout medium supplemented with Aureobasidin A but lacking tryptophan; or SDO/A/X, synthetic dropout medium supplemented with Aureobasidin A and X-a-Gal but lacking tryptophan. pGBKT7 vector was used as negative control.

synthetic drop-out media (SDO) media. Yeast transformants with *VvERF75* grew successfully in the selective media SDO/A and SDO/A/X, while yeast cells transformed with pGBKT7 vector did not (Fig. 3b). These results provide evidence that *VvERF75* activated transcription in yeast cells.

### *VvERF75* activated expression of *VvPAO1* and *VvACS5*

Yeast one-hybrid and luciferase assays were performed to detect any relationship between *VvERF75* and Chl degradation-related genes. The results suggest that *VvERF75* can bind to the promoter of *VvPAO1* (Fig. 4a) and positively regulate its expression (Fig. 4c).

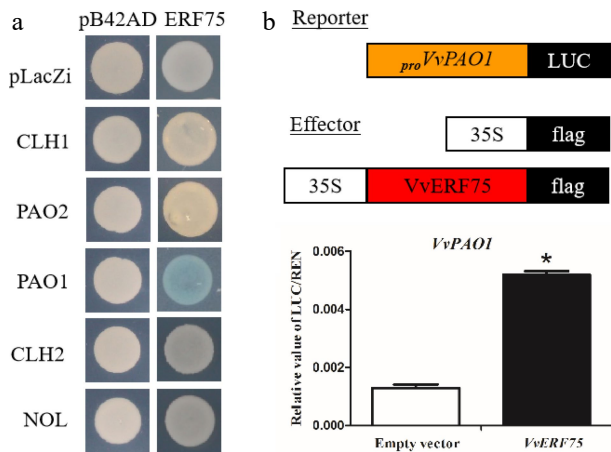
To identify specific *VvERF75* binding sites in the *VvPAO1* promoter sequence, a sequential deletion series was generated and assessed *via* yeast one-hybrid assays and EMSA. The fragment *p5* contains the DRE motif. The results indicated that *VvERF75* can bind to the DRE motif in the promoter of *VvPAO1* (Fig. 5a & b). Mutation of the DRE motif in the fragment *p5-3* disrupted the interaction between *VvERF75* and the *PAO1* fragment in yeast (Fig. 5c). This interaction was further confirmed by EMSA (Fig. 5d), in which the *VvERF75*-GST fusion protein bound directly to the promoter of *VvPAO1* (Fig. 5d), but failed to bind to the mutant DRE motif of *VvPAO1* (Fig. 5d).

ERF transcription factors have been reported to regulate the expression of *ACS* or *ACO*, and thus, regulate ethylene biosynthesis. The increased production of ethylene in grape berries raises the question of whether *VvERF75* can modulate ethylene biosynthesis during berry ripening. *VvACS5* has a DRE/GCC-box motif and is differentially expressed during rachis browning<sup>[36]</sup>, so was selected for further analysis. Yeast one-hybrid and dual-luciferase experiments (Fig. 6b) revealed that *VvERF75* can bind to the promoter of *VvACS5* and positively regulate its expression (Fig. 6a & c). The DRE and GCC motifs in the promoter of *VvACS5* were mutated (m1 and m2), and binding of *VvERF75* on these promoter sequences was assessed by dual-LUC experiments. The LUC/REN expression ratio in the m1 and m2 variants was not significantly different with that of the empty vector, which implied that *VvERF75* binds the intact DRE motif in the promoter of *VvACS5* (Fig. 6d). In the EMSA, the *VvERF75*-GST fusion protein can only bind directly to the DRE motif of *VvACS5* (Fig. 6e), but not the GCC-box motif of *VvACS5* (Fig. 6f). Taken together, these results indicated that *VvERF75* might regulate ethylene biosynthesis and Chl degradation genes during color development in grape berry.

### *VvERF75* affects flowering time and plant height in transgenic tomato

To investigate the function of *VvERF75* during plant development, *VvERF75* was overexpressed in tomato *via* *Agrobacterium*-mediated transformation. *VvERF75*-OE plants were identified by PCR and qRT-PCR (Fig. 7a & b). Three independent lines (75-4, 75-7 and 75-8) that showed higher expression levels of *VvERF75* were selected for further analysis and taken to the T1 generation (Fig. 7c). Flowering time of the *VvERF75*-OE transgenic plants was advanced by about 2-3 d compared with

VvERF75 promotes chlorophyll degradation



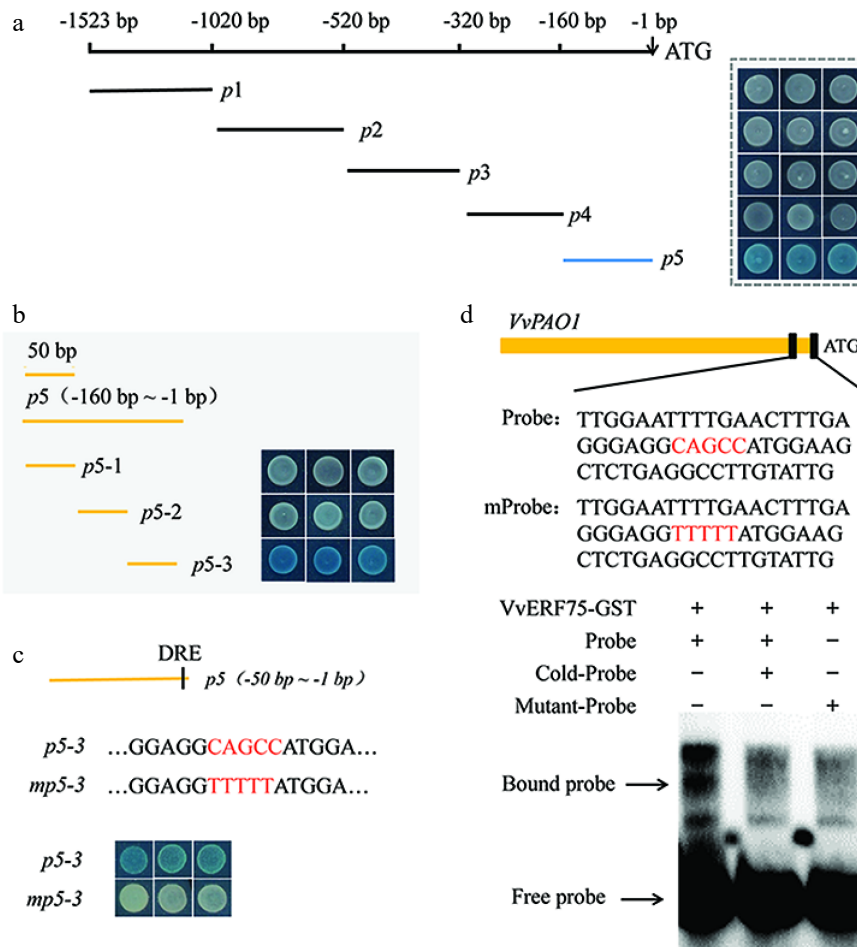
**Fig. 4** Analysis of *VvERF75* regulation of *VvPAO1*. (a) Yeast one-hybrid analysis of *VvERF75* binding to the promoters of chlorophyll catabolic genes. (b) Schematics of the reporter and effector constructs used in dual-luciferase reporter assay. (c) Activation of the *VvPAO1* gene by *VvERF75* is indicated by the LUC/REN ratio. Empty vector: 35S:flag + *proVvPAO1*:LUC; *VvERF75*: 35S:*VvERF75*:flag + *proVvPAO1*:LUC, the empty effector was used as a control.

WT, which flowered at an average of 43 d after sowing (Fig. 7d). In addition, the height of the *VvERF75*-OE plants was significantly decreased compared to the WT plants (Fig. 7e). Our results implied that *VvERF75* plays a critical role in plant development.

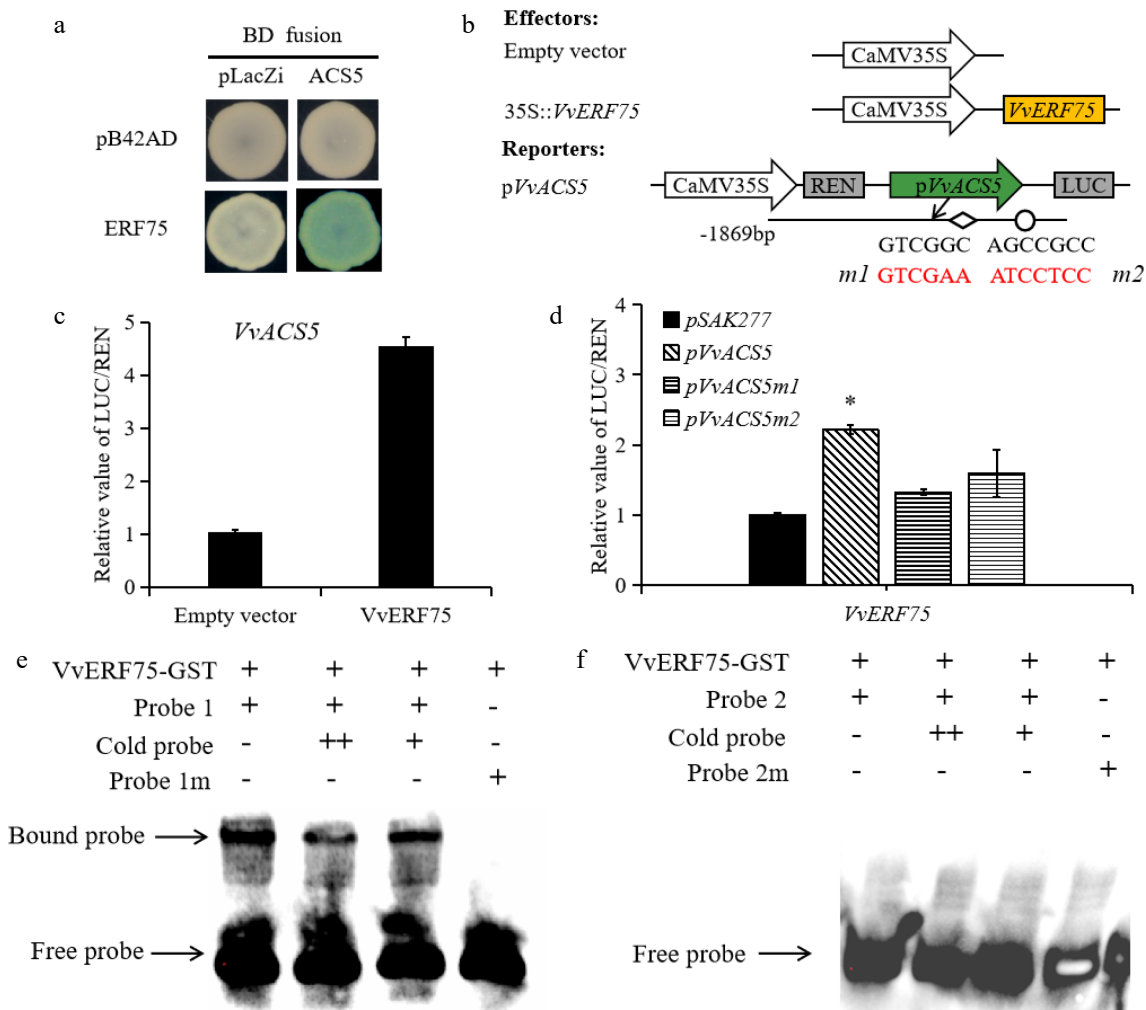
**VvERF75 enhanced ethylene production and chlorophyll degradation in transgenic tomato**

Fruits reached the breaker (Br) stage 42 d post-anthesis (DPA) in WT plants (Fig. 8a), but the *VvERF75*-OE lines began to turn red about 3 d later. Ethylene production, Chl and lycopene contents were monitored from Br to Br+7. In WT plants, ethylene production and lycopene contents increased while Chl content decreased during fruit ripening (Fig. 8b–d). In the *VvERF75*-OE plants, the ethylene production was higher than in WT (Fig. 8b), while the Chl content was lower in the *VvERF75*-OE lines (Fig. 8c) and the lycopene content was increased (Fig. 8d).

Tomato fruits at the Br and Br+3 stage, which showed obvious differences, were selected for gene expression analysis. Expression levels of *SIPAO*, *SIACS2* and *SIACS4* were significantly increased in *VvERF75*-OE plants compared with WT (Fig. 8e–g). Together these data further support that *VvERF75* can regulate



**Fig. 5** Electromobility shift and yeast one-hybrid assays showing the binding of *VvERF75* to the promoter of *VvPAO1*. (a), (b) Sequential deletion series and (c) mutation of *VvPAO1* promoter for use in yeast one-hybrid assays with *VvERF75*. (d) Sequence of the probe derived from the promoter of *VvPAO1* (p5-3) and *VvPAO1m* are shown, with the DRE motif and its mutant indicated in red. (e) EMSA of *VvERF75* protein binding to the promoter sequence of the *VvPAO1* gene. + and - represent the presence or absence of the respective composition in the binding experiment. The labeled 'free probe' and DNA protein complex 'bound probe' positions are indicated, showing competition with an unlabeled fragment for each promoter domain.



**Fig. 6** Regulation of VvACS5 by VvERF75. (a) Yeast one-hybrid assay analysis of VvERF75 binding to VvACS5 promoter. (b) Schematics of the reporter and effector constructs used in the dual-luciferase reporter assay, the DRE motif and its mutant indicated in red. (c) Dual-luciferase assays of VvERF75 on promoter of VvACS5. (d) VvERF75 activation of the wild-type and mutant promoters of the VvACS5 genes. *m1* carries a mutation of the DRE motif and *m2* of the GCC box in the promoter of VvACS5. (e), (f) EMSA of VvERF75 protein binding to motifs 1 and 2 on promoter of the VvACS51 gene. + and - represent the presence or absence of the respective component in the binding experiment. The labeled 'free probe' and DNA protein complex 'bound probe' positions are indicated, showing competition with an unlabeled fragment for each promoter domain.

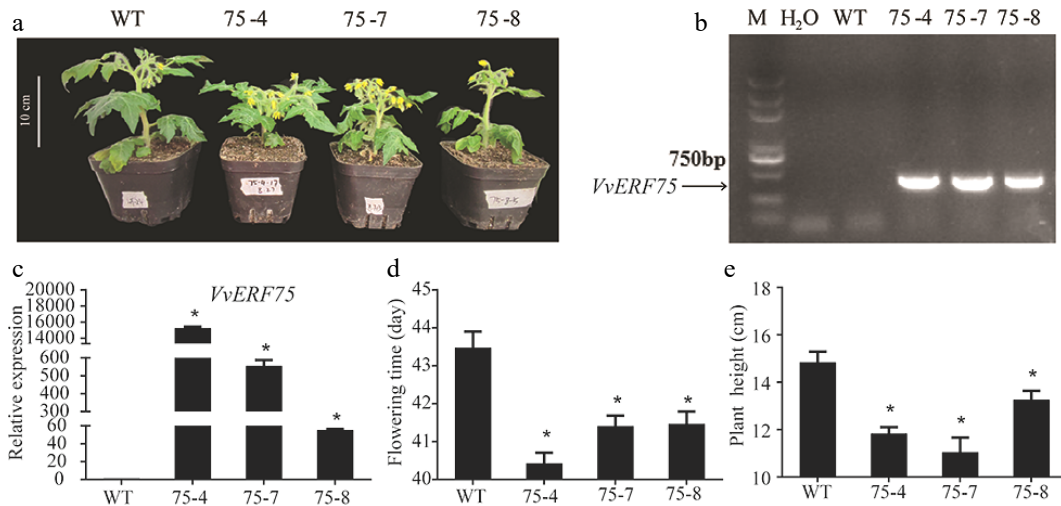
expression of PAO and ACS, thus, modulating chlorophyll degradation and ethylene biosynthesis.

## DISCUSSION

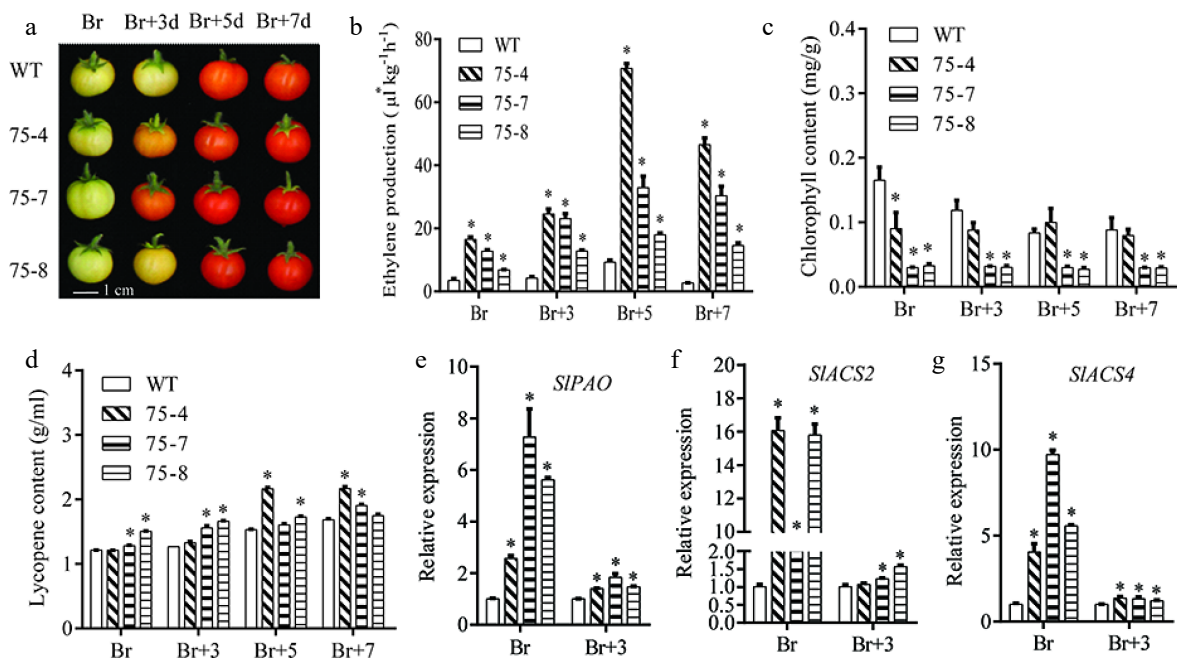
Ethylene has been proven to play vital roles during plant development<sup>[37–39]</sup> and to control downstream transcriptional regulators, the ERFs, that are responsible for the ethylene signal. ERFs are in the AP2/ERF superfamily and carry the AP2/ERF domain, which is 60-70 amino acids long and binds DNA. The superfamily can be further divided into the AP2 family, the ERF family, and the RAV family<sup>[20]</sup>. The ERF family in Arabidopsis can be classified into 12 subfamilies<sup>[20]</sup>. The ERF IX subfamily can divide into three subgroups, IXa, IXb and IXc<sup>[20]</sup>, phylogenetic analysis and sequence alignment revealed that VvERF75 belongs to subfamily IXa (Supplemental Fig. S2a & Sb) and contains an AP2/ERF conserved domain (Supplemental Fig. S2c). ERF transcription factors can recognize and bind to the GCC-box/DRE motifs in promoters of downstream genes to

activate their expression<sup>[2,3,5,40]</sup>. Genes of the ERF IX subfamily participate in resistance to abiotic stress<sup>[41–43]</sup>, cold stress<sup>[44,45]</sup>, and ethylene synthesis and response<sup>[3,46,47]</sup>. There are 40 predicted proteins in the grape ERF IX subfamily<sup>[25]</sup>, some of which have been functionally characterized. VaERF092 from Amur grape regulates cold tolerance<sup>[48]</sup>, VaERF20 enhances resistance in tomato to *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000<sup>[49]</sup>. Our previous research revealed that the Chl content was reduced during rachis degreening and that VvERF95 could modulate Chl degradation in this process<sup>[35]</sup>. Nevertheless, little is known about any roles of genes in the grape ERF IX family during berry ripening.

Ethylene and Chl degradation have been proved to be main aspects of berry coloration during grape fruit ripening<sup>[50,51]</sup>, but the mechanism underlying chlorophyll degradation during fruit ripening remains largely unknown. Our previous report revealed that VvERF95 can activate expression of VvPAO1, thus accelerating Chl degradation during rachis browning, and that the motifs for ERF binding exist in the promoters of many



**Fig. 7** Overexpression of *VvERF75* in tomato changed flowering time and plant height. (a) Transgenic plants of *VvERF75*. Detection of transgenic *VvERF75* plants by (b) PCR and (c) qRT-PCR. (d) Flowering time of transgenic *VvERF75*-OE plants compared to WT. (e) Height of transgenic *VvERF75*-OE plants compared to WT.



**Fig. 8** *VvERF75* enhanced ethylene production and chlorophyll degradation in tomato. (a) Color phenotype of *VvERF75* overexpression lines. (b) Ethylene production, (c) chlorophyll content, (d) lycopene content in WT and *VvERF75* overexpression lines at Br, Br+3, Br+5 and Br+7 stage. Expression of (e) *SIPAO*, (f) *SIACS2* and (g) *SIACS4* in WT and *VvERF75*-overexpression lines at Br and Br+3 stage.

CCGs<sup>[35]</sup>. For example, *CitERF6* can activate expression of *CitPPH* to promote Chl degradation during citrus fruit degreening<sup>[40]</sup>; *VvERF17* regulates the expression of *VvNOL*, *VvPPH*, *VvPAO* and *VvRCCR* to mediate Chl degradation in grape berry coloration<sup>[40]</sup>; apple *ERF17* binds the promoter of *PPH* and *NYC* to regulate Chl degradation during fruit peel degreening<sup>[4,52]</sup>. In this study, expression of *VvERF75* increased during grape berry ripening in response to ethylene treatment, while Chl content decreased, ethylene release increased, and the transcript levels of Chl degradation and ethylene synthesis genes increased. Our data demonstrated that *VvERF75* can activate expression of *VvPAO1* (Fig. 3 & 4), which is like the activity of *VvERF95*<sup>[35]</sup>.

In addition, ERF proteins have been shown to modulate ethylene in banana and apple by regulating the expression of

*ACO* or *ACS*<sup>[3,23]</sup>. Our experiment revealed that *VvERF75* can modulate expression of *VvACS5*. Site mutation of the DRE motifs revealed that *VvERF75* can bind to the promoters of *VvPAO1* (Fig. 4c & d) and *VvACS5* (Fig. 5d) at the DRE motifs, which is consistent with previous reports<sup>[2,3,5,40]</sup>.

To further characterize the role of *VvERF75* in chlorophyll degradation and ethylene production, *VvERF75* was transformed into tomato. Overexpression of *VvERF75* in tomato resulted in decreased content of Chl and increased production of ethylene during fruit development as well as the increased expression of *SIPAO*, *SIACS2* and *SIACS4*, further validating that *VvERF75* activates the expression of *PAO1* and *ACS5*. These results are consistent with results for *VvERF17*<sup>[26]</sup> and *VvERF95*<sup>[35]</sup> in grape as well as ERFs in apple and banana<sup>[3,23]</sup>,

revealing that the ability of ERF transcription factors to regulate chlorophyll degradation and ethylene biosynthesis is conserved among different species.

## CONCLUSIONS

In brief, our findings reveal that VvERF75 belongs to the ERF IXa subfamily, has an AP2 domain, is localized in the nucleus, and has transactivation activity in yeast cells. During grape berry coloration, expression of VvERF75 increased, activating the expression of VvPAO1 by binding to the DRE motif in its promoter. This accelerates degradation of chlorophyll. VvERF75 can also modulate ethylene biosynthesis by activating the expression of VvACS5, leading to increased ethylene production, which induces chlorophyll degradation. In addition, VvERF75-OE tomato plants showed reduced plant height and advanced flowering and fruit ripening times. Our results provide new insight into how VvERF75 impacts chlorophyll degradation and ethylene production during fruit ripening in grape. Our results also expanded the knowledge of the diverse functions of ethylene in non-climacteric fruits.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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## REFERENCES

- Alexander L, Grierson D. 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of Experimental Botany* 53:2039–55
- Zhang Z, Zhang H, Quan R, Wang X, Huang R. 2009. Transcriptional regulation of the ethylene response factor LeERF2 in the expression of ethylene biosynthesis genes controls ethylene production in tomato and tobacco. *Plant Physiology* 150:365–77
- Li T, Jiang Z, Zhang L, Tan D, Wei Y, et al. 2016. Apple (*Malus domestica*) MdERF2 negatively affects ethylene biosynthesis during fruit ripening by suppressing *MdACS1* transcription. *The Plant Journal* 88:735–48
- Han Z, Hu Y, Lv Y, Rose JKC, Sun Y, et al. 2018. Natural variation underlies differences in ETHYLENE RESPONSE FACTOR17 activity in fruit peel degreening. *Plant Physiology* 176:2292–304
- Yin X, Xie X, Xia X, Yu J, Ferguson IB, et al. 2016. Involvement of an ethylene response factor in chlorophyll degradation during citrus fruit degreening. *The Plant Journal* 86:403–12
- Qiao H, Zhang H, Wang Z, Shen Y. 2021. Fig fruit ripening is regulated by the interaction between ethylene and abscisic acid. *Journal of Integrative Plant Biology* 63:553–69
- Gu T, Jia S, Huang X, Wang L, Fu W, et al. 2019. Transcriptome and hormone analyses provide insights into hormonal regulation in strawberry ripening. *Planta* 250:145–62
- Chervin C, El-Kereamy A, Roustan JP, Latché A, Lamon J, et al. 2004. Ethylene seems required for the berry development and ripening in grape, a non-climacteric fruit. *Plant Science* 167:1301–5
- El-Kereamy A, Chervin C, Roustan JP, Cheynier V, Souquet JM, et al. 2003. Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. *Physiologia Plantarum* 119:175–82
- Wang P, Yu A, Ji X, Mu Q, Salman Haider M, et al. 2022. Transcriptome and metabolite integrated analysis reveals that exogenous ethylene controls berry ripening processes in grapevine. *Food Research International* 155:111084
- Hörtensteiner S. 2009. Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence. *Trends in Plant Science* 14:155–62
- Schelbert S, Aubry S, Burla B, Agne B, Kessler F, et al. 2009. Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in *Arabidopsis*. *The Plant Cell* 21:767–85
- Sakuraba Y, Park SY, Kim YS, Wang SH, Yoo SC, et al. 2014. *Arabidopsis* STAY-GREEN2 is a negative regulator of chlorophyll degradation during leaf senescence. *Molecular Plant* 7:1288–302
- Wei Y, Jin J, Xu Y, Liu W, Yang G, et al. 2021. Ethylene-activated MdPUB24 mediates ubiquitination of MdBEL7 to promote chlorophyll degradation in apple fruit. *The Plant Journal* 108:169–82
- Schenk N, Schelbert S, Kanwischer M, Goldschmidt EE, Dörmann P, et al. 2007. The chlorophyllases AtCLH1 and AtCLH2 are not essential for senescence-related chlorophyll breakdown in *Arabidopsis thaliana*. *FEBS Letters* 581:5517–25
- Peng G, Xie X, Jiang Q, Song S, Xu C. 2013. Chlorophyll a/b binding protein plays a key role in natural and ethylene-induced degreening of Ponkan (*Citrus reticulata* Blanco). *Scientia Horticulturae* 160:37–43
- Xie Z, Wu S, Chen J, Zhu X, Zhou X, et al. 2019. The C-terminal cysteine-rich motif of NYE1/SGR1 is indispensable for its function in chlorophyll degradation in *Arabidopsis*. *Plant Molecular Biology* 101:257–68
- Hörtensteiner S, Kräutler B. 2011. Chlorophyll breakdown in higher plants. *Biochimica et Biophysica Acta* 1807:977–88
- Qiu K, Li Z, Yang Z, Chen J, Wu S, et al. 2015. EIN3 and ORE1 accelerate degreening during ethylene-mediated leaf senescence by directly activating chlorophyll catabolic genes in *Arabidopsis*. *PLoS Genetics* 11:e1005399
- Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology* 140:411–32
- Liu W, Karemera NJU, Wu T, Yang Y, Zhang X, et al. 2017. The ethylene response factor AtERF4 negatively regulates the iron deficiency response in *Arabidopsis thaliana*. *PLoS One* 12:e0186580
- Liu W, Li Q, Wang Y, Wu T, Yang Y, et al. 2017. Ethylene response factor AtERF72 negatively regulates *Arabidopsis thaliana* response to iron deficiency. *Biochemical and Biophysical Research Communications* 491:862–68
- Xiao Y, Chen J, Kuang J, Shan W, Xie H, et al. 2013. Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. *Journal of Experimental Botany* 64:2499–510
- Han YC, Kuang JF, Chen JY, Liu XC, Xiao YY, et al. 2016. Banana transcription factor MaERF11 recruits histone deacetylase MaHDA1 and represses the expression of *MaACO1* and *Expansins* during fruit ripening. *Plant Physiology* 171:1070–84
- Licausi F, Giorgi FM, Zenoni S, Osti F, Pezzotti M, et al. 2010. Genomic and transcriptomic analysis of the AP2/ERF superfamily in *Vitis vinifera*. *BMC Genomics* 11:719



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26. Lu S, Zhang M, Zhuge Y, Fu W, Ouyang Q, et al. 2022. VvERF17 mediates chlorophyll degradation by transcriptional activation of chlorophyll catabolic genes in grape berry skin. *Environmental and Experimental Botany* 193:104678
27. Yu Z, Li J, Yang C, Hu W, Wang L, et al. 2012. The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. *Molecular Plant* 5:353–65
28. Lee S, Park JH, Lee M, Yu JH, Kim SY. 2010. Isolation and functional characterization of CE1 binding proteins. *BMC Plant Biology* 10:277
29. Li Z, Zhang C, Guo Y, Niu W, Wang Y, et al. 2017. Evolution and expression analysis reveal the potential role of the HD-Zip gene family in regulation of embryo abortion in grapes (*Vitis vinifera* L.). *BMC Genomics* 18:744
30. Li Z, Jiao Y, Zhang C, Dou M, Weng K, et al. 2021. VvHDZ28 positively regulate salicylic acid biosynthesis during seed abortion in Thompson Seedless. *Plant Biotechnology Journal* 19:1824–38
31. Lu S, Zhang Y, Zhu K, Yang W, Ye J, et al. 2018. The citrus transcription factor CsMADS6 modulates carotenoid metabolism by directly regulating carotenogenic genes. *Plant Physiology* 176:2657–76
32. Xiang J, Liu RQ, Li TM, Han LJ, Zou Y, et al. 2013. Isolation and characterization of two VpYABBY genes from wild Chinese *Vitis pseudoreticulata*. *Protoplasma* 250:1315–25
33. Ye X, Zheng X, Zhai D, Song W, Tan B, et al. 2017. Expression patterns of ACS and ACO gene families and ethylene production in rachis and berry of grapes. *HortScience* 52:413–22
34. Zhang S, Fu M, Li Z, Li J, Hai L, et al. 2022. VvEIL2 and VvEIL4 regulate ethylene synthesis and carotenoid metabolism during senescence of grape rachis. *Postharvest Biology and Technology* 187:111853
35. Li Z, Li J, Ye X, Zheng X, Tan B, et al. 2022. VvERF95 regulates chlorophyll degradation by transcriptional activation of VvPAO1 causing grape rachis degreening after harvesting. *Scientia Horticulturae* 303:111224
36. Gao J, Zhang Y, Li Z, Liu M. 2020. Role of ethylene response factors (ERFs) in fruit ripening. *Food Quality and Safety* 4:15–20
37. Hu Y, Han Z, Wang T, Li H, Li Q, et al. 2022. Ethylene response factor MdERF4 and histone deacetylase MdHDA19 suppress apple fruit ripening through histone deacetylation of ripening-related genes. *Plant Physiology* 188:2166–81
38. Wu B, Shen F, Wang X, Zheng W, Xiao C, et al. 2021. Role of MdERF3 and MdERF118 natural variations in apple flesh firmness/crispness retainability and development of QTL-based genomics-assisted prediction. *Plant Biotechnology Journal* 19:1022–37
39. Wang X, Pan L, Wang Y, Meng J, Deng L, et al. 2021. PpIAA1 and PpERF4 form a positive feedback loop to regulate peach fruit ripening by integrating auxin and ethylene signals. *Plant Science* 313:111084
40. Li S, Xie X, Liu S, Chen K, Yin X. 2019. Auto- and mutual-regulation between two CitERFs contribute to ethylene-induced citrus fruit degreening. *Food Chemistry* 299:125163
41. Catinot J, Huang J, Huang P, Tseng M, Chen Y, et al. 2015. ETHYLENE RESPONSE FACTOR 96 positively regulates Arabidopsis resistance to necrotrophic pathogens by direct binding to GCC elements of jasmonate - and ethylene-responsive defence genes. *Plant, Cell & Environment* 38:2721–34
42. Warmerdam S, Sterken MG, Van Schaik C, Oortwijn MEP, Lozano-Torres JL, et al. 2019. Mediator of tolerance to abiotic stress ERF6 regulates susceptibility of Arabidopsis to *Meloidogyne incognita*. *Molecular Plant Pathology* 20:137–52
43. Lestari R, Rio M, Martin F, Leclercq J, Woraathasin N, et al. 2018. Overexpression of *Hevea brasiliensis* ethylene response factor HbERF-IXc5 enhances growth and tolerance to abiotic stress and affects laticifer differentiation. *Plant Biotechnol J* 16:322–36
44. Illgen S, Zintl S, Zuther E, Hincha DK, Schmülling T. 2020. Characterisation of the ERF102 to ERF105 genes of *Arabidopsis thaliana* and their role in the response to cold stress. *Plant Molecular Biology* 103:303–20
45. Bolt S, Zuther E, Zintl S, Hincha DK, Schmülling T. 2017. ERF105 is a transcription factor gene of *Arabidopsis thaliana* required for freezing tolerance and cold acclimation. *Plant, Cell & Environment* 40:108–20
46. Kim NY, Jang YJ, Park OK. 2018. AP2/ERF family transcription factors ORA59 and RAP2.3 interact in the nucleus and function together in ethylene responses. *Frontiers in Plant Science* 9:1675
47. Khan MA, Imtiaz M, Hussain A, Jalal F, Hayat S, et al. 2019. Isolation and functional characterization of an Ethylene Response Factor (RhERF092) from rose (*Rosa hybrida*). *Plant Cell, Tissue and Organ Culture* 140:157–72
48. Sun X, Zhang L, Wong DCJ, Wang Y, Zhu Z, et al. 2019. The ethylene response factor VaERF092 from Amur grape regulates the transcription factor VaWRKY33, improving cold tolerance. *The Plant Journal* 99:988–1002
49. Wang M, Zhu Y, Han R, Yin W, Guo C, et al. 2018. Expression of *Vitis amurensis* VaERF20 in *Arabidopsis thaliana* improves resistance to *Botrytis cinerea* and *Pseudomonas syringae* pv. Tomato DC3000. *International Journal of Molecular Sciences* 10:696
50. Wang L, Luo Z, Li J, Yang M, Yan J, et al. 2019. Morphological and quality characterization of grape berry and rachis in response to postharvest 1-methylcyclopropene and elevated oxygen and carbon dioxide atmospheres. *Postharvest Biology and Technology* 153:107–17
51. Li L, Kaplunov T, Zutahy Y, Daus A, Porat R, et al. 2015. The effects of 1-methylcyclopropane and ethylene on postharvest rachis browning in table grapes. *Postharvest Biology and Technology* 107:16–22
52. Wang S, Wang T, Li Q, Xu C, Tian J, et al. 2022. Phosphorylation of MdERF17 by MdMPK4 promotes apple fruit peel degreening during light/dark transitions. *The Plant Cell* 34:1980–2000



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