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PpERF3 positively regulates ABA biosynthesis by activating *PpNCED2/3* transcription during fruit ripening in peach

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Abstract

The plant hormone ethylene regulates ripening in climacteric fruits. The phytohormone abscisic acid (ABA) affects ethylene biosynthesis, but whether ethylene influences ABA biosynthesis is unknown. To explore this possibility, we investigated the interactions between the ABA biosynthesis genes *PpNCED2/3* and the ethylene response transcription factor PpERF3 in peach fruit. The ABA content increased during fruit maturation and reached a peak at stage S4 III. The increase was greatly inhibited by the ethylene inhibitor 1-MCP, which also suppressed *PpERF3* expression. *PpERF3* shared a similar expression profile with *PpNCED2/3*, encoding a rate-limiting enzyme involved in ABA biosynthesis, during fruit ripening. A yeast one-hybrid assay suggested that the nuclear-localized PpERF3 might bind to the promoters of *PpNCED2/3*. PpERF3 increased the expression of *PpNCED2/3* as shown by dual-luciferase reporters, promoter-GUS assays and transient expression analyses in peach fruit. Collectively, these results suggest that ethylene promotes ABA biosynthesis through PpERF3's regulation of the expression of ABA biosynthesis genes *PpNCED2/3*.

Introduction

Ethylene is the key plant hormone that regulates the fruit ripening process. This regulation is mediated through ethylene-responsive genes, particularly those encoding transcription factors. These transcription factors regulate multiple biochemical pathways that underpin fruit ripening traits, including the levels of sugars, acids, pigments, and flavor and aroma compounds and fruit firmness and texture^{1,2}. In the past few decades, our understanding of the mechanisms by which plants respond to ethylene has greatly increased^{3,4}. Ethylene biosynthesis is catalyzed by two key enzymes: ACC synthase and ACC oxidase. The expression of the genes encoding these two ethylene

enzymes is modulated by ethylene-responsive transcription factors, thereby forming a feedback loop. Depending whether the transcription factor acts as a repressor or activator of transcription, the feedback loop may be autoinhibitory or autostimulatory. The autoinhibitory loop, also known as system 1 ethylene production, functions during the early stage of fruit development and is responsible for producing basal levels of ethylene. The autostimulatory loop, also known as system 2 ethylene production, functions during climacteric fruit ripening, and is responsible for producing high levels of ethylene⁵.

Abscisic acid (ABA) is another plant hormone that has regulatory roles during fruit development and ripening⁶, although it also controls plant wilting and stomatal closure⁷. ABA biosynthesis requires the cleavage of C40 carotenoids by 9-*cis*-epoxycarotenoid dehydrogenase (NCED) to form its direct precursor, xanthoxin. This cleavage process is a key rate-limiting step in ABA biosynthesis⁸. ABA levels sharply increase preceding the release of ethylene. The process of fruit ripening can be

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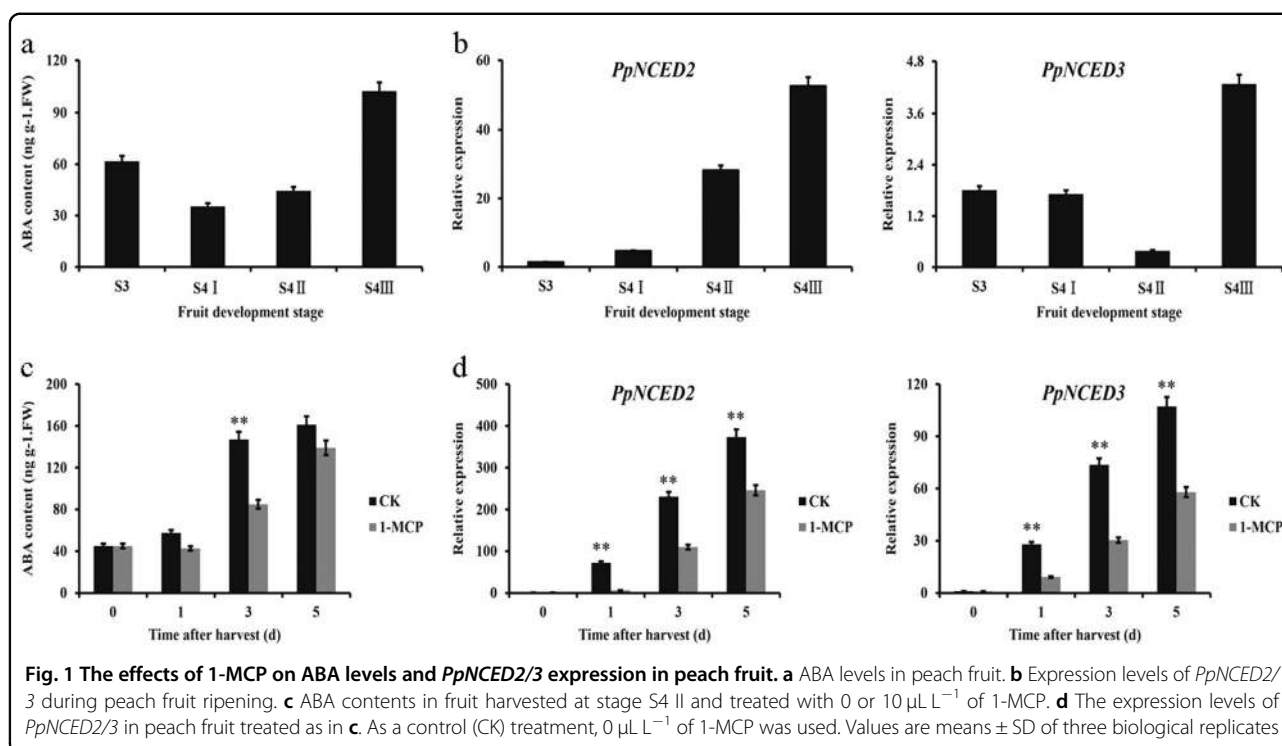
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induced by the application of exogenous ABA⁹. Transgenic tomato (*Solanum lycopersicum*) fruits containing the *SINCE1-RNAi* construct (to silence *SINCE1*) exhibited inhibited cell wall degradation due to reduced levels of endogenous ABA, indicating that ABA has crucial roles in fruit ripening^{10,11}. Many studies suggest that ABA affects ethylene production, likely by regulating the levels of ACC synthase (ACS) and ACC oxidase (ACO)^{12,13}. However, little is known about whether ethylene affects ABA biosynthesis.

Ethylene response factors (ERFs) are plant-specific transcription factors. Many of them can mediate the transcription of ethylene-dependent genes^{3,14,15}. Because ERFs comprise a family of transcription factors, different members of the family allow diverse and specific ethylene responses to occur in different plants¹⁶. ERF proteins can bind to DNA *cis*-acting elements such as a DRE (CCGAC), a GCC box (AGCCGCC), (A/G)CC(G/C)AC, and AA(T)TTCAAA motifs through its conserved ERF domain. These elements are found in the promoters of many ethylene-responsive genes, confirming that ERFs are involved in ethylene signaling or biosynthesis^{3,4,16,17}. For example, tomato LeERF interacts with the GCC box and activates the expression of ethylene biosynthesis genes¹⁸. In banana (*Musa acuminata*), MaERF11 represses the expression of *MaACO1* and expansin genes via recruiting the histone deacetylase MaHDA1⁴. Apple (*Malus domestica*) MdERF2 interacts with the DRE motif in the promoter of the *MdACS1* gene and suppresses its

transcription, thereby inhibiting ethylene biosynthesis in ripening fruit³. Although it well known that ERFs regulate fruit ripening through ethylene¹, it is unclear whether they also control fruit ripening by the transcriptional regulation of ABA biosynthesis genes.

The peach (*Prunus persica*) gene *PpERF3* (Prupe.7G194400) shares similar expression patterns with *PpACS1* and is regulated by 1-MCP¹⁹, but the target genes of *PpERF3* are unknown. In the present study, we found that *PpERF3* directly bound to the promoters of *PpNCED* genes and enhanced their transcription. We also found that *PpNCED* promoter activity is positively regulated by ethylene. Our results show that ERFs regulate ABA biosynthesis in ripening peach fruit by targeting *PpNCED* promoters.

Results

ABA levels and *PpNCED2/3* expression during peach fruit ripening

During the maturation of 'CN13' peach fruit, the ABA content decreased slowly from S3 to S4 I, which was followed by a gradual increase from S4 I to S4 II and a marked increase from S4 II to S4 III (Fig. 1a). Since *PpNCED* functions in a rate-limiting step in ABA biosynthesis, we analyzed the transcript profiles of *PpNCED* genes using transcriptome data and verified the results by qRT-PCR. Three *NCED* genes were found in peach, Prupe.1G061300, Prupe.4G082000, Prupe.4G150100, which were named *PpNCED1*, *PpNCED2*, and *PpNCED3*, respectively. The mRNA transcript level of *PpNCED2*

remained low at stage S3 and then increased sharply from S4 I to S4 III. The mRNA transcript level of *PpNCED3* decreased at stage S4 II and then increased markedly at stage S4 III (Fig. 1b). In contrast, the *PpNCED1* transcript level remained low throughout fruit ripening (Supplementary Table S1).

We analyzed the correlation between ABA content and *PpNCED2/3* expression level in peach fruits subjected to 1-MCP treatment. We treated 'CN13' peach fruit harvested at stage S4 II with 1-MCP (0, 10 $\mu\text{L L}^{-1}$) at 20 °C for 1 d to delay fruit ripening. ABA content was lower in fruit treated with 10 $\mu\text{L L}^{-1}$ 1-MCP than in control fruit receiving 0 $\mu\text{L L}^{-1}$ on days 1, 3, and 5, and a significant difference in ABA content was detected on day 3 (Fig. 1c). To identify the possible roles of *PpNCED2/3* in ABA biosynthesis, we analyzed their expression profiles in peach fruit after treatment with 1-MCP. Consistent with the above results, the expression levels of *PpNCED2/3* were strongly suppressed in 'CN13' fruit under 1-MCP treatment on days 1, 3, and 5 (Fig. 1d).

Promoter elements identified using bioinformatics analysis

The *cis*-elements in the promoter sequences (i.e., in the 2, 2.9 kb region upstream of the translation start site of *PpNCED2* and *PpNCED3* genes, respectively) involved in plant hormone (especially ethylene) responses were identified using PlantCARE and a manual search to understand the transcriptional regulation of the *PpNCED2/3* genes. ERF (ethylene response factor) binding site, MeJA-responsive element, and abscisic acid-responsive element were found in the promoter of *PpNCED2*. These elements were also found in the promoter of *PpNCED3*, in addition to auxin-responsive element, gibberellin-responsive element, and MYB binding site (Table 1).

PpNCED2/3 promoter activity assays

To further demonstrate that *PpNCED2/3* expression levels were enhanced by ethylene, we fused the *PpNCED2/3* promoters with the *GUS* reporter gene and transiently expressed these genes in tomato fruits. After 3 days, we treated the transiently transformed tomato fruits with ethylene and used untreated fruits as controls. As expected, *GUS* driven by the *PpNCED2/3* promoter was highly expressed in fruits treated with ethylene and not strongly expressed in untreated fruits. By contrast, *CaMV35Spro::GUS* was ubiquitously expressed in fruits regardless of ethylene treatment (Fig. 2). These observations suggested that *PpNCED2/3* expression is affected by ethylene.

Phylogenetic tree analysis of PpERF3 and other ERFs

ERFs comprise one of the largest families of plant transcription factors. Phylogenetic analysis of PpERF3 and ERFs from other fruits, including tomato (*Solanum lycopersicum*), banana (*Musa acuminata*), kiwifruit (*Actinidia deliciosa*),

melon (*Cucumis melo*), and apple (*Malus domestica*), revealed that PpERF3 clustered with MaERF9 from banana (Supplementary Fig. S1a). PpERF3 aligned with many functionally characterized AP2/ERF-type proteins from various plant species, revealing high homology within the AP2/ERF domain (Supplementary Fig. S1b).

PpERF3 is localized to the nucleus

To examine the subcellular localization of PpERF3 in vivo, we cloned *PpERF3* and fused the full-length coding sequence without the termination codon in-frame with *GFP*. We transformed *Arabidopsis thaliana* plants with the *PpERF3::GFP* vector via *Agrobacterium*-mediated transformation and found that the fluorescent signals from the *PpERF3::GFP* fusion protein were exclusively localized to the nucleus. By contrast, fluorescent signals from the GFP control were ubiquitously distributed throughout the cell (Fig. 3).

PpERF3 might bind to *PpNCED2/3* promoters

Because the expression levels of *PpERF3* and *PpNCED2/3* could be enhanced by ethylene treatment and because all of these genes have important roles in fruit ripening, we investigated whether PpERF3 regulates the expression of *PpNCED2/3* during fruit ripening. First, we performed a search for *cis*-acting regulatory motif in the *PpNCED2/3* promoters. We found an ERF-binding motif ACCGAC (CCGAC) located at -1121 bp in the *PpNCED2* promoter and four ERF-binding motifs, ATTTCAAA, ACCGAC (CCGAC), and GCCGAC (CCGAC) located at -739, -2308, and -2740 bp, respectively, in the *PpNCED3* promoter (Fig. 4a).

We further performed a Y1H experiment. The CDS of *PpERF3* were cloned into the pGADT7 vector for the effector construct, and the *PpNCED2* or *PpNCED3* promoter fragment was cloned into the pAbAi vector immediately in front of the reporter gene, *AURI-C*, for the reporter construct (Fig. 4a). When plasmids carrying cassettes constitutively expressing the *PpERF3* effectors were transformed into the Y1H reporter strains, yeast cells harboring the *PpNCED2/3* promoters grew well with aureobasidin A (50 ng mL⁻¹) (Clontech, San Francisco, USA), whereas cells co-transformed with empty pGADT7 vector did not (Fig. 4b). These results suggested that PpERF3 binds to the promoters of *PpNCED2/3*.

PpERF3 enhanced the activity of *PpNCED2/3* promoters

To determine whether PpERF3 can enhance *PpNCED2/3* promoter activity given they can bind together, we carried out transient expression assays in tobacco leaves using dual-luciferase reporters. The assays showed that the interaction of PpERF3 with the *PpNCED2* promoter lead to a nearly 1.5-fold increase in the relative LUC/REN ratio, and the interaction of PpERF3 with the *PpNCED3*

Table 1 Cis-acting regulatory elements were predicted in the promoter regions of *PpNCED2/3* related to fruit development and ripening in peach

Genes	cis-element ^z	Sequence	Probable function
<i>PpNCED2</i>	CRT/DRE	A/GCCGAC	ERF (ethylene response factor) binding site
	CGTCA-motif	CGTCA	MeJA-responsive element
	TGACG-motif	TGACG	MeJA-responsive element
		CACGTG	
		TACGTG	
	ABRE	GCAACGTGTC	Abscisic acid-responsive element
		GCCACGTACA	
		ACGTGGC	
		ACGTGGC	
	<i>PpNCED3</i>	ERE	ATTTCAA
CRT/DRE		A/GCCGAC	ERF (ethylene response factor) binding site
TGA-element		AACGAC	Auxin-responsive element
P-box		CCTTTTG	Gibberellin-responsive element
TATC-box		TATCCCA	Gibberellin-responsive element
GARE-motif		TCTGTTG	Gibberellin-responsive element
ABRE		T/CACGTG	Abscisic acid-responsive element
CGTCA-motif		CGTCA	MeJA-responsive element
TGACG-motif		TGACG	MeJA-responsive element
MBS		CGGTCA	MYB binding site
MBSI		aaaAaaC(G/C)GTTA	MYB binding site

promoter lead to a nearly twofold increase in the relative LUC/REN ratio (Fig. 5a). These results suggest that PpERF3 enhances the transcription of *PpNCED2/3* by directly interacting with their promoters.

We further performed a GUS transactivation experiment by infiltrating the *35S::PpERF3* and *PpNCED2/3::GUS* plasmids into tobacco leaves. We observed significantly higher GUS activities driven by *PpNCED2/3* promoters in the assays with the *35S::PpERF3* construct than in the control assays without the *35S::PpERF3* construct (Fig. 5b). These results suggest that PpERF3 is a transcriptional activator that regulates the expression of *PpNCED2/3*.

Transient over-expression PpERF3 in peach fruit

To verify the function of PpERF3 in regulating the expression of *PpNCED2/3* in peach, 'CN13' fruit at stage S3 were transiently transformed using the *35S::PpERF3* construct or a control vector without *PpERF3*. The transcript of *PpERF3* was not detectable in fruit transformed with the control vector, indicating that *PpERF3* was not expressed in the fruit at stage S3. However, the *PpERF3*

transcript level was significantly increased in same-stage fruit transformed with the *35S::PpERF3* construct, indicating the over-expression of *PpERF3*. The transcript levels of *PpNCED2* and *PpNCED3* were increased sixfold and ninefold, respectively, in fruit transformed with *35S::PpERF3* relative to their levels in fruit transformed with the control vector (Fig. 6). This result indicated that PpERF3 can activate the expression of both *PpNCED2* and *PpNCED3* genes in peach fruit.

Discussion

ABA levels and the expression profiles of *PpNCED2/3* in peach fruit

ABA has critical roles in early fruit development and ripening^{9–13}. Depending on the physiological stage of the fruit, the use of exogenous ABA affects the progress of fruit ripening, leading to less or more ripe fruit¹³. When exogenous ABA was applied to peach fruit at stage S4, the expression levels of ethylene- (ACS1, ACO1), cell wall- (endo-polygalacturonase, pectin methylesterases inhibitors) and auxin-related genes were upregulated, suggesting an acceleration of ripening¹³. In our study, a marked

increase in ABA concentration during peach fruit ripening was observed, suggesting that ABA accumulation at stage S4 III might accelerate the softening and ripening of peach fruit.

In fruit, the concentration of endogenous ABA is determined by the kinetic equilibrium of biosynthesis (NCED), catabolism (ABA 8'-hydroxylase), and reactivation (β -glucosidases/glucosyltransferases) genes. The spatio-temporal expression of these genes is regulated at the transcriptional

level by ABA content⁶. Since the gene encoding NCED was first separated from the maize vp14 mutant²⁰, it has been researched in various climacteric fruit species, including peach¹³, tomato²⁰, apple²¹, and melon²², and non-climacteric fruits, such as orange²³ and grape²⁴. NCED is a key rate-limiting step in ABA biosynthesis⁸. In transgenic tomato fruits with *SINCE1* silenced, cell wall degradation is inhibited due to the reduction in endogenous ABA levels. The shelf life of these fruits is longer than those of control fruits^{10,11}. In strawberry, fruit ripening is affected by the downregulation of *FaNCED* genes²⁵. In this study, we identified three *NCED* genes (Prupe.1G061300, Prupe.4G082000 and Prupe.4G150100) in the peach genome, and we examined the expression levels of two *PpNCED* genes in parallel with ABA content. The results suggest that these two genes may be important participants in ABA accumulation during fruit ripening. Compared with the *PpNCED2* promoter, the *PpNCED3* promoter contained many more binding sites for ERFs and other hormone-responsive proteins. This suggests that *PpNCED3* may be regulated by more complicated signal transduction pathways than is *PpNCED2*.

PpERF3 directly activates the expression of *PpNCED2/3*

ERF transcription factors function as downstream components of ethylene signaling by regulating the expression of ethylene-responsive genes³. ERFs can direct regulate the expression of ripening-related genes by binding to *cis*-elements, such as DRE (CCGAC), (A/G)CC (G/C)AC, GCC box (AGCCGCC), and AA(T)TTCAAA, and affect the fruit ripening process^{3,4,16,17}. There are few reports addressing whether ERFs can regulate ABA biosynthesis. Accumulating evidence supports the idea that ABA regulates ethylene biosynthesis and signaling by regulating the expression of *LeACS4* and *LeACO1*^{12,13}. Kumar speculated that ethylene may affect the biosynthesis of ABA via regulating the expression of NCED²⁶. In

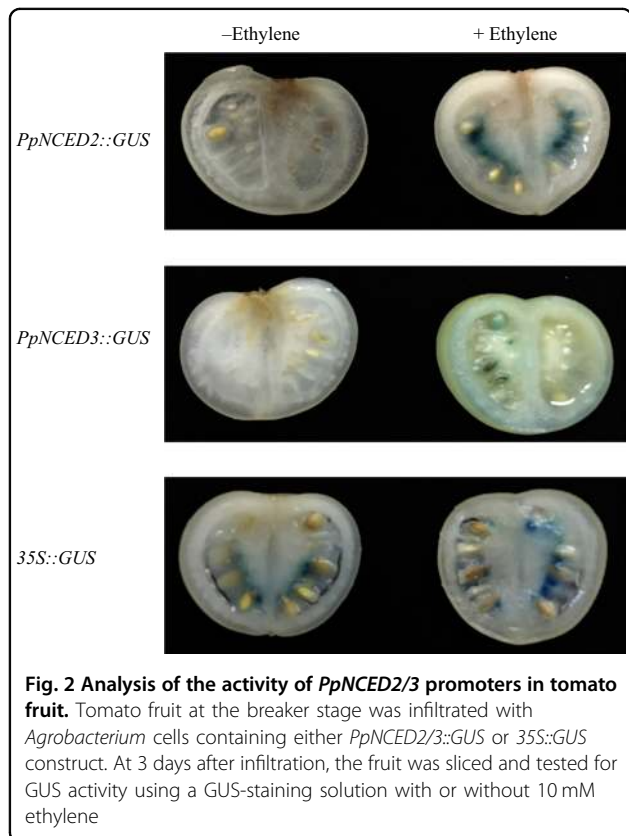


Fig. 2 Analysis of the activity of *PpNCED2/3* promoters in tomato fruit. Tomato fruit at the breaker stage was infiltrated with *Agrobacterium* cells containing either *PpNCED2/3::GUS* or *35S::GUS* construct. At 3 days after infiltration, the fruit was sliced and tested for GUS activity using a GUS-staining solution with or without 10 mM ethylene

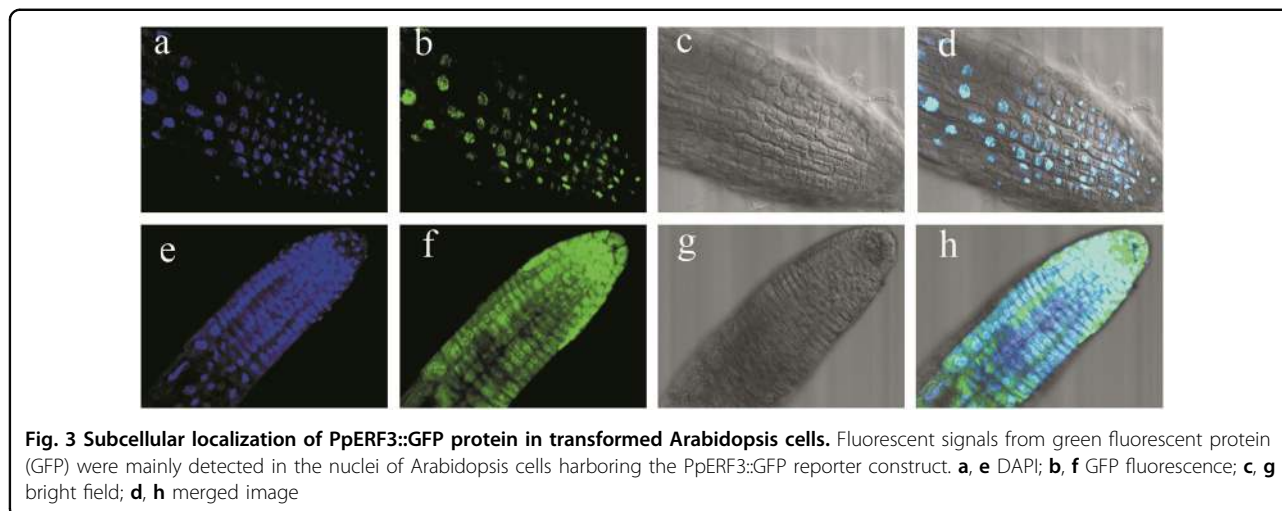
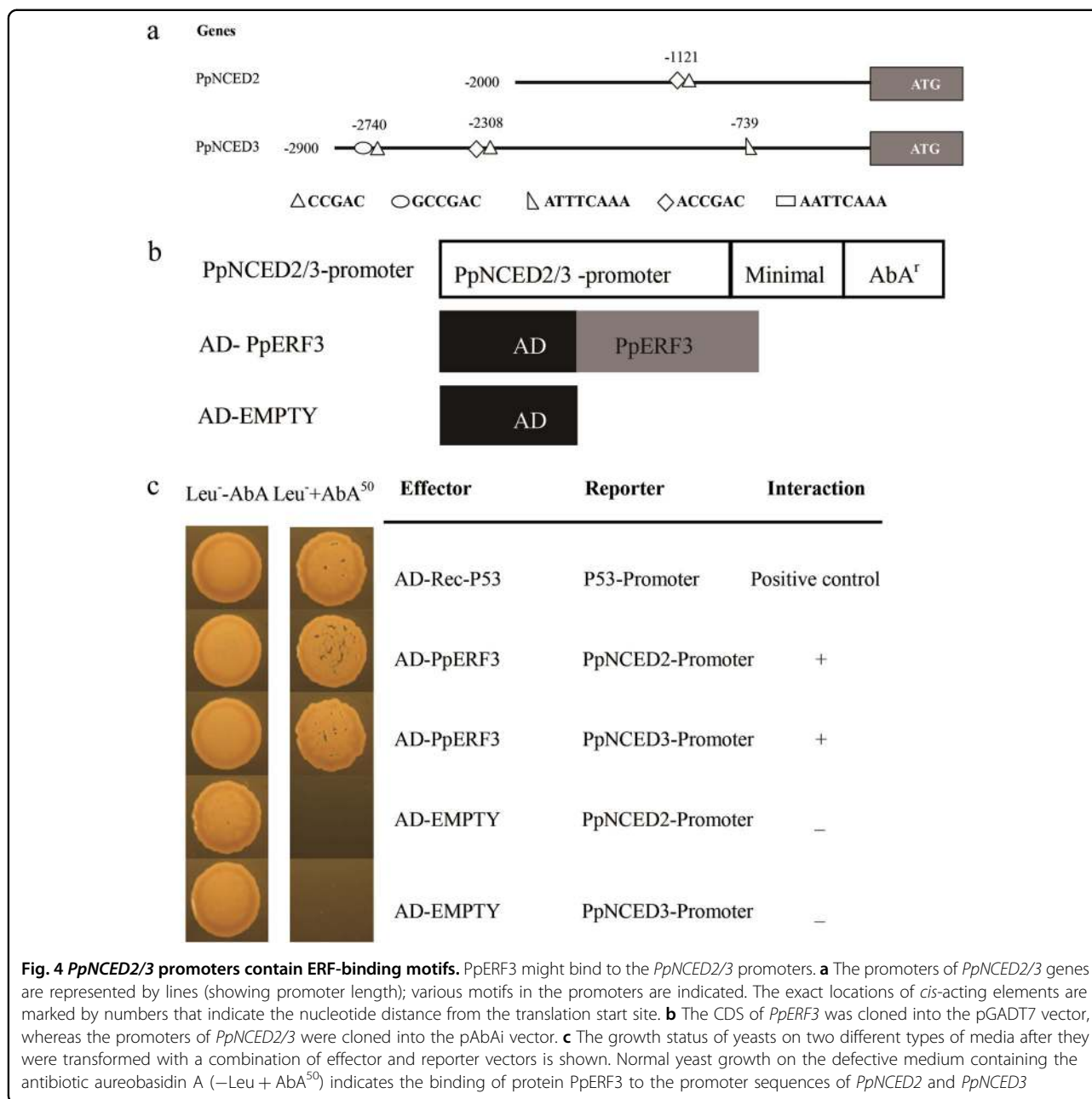


Fig. 3 Subcellular localization of PpERF3::GFP protein in transformed Arabidopsis cells. Fluorescent signals from green fluorescent protein (GFP) were mainly detected in the nuclei of Arabidopsis cells harboring the PpERF3::GFP reporter construct. **a, e** DAPI; **b, f** GFP fluorescence; **c, g** bright field; **d, h** merged image

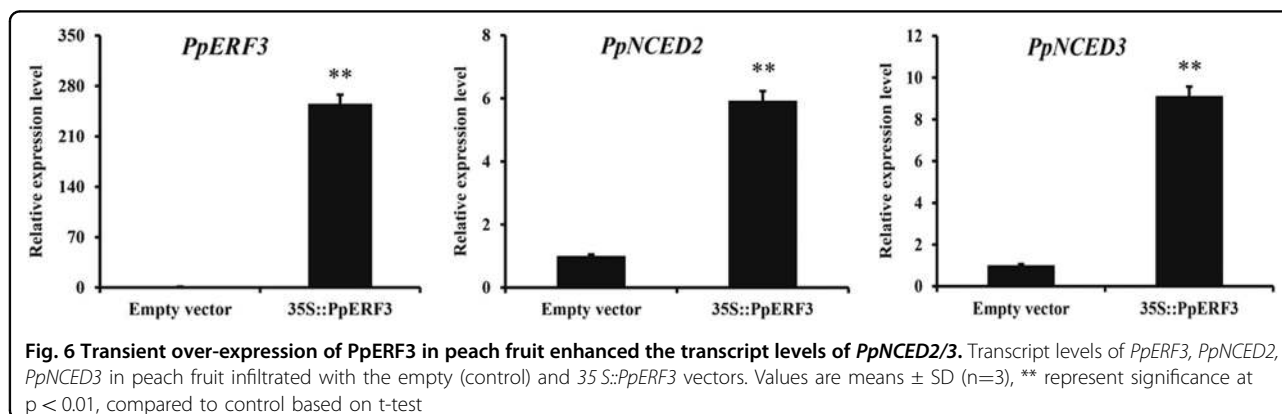
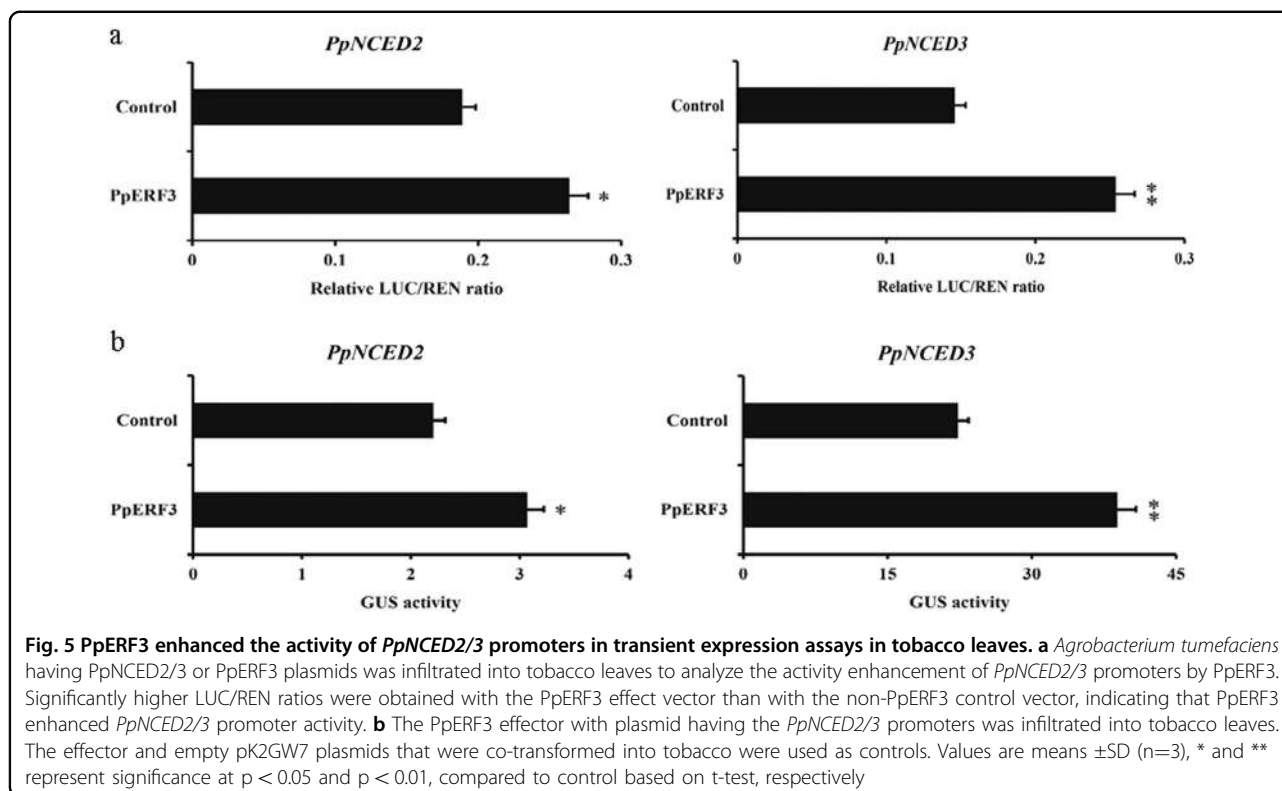


this study, phylogenetic analysis showed that PpERF3 clustered with MaERF9 (Supplementary Fig. S1a). MaERF9 from banana activates *MaACO1* promoter activity, and its expression is strongly correlated with the increase in ethylene production associated with climacteric fruit ripening¹⁵. We have shown here that *PpNCED2/3* promoters contain ERF-binding sites (Table 1) and that PpERF3 can bind to the *PpNCED2/3* promoter and enhance both promoter activity in tobacco leaves and the transcript levels of *PpNCED2/3* in peach fruit. In addition, *PpNCED2/3* expression is responsive to ethylene signaling. Taken together, our results suggest

that ethylene may affect ABA biosynthesis via regulating the expression of *PpNCED2/3* genes by PpERF3 in peach fruit.

Crosstalk between ABA and ethylene signaling

Fruit ripening is regulated by a complicated network of feedback and crosstalk among various phytohormone signaling pathways²⁶. Increasing evidence suggests that the combined actions of auxin, gibberellins (GAs), cytokinin, ethylene, and ABA have crucial roles in the regulation of fruit ripening²⁶. The present study showed that ABA content was markedly reduced by the ethylene



inhibitor 1-MCP (Fig. 1c) and that the promoter activity of the ABA biosynthesis gene *PpNCED2/3* was strongly enhanced in tomato fruits by ethylene (Fig. 2). These results suggest that ethylene can regulate the biosynthesis of ABA. It is generally accepted that ethylene and ABA have important roles during fruit ripening. Fruit ABA content increased during ripening, and the initiation of fruit ripening was promoted by exogenous application of ABA but was delayed by fluridone (ABA inhibitor), indicating that ABA has a major role in fruit ripening process¹⁸. ABA, synergistically with ethylene, promotes softening in banana²⁷. Overall, ABA and ethylene have

important roles in the fruit ripening process, and the feedback regulation between ABA and ethylene biosynthesis may contribute to rapid fruit softening and a short shelf life of peach fruit.

In conclusion, *PpNCED2/3* share the same expression pattern with *PpERF3*, and the expression of these three genes can be greatly inhibited by 1-MCP treatment. PpERF3, as a transcriptional activator, regulates *PpNCED2/3* expression by binding to ERF response elements in the promoter region of *PpNCED2/3* genes, thereby increasing ABA levels. Our findings indicate that PpERF3 regulates fruit ripening by controlling the

transcription of *PpNCED2/3* and provide insight into the regulatory network linking ethylene and ABA signaling.

Materials and methods

Fruit tissue collection

The peach cultivar 'CN13' grown in the research orchard of Zhengzhou Fruit Research Institute, Zhengzhou, China, was used in this study. 'CN13' (melting flesh; MF) fruits were harvested at 87, 90, 93, and 96 DAFB (days after full bloom). These time points corresponded to fruit development stages S3, S4 I, S4 II, and S4 III. At stage S3, the fruit is still green and at the end of the second exponential growth phase. At S4 I, the fruit is no longer inflated and does not release ethylene. At S4 II, the fruit releases low amounts of ethylene. At S4 III, the fruit release much higher levels ethylene and begins to soften rapidly²⁸. Twenty fruits were collected from five different trees at each time point. Fruits collected at S4 II were treated with a solution containing 0 or 10 $\mu\text{L L}^{-1}$ 1-MCP at 20 °C for 24 h. The treated fruits were then stored at 20 °C for 1, 3, and 5 days. Mesocarp tissues of the fruits were directly collected into liquid nitrogen, and stored in a -80 °C freezer for future DNA, RNA, and ABA extraction.

Quantitation of ABA content in 'CN13' fruit

ABA was extracted from 'CN13' fruit tissues collected at the four stages using the method described by He²⁹. In brief, extraction was carried out in a solution of methanol (80%, v/v) and butylated hydroxytoluene (1 mmol L⁻¹) overnight at 4 °C. The extracts were centrifuged at 10,000 \times g (4 °C) for 20 min, passed through a C 18 Sep-Pak cartridge (Waters, Milford, MA) and dried in N₂. The dried samples were dissolved in PBS buffer (0.01 mol L⁻¹, pH 7.4). ABA content was determined using an enzyme-linked immunosorbent assay (ELISA), and the ELISA data were analyzed according to Weiler et al.³⁰ In brief, the samples, an ABA standard, and ABA antibodies were loaded into the wells of a microtitration plate (Nunc). The plate was maintained at 37 °C for 45 min before horseradish peroxidase-labeled goat anti-rabbit immunoglobulin was added. The plate was then maintained at 37 °C for 1 h before buffered substrate (orthophenylenediamine) was applied. The enzymatic reaction was carried at 37 °C for 15 min in the dark, and then 3 mol L⁻¹ H₂SO₄ was added to terminate the reaction. The plate was then read at 490 nm.

RNA extraction and cDNA synthesis

RNA was extracted from the collected peach fruit tissues using a Total RNA Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. RNA samples were evaluated in 1% agarose gels to determine the levels of degradation and contamination and then analyzed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) to determine RNA

quantity and quality. First-strand cDNA was synthesized from the RNA samples with a Reverse Transcriptase kit (Tiangen, Beijing, China). The cDNA products were diluted to 20 ng μL^{-1} for subsequent quantitative reverse-transcription PCR (qRT-PCR) analyses.

Gene expression analysis by qRT-PCR

The relative expression levels of the *NCED* and *AP2/ERF* genes were analyzed together with an *Actin* gene as a reference³¹ using qRT-PCR. The analyses were performed with three independent biological replicates. All data were analyzed using the 2^{- $\Delta\Delta\text{Ct}$} method³². PCR primers were designed based on the sequences of the 3'-UTRs or 5'-UTRs of individual genes to ensure gene-specificity using Primer 5 software. The designed primers are listed in Supplementary Table S2. These primers produced 60 to 200 bp DNA fragments that were then cloned into pTOPO-blunt vectors and sequenced to confirm the correct amplicons of each primer pair³³.

Gene cloning and promoter analysis

DNA was extracted from peach leaves using a DNeasy Plant Mini Kit (Tiangen, Beijing, China). From this extracted DNA, the promoter regions of *PpNCED* genes were amplified by PCR using Phanta HS Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China), cloned into the pTOPO-blunt vector (Aidlab, Beijing, China) and sequenced. DNA sequence alignments of the cloned promoters were performed using MEGA 5.0 software. The motifs or *cis*-elements in the promoter sequences were identified using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>)³⁴.

Promoter activity assay

The promoter DNA fragment (~3 kb) of *PpNCED2/3*, amplified as described above, was ligated into pCAMBIA1301 to replace the cauliflower mosaic virus (CaMV) 35S promoter. The resulting *PpNCED2/3 pro::GUS* constructs or empty vector (pCAMBIA1301) was transferred into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Then, a liquid culture of *A. tumefaciens* containing the *PpNCED2/3 pro::GUS* construct or control vector was injected into Micro Tom tomato fruit at the breaker stage through the fruit stalk until the whole fruit was infiltrated. After infiltration, the transformed fruits were sliced into discs that were then immersed in a GUS-staining solution (50 mM Na-phosphate, pH 7.2, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, and 0.5% Triton X-100, 0.5 mg L⁻¹ x-gluc) with or without 10 mM ethephon overnight at 37 °C.

Gene characteristics and structural analysis

Neighbor-joining phylogenetic trees were constructed from the protein sequences of PpERF3 and other ERFs affecting ethylene biosynthesis and fruit ripening using the

ClustalW tool in conjunction with MEGA 5.0³⁵ software with 1000 bootstrap replicates. The AP2/ERF domains of these genes were aligned together with several other functionally characterized AP2/ERF-domain proteins. The deduced amino acid sequences of the AP2/ERF genes used to perform the phylogenetic analysis are listed in Table S3.

Subcellular localization of PpERF3

To investigate the subcellular localization of PpERF3, a PpERF3::GFP fusion protein was constructed. The PCR product of the *PpERF3* coding sequence was cloned into the pBI121::GFP³⁶ vector after digestion with the *Xba*I and *Bam*HI restriction endonucleases. The resulting fusion construct, pBI121-PpERF3::GFP, harbored *PpERF3::GFP* driven by the 35S promoter. The pBI121-PpERF3::GFP vector was transferred into *Agrobacterium tumefaciens* strain GV3101. Arabidopsis was transformed by the floral dip method as described previously³⁷. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. The subcellular localization of PpERF3-GFP was observed under a confocal laser-scanning fluorescence microscope.

Yeast one-hybrid assay

The CDS region of *PpERF3*, amplified as described above, was cloned into the pGADT7 vector. The promoter fragment of *PpNCED2* or *PpNCED3*, amplified as described above, was ligated into the pAbAi vector. A transcription factor and promoter interaction assay was conducted using a Matchmaker™ Gold Yeast One-Hybrid Library Screening System Kit (Clontech, San Francisco, USA).

GUS analysis

The effector construct was constructed by recombining the CDS of *PpERF3* with the flanking *attB* sites into the *attP* site of pDONR201 using GATEWAY™ BP Clonase™ Enzyme Mix (Invitrogen), followed by moving the *PpERF3* fragment from pDONR201 to the pK2GW7 destination vector containing the *attR* sites by mixing both plasmids using GATEWAY™ LR Clonase™ Enzyme Mix (Invitrogen). The *pK2GW7-PpERF3* fusion was driven by the 35S promoter. The reporter constructs were the *PpNCED2/3 pro::GUS* constructs described in the promoter activity assay. The reporter and effector constructs were transferred into *Agrobacterium tumefaciens* strain GV3101 and co-infiltrated into tobacco leaves. Each reporter-effector combination was infiltrated at least three times. Three days after infiltration, the leaves were used for GUS activity analyses. A fluorimeter (SpectraMax® i3x Platform, USA) was used to measure fluorescence after the proteins were extracted from the infected tobacco leaves³⁸.

Dual-luciferase reporter assay

The promoter sequences of *PpNCED2* and *PpNCED3* were cloned into the pGreenII 0800-LUC³⁹ vector, and

the CDS of *PpERF3* was cloned into the pK2GW7 vector driven by the 35S promoter as an effector. A dual-luciferase assay kit was used to measure LUC and REN luciferase activity, and analysis was performed using the SpectraMax® i3x Platform (USA) at 560 and 465 nm, respectively. The LUC to REN ratio was calculated. No fewer than six measurements were performed per assay.

Transient expression in peach

The expression constructs *pK2GW7-PpERF3* were transferred into *A. tumefaciens* GV3101, and the empty vector (pK2GW7) was used as a control. Peach fruits were harvested at the S3 stage and used for transient expression assay as described by Liu et al.⁴⁰ Briefly, infiltration was performed by submerging peach flesh cubes (1 cm thick) into *Agrobacterium* suspension and applying a vacuum (−70kPa) for 30 min. Then, the flesh cubes were sampled for RNA and subsequent expression analysis after culture on MS medium for 2 days.

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W.X. and Z.W. conceived the experiments; Y.D. and Y.W. collected plant materials and conducted the experiments; L.N., L.P., Z.L. and G.C. performed phenotyping; G.H. and W.Z. analyzed the data; W.X., J.L. and Z.W. wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

- Klee, H. J. & Giovannoni, J. J. Genetics and control of tomato fruit ripening and quality attributes. *Annu. Rev. Genet.* **45**, 41–59 (2011).
- Martel, C., Vrebalov, J., Tafelmeyer, P. & Giovannoni, J. J. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. *Plant Physiol.* **157**, 1568–1579 (2011).
- Li, T. et al. Apple (*Malus domestica*) MdERF2 negatively affects ethylene biosynthesis during fruit ripening by suppressing MdACS1 transcription. *Plant J.* **88**, 735–748 (2016).
- Han, Y. et al. Banana transcription factor MaERF11 recruits histone deacetylase MaHDA1 and represses the expression of MaACO1 and expansins during fruit ripening. *Plant Physiol.* (2016). <https://doi.org/10.1104/pp.16.00301>.
- McMurtrie, E. J., McGlasson, W. B. & Eaks, I. L. Treatment of fruit with propylene gives information about the biogenesis of ethylene. *Nature* **237**, 235 (1972).
- Leng, P., Yuan, B. & Guo, Y. The role of abscisic acid in fruit ripening and responses to abiotic stress. *J. Exp. Bot.* **65**, 4577–4588 (2014).
- Wright, S. T. C. & Hiron, R. W. P. (+)-Abscisic acid, the growth inhibitor induced in detached wheat leaves by a period of wilting. *Nature* **224**, 719 (1969).
- Estrada-Melo, A. C., Reid, M. S. & Jiang, C. Z. Overexpression of an ABA biosynthesis gene using a stress-inducible promoter enhances drought resistance in petunia. *Hortic. Res.* **2**, 15013 (2015).
- Giribaldi, M., Gény, L., Delrot, S. & Schubert, A. Proteomic analysis of the effects of ABA treatments on ripening *Vitis vinifera* berries. *J. Exp. Bot.* **61**, 2447–2458 (2010).
- Sun, L. et al. Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. *Plant Physiol.* **158**, 283–298 (2012).
- Sun, L., Yuan, B., Zhang, M., Wang, L. & Cui, M. Fruit-specific RNAi-mediated suppression of SINCED1 increases both lycopene and β -carotene contents in tomato fruit. *J. Exp. Bot.* **63**, 3097–3108 (2012).
- Mou, W. et al. Comprehensive analysis of ABA effects on ethylene biosynthesis and signaling during tomato fruit ripening. *PLoS ONE* **11**, e0154072 (2016).
- Soto, A., Ruiz, K. B., Ravaglia, D., Costa, G. & Torrigiani, P. ABA may promote or delay peach fruit ripening through modulation of ripening-and hormone-related gene expression depending on the developmental stage. *Plant. Physiol. Biochem.* **64**, 11–24 (2013).
- Shin, S., Lv, J., Fazio, G., Mazzola, M. & Zhu, Y. Transcriptional regulation of ethylene and jasmonate mediated defense response in apple (*Malus domestica*) root during *Pythium ultimum* infection. *Hortic. Res.* **1**, 14053 (2014).
- Xiao, Y. Y. et al. Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. *J. Exp. Bot.* **64**, 2499–2510 (2013).
- Kuang, J. F. et al. The transcriptional regulatory network mediated by banana (*Musa acuminata*) dehydration-responsive element binding (MaDREB) transcription factors in fruit ripening. *New Phytol.* **214**, 762–781 (2017).
- Yin, X. R., Allan, A. C., Chen, K. S. & Ferguson, I. B. Kiwifruit EIL and ERF genes involved in regulating fruit ripening. *Plant Physiol.* **153**, 1280–1292 (2010).
- Zhang, Z., Zhang, H., Quan, R., Wang, X. C. & Huang, R. Transcriptional regulation of the ethylene response factor LeERF2 in the expression of ethylene biosynthesis genes controls ethylene production in tomato and tobacco. *Plant Physiol.* **150**, 365–377 (2009).
- Wang, X. et al. Genes involved in ethylene signal transduction in peach (*Prunus persica*) and their expression profiles during fruit maturation. *Sci. Hortic.* **224**, 306–316 (2017).
- Burbidge, A., Grieve, T. M., Jackson, A., Thompson, A. & McCarty, D. R. Characterization of the ABA-deficient tomato mutant notabilis and its relationship with maizeVp14. *Plant J.* **17**, 427–431 (1999).
- Lara, I. & Vendrell, M. Changes in abscisic acid levels, ethylene biosynthesis, and protein patterns during fruit maturation of Granny Smith' apples. *J. Am. Soc. Hortic. Sci.* **125**, 183–189 (2000).
- Sun, Y. et al. Transcriptional regulation of genes encoding key enzymes of abscisic acid metabolism during melon (*Cucumis melo* L.) fruit development and ripening. *J. Plant. Growth Regul.* **32**, 233–244 (2013).
- Rodrigo, M. J., Alquezar, B. & Zacarías, L. Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *J. Exp. Bot.* **57**, 633–643 (2006).
- Wheeler, S., Loveys, B., Ford, C. & Davies, C. The relationship between the expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the promotion of *Vitis vinifera* L. berry ripening by abscisic acid. *Aust. J. Grape Wine Res.* **15**, 195–204 (2009).
- Ji, K. et al. Non-climacteric ripening in strawberry fruit is linked to ABA, FaNCED2 and FaCYP707A1. *Funct. Plant Biol.* **39**, 351–357 (2012).
- Kumar, R., Khurana, A. & Sharma, A. K. Role of plant hormones and their interplay in development and ripening of fleshy fruits. *J. Exp. Bot.* **65**, 4561–4575 (2014).
- Lohani, S., Trivedi, P. K. & Nath, P. Changes in activities of cell wall hydrolases during ethylene-induced ripening in banana: effect of 1-MCP, ABA and IAA. *Postharvest Biol. Technol.* **31**, 119–126 (2004).
- Tonutti, P., Bonghi, C., Ruperti, B., Tomielli, G. B. & Ramina, A. Ethylene evolution and 1-aminocyclopropane-1-carboxylate oxidase gene expression during early development and ripening of peach fruit. *J. Am. Soc. Hortic. Sci.* **122**, 642–647 (1997).
- He, Z. in *Guidance to Experiment on Chemical Control in Crop Plants* (ed He, Z. P.) 60–68 (Beijing Agricultural University Publishers, Beijing, 1993).
- Weiler, E. W., Jourdan, P. S. & Conrad, W. Levels of indole-3-acetic acid in intact and decapitated coleoptiles as determined by a specific and highly sensitive solid-phase enzyme immunoassay. *Planta* **153**, 561–571 (1981).
- Tatsuki, M. et al. Increased levels of IAA are required for system 2 ethylene synthesis causing fruit softening in peach (*Prunus persica* L. Batsch). *J. Exp. Bot.* **64**, 1049–1059 (2013).
- Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**, 402–408 (2001).
- Zhang, B. et al. Differential expression within the LOX gene family in ripening kiwifruit. *J. Exp. Bot.* **57**, 3825–3836 (2006).
- Lescot, M., Déhais, P., Thijs, G., Marchal, K. & Moreau, Y. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **30**, 325–327 (2002).
- Liu, C., Zhao, A., Zhu, P., Li, J. & Han, L. Characterization and expression of genes involved in the ethylene biosynthesis and signal transduction during ripening of mulberry fruit. *PLoS ONE* **10**, e0122081 (2015).
- Wei, Q. et al. A wheat MYB transcriptional repressor TaMyb1D regulates phenylpropanoid metabolism and enhances tolerance to drought and oxidative stresses in transgenic tobacco plants. *Plant Sci.* **265**, 112–123 (2017).
- Clough, S. J. & Bent, A. F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).
- Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907 (1987).
- Hellens, R. P. et al. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods* **1**, 13 (2005).
- Liu, H. et al. UV-B irradiation differentially regulates terpene synthases and terpene content of peach. *Plant Cell Environ.* **40**, 2261–2275 (2017).