Effects of exogenous GA, IAA, ABA and ethylene on pear fruit during different development stages

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Abstract

Phytohormones are very important for fruit development and ripening. However, it is unclear the role of phytohormones in pear fruit quality. In this study, gibberellin (GA), Indole-3-acetic acid (IAA), abscisic acid (ABA), and ethephon were selected to treat pear fruit at different stages, including 30, 45, 60, 75, and 90 days after flowering blooming (DAFB). As a result, exogenous GA treatment could promote fruit enlargement, decrease fruit firmness, and inhibit the accumulation of organ acids in ripening fruit (110DAFB). Exogenous IAA or ABA treatment could also promote fruit enlargement. Exogenous ABA or ethephon could promote the accumulation of soluble solids in ripening fruit (110DAFB), indicating the involvement of ABA and ethylene in fruit ripening. Quantitative real-time PCR (qPT-PCR) analysis suggested that PbZEP1, PbNCED.B, PbSDR4, and PbAO3 are the crucial genes for ABA biosynthesis, and PbACS1b and PbACO1 are the crucial genes for ethylene biosynthesis in pear fruit. EMSA and dual-luciferase assay suggested that PbABF.B and PbABF.C.2 directly bind to the PbACS1b promoter to enhance the activity, while PbABF.E.1 and PbABF.E.2 directly bind to the PbACO1 promoter to enhance the activity. This result indicates that the four ABF proteins may be involved in ethylene biosynthesis during fruit ripening. Our study provides a foundation for the roles of GA, IAA, ABA and ethylene in pear fruit and reveals the ABA–ethylene cross-talking during fruit ripening.

1. Introduction

Fruit development and ripening are crucial stages for the whole growth of the fruit and play important roles of fruit quality that strongly influence consumer choice (Shinozaki et al., 2018; Chen et al., 2020). Most fruit quality attributes emerge and change during fruit development and ripening including fruit size, color, texture, sugar, acidity, aroma, hormones and others (Liu et al., 2020). In recent decades, it has been reported that fruit development and ripening regulated by a complex network of phytohormones and transcription factors (TFs) at multiple levels (Guo et al., 2019; Kou et al., 2021, Xiao et al., 2020).

Plant hormones are obviously and constantly change with the fruit growth and ripening biological processes, including IAA, GA, ethylene, JA, ABA and other hormones (Cong et al., 2019; Fenn et al., 2021; Kumar et al., 2014). Ethylene is a phytohormone that plays critical role in the regulation of ripening. Ethylene biosynthesis comprises two main steps: First, based on the substrate of S-adenosyl methionine (SAM), 1-aminocyclopropane-1-carboxyla acid (ACC) was catalyzed by 1-aminocyclopropane-1-carboxyla synthase (ACS). Then, ACC oxidase (ACO) leads to the transformation of ACC into ethylene (Gu et al., 2017; Yang et al., 1984). So far, ACS and ACO are serve as the core regulator for climacteric fruit ripening. At least, 12 ACS genes and 7 ACO genes were found in tomato (Barry et al., 2000). 6 ACS members and 32 ACO members were identified in peach, with PpACS1 and PpACO1 participating in and regulating ethylene synthesis (Gu et al., 2021). 13 ACS and 11 ACO members were identified in pear (Pyrus ussuriensis) (Yuan et al., 2019). 9 ACS genes were found in apple (Malus domestica), of which MdACS1 can be highly expressed in mature fruits, and its expression is inhibited after 1-MCP treatment (Li et al., 2013). ACS and ACO have been identified in many other horticultural fruits such as bananas, kiwifruit, and persimmon indicating a key role in regulating fruit ripening (Shan et al., 2000; Wu et al.,...
Ethylene is perceived by ethylene receptors and subsequently initiates the ethylene signal transduction. The factors in the ethylene signal including ETR, CTR, EIN, EIL, EBF, ERF also play crucial role in the regulation of ethylene in fruit ripening (Dolgikh et al., 2019; Li et al., 2015; Wang et al., 2023). In addition to ethylene, many studies have addressed the important functions of abscisic acid (ABA) for fruit development and ripening. In the synthesis of ABA process, zeaxanthin is converted into 9-cis-Neoxanthin and/or 9-cis-Violaxanthin by zeaxanthin epoxidase (ZEP) in the plastids (North et al., 2007). The 9-cis-epoxycarotenoid dioxygenase (NCED) is involved by assisting epoxycarotenoid precursor to form xanthoxin (XHT). XHT further converts to abscisic aldehyde through the catalytic action of short chain dehydrogenase/reductase (SDR), then oxidized by abscisic aldehyde oxidase (AAO) to form ABA (Schwartz et al., 1997; Gupta et al., 2022). NCED is considered a key enzyme in the ABA Biosynthesis (Qin et al., 1999). The expression of CsNCED1 is accordance with ABA accumulation, suggesting CsNCED1 participates in ABA biosynthesis (Rodrigo et al., 2006). PpNCED1 and PpNCED5 cooperatively regulate ABA biosynthesis in peach fruits (Wang et al., 2021). ABA signal transduction has been reported and summarized a core pathway: PYR/PYL/RCAR—PP2C—SnRK2, SnRK2 makes phosphorylation with ABA response factors ABEBs and ABFs. Finally, ABFs/AREBs could be bind the elements ABRE (ACGTG) of the promoters for ABA response factors to regulate fruit development and ripening (Fujita et al., 2013). SIPP2C1 plays a negative role in regulating ABA signaling and fruit ripening, and silencing of SIPP2C1 in tomato improves ABA levels (Zhang et al., 2018). SIPYL1, SIPYL2, SIPYL3, and SIPYL6 express mainly during fruit development and ripening. The expression level of SIPYL3 is higher in the small green stage and green mature stage and the expression level of SIPYL6 is higher in the small green and red mature stages (Sun et al., 2011). 10 ABF/AREB members were identified in tomato, and SIABF2 and SIABF10 play an important role in fruit ripening based on high expression profiles.

Ethylene and abscisic acid are both important factors during fruit development and maturation, while significant progress for the crosstalk between ethylene and abscisic acid has been reported in recent years. In climacteric fruits, the accumulation of ABA precedes ethylene release, suggesting that ABA may act as an upstream regulator of ethylene (Soto et al., 2013). ABA inhibited the ethylene synthesis obviously while NDGA promoted them when treated the immature fruit. At the breaker, NDGA treatment cannot block ABA accumulation and ethylene synthesis. concluding that ABA plays different role in ethylene synthesis system in different stages of tomato fruit ripening. (Zhang et al., 2009). Qiao et al. (2021) found that fig fruit was be retard ripening with downregulation of FcACO2 or FcPYL8 at the degreening stage. While downregulation of FcAAO3 fruit accelerate ripening but inhibited ripening only before the degreening stage, indicating ethylene regulates the fig fruit ripening in an ABA-dependent manner. Silencing of SIPP2C1 made improvement of ABA levels and acceleration for ethylene release, resulting in early fruit ripening, SIAREB1, can regulate fruit ripening by activating the expression of ethylene biosynthesis genes in tomato. (He et al., 2023, Zhang et al., 2018).

Fruit ripening regulated by ethylene and ABA was characterized in the hot pepper (Capsicum frutescens). Down-regulation of ACO3 and NCED1/3 expression inhibited and promoted the changes in carotenoid,
ABA, and ethylene levels, carotenoid biosynthesis-related gene expression, respectively. The retarded colouration in AC03-VIGS fruits was rescued by exogenous ethylene (Hou et al., 2018). The expression levels of NOR, SIACS2, SIACS4 and SIAC01, are increase in SIAREB1-overexpressing fruit, which indicates that SIAREB1 can promote the expression of NOR and further improve ethylene synthesis levels by mediating ABA signaling in tomato (Mou et al., 2018). In brief, the crosstalk interaction between ABA and ethylene performed different critical roles at different stages for fruit development and ripening.

Pear is cultivated in the widely and typically climacteric fruit that are accompanied with sudden increase of respiration and the release of a large amount ethylene during ripening. In previous studies, we had made some progress about pear fruit, including completed the transcriptome sequencing of the different stages of fruits for pear cultivars ‘Cui guan’ (Gu et al., 2021; Wang et al., 2022), and identified the ethylene biosynthetic genes PbACS1b and PbAC01, ABA biosynthetic genes such as PbZEP1, PbSDR1, PbNCED.A1 (Hao et al., 2018; Qi et al.,2019). Some transcription factors about ethylene and ABA signal transduction were researched. PbrEBF3 interacts with PbrEIL1, PbrEIL2, and PbrEIL3 proteins which trigger a transcriptional activity of PbrERF24 and finally affect ethylene synthesis. PuERF2, PuERF3, PuBZR1 also regulate ethylene biosynthesis during pear fruit ripening (Hao et al., 2018, Ji et al., 2021; Wang et al., 2023). A previous study isolated 11 PYL genes and 118 PP2C genes in pear (Wang et al., 2021, 2022), however, it is unclear how the ABA regulate fruit ripening and which intricate mechanisms of the crosstalk interaction between ethylene and ABA during fruit development and ripening. In this study, we performed exogenous hormones with different concentrations at six stages of pear fruits and investigated traits in two or three years. We found that GA has multiple actions at different development stages of pear fruit. IAA treatment with 10 mg/L could promote the fruit growth in the early stages of development. Lower concentration of ABA could promote fruit ripening. Ethylene and ABA synthesis genes were be analyzed their expression during whole growth processes. Four PbABF genes were highly expressed at 30 and 45 DAFB, and played importance roles in the earlier development stages. Further analysis elucidated they directly bind to PbACS1b and PbAC01 promoters to regulate ethylene synthesis and are involved in fruit ripening by using LUC assay and EMSA. Our findings provide a foundation for understanding the roles of GA, IAA, ABA and ethylene in fruit development and propose preliminary ABA–ethylene crosstalk involving in fruit ripening.

2. Materials and methods

2.1. Plant materials and growth conditions

Pear cultivars ‘Cuiguan’ fruit were collected from the Baima experimental station of Nanjing Agricultural University, in Nanjing, China. Fruit samples were collected every 15 days from 45 days after flower blooming (DAFB) to fruit ripening. Each sample was contained at least five fruits. All pear fruits had not been squeezed or damaged during picking, transportation and storage. The mixed sample is immediately frozen in liquid nitrogen and stored at −80 °C according to Hao et al. (2018). Nicotiana benthamiana plants were in growth chambers at 25 °C with 14 h of daylight. The tobacco with six-leaf stage can be in infiltration.
2.2. Phytohormones treatment of pear fruits

Hormones treatments were performed many years in Baima experimental station including Ethephon, 1-MCP (1-Methylcyclopropene), IAA, GA, and ABA. 'Cui guan' fruit was chosen and each treated tree had experiment groups and control group. Ethephon treated pear fruits with 50 mg/L and 1-MCP treated with 1.5µl/L at 75, 90 and 110 DAFB, respectively. GA at concentrations of 0, 10, 100, 1000 and 10000 mg/L were applied for five stages: 30, 45, 60, 75, and 90 DAFB. IAA and ABA were applied at concentrations of 0, 0.1, 1, 10, 100 and 1000 mg/L respectively and performed at the same stages as GA treatment. At least twenty fruits were used in every group. Treated and untreated fruits were allowed to measure and sample for each treatment at the all stages.

2.3. Measurement of fruit quality traits

The phenotypes of the treated and control fruits were investigated and physiological traits of fruits were measured. Fruit weight was measured by electronic balance (0.1 g precision), transverse diameter and longitudinal diameter of the whole fruits were measured using Vernier caliper (0.01mm precision). Moreover, Fruit firmness, soluble solids and titratable acid were measured according to Zhu et al. (2015). Each group was contained at least ten fruits.

2.4. RNA extraction and RT-qPCR analysis

The total RNA of pear flesh was extracted according to the instruction of RNAPrep Pure Polysaccharide Polyphenol Plant Total RNA Extraction Kit (DP441, Tiangen, Beijing, China). The specific operation steps were as previous study (Hao et al., 2018). Reverse transcription was conducted with 1 µg RNA by TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TRANSGEN, Beijing China). The Light Cycler FastStart DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) and the Light Cycler 480 (Roche, Germany) was used for qPCR reactions. The specific primers were designed by Primer Premier 5.0. and all of them were listed in Table S1. The data were evaluated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). $PbTUB$ was used as the reference gene in accordance with previous reports(Wang et al., 2022). All reactions were performed with four independent biological replicates and three technical replicates.

2.5. Dual luciferase reporter assay

The CDS of $PbABF.B$, $PbABF.E.1$, $PbABF.E.2$ and $PbABF.C.2$ were inserted into the pSAK277 vector respectively using related enzyme sites HpaI and XbaI to generate effector construct with expression driven by the $35S$ promoter. To generate the reporters, the 2000-bp sequences upstream of $PbACS1b$ and $PbACO1$ were cloned and constructed with the pGreen II0800-LUC vector, the empty vectors were used as negative controls. For the Dual luciferase reporter assays to test the regulation of $PbABF.B$, $PbABF.E.1$, $PbABF.E.2$ and $PbABF.C.2$ on the $PbACS1$ and $PbACO1$ promoters, all plasmids were transformed Agrobacterium and resuspended in infiltration buffer (10 m M MgCl$_2$, 10 mM MES, pH = 5.7, 200 µM AS). Agrobacterium resuspension was mixed in a 1:9 (v/v) ratio and injected into the N. benthamiana leaves. After infiltrating, the tobaccos were cultured in the room for 20–36 h. A Dual Luciferase Reporter Assay
Kit (DL101-01; Vazyme) and MD ID5 microplate reader (Spectramax ID5, Molecular, USA) were used to measure the activities of LUC and REN. The assay was performed as previously described (Gu et al., 2019). Primers used are listed in Table S1. Six independent biological replicates were performed.

### 2.6. Electrophoretic mobility shift assay (EMSA)

The full-length CDS of *PbABF.B*, *PbABF.E.1*, *PbABF.E.2* and *PbABF.C.2* were amplified and inserted into the pCold-TF expression vector containing a His-tag protein, respectively. The construct was transferred into *Escherichia coli* BL21, and the recombinant was extracted and purified using Ni-NAT His-binding. For EMSA, the analysis of *cis*-elements in the *PbACS1b* promoter and *PbACO1* promoter were performed and integrated by PlantCare online database. The sequences that contained ABRE elements ‘ACGTG’ were biotin labeled. Then, EMSA assay were performed according to the LightShift Chemiluminescent EMSA Kit (Cat.no.20148, Thermo Scientfic) and detected as described previously (Guo et al., 2021). All primer sequences are listed in Table S1.

### 2.7. Data analysis

Data were analyzed using SPSS software. Data are displayed as the mean ± SD using three or six independent biological replicates. All experiments were repeated with at three or six biological replicates. Standard errors and analysis of variance are calculated using Student’s t-test (*P < 0.05, **P < 0.01).

### 3. Results

#### 3.1 Effect of GA on pear fruit quality

To explore the functions of GA on fruit development and ripening, five concentrations of GA treatments were used to treat the ‘Cuiguan’ fruits at different stages. We precisely assessed the effects of exogenous GA treatment on physiological indexes of fruits collected at ripening stages. As shown as Fig. 1, at 30 DAFB, compared to the control fruits, the transverse diameters, longitudinal diameters and fruit weights were increased in the fruits with the 10 or100 mg/L GA treatments, soluble solids were increased in the fruits with the 1, 10, or 100 mg/L GA treatments, fruit firmness was increased in the fruits with the 100 mg/L GA treatments, and titratable acid was decreased in the fruits with the 10 or 10000 mg/L GA treatments. However, these trends were not detected in the second year. These results suggest that the exogenous GA treatment at 30 DAFB can hardly change fruit quality.

At 45 DAFB, compared to the control fruits, transverse diameter, longitudinal diameter and fruit weight were increased in the fruit with the 100 mg/L GA treatment, soluble solids and titratable acid were respectively decreased in the fruit with the 10 and 1000 mg/L GA treatments, and fruit firmness was increased in the fruit with the 10000 mg/L GA treatments in first year. However, these trends were not detected in the second year. It is noteworthy that titratable acid was also decreased in the fruits with the 100 or 1000 mg/L GA treatments in both years, indicating that the exogenous GA treatment at 45 DAFB can inhibit accumulation of organ acids.
At 60 DAFB, compared to the control fruits, transverse diameter, longitudinal diameter, and fruit weight was increased in the fruits with the 1 mg/L GA treatment, soluble solids were increased in the fruits with the 1 or 100 mg/L GA treatment and decreased in the fruits with the 100 mg/L GA treatment, fruit firmness was decreased in the fruits with the 10000 mg/L GA treatment, and titratable acid was decreased in the fruits with the 10, 100, or 1000 mg/L GA treatment in the first year. However, these trends were not found in the second year. It is noteworthy that transverse diameter and fruit weight was increased in the fruits with the 1000 mg/L GA treatment and fruit firmness was decreased in the fruits with the 10 mg/L GA treatment in both two years, indicating that the exogenous GA treatment at 60 DAFB can promote fruit enlargement and decrease fruit firmness.

At 75 DAFB, compared to the control fruits, fruit weight was increased in the fruits with the 1 or 10000 mg/L GA treatment, soluble solid were increased in the fruits with the 10 or 1000 mg/L GA treatment, and fruit firmness was decreased in the fruits with the 1000 mg/L GA treatment in the first year. However, these trends were not detected in the second year. It is noteworthy that titratable acid was decreased in the fruits with the 1000 mg/L GA treatment in both years, indicating that the exogenous GA treatment at 75 DAFB can inhibit accumulation of organ acids, too.

At 90 DAFB, compared to the control fruits, transverse and longitudinal diameters were increased in the fruits with the 1 or 1000 mg/L GA treatment, fruit weight was increased with the 1 mg/L GA treatment, and soluble solid were increased in the fruits with the 10 or 100 mg/L GA treatment in the first year. However, these trends were not detected in the second year. These results indicate that the exogenous GA treatment at 90 DAFB can hardly affect fruit quality, too. Taken together, the highlight of the exogenous GA treatment is to improve pear fruit quality by promoting fruit enlargement.

### 3.2 Effect of IAA on pear fruit quality

To investigate the roles of IAA on fruit development and ripening, similarly, five concentrations of IAA were used to treat the ‘Cuiguan’ fruits at different stages. As shown in Fig. 2, compared to the control fruits, transverse diameter, longitudinal diameter, and fruit weight were increased in the fruits with the 10 or 100 mg/L IAA treatment at 30 DAFB, and soluble solids were increased in the fruits with 1, 10, or 100 mg/L IAA treatment at 45 DAFB and in the fruits with 0.1, 1, 10, 100, and 1000 mg/L IAA treatment at 90 DAFB in the first year. To validate the reliability, these treatments were again performed in the second year. The result showed that transverse diameter, longitudinal diameter, and fruit weight were increased in the fruits with the 10 mg/L IAA treatment, but were hardly changed in the fruits with the 100 mg/L IAA treatment at 30 DAFB in the second year. Soluble solids were hardly changed in the fruits with any concentration treatment at 45 and 90 DAFB. Moreover, fruit firmness was decreased in the fruits with the 100 mg/L IAA treatment at 30 DAFB, in the fruits with the 0.1 or 1 mg/L IAA treatment at 45 DAFB, and in the fruits with the 1 mg/L IAA treatment at 75 DAFB, but was increased in the fruits with the 0.1, 100, and 1000 mg/L IAA treatment at 90 DAFB in the first year. Meanwhile, titratable acid was decreased in the fruits with the 100 mg/L IAA treatment at 30 DAFB and in the fruits with the 0.1 mg/L IAA treatment at 45 DAFB in the first year. However, these trends were not found in the second year. These results suggest
that the highlight of the exogenous IAA treatment is to improve pear fruit quality by promoting fruit enlargement, too.

### 3.3 Effect of ABA on pear fruit quality

To research the roles of ABA play in fruit development and ripening, five concentrations of ABA were used to treat the 'Cuiguan' fruits at different stages. As shown in Fig. 3, compared to the control fruits, transverse diameter, longitudinal diameter, and fruit weight were increased in the fruits with the 10 mg/L ABA treatment at 30 DAFB and in the fruits with the 0.1 or 1 mg/L ABA treatment at 45 DAFB, and soluble solids were increased in the fruits with 10 mg/L ABA treatment at 75 DAFB and in the fruits with 0.1, 1, 10, 100, and 1000 mg/L ABA treatment at 90 DAFB in the first year. To validate the reliability, these treatments were again performed in the second year. The result showed that transverse diameter, longitudinal diameter, and fruit weight were increased in the fruits with the 1 mg/L ABA treatment at 45 DAFB, but were hardly changed in the fruits with the 10 mg/L ABA treatment at 30 DAFB and in the fruits with the 0.1 mg/L ABA treatment at 45 DAFB in the second year. Soluble solids were increased in the fruits with the 10 mg/L ABA treatment at 75 DAFB and in the fruits with the 1 and 10 mg/L ABA treatment at 75 DAFB, but were hardly changed in the fruit with the 0.1, 100, and 1000 mg/L ABA treatment at 90 DAFB in the second year. Moreover, fruit firmness was decreased in the fruits with the 10 mg/L ABA treatment at 75 DAFB and increased in the fruits with the 1000 mg/L ABA treatment at 90 DAFB, and titratable acid was increased in the fruits with the 1, 10, 100, and 1000 mg/L ABA treatment at 90 DAFB in the first year, but these trends were not found in the second year. These results suggest that the highlight of the exogenous ABA treatment is to improve pear fruit quality by promoting fruit enlargement and accumulation of soluble solids.

### 3.4 Role of ethylene in fruit ripening

To clarify the role of ethylene in fruit ripening, the ethylene production was investigated in pear fruits at six stages, from fruitlet (30 DAFB) to ripening (110 DAFB), and was significantly increased in the ripening fruits than in the developing fruits (Fig. 4A). Subsequently, ethephon and 1-MCP were used to treat the fruits at 75, 90, and 100 DAFB. The result showed that soluble solids were increased in the fruits with ethephon treatment at 75 DAFB and decreased in the fruits with 1-MCP treatment at 90 DAFB in the both years (Fig. 4B). In contrast, fruit firmness was hardly changed in the fruits with any treatment at any stage (Fig. 4B). These results suggest that ethylene positively regulates fruit ripening by promoting the accumulation of soluble solids in pear.

### 3.5 Identification of the crucial genes involved in ethylene and ABA biosynthesis

To identify the crucial genes involved in ABA and ethylene biosynthesis, the expression patterns of ABA and ethylene biosynthetic genes were investigated based on the transcriptome data in a previous study (Gu et al., 2021). The result showed that 3 ZEP, 3 NECD, 3 SDR, 5 AAO, 2 ACS, and 3 ACO genes were
detected in ‘Cuiguan’ fruit (Table S2). The qRT-PCR analysis showed that the expression level among the different members in each family was similar to the result of transcriptome analysis (Fig. 5). Of these genes, *PbZEP1, PbNCED.B, PbSDR4,* and *PbAO3* were higher expressed in pear fruit than the other members in the corresponding family, suggesting that these genes are the crucial genes for ABA biosynthesis in pear fruit. Moreover, *PbACS1b* and *PbACO1* were higher expressed in pear fruit than the other members in the corresponding family, suggesting that the two genes are the crucial genes for ethylene biosynthesis in pear fruit. In addition, the expression patterns of *PbZEP1, PbZEP2, PbZEP3, PbNECD.A2, PbSDR1, PbSDR2,* and *PbACS6* tested by the qRT-PCR analysis was positively correlated to that tested by transcriptome analysis, while the other 11 genes had the different expression patterns (Fig. 5). This result may be caused by the mismatch of transcriptomic reads mapping to the pear reference genome.

### 3.6 Regulation of PbABFs on the promoters of PbACS1b and PbACO1

A total of 8 ABF genes were identified from pear genome, of these, only *PbABF.B, PbABF.C.2, PbABF.E.1,* and *PbABF.E.2* could be detected in ‘Cuiguan’ fruit (Wu et al., 2022). To test whether these four *ABFs* can enhance the activities of ethylene biosynthetic genes, the PbACS1b pro-LUC and PbACO1 pro-LUC recombinants were used as the reporters, and the CaMV35S-PbABF.B, CaMV35S-PbABF.C.2, CaMV35S-PbABF.E.1, and CaMV35S-PbABF.E.2 recombinant plasmids were used as the effectors (Fig. 6A). Dual-luciferase assay showed that the activity of *LUC* driven by the *PbACS1b* promoter (PbACS1b pro-LUC) was increased in the tobacco leaves over-expressing PbABF.B or PbABF.C.2, and the activity of *LUC* driven by the *PbACO1* promoter (PbACO1 pro-LUC) was increased in the tobacco leaves over-expressing PbABF.E.1 or PbABF.E.2, compared to the controls (Fig. 6B). These results indicated that PbABF.B and PbABF.C.2 can promote the activity of *PbACS1b*, PbABF.E.1, and PbABF.E.2 can enhance the activity of *PbACO1*, indicating that *PbABF* genes could promote the activity of *PbACO1* and *PbACS1b* to regulate ethylene synthesis. In addition, we tested the expression patterns of these four *ABF* genes during pear fruit development and ripening. The result showed that all tested *ABF* genes were higher expressed in the fruits at 30 and 45 DAFB than in the fruits at other stages, but only *PbABF.B* and *PbABF.E2* were significantly high expressed in the fruit at 110 DAFB compared to those at 90 DAFB (Fig. 6C). This result indicated *PbABF.B* and *PbABF.E2* may be involved in the accumulation of soluble solids that was significantly increased in ripening fruit than in non-ripening fruits of ‘Cuiguan’ fruit (Shi et al, 2007).

### 3.7 Binding of PbABF proteins to the PbACS1b or PbACO1 promoter

To further investigate whether PbABF proteins directly bind to the promoters of *PbACS1b* and *PbACO1*, we predicted the *cis*-elements in both promoters and found that two and three ABA-responsive elements (ABERs) presented in the promoters of *PbACS1b* and *PbACO1*, respectively (Fig. 7A). The potential binding region containing any ABRE was used to synthesize probe for EMSA. Moreover, the full-length
CDS of PbABFs were amplified and inserted into the pCold-TF vectors with a His-tag. The recombinant PbABF.B-His, PbABF.C2-His, PbABF.E1-His and PbABF.E2-His were extracted from Escherichia coli BL21 and purified in vitro (Fig. S1). EMSA showed that the recombinant protein PbABF.B and PbABF.C2 could bind to the PbACS1b promoter, while PbABF.E1 and PbABF.E2 could bind to the PbACO1 promoter (Fig. 7B). The binding signals were weakened with the increasing concentration of cold probes and vanished with the mutant probes (Fig. 7B). These results suggest that the PbABFs, which are expressed in pear fruit, directly bind to the promoters of PbACS1b and PbACO1.

4. Discussion

Fruit development and ripening is an elaborate bioprocess which is regulated by many multiple aspects. On the one hand, fruit growth commences after fruit set and creases before fruit maturation (Fenn et al., 2021). During fruit development, cell division and cell expansion are the two stages of fruit growth, which decide to fruit shape, size, and mass. Apple initiates with the combination of cell division and expansion, growth with cell division subsiding, and a subsequent stages of predominant cell expansion is allowing (Devoghalaeere et al., 2012; Fenn et al., 2021). Pear fruit exhibit a single sigmoid pattern while peach and strawberry fruits exhibit a double sigmoid pattern during development both single and double sigmoid patterns resulted from cell expansion, but not cell division (Pei et al., 2020). On the other hand, fruit ripening is a complex and closely coordinated developmental process that involves various physiological and metabolic changes, including color, stone cells, flavor, aroma, texture, hormones and so on, making the fruit attractive, delicious, and nutritious (Giovannoni et al., 2004, Gu et al., 2016, Forlani et al., 2019, Gao et al., 2020). During ‘Nanguo’ fruit ripening, the color of fruit is from green to yellow and large amounts of ethylene is produced, accompanied by rapid softening, but the soluble solids contents and the titratable acidities of the fruits show no obvious changes (Yuan et al., 2017). Fruit aroma-related compounds are change in order to the optimal taste. (Li et al., 2022).

Phytohormones are crucial to the regulation of fruit development and maturation. GA and auxin are the primary regulators which involved in and accumulate in fruit set initiation and early stages of development (Feen et al., 2021; Gustafson et al., 1936, Kumar et al., 2014). The influence of GA and IAA has been widely reported in studies of horticultural fruits, including apples, grapes, and watermelons (Liu et al., 2022; Wang et al., 2021; Zheng et al., 2022), yet little is known about their exact roles in pear. In this study, we found that exogenous treatment of GA could promote fruit enlargement and inhibit the accumulation of organ acids in pear (Fig. 1), and exogenous treatment of IAA increased fruit size (Fig. 2). This result is consistent with a previous study, in which, exogenous IAA caused an increase in cell size at the start of cell expansion (Devoghalaeere et al., 2012). However, auxins also trigger fruit maturation in tomato, which down-regulation of DR12 results in dark green and blotchy ripening (Jones et al., 2002, Forlani et al., 2019).

It is reported that ethylene positively regulates fruit ripening (Guo et al, 2019; Chen et al, 2020). As the ethylene biosynthetic genes, PbACO1 and PbACO4 were higher expressed in the fruits at 30 and/or 60 DAFB than in the ripening fruit (110 DAFB; Fig. 5). This pattern is inconsistent with the metabolic pattern
that ethylene was hardly detected in developing fruit, but dramatically increased in ripening fruit (Fig. 5A). The result may be caused by the fact that \(PbACS1\) was hardly expressed in developing fruit, and \(PbACS6\) presents the extremely low level of expression in pear fruit (Fig. 5A). Further analyses showed that the ABA responsive factors \(PbABF.B\) and \(PbABF.C2\) could bind to the \(PbACS1b\) promoter to enhance the \(PbACS1b\) expression, and \(PbABF.E1\) and \(PbABF.E2\) could bind to the \(PbACO1\) promoter to enhance the \(PbACO1\) expression (Figs. 6B and 7B). The result indicates that ABA may play important role in ethylene biosynthesis in pear fruit.

Abscisic acid (ABA) plays a crucial role in various aspects, including seed dormancy, fruit development and ripening, involvement in response to environmental stresses (Lee et al., 2006; Li et al., 2022; Qi et al., 2019). In this study, we found that ABA can increase accumulation of soluble solids (Fig. 3), indicating that ABA may promote fruit ripening. This result is consistent with the previous studies, in which ABA plays a pivotal role in the ripening process of non-climacteric as well as climacteric fruits (Li et al., 2021; Qiao et al., 2021; Wang et al., 2019; Wang et al., 2021). The relationship between ABA and ethylene was also observed. It also reported in tomato that ABA roles in triggering ethylene biosynthesis and ripening that \(LeNCED1\) was highly expressed only at the breaker stage when the ABA content becomes high, \(LeACS2, LeACS4\), and \(LeACO1\) genes were expressed with some delay (Zhang et al., 2009). The consistent results were obtained in our studies that the expression of \(PbAO6, PbAO7\) were highest at 90 DAFB, earlier than the expression of \(PbACS1b, PbACS3, PbACO2\) (Fig. 5), indicating that ABA play roles in the development and pre-ripening and maybe incite ethylene accumulation and release. The cross-talking regulation of ABA and ethylene has been reported in many fruits. In hot pepper, \(ACO3\) and \(NCED1/3\) gene expression determined ethylene and ABA levels, respectively. Downregulation of \(ACO3\) and \(NCED1/3\) expression inhibited and promoted coloration, as well as carotenoid biosynthesis-related gene expression (Hou et al., 2009). Silencing SIZFP2 showed that \(LeACS1\) and \(LeACO1\) were positively regulated by ABA during early fruit growth, and SIZFP2 direct suppression of ABA biosynthetic genes and CNR, indicating that SIZFP2 modulates the cross-talk between ABA and ethylene in regulation of fruit development and ripening (Weng et al., 2015). Block of ABA signaling inhibited the transcription of ethylene biosynthetic genes and other related regulators, such as \(ACS2, ACS4, RIN, NOR\), and \(E8\), and then significantly decreased ethylene production at the early stage of fruit ripening in tomato, suggesting ABA signaling works upstream of ethylene for regulation of fruit ripening (Zou et al., 2022). In the study, four \(PbABFs\) were higher expressed at 30 and 45 DAFB but had lower expression at the 60, 75, 90, and 110 DAFB (Fig. 6A and B), suggesting that four \(PbABF\) genes play important roles in the early development stages. Interestingly, \(PbABFB\) and \(PbABFE2\) increased significantly at 110 DAFB compared to those at 90 DAFB (Fig. 6B), implying that \(PbABFB\) and \(PbABFE2\) may be also involved in fruit ripening. Further analysis show that PbABFs can directly bind to ABRE elements of \(PbACS1b\) and \(PbACO1\) promoters to promote their activities (Fig. 6C and D), indicating that these ABFs may regulate ethylene and fruit ripening. The investigation reveals the cross-talking between ABA and ethylene during fruit ripening. However, studies in interaction between ABA and ethylene in pear are still scarce. A further dissection of cross-talking between regulation of ABA and ethylene may open up new understanding fruit ripening and quality maintenance.
5. Conclusion

Fruit development and ripening is synergistically regulated by multiple phytohormones, which are play roles in different stages and cross-talking with each other. Our study found that phytohormones, including GA, IAA, ABA and ethylene, are involved in regulating fruit development and ripening. *PbABFs* can directly bind ABRE elements of *PbACS1b* and *PbACO1* promoters to promote their activities. Our study provides a foundation for further research about roles of GA, IAA, ABA and ethylene in pear fruit and reveals the ABA—ethylene cross-talking during fruit ripening.

Declarations

Data availability

All relevant data analyzed during this study are included in this article and in Additional files.

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Contributions

GC and ZSL designed the experiment and revised the manuscript. GZH drafted the manuscript, qRT-PCR, and data analysis. GZH carried out the treatments with the help from LH, WXP, XZH, ZZM, LJR, LJM, and LSY. LH conducted the dual-luciferase assay and EMSA. All authors have read and approved the final manuscript.

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Ethics declarations

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

References


Figures
Figure 1

Physiological changes of pear fruit with the exogenous GA treatment. The time for GA treatment was performed in the fruit at 30, 45, 60, 75, and 90 DAFB in two years. Each background represents one group that contains control and treatment. Means and standard errors were calculated using ANOVA. Asterisk represents the level of significance at $P < 0.05$. 

Figure 2

Physiological changes of pear fruit with the exogenous IAA treatment. The time for IAA treatment was performed in the fruit at 30, 45, 60, 75, and 90 DAFB in the first year, and in the fruit at 30, 45, 75, and 90 DAFB in the second year. Each background represents one group that contains control and treatment. Means and standard errors were calculated using ANOVA. Asterisk represents the level of significance at $P < 0.05$. 


Figure 3

Physiological changes of pear fruit with the exogenous ABA treatment. The time for ABA treatment was performed in the fruit at 30, 45, 60, 75, and 90 DAFB in the first year, and in the fruit at 30, 45, 75, and 90 DAFB in the second year. Each background represents one group that contains control and treatment. Means and standard errors were calculated using ANOVA. Asterisk represents the level of significance at $P < 0.05$. 
Figure 4

Ethylene mediates the accumulation of soluble solids in pear fruit. (A) Ethylene production was measured in the fruit at 30, 45, 60, 75, 90, and 110 DAFB. (B) Soluble solids and fruit firmness were measured in the fruit with the ethephon or 1-MCP treatment. The time for ethephon or 1-MCP treatment was performed in the fruit at 75, 90, and 100 DAFB in the first year, and in the fruit at 75 and 90 DAFB in the second year.
Means and standard errors were calculated using ANOVA. Asterisk represents the level of significance at $P < 0.05$.

**Figure 5**

Expression patterns of ethylene and ABA biosynthetic genes during fruit development and ripening. The fruit for qRT-PCR analysis was collected at 30, 45, 60, 75, 90, and 110 DAFB. Means and standard errors were calculated using ANOVA.
Figure 6

PbABF proteins enhance the activities of the *PbACS1b* and *PbACO1* promoters. (A) The constructed reporters and effectors for dual-luciferase assay. (B) Dual-luciferase assay showing the LUC activities driven by the *PbACS1b* or *PbACO1* promoter in the tobacco leaves infiltrating each effector or an empty vector. *PbACS1bpro* and *PbACO1pro* represent the *PbACS1b* and *PbACO1* promoters, respectively. Six replicates were performed and used for calculating means and standard errors. Double asterisk represents the level of significance at $P < 0.01$. 
Figure 7

PbABF proteins directly bind to the *PbACS1b* or *PbACO1* promoter. (A) The *cis*-elements for ABA responsiveness were predicted from the *PbACS1b* and *PbACO1* promoters. (B) EMSA showing the binding of the recombinant PbABF.B, PbABF.C.2, PbABF.E.1, and PbABF.E.2 to the *PbACS1b* or *PbACO1* promoter. + and − indicate the presence and absence of the recombinant protein, biotin-labeled probe, cold probe, or
mutant probe, respectively. Cold probe concentrations were 10-fold (10×), 20-fold (20×), 50-fold (50×), 100-fold (100×), and 200-fold (200×) of labeled probes.

**Supplementary Files**

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