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RESEARCH REPORT

# The miR156x + p/*SPL13-6* module responds to ABA, IAA, and ethylene, and SPL13-6 participates in the juvenile–adult phase transition in *Pyrus*

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## Abstract

Perennial woody fruit trees usually have a long juvenile period, which greatly restricts the breeding process. The miR156/squamosa promoter binding protein-like (SPL) module and hormones participate in phase transition. To determine which miR156s and SPLs play roles in the juvenile–adult phase transition in pear (*Pyrus*) trees, and whether hormones affect the expressions of miR156 and target *SPLs*, we constructed and sequenced juvenile and adult sRNA libraries and then analyzed the expression patterns of differentially expressed miRNAs/targets pairs in the process of ontogenic development as well as under abscisic acid (ABA), indole-3-acetic acid (IAA) and ethylene precursor 1-aminocylopropane-1-carboxylic acid (ACC) treatments. *SPL13-6* was identified as a candidate gene, and the function of its product was verified in transgenic Arabidopsis. Compared with in the juvenile phase, 65/62 miRNAs were up-/down-regulated in the adult phase, including two miR156 members, miR156x and miR156p. According to the mRNA database for the juvenile–adult phase transition, 11 pairs of miRNAs/targets participate in the juvenile–adult phase transition, including four pairs of miR156/*SPLs*. With ontogenic development, the expression level of miR156x + p decreased and those of four *SPLs* increased. ABA and IAA inhibited the expression of miR156, while low and high concentrations of ACC increased and decreased miR156 expression, respectively, compared with control levels. Among the four *SPLs*, *SPL13-6* displayed an opposite expression patterns compared with that of miR156 under hormone treatments. Overexpression of *SPL13-6* in Arabidopsis resulted in earlier abaxial trichome production and flowering phenotypes compared with the wild-type phenotype. These results suggest that the miR156x + p/*SPL13-6* module responds to ABA, IAA, and ethylene and that SPL13-6 participates in the juvenile–adult phase transition. This research lays a foundation for further studies on regulation mechanisms involved in the juvenile–adult phase change in pear trees.

**Keywords** Juvenile-adult phase transition · ABA · IAA · Ethylene · miR156/*SPL* · *Pyrus*

## 1 Introduction

In the ontogenetic development of plants, three phases are frequently distinguished during postembryonic development: the juvenile vegetative phase, adult vegetative phase, and adult reproductive phase (Poethig 2003). In the adult phase, seedlings can respond to floral inductive signals and initiate flowering. Perennial woody fruit trees, including pear (*Pyrus*) trees, usually have a long juvenile period, which greatly restricts the breeding process. Analysis of juvenile–adult phase transition mechanisms could help to shorten the breeding cycle and improve breeding efficiency.

The miR156/squamosa promoter binding protein-like (SPL) module is a major factor regulating the juvenile–adult phase transition and is highly conserved in different plant species (Huijser and Schmid 2011). SPL is a positive regulator of the juvenile–adult phase transition. The expression of *SPL* is inhibited by miR156, which is highly expressed in the juvenile stage and gradually decreases with growth (Wu and Poethig 2006; Wang et al. 2009; Wu et al. 2009; Yamaguchi

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et al. 2009). In Arabidopsis, two miR156 members, miR156a and miR156c, play major roles in the juvenile–adult phase transition (Yang et al. 2013). Ten of the 17 *SPL* genes are targeted by miR156 (Preston and Hileman 2013). Among the products of these *SPLs*, SPL2, SPL9, SPL10, SPL11, SPL13, and SPL15 are involved in the juvenile–adult phase transition and floral induction, with SPL9, SP13, and SPL15 being more important for these processes (Xu et al. 2016; Cardon et al. 1997; Wu and Poethig 2006). Compared with Arabidopsis (12 members) and rice (12 members), there are 22 and 31 miR156 members in pear (*Pyrus pyrifolia*) and apple, respectively (Xia et al. 2012; Niu et al. 2013), suggesting that there are more non-conserved miR156 s in pear and apple trees than in other plants. Through analysis of the *SPL* gene family in Rosaceae species, 11 out of 19 *PpSPLs* in *P. pyrifolia* Nakai, 15 of 27 *MdSBP* genes in apple, 5 of 14 *FvSPL* genes in strawberry, 9 of 17 *PrpSPLs* in *Prunus persica*, and 8 of 15 *RoSPLs* in *Rubus occidentalis* were identified as potential targets of miR156 (Jiang et al. 2021). However, we still do not know which miR156s and SPLs play roles in the juvenile–adult phase transition in most Rosaceae species.

Hormones also play roles in regulating phase transitions (Davis 2009; Kazan and Lyons 2016; Conti 2017), such as gibberellin (GA; Wilson et al. 1992; Blázquez et al. 1998; Srikanth and Schmid 2011), ethylene (Achard et al. 2006), brassinosteroid (BR; Domagalska et al. 2007), salicylic acid (SA; Jin et al. 2008), cytokinins (CK; D'aloia et al. 2011), auxin (Shimada et al. 2005), abscisic acid (ABA; Barrero et al. 2005; Achard et al. 2006; Riboni et al. 2016; Endo et al. 2017), and jasmonic acid (JA; Hibara et al. 2016). However, these reports are mainly related with the flowering process. Less research has been conducted on hormones involved in the juvenile–adult phase transition process. In 1975, a study found that exogenous $GA_3$ treatment could lead to reversion from the adult to the juvenile form in *Hedera helix* (Rogler and Hackett 1975). Other studies have mainly focused on hormone contents during different stages. For example, the ABA content in juvenile samples was higher than that in adult samples in *Hedera helix* (Hillman et al. 1974). In apple trees, the leaf ABA content was significantly higher in the adult phase than in the juvenile phase from March to May, but this was reversed in July and August; when the seedlings developed to the adult phase, auxin and CK levels increased and the GA level decreased (Xing et al. 2014). In *Pyrus*, the contents of ethylene, $GA_4$ and JA were lower in the adult phase than in the juvenile phase; whereas the auxin, $GA_1$ and ABA contents were higher in adult phase (Song et al. 2020). These reports all indicate that hormones affect phase transition, with much work needed to establish the molecular pathways of this input.

In our previous study, we concluded that *SPL* genes as well as hormone biosynthesis and signaling accompany the juvenile–adult phase transition in *Pyrus* (Song et al. 2020). But little is known about which miR156 and *SPL* genes play roles in this process and whether hormones affect the expressions of miR156 and its target *SPLs*. Therefore, in this study, we analyzed the expression profile of miRNA during the juvenile–adult phase transition and identified which miR156 participated in the process. Then, we detected the expression patterns of miR156 and its target *SPLs* under ABA, indole-3-acetic acid (IAA), and ethylene precursor1-aminocylopropane-1-carboxylic acid (ACC) treatments, and we selected a candidate *SPL13-6* gene which displayed an opposite expression pattern compared with that of miR156. Through over-expression and functional verification in Arabidopsis, we proved that SPL13-6 regulates the phase transition process. Therefore, we suggest that the miR156x + p/*SPL13-6* module responds to ABA, IAA, and ethylene treatment and that SPL13-6 participates in the juvenile–adult phase transition in *Pyrus*. These results lay a foundation for further studies on regulation mechanisms involved in the juvenile–adult phase transition in pear trees.

## 2 Materials and methods

### 2.1 Plant materials and treatments

Leaf samples were collected in April 2017, from three independent 6-year-old hybrid offspring (*Pyrus pyrifolia* Nakai 'Whangkeumbae' × *P. bretschneideri* Rehd. 'Zaosu') planted with 1.0-m row spacing under a normal water and fertilizer management level in the pear experimental plot of Qingdao Agricultural University. For miRNA sequencing analysis, adult phase leaves were collected from above of the first flower node and juvenile phase leaves were collected from the basal part between the 1st and 60th node of the tree. To reduce the differences caused by leaf age, fully expanded leaves were collected. For qPCR analysis, leaves representing different ontogenetic phases were collected from the following positions at different spans (1, 1st–30th node; 2, 30th–60th node; 3, 60th–90th node; 4, 90th–120th node; and 5, above the 120th node) (the average first flower node number was 96.3) (Song et al. 2020). Three replicates were collected for each sample. *Pyrus bretschneideri* Reld. 'Laiyang chili' plantlets sub-cultured for 14 d were transferred to MS medium supplemented with different concentrations (0, 0.01, 0.1, and 1 mg $L^{-1}$) of IAA, ACC and ABA for 30 d. Then, the plantlets were collected for qPCR analysis of miR156 and *SPLs*. Three replicates were collected for each treatment. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

### 2.2 Small RNA library construction, sequencing, and data analysis

Total RNA from adult and juvenile leaves was isolated using an RNAprep Pure Plant kit (Tiangen, Beijing, China). Three micrograms of RNA per sample was used as input material for the small RNA library construction. Sequencing libraries were generated using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations. Briefly, after ligation of 3′ and 5′ adapters and RT-PCR amplification, PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). DNA fragments corresponding to 140–160 bp (the length of small noncoding RNA plus the 3' and 5' adaptors) were recovered and dissolved in 8 µL elution buffer. Then, the library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips, and the mixed sRNA libraries were sequenced on an Illumina Hiseq 2500 platform. Raw data from sRNA sequencing was submitted to the Sequence Read Archive (SRA ID: PRJNA778937).

After filtering and adapter cutting, clean reads were obtained. Then, the rRNAs, scRNAs, snoRNAs, snRNAs, and tRNAs were subsequently removed through searching the GenBank and Rfam 10.0 databases (Kozomara and Griffiths-Jones 2014). The remaining small RNA tags were mapped to the miRBase database (www.mirbase.org/) and pear genome sequence (http://peargenome.njau.edu.cn:8004/default.asp?d=4&m=2) (Wu et al. 2013) using Bowtie (Langmead et al. 2009) without mismatch to analyze their expression and distribution on the genome.

The miRNA expression levels were estimated by TPM (transcript per million) through the following formula (Zhou et al. 2010): normalized expression = mapped miRNA read count/total number of clean reads × 1,000,000. Differential expression analysis of two groups was performed using the DESeq R package (1.8.3). The *P*-values were adjusted using the Benjamini and Hochberg method (1997). A corrected *P*-value of 0.05 was set as the threshold for significant differential expression by default.

### 2.3 Prediction of differently expressed miRNAs targeted genes

Predicting the target gene of differentially expressed miRNAs was performed using psRobot_tar in psRobot (Wu et al. 2012). Data were combined with the RNA-seq database for juvenile–adult phase transition (Song et al. 2020), and pairs of miRNA/mRNA with opposite expression patterns were regarded as participating in the juvenile–adult phase transition.

### 2.4 RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted using a FastPure Plant Total RNA Isolation Kit (Vazyme, China). For expression analysis of miRNAs, miRNA cDNA was synthesized using an miRNA 1st Strand cDNA Synthesis Kit (Vazyme, China) following the manufacturer's instructions. The qPCR analysis was performed using miRNA Universal SYBR qPCR Master Mix (Vazyme, China) and a Roche 480 real-time PCR system (Roche, Switzerland). Each sample was analyzed three times, and 5S rRNA was used as the internal control (Wu et al. 2014). For expression analysis of miRNA target genes, cDNAs were synthesized from 1 µg total RNA using a HiScript 1st Strand cDNA Synthesis Kit (Vazyme, China) following the manufacturer's instructions. qPCR was performed using the Roche 480 real-time PCR system (Roche, Switzerland) in standard mode with a FastStart Essential DNA Green Master kit (Roche, Switzerland). The relative expression levels of detected genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The qPCR primers are listed in Supplementary Table 1.

### 2.5 Function analysis of SPL13-6 in the juvenile–adult phase transition

Columbia wild type Arabidopsis (*Arabidopsis thaliana*) was used for SPL13-6 function analysis. The ORF sequence of *SPL13-6* was amplified using specific primers with *Xba* I and *Kpn* I sites. The primers are listed in Supplementary Table 1. The PCR products were digested with *Xba* I and *Kpn* I and then inserted into the binary vector pBI121 to construct the 35S::*SPL13-6* expression vector. Plasmids containing 35S::*SPL13-6* were then transferred into *Agrobacterium* EHA105. The transformations of Arabidopsis were performed using the flower dip method described by Clough and Bent (1998). Then, the transformed Arabidopsis plants were cultured in a greenhouse under 22 ± 1 °C, 16/8 h (light/dark), and 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ illumination. The plants were watered once every 4 days. The seeds collected from transformed plants were germinated on MS medium containing 50 mg $L^{-1}$ kanamycin. $T_2$ homozygous transgenic plants were obtained after screening for two generations and used to characterize the phenotype affected by the *SPL13-6* gene. Transgenic and control Arabidopsis plants were photographed at 30 days after sowing and the first leaf with abaxial trichomes was recorded.

## 3 Results

### 3.1 Analysis of differently expressed miRNAs in juvenile and adult phases

To verify whether miR156 plays an important role in the juvenile–adult phase transition and identify which miR156 member participates in this process, juvenile and adult sRNA libraries from the leaves of *Pyrus* plants were constructed and sequenced. A total of 8,871,501–10,522,819 raw reads were acquired through high throughput sequencing. After removing the low-quality raw reads, the remaining 8,739,287–9,711,389 clean reads were used for predicting miRNAs (Supplementary Table 2). A total of 232 miRNAs were identified in the sRNA library. Among these miRNAs, nine and four miRNAs were specifically expressed in the juvenile and adult phase, respectively; and 219 miRNAs were expressed in both the juvenile and adult phases (Fig. 1A). A total of 127 miRNAs were differentially expressed in the adult vs. juvenile stage, with 65 and 62 miRNAs up- and down-regulated, respectively (Fig. 1B), including miR156, miR172i, miR159c, and miR396. miR156p, 156x, and 159c were significantly down-regulated and miR172i, 396a, and 396b were significantly up-regulated. Of the miR156 and miR396 members, miR156x and miR156p, as well as miR396a and miR396b, had high sequence similarity. Therefore, conserved primers were designed to analyze the total expression of miR156x + p and miR396a + b. Through qPCR, we confirmed that the expression profiles of detected miRNAs were consistent with the RNA-seq results (Fig. 2). In summary, the diverse expression patterns of miRNAs between adult and juvenile phases implied their potential functions during the juvenile–adult phase transition, and the two miR156 family members, miR156x and miR156p, might play roles in this process.

### 3.2 Expression pattern analysis of candidate miRNA-target pairs in different ontogenetic phases

Through targeted gene predictions, a total of 496 genes were identified as potential targets of differentially expressed miRNAs. By combining our results with the RNA-seq database for juvenile–adult phase transition (Song et al. 2020), we screened 11 pairs of miRNAs and mRNAs with opposite expression patterns, including miR159c/*YUCCA4*, miR172i/*ethylene responsive transcription factor RAP2-7*, miR396/*transcription factor EMB1444*, miR396/*cytochrome P450 71A1*, miR7121/*MATE efflux family protein ALF5*, and four pairs of miR156/*SPL* (Table 1).

Through qPCR analysis, we determined the expression patterns of miR156, miR159c, miR172i, miR396 and their predicted target genes in five different ontogenetic phases. The results showed that the expression level of miR156 was

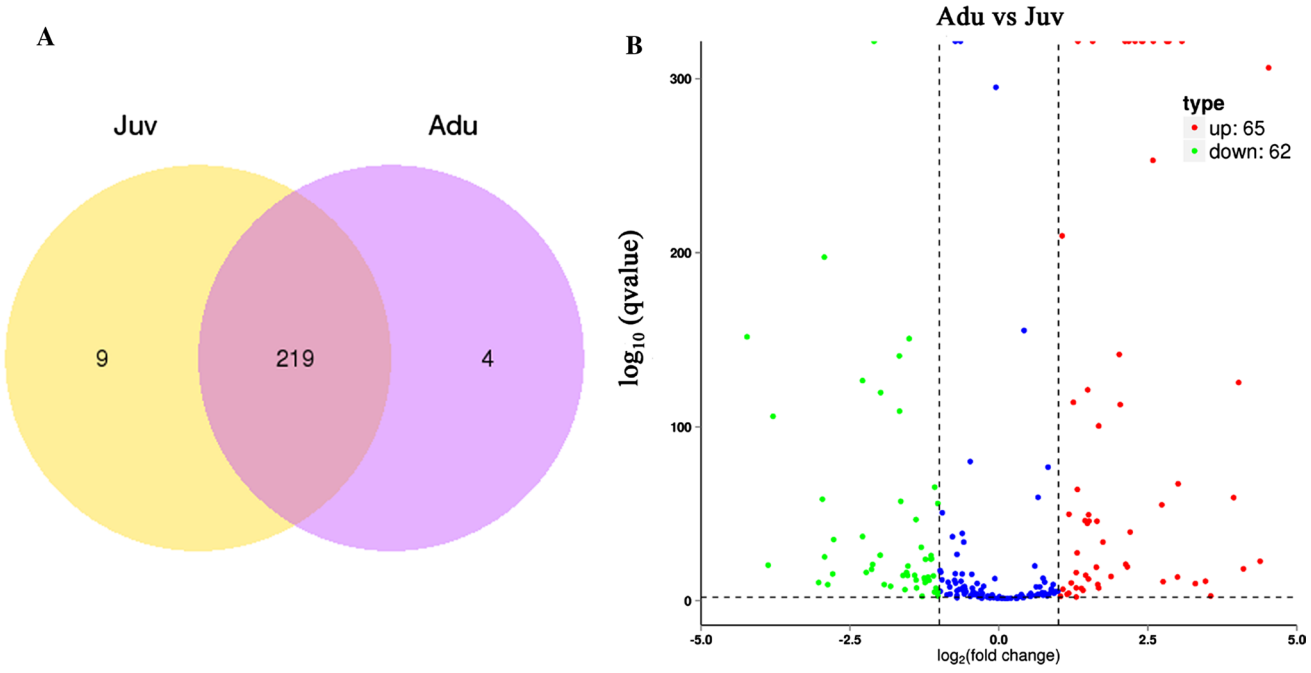


**Fig. 1** Distribution and expression analysis of miRNAs in juvenile and adult phases. **a**. Venn diagram showing the numbers of miRNAs expressed in juvenile (Juv) and adult (Adu) phases. **b**. miRNAs differentially expressed between juvenile and adult phases

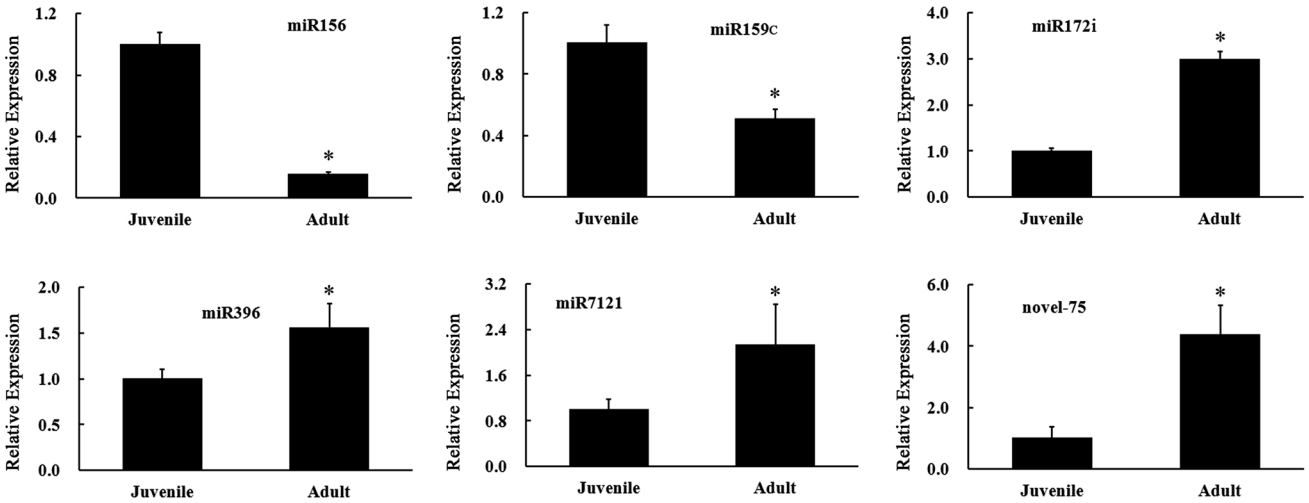


**Fig. 2** qPCR analysis of differentially expressed miRNAs in juvenile and adult phases. All columns and asterisks (*) represent mean ± standard error (SE) and significantly differences compared with the juvenile (Duncan' s multiple range test, $P < 0.05$)

**Table 1** Differential expression analysis of 11 pairs of predicted miRNAs/targets between adult and juvenile leaves

| miRNA | $Log_2$(fold change) | *P*-value | Gene ID | Protein description | $Log_2$(fold change) | *P*-value |
|---|---|---|---|---|---|---|
| miR156x | − 6.4286 | 4.99E-12 | LOC103944181 | Squamosa promoter-binding-like protein 13–2 | 3.0675 | 2.41E-05 |
| miR156x | − 6.4286 | 4.99E-12 | LOC103932829 | Squamosa promoter-binding protein 5 | 1.7334 | 0.039912 |
| miR156x,miR156p | − 6.4286 | 4.99E-12 | LOC103961451 | Squamosa promoter-binding-like protein 2 | 1.7362 | 0.0027479 |
| miR156x,miR156p | − 6.4286 | 4.99E-12 | LOC103936787 | Squamosa promoter-binding-like protein 13–6 | 1.0732 | 0.047602 |
| miR159c | − 5.5806 | 2.12E-07 | LOC103962018 | Probable indole-3-pyruvate monooxygenase YUCCA4 | | 0.014522 |
| miR172i | 2.998 | 1.76E-13 | LOC103926659 | Ethylene-responsive transcription factor RAP2-7-like | − 2.7227 | 0.014579 |
| miR172i | 4.385 | 1.08E-22 | LOC103966662 | Ethylene-responsive transcription factor RAP2-7-like | − 1.8271 | 0.0016402 |
| miR396a, miR396b | 2.8327 | 4.12E-09 | LOC103950151 | Transcription factor EMB1444-like | − 1.4564 | 0.042641 |
| miR396b | 2.8327 | 4.12E-09 | LOC103964280 | Cytochrome P450 71A1-like | − 1.7135 | 0.016306 |
| miR7121 | 2.735 | 2.44E-55 | LOC103932868 | MATE efflux family protein ALF5-like | − 2.0292 | 0.01054 |
| novel_154 | − 3.0244 | 3.09E-10 | LOC103942953 | Uncharacterized | 1.35 | 0.028043 |

higher in younger stages (span 1 and 2), then decreased at span 3, with the lower level in span 3 to 5, which represented the adult phase (Fig. 3A). The expression levels of predicted targets of miR156, *SPLs*, were all high in at least one span in the adult phase. *SPL13-6* (LOC103936787) expression was induced at span 3 and peaked at span 5 (Fig. 3B). The expression levels of *SPL2* (LOC103961451) and *SPL13-2* (LOC103944181) peaked at span 3 and 4, respectively (Fig. 3C, D). *SPL5* (LOC103932829) expression was induced at span 4 and peaked at span 5 (Fig. 3E). The expression level of miR159c was highest in span 2, and then decreased at span 3, with a relatively lower expression in span 4 and 5 (Fig. 3F). Its predicted target, *YUCCA4*, had low expression in span 2 and high expression in span 3 and 4 (Fig. 3G). miR172i expression increased during ontogenic development and peaked at span 5 (Fig. 3H). One predicted target of miR172i, *ethylene-responsive transcription factor RAP2-7-like* (LOC103926659), displayed a relatively lower expression at span 2 to 5 compared with at span 1 (Fig. 3I). The other target of miR172i, *RAP2-7-like* (LOC103966662), showed the highest expression level in span 3 (Fig. 3J). The expression of miR396 decreased from span 2 to 4, and then peaked at span 5 (Fig. 3K). The expression of *cytochrome P450 71A1-like* (LOC103964280), the predicted target of

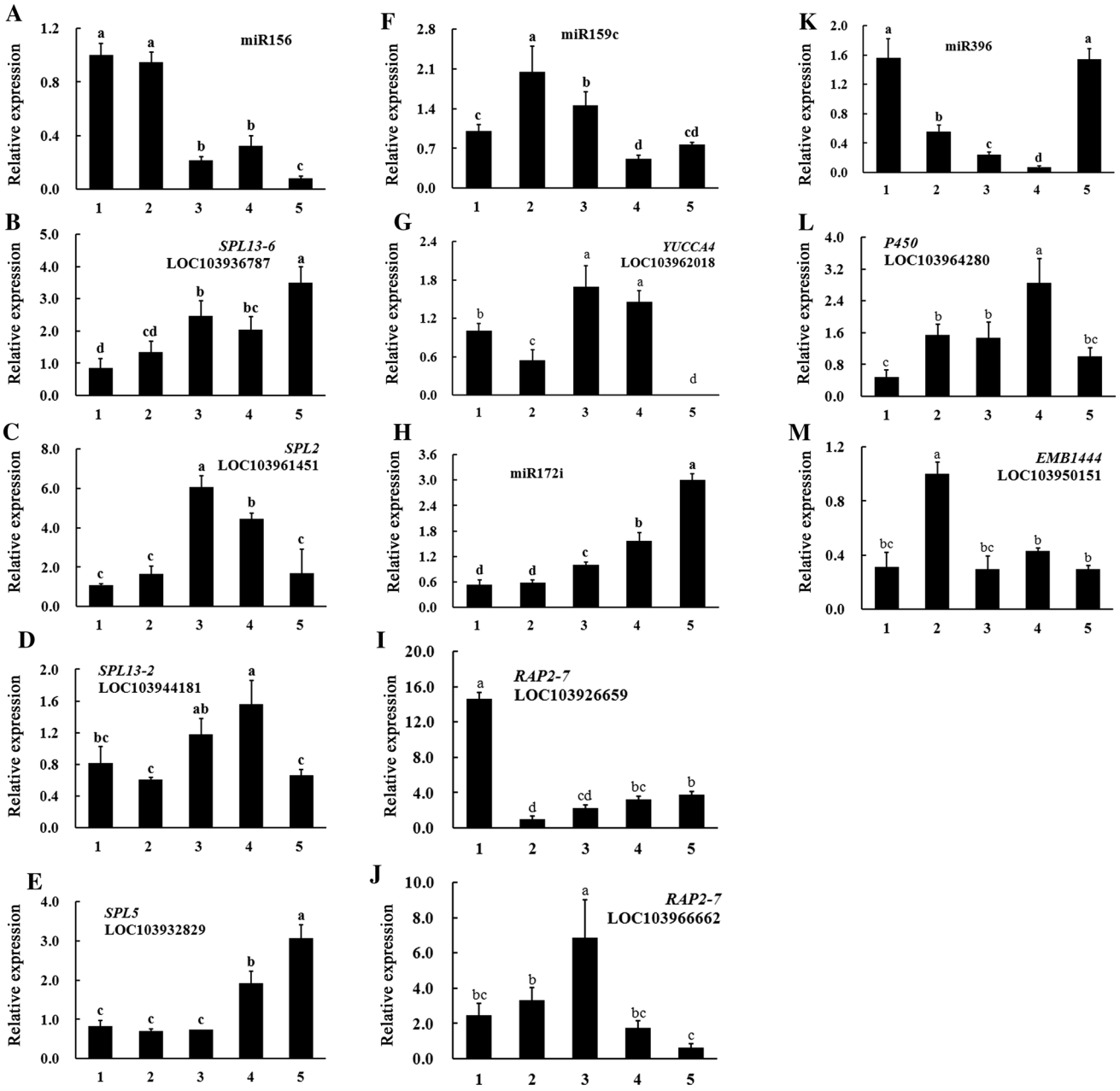


**Fig. 3** Expression patterns of differentially expressed miRNAs/targets in different ontogenetic phases. 1, 2, 3, 4, 5, represent different node spans (1, 1st–30th node; 2, 30th–60th node; 3, 60th–90th node; 4, 90th–120th node; and 5, above 120th node) of pear trees. All columns and Different lowercase letters represent mean ± standard error (SE) and significantly differences, which were determined by Duncan' s multiple range test ($P < 0.05$)

miR396, was high from span 2 to span 4 (Fig. 3L). Another miR396 target, *EMB1444-like* (LOC103950151), had a high expression at span 2 and then its expression decreased (Fig. 3M). In conclusion, a series of miRNAs and their targets displayed diverse expression patterns during ontogenic development, suggesting that they either accompanied the juvenile–adult phase transition or participated in regulating this process. Our results indicate that miR156x + p and the products of its target genes, SPL2, SPL13-2, SPL13-6, and SPL5, are key participants in juvenile–adult phase transition regulation in *Pyrus*.

### 3.3 Expression patterns of miR156x + p and targeted *SPL* genes under hormone treatments

A previous study showed that ABA and auxin might play positive roles and ethylene might play a negative role in

*Pyrus* juvenile–adult phase transition (Song et al. 2020). However, there have been no experiments to prove this. Therefore, we explored whether ABA, auxin and ethylene affect the expression of miR156x + p and its target *SPLs*. As shown in Fig. 4, 0.01 mg $L^{-1}$ ABA and ACC treatments had no obvious effects on the expression of miR156. However, under 0.1 and 1 mg $L^{-1}$ ABA, the expression levels of miR156 were lower than those in control plants. Interestingly, 0.1 and 1 mg $L^{-1}$ ACC resulted in higher and lower expression levels of miR156 compared with the control, respectively. Under the IAA treatment, the expression level of miR156 was lower than that in control plants regardless of concentration. *SPL13-6* displayed an opposite expression pattern compared with that of miR156 under ABA, ACC and IAA treatments, with higher expression levels under 0.1 and 1 mg $L^{-1}$ ABA, 1 mg $L^{-1}$ ACC, as well as all concentrations of IAA, and lower levels under 0.1 mg $L^{-1}$ ACC compared with levels in control plants. The expression levels of *SPL13-2* and *SPL2* were higher under 0.1 and 1 mg $L^{-1}$ ABA treatments than in control plants, which was opposite to the results of miR156. *SPL13-2* expression was also higher under 0.01 and 0.1 mg $L^{-1}$ ACC but not under 1 mg $L^{-1}$ ACC compared with expression in control plants. Regarding IAA treatment, the expression level of *SPL13-2* was higher under 0.1 mg $L^{-1}$ IAA than in control plants, and peaked under 1 mg $L^{-1}$ IAA. The 1 mg $L^{-1}$ ACC and 0.1 mg $L^{-1}$ IAA treatments resulted in high expression of *SPL2*. IAA treatment at 1 mg $L^{-1}$ resulted in higher expression of

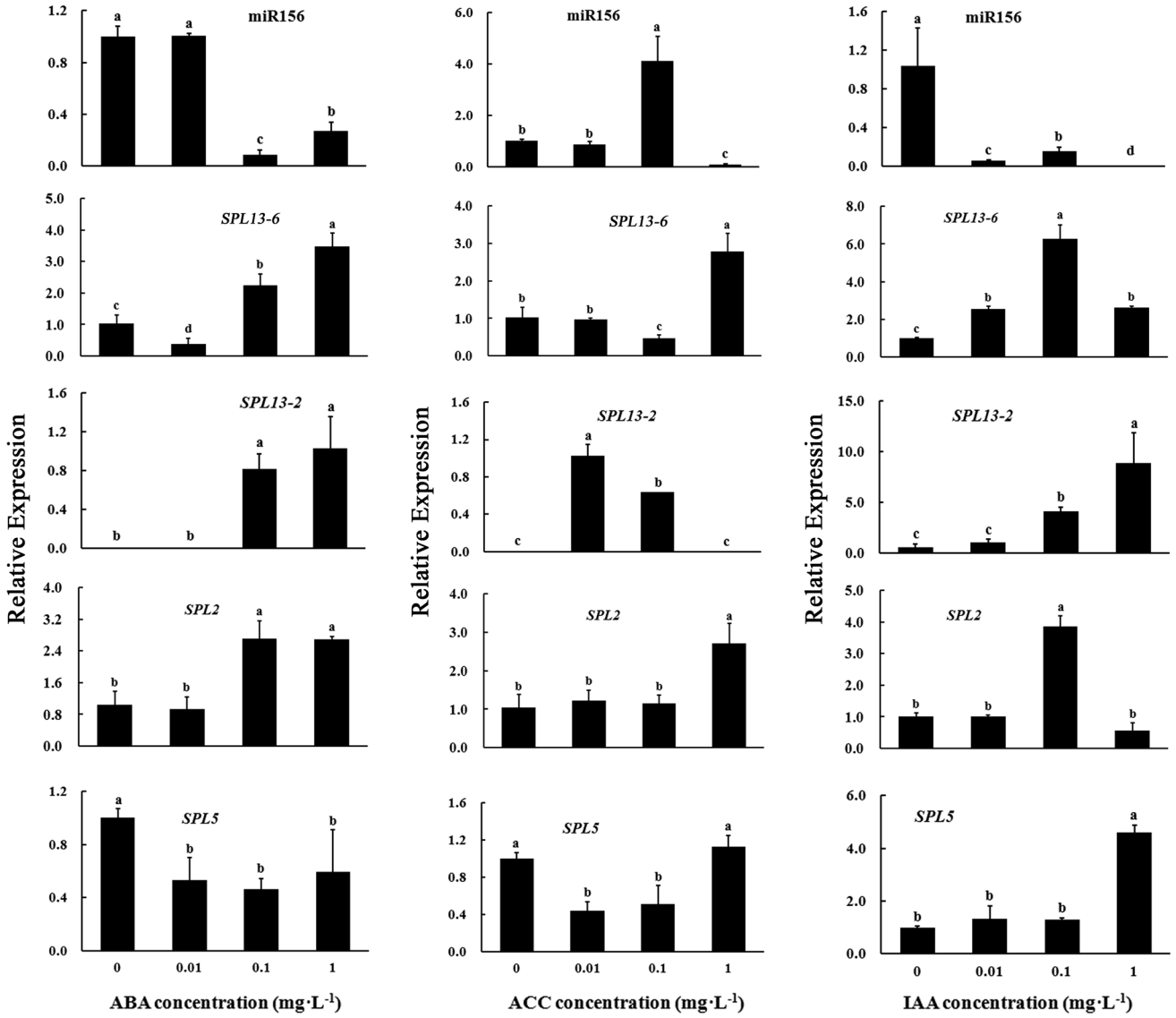


**Fig. 4** Expression patterns of miR156/*SPLs* under different concentrations of ABA, ACC, and IAA. Each column represents mean ± SE. Different lowercase letters indicate significant differences, which were determined by Duncan' s multiple range test ($P < 0.05$)

*SPL5* than that in control plants, whereas 0.01 and 0.1 mg $L^{-1}$ ABA and ACC resulted in lower expression of *SPL5* than that in control plants. These results suggest that ABA, ACC, and IAA affect the expression levels of *SPLs* through miR156x + p, especially that of *SPL13-6*, which display an opposite expression pattern compared with that of miR156 under all three hormone treatments.

### 3.4 Function analysis of SPL13-6 in Arabidopsis

*SPL13-6* was identified as a candidate gene and transferred into Arabidopsis. Two transgenic Arabidopsis lines were selected for further analysis. No expression of *SPL13-6* was detected in control plants. However, the expression levels of *SPL13-6* were high in transgenic lines (Fig. 5A). Transgenic plants bolted at about 15 d after germination, whereas the control plants bolted at about 21 d after germination. The transgenic plants flowered earlier than control plants (Fig. 5B). Moreover, the transgenic plants first produced abaxial trichomes on leaf 7.6 whereas the control plants first produced abaxial trichomes on leaf 10.2 (Fig. 5C). These results suggest that SPL13-6 plays a role in the juvenile–adult phase transition process.

## 4 Discussion

During plant growth and development, plants undergo a gradual and continuous transition from the juvenile to the adult to the reproductive phase. Phase transition, which affects breeding efficiency, is particularly important for perennial woody species, including fruit trees. For perennial woody plants, transition from the vegetative to the reproductive phase is easy to recognize and is commonly marked by flowering. Some studies have reported that morphological changes can be detected in the juvenile–adult phase transition, such as leaf shape and size, leaf arrangement, thorniness, and angles between lateral shoots and the central axis (Hackett 1985; James and Bell 2001; Moreno-Alías et al. 2009; Xing et al. 2014; Lawrence et al. 2021). However, these changes are species-specific and are not common morphological markers. For most fruit trees, the juvenile–adult phase transition is difficult to observe because it usually lacks visual changes. However, studies have concluded that in fruit tree, adult characteristics appear first in the periphery and upper portions of the seedling, whereas the interior portion, particularly the basal part of the stem, permanently remains in the juvenile stage; this area has been termed the juvenile zone (Muckadell 1954; Jay-Allemand et al. 1988; Amo-Marco et al. 1993). In our research, although the juvenile phase could not be identified accurately, we considered the basal and upper leaves to be juvenile and adult phase leaves, respectively, and used these to screen differentially expressed mRNAs (Song et al. 2020) and miRNAs. Then, we further verified the expression patterns of related miRNAs and genes throughout the ontogenetic development of *Pyrus* by taking samples at five continuous node spans from the bottom to the top of the plants. We consider this method appropriate to screen miRNAs or mRNAs participating in the juvenile–adult phase transition.

Previous studies have shown that miR156 and its target genes play important roles in the process of phase transition (Fornara and Coupland 2009). The functions of miR156 and its target *SPL* genes in phase transition have also been verified in some perennial woody plants. For example, in *Malus*, the expression of miR156 in adult tissues was significantly lower than that in juvenile tissues (Jia et al., 2017; Xu et al., 2017). Ectopic expression of apple miR156h in Arabidopsis delayed flowering time through reducing the expression of *AtSPL9* and *AtSPL15* (Sun et al. 2013). *Paeonia delavayi PdSPL9* and grapevine *VpSBP11* can shorten juvenility and promote flowering time in transgenic Arabidopsis (Hou

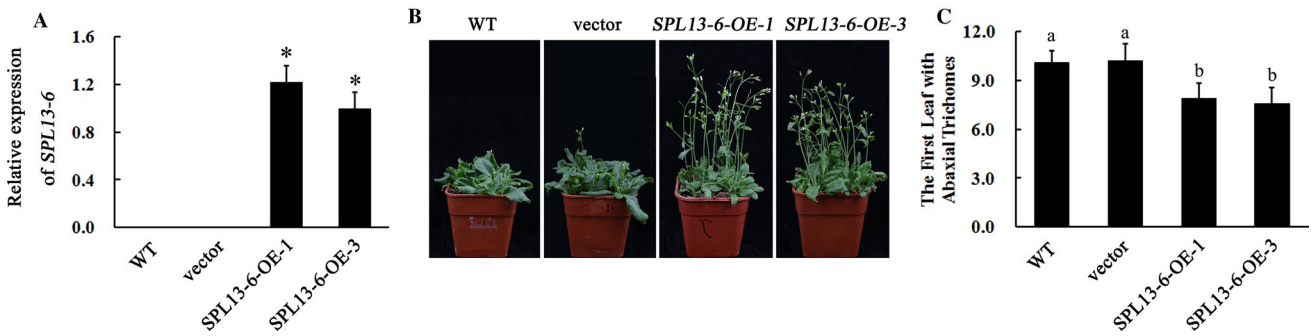


**Fig. 5** Function of SPL13-6 in Arabidopsis. a. Relative expression level of *SPL13-6* in the wild type (WT; control), vector, and two transgenic lines, *SPL13-6-OE-1* and *SPL13-6-OE-3*. b. Phenotypes of 30-day-old WT, vector, *SPL13-6-OE-1* and *SPL13-6-OE-3* plants. c. The first leaf with abaxial trichomes in WT, vector, *SPL13-6-OE-1* and *SPL13-6-OE-3* plants. Each column represents mean ± standard error (SE). Asterisks (*) and different lowercase letters represent significant differences, which were determined by Duncan' s multiple range test ($P < 0.05$)

et al. 2017; Zhu et al. 2018). In *Populus tremula* × *Populus alba*,, the differences between adult and juvenile leaves were analyzed by over- and under-expressing miR156 (Lawrence et al. 2021). In our research, we identified two differentially expressed miR156s, miR156x and miR156p, participating in the juvenile–adult phase transition process. Combining target prediction analyses and transcriptome data of the juvenile–adult phase transition, we screened *SPL2*, *SPL13-2*, *SPL13-6*, and *SPL5* as the target genes of miR156. According to qPCR analysis, the expression of miR156 decreased during the phase transition process whereas that of the four *SPLs* increased (Fig. 3A-E), indicating that the miR156/*SPL* module is an evolutionarily conserved regulator of the juvenile–adult phase transition in perennial *Pyrus* trees. These results are in accordance with those of Wang et al. (2011).

Plant hormones play important roles in the regulation of plant growth and development (Santner and Estelle 2009; Wolters and Jürgens 2009). In *Pyrus*, hormone contents differ between the juvenile and adult phases (Song et al. 2020). However, the functions of hormones in the juvenile–phase transition process are still unknown. It is possible that these hormone content differences just accompany the phase transition process. Therefore, to clarify whether hormones play roles in the juvenile–adult phase transition process, we further analyzed the expression patterns of miR156x + p and the four target *SPLs* under different ABA, ACC and IAA treatments. We found that ABA and IAA inhibited the expression of miR156. Low and high concentrations of ACC increased and decreased the expression of miR156, respectively. Most of the *SPLs*, particularly *SPL13-6*, displayed an opposite expression pattern compared with that of miR156 under the hormone treatments. These results indicate that ABA and IAA play positive roles in the juvenile–adult phase transition process, and that ethylene might participate in this process in a more complex way. ABA and ethylene are both regarded as stress related hormones. Many plant species belonging to a wide range of taxonomic groups can be induced to flower by responding to stress factors. For example, SA plays a role in stress-induced flowering (Wada and Takeno 2010). Therefore, we postulate that stress factors affect the juvenile–adult phase transition through ABA or ethylene.

We also analyzed the function of SPL13-6 through heterologous transformation of Arabidopsis. *SPL13-6* is homologous with Arabisopsis *SPL13*, which is a single member of one clade based on the amino acid sequence of their conserved DNA binding domain (Preston and Hileman 2013). In Arabidopsis, *SPL13* promotes both vegetative phase change and floral induction (Xu et al. 2016). In our results, transgenic Arabidopsis overexpressing *SPL13-6* displayed earlier abaxial trichome production and flowering than control (wild-type) plants, suggesting that *Pyrus* SPL13-6 plays a role in the juvenile–adult phase transition and floral induction.

In addition, among the differentially expressed miRNAs between juvenile and adult phases, miR172i, miR396, miR159c, and a series of novel miRNAs were also identified. In Arabidopsis, miR172 and miR159 participate in phase transition. The expression levels of miR172 mirror those of *SPLs*, as miR172 is induced by the SPL protein. Moreover, miR172 positively regulates flowering time through targeting the *APETALA2* (*AP2*)*-like* genes, which repress the expression of flowering related genes, including *FT*, *SOC1*, *FRUITFUL* (*FUL*) as well as *APETALA1* (Zhang and Chen 2021). In *Malus*, the expression levels of miR172 members were significantly higher in adult tissues than in juvenile tissues, and the targets of miR172, *AP2* and *AP2-like* genes, displayed higher expression levels in juvenile tissues than in adult tissues (Xing et al. 2014). In our research, the expression of miR172i was up-regulated in the adult phase, implying that it was also active in adult stage maintenance. Two ethylene-responsive transcription factor *RAP2-7-like* genes, which were predicted as the targets of miR172i, were down-regulated in the adult phase. Through qPCR analysis in the ontogenesis development process, we observed that the expression patterns of miR172i and one *RAP2-7-like* gene (LOC103926659) were opposite (Fig. 3H, I). Therefore, miR172i and the RAP2-7-like protein might play roles in phase transition regulation in *Pyrus*; however, this needs further research. In Arabidopsis, miR159 controls the timing of the juvenile–adult phase change by repressing *MYB33*, the product of which in turn promotes the expression of miR156 directly or through *ABI5* (Guo et al. 2017, 2021). In our research, the expression level of miR159c was lower in the adult than in the juvenile phase, which was similar to the expression pattern of miR156. In *Malus*, miR159 showed no significant differences in expression levels between the adult and juvenile phases (Xing et al. 2014). Therefore, it is unclear whether miR159 participates in the juvenile–adult phase transition in *Pyrus*; this requires further research. The function of miR159 might be different in annual and perennial woody plants. Meanwhile, during the ontogenetic development process in *Pyrus*, the expression of miR396 decreased from span 2 to 4, and was then higher at span 5 (Fig. 3K). Its target gene, *P450* (LOC103964280), displayed the opposite expression pattern (Fig. 3L). Cytochrome P450 regulates phase transitions in leaf and reproductive development in Arabidopsis (Sotelo-Silveira et al. 2013). Therefore, we speculated that during the *Pyrus* ontogenetic development process, miR396 might participate in leaf and reproductive development regulation through *P450*. Meanwhile, 92 novel miRNAs were identified, which suggested that during the juvenile–adult phase transition process, there might still be some unknown physiological changes or regulatory mechanisms.

## 5 Conclusion

We analyzed the expression of miR156x + p and its target *SPLs* during ontogenesis development and under hormone treatments, as well as the function of SPL13-6. Our results indicate that the miR156x + p/*SPL13-6* module responds to ABA, IAA, and ethylene treatment, and that SPL13-6 participates in the juvenile–adult phase transition in *Pyrus*.

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**Author contribution** MS, AL, and YY performed the experiments and wrote the article. LS, ZW, RW, and CZ helped with experiments. RW, JS, and DL modified the article.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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