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ORIGINAL ARTICLE

Overexpression of herbaceous peony miR156e-3p improves anthocyanin accumulation in transgenic *Arabidopsis thaliana* lateral branches

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Abstract microRNAs (miRNAs) play critical regulatory roles in plant growth and development. In the present study, the function of herbaceous peony (*Paeonia lactiflora* Pall.) miR156e-3p in the regulation of color formation has been investigated. Firstly, *P. lactiflora* miR156e-3p precursor sequence (pre-miR156e-3p) was isolated. Subsequently, the overexpression vector of pre-miR156e-3p was constructed and transformed into *Arabidopsis thaliana*. Moreover, the medium screening, GUS staining, polymerase chain reaction (PCR) of the GUS region and real-time quantitative PCR (qRT-PCR) of miR156e-3p all confirmed that the purpose gene had been successfully transferred into *Arabidopsis* plants and expressed, which resulted in apparent purple lateral branches. And this change in color was caused by the improved anthocyanin accumulation. In addition, expression analysis had shown that the level of miR156e-3p transcript was increased, while transcription level of target gene squamosa promoter binding protein-like gene (*SPL1*), encoding *SPL* transcription factor that negatively regulated anthocyanin accumulation, was repressed in miR156e-3p-overexpressing transgenic plants, and its downstream gene dihydroflavonol 4-reductase gene (*DFR*) that was directly involved in anthocyanin biosynthesis was strongly expressed, which resulted in anthocyanin accumulation of *Arabidopsis* lateral branches. These findings would improve the understanding of miRNAs regulation of color formation in *P. lactiflora*.

Keywords *Paeonia lactiflora* · miR156 · Lateral branch · Anthocyanin accumulation

Introduction

microRNAs (miRNAs) are non-coding small RNAs (sRNAs) with approximately 21-nucleotides (nt) length, which are widely distributed in animals and plants and negatively regulate gene expression by either mRNA degradation or translation inhibition (Rogers and Chen 2013). Since they are discovered in *Caenorhabditis elegans* firstly (Lee et al. 1993), a mass of miRNAs in higher plants have been continually identified, including *Arabidopsis thaliana* (Sunkar and Zhu 2004), *Oryza sativa* (Jian et al. 2010), *Triticum aestivum* (Yao et al. 2007), *Zea mays* (Zhang et al. 2006) and so on. And the latest version of miRBase 21.0 until 2014 includes 28,645 entries hairpin precursor miRNAs (pre-miRNAs), expressing 35,828 mature miRNA products in 223 species (Kozomara and Griffiths-Jones 2014). miRNAs have an important regulatory role in plant growth and development, including root, stem, leaf, flower and fruit growth and development (Hu et al. 2014; Spanudakis and Jackson 2014; Li et al. 2010; Wang et al. 2010a, b). However, to the best of our knowledge, in color formation, only miR156, miR828, miR858 and miR778 have been reported to play important roles in anthocyanin accumulation (Shen 2015; Wang et al. 2015; Yang et al. 2013; Gou et al. 2011); miR1857 plays a major role in carotenoid accumulation (Xu et al. 2010), and miR826 and miR5090 have vital roles in chlorophyll

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accumulation (He et al. 2014), but their mechanisms are different.

Herbaceous peony (*Paeonia lactiflora* Pall.) is a traditional flower in China that belongs to the Paeoniaceae family. Because of its straight stem, elegant flower shape, rich color and beautiful appearance, *P. lactiflora* is loved by people at home and abroad. As far as the flower color is concerned, it can be divided into nine color categories, including black, purple, red, pink, white, blue, green, yellow and double color (Wang and Zhang 2005). In addition, in *P. lactiflora* flower coloration, numerous studies have examined chemical constituents, flavonoids including anthocyanins and multiform glycosides of flavones and flavonols determine its color (Zhao et al. 2014, 2016). Furthermore, many key genes regulating *P. lactiflora* color formation are observed on the transcription level, for example, the dihydroflavonol 4-reductase gene (*PlDFR*) and anthocyanidin synthase gene (*PlANS*) result in the shift from white to pink and red in *P. lactiflora* flowers (Zhao et al. 2016). And on post-transcriptional level, only our previous study reported that the yellow formation might be under the regulation of an miR156e-3p-targeted squamosa promoter binding protein-like gene (*SPL1*) (Zhao et al. 2017), but this result has only been obtained according to their dynamic expression patterns. However, there is no more direct evidence that *P. lactiflora* miR156e-3p regulates color formation. To validate the miR156e-3p regulation mechanism of the color formation in *P. lactiflora*, the miR156e-3p precursor sequence (pre-miR156e-3p) was isolated, and an ectopic expression analysis of its function in *Arabidopsis* was also conducted. These results would improve the understanding of miRNAs regulation of *P. lactiflora* color formation.

Materials and methods

Plant materials

Paeonia lactiflora cv 'Jinhui' was grown in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32°30′N, 119°25′E), and the ground plants grew well with sufficient light and water supply. The young petal samples in initiating bloom stage were used for the isolation of pre-miR156e-3p. Seeds from transgenic and wild-type *Arabidopsis* (Col-0) sown in half-strength Murashige-Skoog (MS) medium were maintained at 4 °C for 3 days in the dark to break residual dormancy; the germinated seedlings were then grown in a culture room at 22 °C under a 14/10 h light/dark photoperiod and 70% relative humidity. All samples were immediately frozen in liquid nitrogen and stored at − 80 °C until further analysis.

Isolation of pre-miR156e-3p

Small RNA was extracted by TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan), and the cDNA was, respectively, synthesized using PrimeScript® RT reagent Kit With gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). pre-miR156e-3p was isolated by polymerase chain reaction (PCR) technology with specific primers (forward primer: 5′-CGAAGAAGAGAAAGAAATGTTGAC-3′, reverse primer: 5′-ACCCTTTAGCTGATCCCGGGTTG TG-3′). PCR products were separated by 1% agarose gel electrophoresis and sequenced.

Vector construction, transformation and transgenic plants identification

pre-miR156e-3p was amplified by PCR and using primers with restriction enzyme cutting sites of BamH I and Kpn I (forward primer: 5′-CGCGGATCCACGAAGAAGA-GAAAGAAATGTTGAC-3′, reverse primer: 5′-CGGGGTACCACCCTTTAGCTGATCCCGGGTTGTG-3′). After double enzymes restriction, pre-miR156e-3p was inserted into the pCAMBIA1301 vector (p1301) by T4 DNA Ligase (TaKaRa, Japan). Luria–Bertani (LB) solid medium containing 0.05% kanamycin (Kan) was used to cultivate *Escherichia coli* DH5a cells (Trans, China), and the plasmid extracted from the positive monoclonal coliform bacteria strains was used for double digestion with the restriction enzymes. Additionally, the final binary vector pCAMBIA1301-Pre-miR156e-3p was transferred into *Agrobacterium tumefaciens* (EHA105) cells using the freezing/heat shock method. And the transformation of *Arabidopsis* was performed by the floral dip method using EHA105 cells (Clough and Bent 1998). Seeds from transgenic *Arabidopsis* and Col-0 were harvested from individual plant and sown again.

To identify transgenic plants, the screening medium [1/2 MS + 30 g/L sucrose + 6.5 g/L agar + 25 mg/L ampicillin (Amp) + 25 mg/L hygromycin (Hyg), pH 5.8] and GUS staining method were used to screen transgenic *Arabidopsis*, and when the screening transgenic *Arabidopsis* grew to the bolting stage, leaves were collected for GUS region amplification (forward primer: 5′-CTGCGTTTCGATGCGGTCAC-3′, reverse primer: 5′-CTCCCTGCTGCGGTTTTTCA-3′).

Qualitative and quantitative analysis of flavonoids

The lateral branches of each plant (0.1 g fresh weight) were extracted with 1.0 mL of acidic methanol solution (70: 0.1: 29.9; v/v/v, CH_3OH: HCl: H_2O) at 4 °C for 24 h. Qualitative and quantitative analysis of flavonoids was performed using high-performance liquid chromatography–electrospray

Table 1 Gene-specific primers used in qRT-PCR analysis for transgenic *Arabidopsis* plants

Gene	Genbank number	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	T_m (°C)
miR156e-3p	–	CGCGCTCTCTCTTCGTCT	GTGCAGGGTCCGAGGT	56
SPL1	NM_180137	ATTCAGGAAACAACACACGA	TTCAACCACTAAGAAAGGGA	56
DFR	NM_123645	GTTAGCGGAGAAAGCAGCGT	CCGAGTGATAGGAGAGAGCG	63
Actin	NM_112764	TCTCCCGCTATGTATGTCGC	TAAGGTCACGTCCAGCAAGG	62

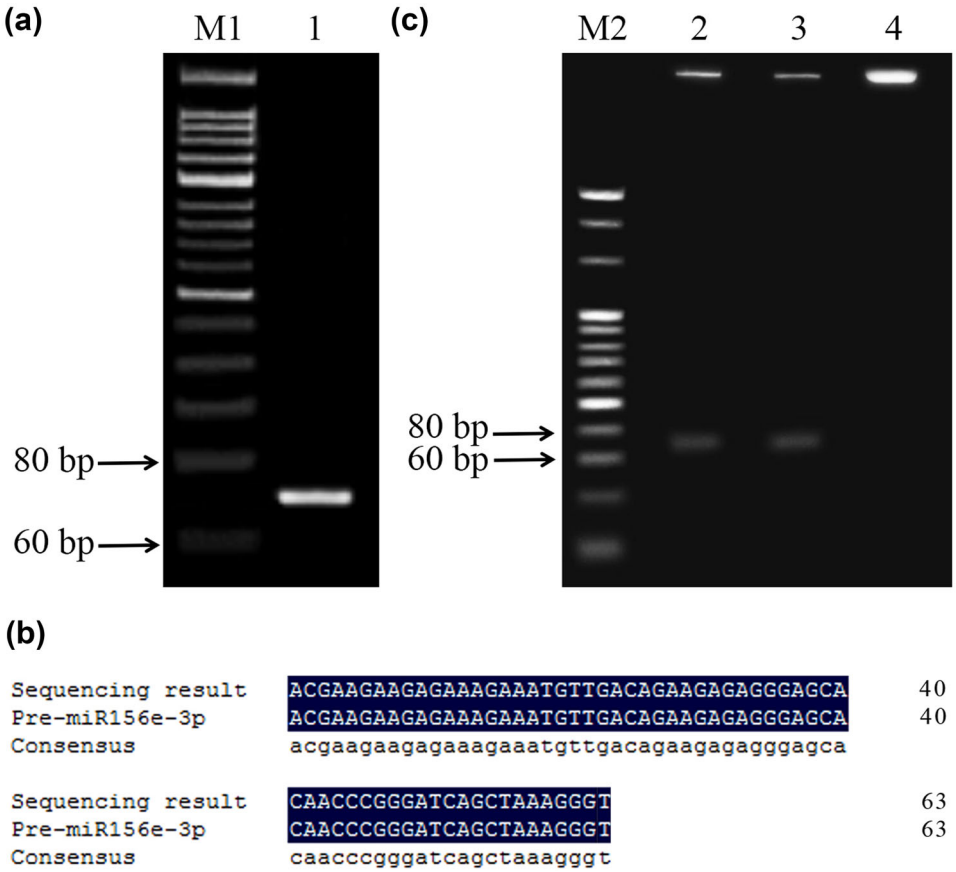

Fig. 1 PCR amplification of miR156e-3p precursor and vector construction. **a** PCR amplification of miR156e-3p precursor. **b** Sequence comparison of miR156e-3p precursor. **c** Double digestion of the recombinant plasmid miR156e-3p. M1: 50 bp DNA Ladder Marker (Dye Plus). 1: PCR product of miR156e-3p precursor. M2: 20 bp DNA Ladder Marker. 2, 3: Double digestion of the recombinant plasmid. 4: negative control

ionization–ion trap mass spectrometry (HPLC–ESI–MS^n) (LCQ Deca XP MAX, Thermo) coupled with photodiode array and mass spectrometry detectors (HPLC–PDA–MS, Thermo company) with a three-dimensional quadrupole ion trap mass spectrometer. The HPLC column was TSK gel ODS-80Ts QA (4.6 × 250 mm) (Tosoh, Japan). The specific conditions were the same as the report of Zhao et al. (2014). Each peak area of anthocyanins and anthoxanthins detected under 525 and 350 nm was recored. The amount of total anthocyanins and anthoxanthins was calculated in milligrams per gram fresh weight (as a quantity of Malvidin-3,5-di-*O*-glucoside (Mv3G5G) mg/g and as a quantity of Rutin mg/g, respectively). Additionally, total contents of flavonoids were the sum of anthocyanins and anthoxanthins.

Gene expression analysis

The transcript levels of genes were analyzed using real-time quantitative PCR (qRT-PCR) with a BIO-RAD CFX96™ Real-Time System (Bio-Rad, USA). Total RNA and small RNA were extracted according to TaKaRa RNAiso Plus (Total RNA extraction) and TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan), respectively. Subsequently, the cDNA was, respectively, synthesized using PrimeScript® RT reagent Kit With gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) and miRNA First Strand cDNA Synthesis Kit with the specific primer of miR156e-3p for inverse transcription (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT

GGATACGACGATGAC-3′) (Sangon Biotech, China). All gene-specific primers for qRT-PCR are shown in Table 1. qRT-PCR was performed using the TransStart® Tip Green qPCR SuperMix (Trans, Chin8ia), and the relative expression levels were calculated by the $2^{-\triangle\triangle Ct}$ comparative threshold cycle (Ct) method (Schmittgen and Livak 2008), and the expression level of Col-0 was used as the control.

Sequence and statistical analysis

Sequence analysis was performed by DNAMAN 5.0 software. All data were average values of three replicates at least with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA).

Results

In our previous study, 'jinhui' was used for the small RNA sequence to identify miRNAs related to yellow formation (Accession numbers in NCBI: SRX1996909 and SRX1996992), and miR156e-3p was screened as the key miRNA, its sequence and precursor sequence were 21 bp (5′-GCTCTCTCTTCGTCTGTCATC-3′) and 63 bp (5′-ACGAAGAAGAGAAAGAAATGTTGACAGAAGAGAGGGAGCACAACCCGGGATCAGCTAAAGGGT-3′), respectively. Based on this, Pre-miR156e-3p was isolated, and an approximately 70-bp band was obtained by PCR technology with specific primers (Fig. 1a). This band was later extracted and sent to be sequenced, and the result showed that this band was 63 bp and exhibited 100% similarity with the small RNA sequencing result (Fig. 1b). Subsequently, the extracted product of Pre-miR156e-3p was cloned into vector p1301 and transformed into competent *E. coli* DH5a cells, and the screening positive transformants were later identified by double digestion with the restriction enzymes. Figure 1c shows that the successful construction of the recombinant plasmid had been cut into an approximately 70-bp band and a more than 15,000-bp band, but the negative control only had a more than 15,000-bp band. The constructed vector diagram of pCAMBIA1301-Pre-miR156e-3p is shown in Supplemental Fig. 1, and this vector was used for subsequent

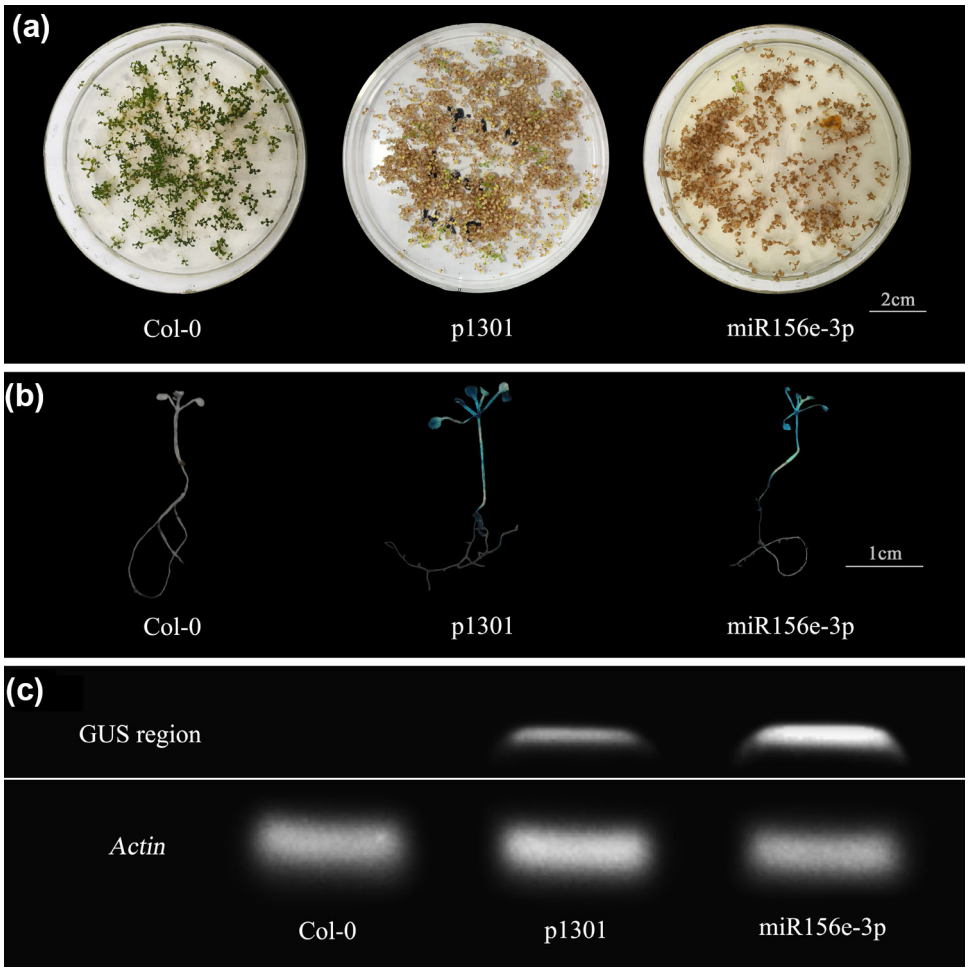

Fig. 2 Identification of transgenic *Arabidopsis* plants. **a** Medium screening. **b** GUS staining. **c** PCR identification of GUS region

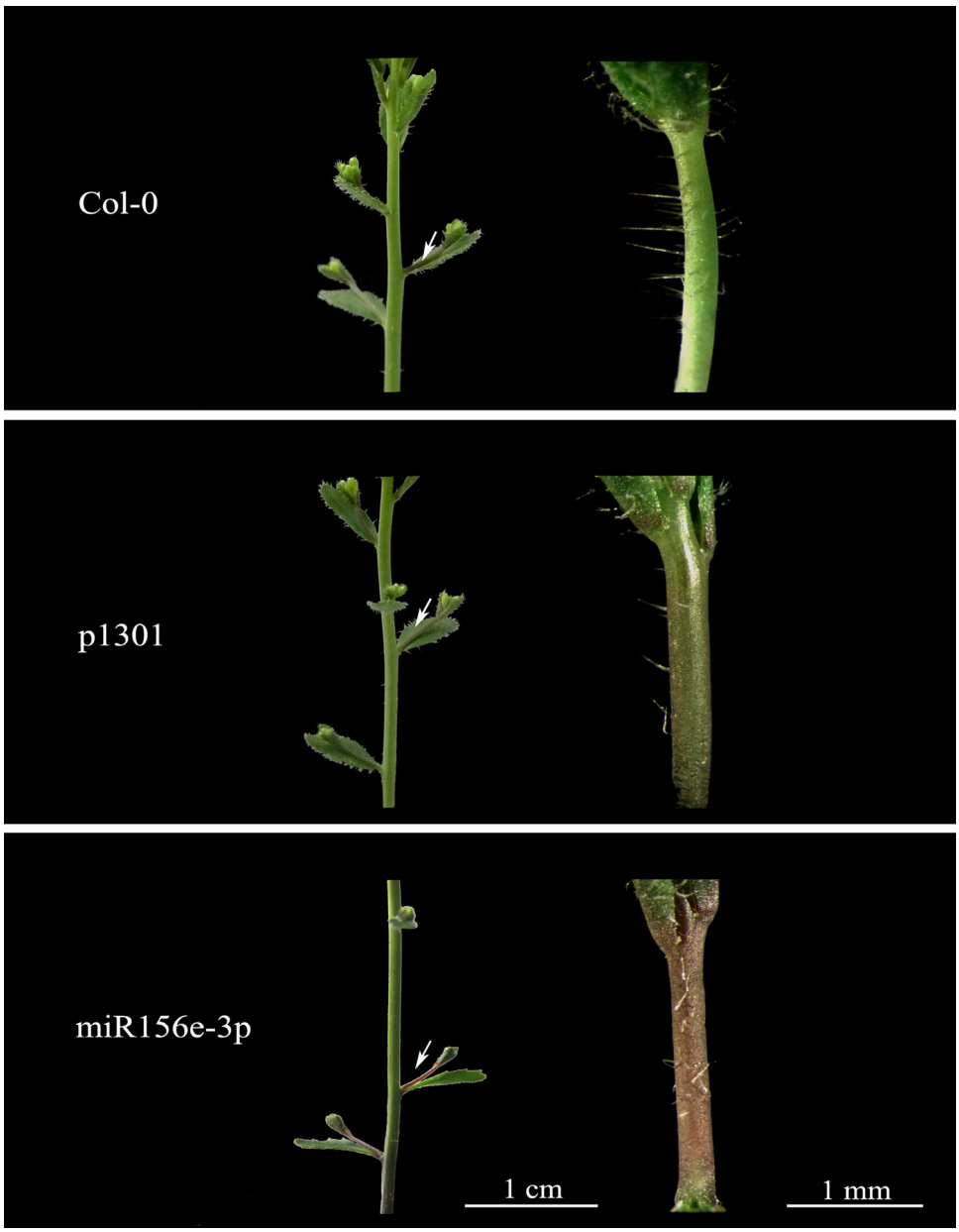

Fig. 3 Phenotype of transgenic *Arabidopsis* lateral branches

Agrobacterium tumefaciens-mediated transformation of *Arabidopsis* plants.

To identify transgenic *Arabidopsis* plants, the harvested seeds were firstly sowed on the screening medium with antibiotics. As shown in Fig. 2a, Col-0 plants grew well in the medium without antibiotics, and the successful miR156e-3p-overexpressing transgenic plants and p1301 transgenic plants all retained robust growth and were green, while non-transgenic plants showed yellowing and death. Furthermore, the identified transgenic plants according to the screening medium were randomly selected for GUS staining. As shown in Fig. 2b, Col-0 plants remained white, and the successful miR156e-3p-overexpressing transgenic plants and p1301 transgenic plants all turned blue in the GUS staining buffer. In addition, when screening transgenic plants grew at the bolting stage, DNA extracted from the leaves was used as the template to amplify the GUS region of the p1301 vector, and the result showed that one clear and bright band could be observed in miR156e-3p-overexpressing transgenic plants and p1301 transgenic plants, whereas there was no specific band in the Col-0 plants (Fig. 2c), which was consistent with the GUS staining result.

When *Arabidopsis* plants grew to the bolting stage, there were no obvious differences between miR156e-3p-overexpressing transgenic plants, p1301 transgenic plants and Col-0 plants with respect to morphology, but the color of their lateral branches had dramatically changed. As shown

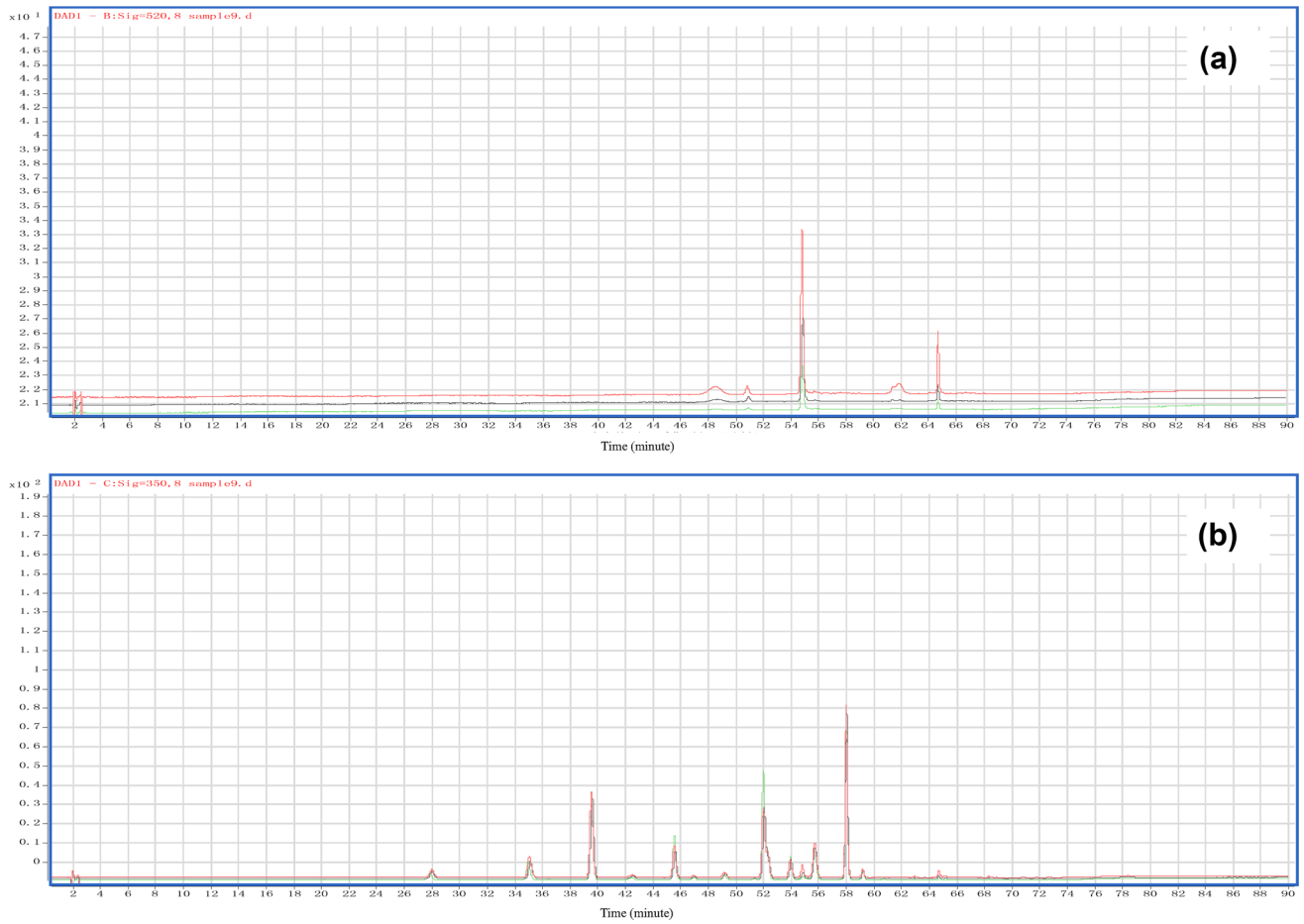

Fig. 4 HPLC chromatograms of anthocyanins (detected at 525 nm) and anthoxanthins (detected at 350 nm) in transgenic *Arabidopsis* lateral branches. **a** Anthocyanin content. **b** Anthoxanthin content. Green line: HPLC chromatograms of Col-0 plants. Black line: HPLC chromatograms of p1301 transgenic plants. Red line: HPLC chromatograms of miR156e-3p-overexpressing transgenic plants

in Fig. 3, the lateral branches of miR156e-3p-overexpressing transgenic plants had an apparent purple, whereas Col-0 plants were still green, and p1301 transgenic plants were slightly red. Subsequently, qualitative and quantitative analysis of flavonoids in the lateral branches was performed using HPLC–ESI–MS^n. On the basis of the ultraviolet–visible absorption characteristics, anthocyanins and anthoxanthins were detected under the wavelength of 525 and 350 nm, respectively. As shown in Fig. 4, there were several differences in the chromatographic peaks of miR156e-3p-overexpressing transgenic plants, p1301 transgenic plants and Col-0 plants. At 525 nm, a very small amount of anthocyanin was found in Col-0 plants and p1301 transgenic plants, whereas there were obvious peaks in miR156e-3p-overexpressing transgenic plants. At 350 nm, the main peaks were largely identical in the three plants and only several minor differences existed. The statistic on the flavonoid content indicated that the highest anthocyanin content was in miR156e-3p-overexpressing transgenic plants, which was approximately 24 times that of the p1301 transgenic plants, and it was largely undetected in Col-0 plants (Fig. 5). However, when anthoxanthin and flavonoid contents were concerned, the tree plants had similar contents, which were below the very significant levels.

To identify the expression pattern of miR156e-3p in *Arabidopsis* lateral branches, the expression analysis of miR156e-3p was first performed using qRT-PCR (Fig. 6). The results revealed that the miR156e-3p transcript was detected in all the plants, but the expression levels were different from each other. Its expression level in miR156e-3p-overexpressing transgenic plants was very significantly higher than that of the Col-0 plants and p1301 transgenic plants, and the former was approximately 2.6 times that of the latter two. Based on this information and to further clarify the role of miR156e-3p in the color formation of *Arabidopsis* lateral branches, the expression pattern of miR156e-3p corresponding target gene was subsequently studied. In a previous study, Cui et al. (2014) reported that the *miR156-SPL9-DFR* pathway coordinated anthocyanin

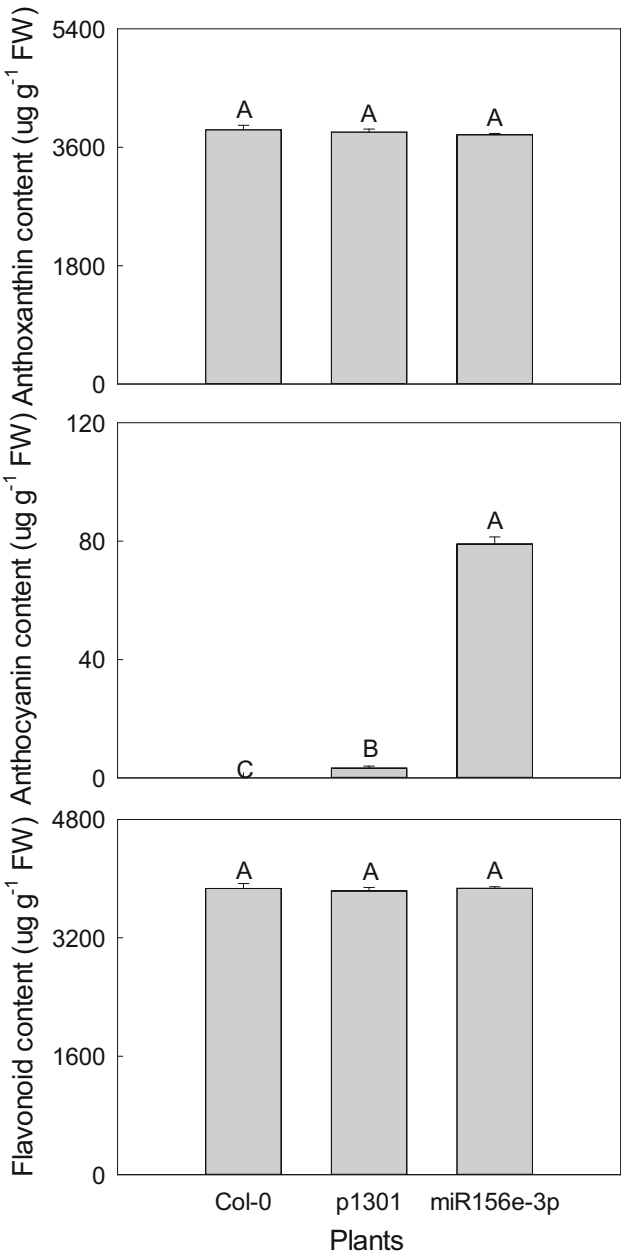

Fig. 5 Flavonoid content of transgenic *Arabidopsis* lateral branches

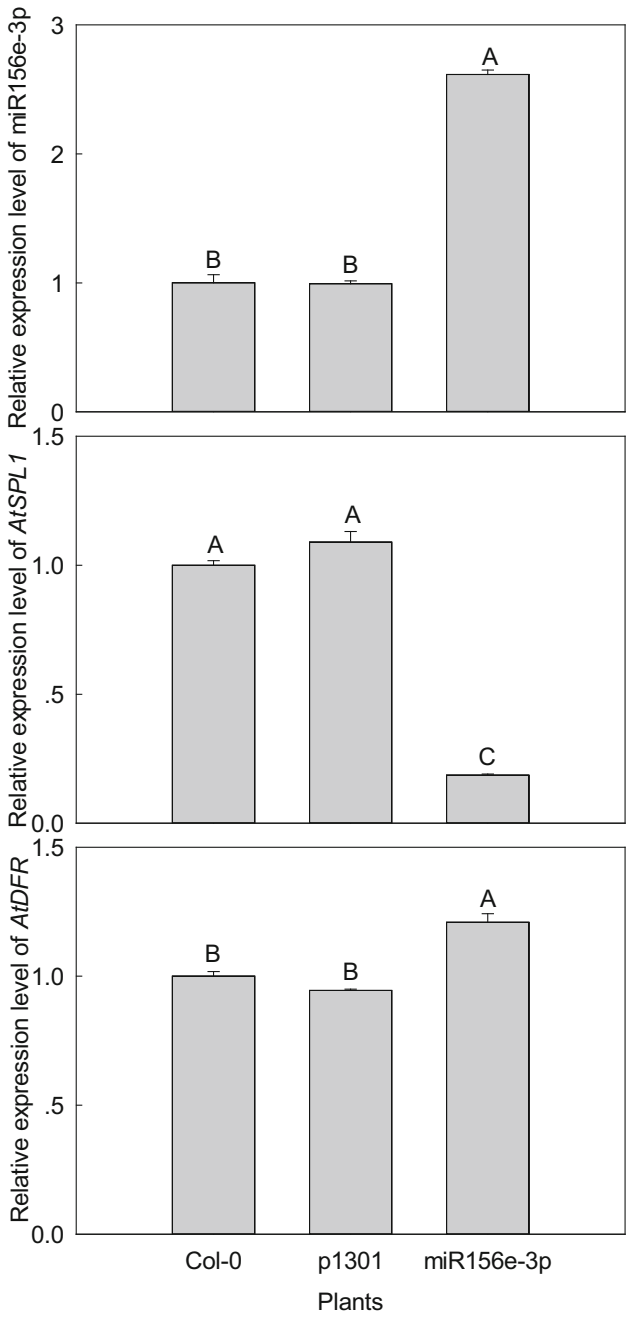

Fig. 6 Expression patterns of miR156e-3p and related genes in transgenic *Arabidopsis* lateral branches

accumulation in *Arabidopsis*. We found an *AtSPL1* (NM_180137) in *Arabidopsis*, which had the highest homology with miR156e-3p corresponding target gene *PlSPL1*, and only an *AtDFR* (NM_123645) in *Arabidopsis*. Regarding the single gene expression level, the expression level of *AtSPL1* in miR156e-3p-overexpressing transgenic plants was very significantly lower than that of the Col-0 plants and p1301 transgenic plants, whereas the expression level of *AtDFR* presented an opposite tendency. Additionally, the expression levels of miR156, *AtSPL1* and *AtDFR* in Col-0 plants and p1301 transgenic plants were largely the same, and there were no very significant differences between them.

Discussion

Regulation of plant color formation by miRNAs has been partially reported in a number of studies. Overexpression of miR828 could reduce anthocyanin accumulation in

Arabidopsis (Yang et al. 2013). miR858 up-regulated the expression of target genes *MYB12* and *Sly-myb-like* in transgenic tomato plants, and flavonoid biosynthetic genes expressions were also up-regulated, which resulted in anthocyanin accumulation (Shen 2015). Overexpression of miR778 moderately enhanced anthocyanin accumulation under Pi deficient conditions (Wang et al. 2015). In addition, miR156 regulating plant color formation had been deeply studied (Gou et al. 2011). miR156 was first observed in *Arabidopsis* with 20 nucleotides in length, and it was considered to be the most conservative miRNA and existed in monocotyledons, dicotyledons, ferns and mosses (Franco-Zorrilla et al. 2007). A previous study revealed that *SPL* family was the target gene of miR156 (Rhoades et al. 2002), 11 of 17 *SPL* in *Arabidopsis* were putative targets of miR156 and 11 of 19 *SPL* in *O. sativa* were putative targets of miR156, and these results all indicated that miR156 played a crucial role in *SPL* family expression and regulation (Chen et al. 2015; Schwab et al. 2005). In our previous study, miR156e-3p was screened as the key miRNA related to color formation in *P. lactiflora*, and its target gene was *SPL1* (Zhao et al. 2017). In addition, an increasing number of studies suggested that the miR156 and *SPL* family were involved in many processes of growth and development, metabolism regulation, and abiotic stresses, and they had been become the regulatory hub of growth and development of plants (Lei and Liu 2016). In the present study, pre-miR156e-3p was isolated and introduced into transgenic *Arabidopsis* to validate that the color formation was under the regulation of miR156e-3p-targeted *SPL1*. This method had not only been widely used in model plants, including *Arabidopsis*, *Nicotiana tabacum* and *O. sativa*, but had also been successfully applied in *Brassica campestris* (Jiang 2014) and *Lycopersicon esculentum* (Jia et al. 2013). Subsequently, the medium screening, GUS staining, PCR of the GUS region and qRT-PCR of miR156e-3p all confirmed that the purpose gene had been successfully transferred into *Arabidopsis* plants and expressed, which indicated that this method could also be used to verify the function of *P. lactiflora* miRNAs.

In the anthocyanin accumulation, Gou et al. (2011) constructed overexpression and silencing expression vectors (Pro35S:MIR156, Pro35S:MIM156) to study the miR156 function in *Arabidopsis* and observed that increased miR156 activity promoted anthocyanin accumulation, whereas reduced miR156 activity resulted in high levels of flavonols. Our study obtained similar results, i.e., miR156e-3p-overexpressing transgenic plants resulted in apparent purple lateral branches, which were caused by the accumulation of anthocyanins, and anthocyanins were largely undetected in Col-0 plants. Moreover, Cui et al. (2014) found that the anthocyanin accumulation was under the regulation of miR156-targeted *SPL*, and *SPL* negatively regulated anthocyanin accumulation by directly preventing the expression of *DFR*. In this study, miR156e-3p was expressed most heavily in miR156e-3p-overexpressing transgenic plants, and the expression level of its target gene *SPL1* was clearly decreased, and its downstream gene *DFR* was strongly expressed, which resulted in increased anthocyanin accumulation of lateral branches, and this result was consistent with a previous study (Cui et al. 2014). These findings would improve the understanding of the miRNAs regulation of color formation in *P. lactiflora*.

Conclusion

Overexpression of *P. lactiflora* miR156e-3p changed the color of transgenic *Arabidopsis* lateral branches to purple. This change in color was caused by the improved anthocyanin accumulation, which was regulated by miR156-targeted *SPL*, and *SPL* negatively regulated anthocyanin accumulation by directly preventing the expression of *DFR*.

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Compliance with ethical standards

Conflict of interest Author declares that there is no competing interest towards the publication of this manuscript.

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