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ORIGINAL ARTICLE

Isolation of *PlANS* and *PlDFR* genes from herbaceous peony (*Paeonia lactiflora* Pall.) and its functional characterization in Arabidopsis and tobacco

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Abstract

Flower colour, which is largely determined by anthocyanin, is an important ornamental value of herbaceous peony (*Paeonia lactiflora* Pall.). Anthocyanin synthesis genes encode enzymes acting at different stages of flavonoid biosynthetic pathway. In this research, we cloned anthocyanin synthesis genes *PlANS* and *PlDFR* from red dominated herbaceous peony cultivar 'Hongyanzhenghui' and explore their functionality with respect to colour formation by genetic engineering techniques. The over-expression vectors pB1301-*PlANS* and pB1301-*PlDFR* were constructed and transformed as heterologous genes into *Arabidopsis thaliana* (*A. thaliana*) and tobacco (K326). The quantitative real-time PCR results showed the over expression of *PlANS* and *PlDFR* in transgenic plants from 10 to 1600 times. Phenotypic observation and HPLC detection at OD 525 nm also revealed the elevated anthocyanin content in the transgenic Arabidopsis. The a* and c* value of transgenic flower increased 3–4 times compared with control tobacco which are consistent with the observed colour change. Taken together, our findings showed that *PlANS*, *PlDFR* is participated in the anthocyanin biosynthesis pathway which could provide a theoretical basis for further research of flower colour in herbaceous peony.

Key message

Our results provided an important research work of two *P. lactiflora* genes which are relatedto the red petal colour formation. It provides a theoretical basis for further identification of flowercolour-related gene function study in *P. lactiflora*.

Keywords Anthocyanins · *Paeonia lactiflora* pall. · Flavonoid biosynthetic pathway · Expression analysis

Introduction

Paeonia lactiflora Pall. (*P. lactiflora*) is a world well-known flower which is famous for its ornamental, nutritional and medicinal value (Zhao et al. 2016). Especially in China, it is distributed broadly with rich resources and abundant cultivars. Because of its unique ornamental value of bright flower colour and large flowers diameter, it is widely used as cut flower for cultivation and garden landscaping (Hong et al. 2010; Walton et al. 2010). Flower colour is mainly consisted of flavonoids, carotenoids, betalains and other pigments, and flavonoids are discovered to be directly involved in flower pigmentation (Winkel-Shirley 2001; Koes et al. 2005).

Anthocyanins are considered one of the subgroups of water soluble flavonoids. It acts as the main pigments (red to blue) in plants, conferring intense red-to-blue cyanic colours on petals (Mazza 2018). The flavonoid biosynthetic pathway is responsible for synthesis of anthocyanins. High anthocyanins content was found in many fruits such as berries and grapes, coloured leafy vegetables and flowers. In roses, the pigments are composed mainly of anthocyanidin 3,5-*O*-diglucoside (anthocyanin), which can be further catalysed through glycosylation at different sites of the anthocyanidin molecule (Ogata et al. 2005). In *P.*

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lactiflora, most of flower colours were still concentrated on white, pink, red and their derivative, which is determined by flavonoids including anthocyanins and flavones and flavonols (Wu et al. 2016). Our group have reported that there were main anthocyanin composition (peonidin 3,5-di-*O*-glucoside) (Pn3G5G) and five main anthoxanthin compositions (kaempferol di-hexoside, kaempferol-3-*O*-malonylglucoside- 7-*O*-glucoside,quercetin-3-*O*-galactoside, luteolin-7-*O*-glucoside and isorhamnetin-3-*O*-glucoside) were found in double colours herbaceous peony (Wu et al. 2016). Among the 41 *P. lactiflora* cultivars, only one white *P. lactiflora* cultivar can not detect anthocyanins while Pn3G5G is identified in the other 3 white *P. lactiflora* cultivars. In the deep purple or reddish purple *P. lactiflora* cultivars, they contain four to five anthocyanins, and their contents are much lower than those of purple ones (Jia et al. 2008). However, the molecular mechanism of flower colour formation in *P. lactiflora* is very few discussed.

Anthocyanins are structurally diverse but all are based on 17 basic anthocyanidin structures in nature. In the biosynthesis pathway of anthocyanin pigments, the first coloured compound is anthocyanidin (pelargonidin, cyanidin and delphinidin), which is derived from colourless leucoanthocyanidin (Saito et al. 2013). Dihydroflavonol 4-reductase (DFR) provides the first step for anthocyanin production with the formation of leucoanthocyanidins which later are converted to anthocyanidin by anthocyanidin synthase (ANS) (Sakakibara et al. 2019). Recent study indicated *CRISPR/Cas9*-targeted mutagenesis of *OsDFR* and two other genes lead to black rice through anthocyanin biosynthesis pathway (Jung et al. 2019). When overexpressing wheat *TaDFR* in an Arabidopsis *dfr* mutant, it resulted in significant accumulation of anthocyanins in the respective transgenic plants (Shin et al. 2015). ANS catalyzes the conversion of leucoanthocyanidin to anthocyanidin, a key committed step in biosynthesis of anthocyanins. A 5 bp insertion in the coding region of ANS results in loss of visible and detectable anthocyanin pigments in raspberry (Rafique et al. 2016).

Li et al. introduced the *MaANS* gene from *Morus alba L.* into tobacco, which increased flavonoids, anthocyanidin accumulation, and antioxidant potential in transgenic tobacco (Li et al. 2018). The current study has also analyzed the anthocyanin related gene expression patterns of several ornamental plants such as *Petunia hybrida* and *Gentiana scabra*, however, there are still many mechanisms for the colour formation of ornamental plants remains further exploration. At present, the research on the colour of peony is mainly focused on the physiological and transcriptome levels (Zhao et al. 2016, 2012). A comprehensive and systematic study of the genes functions which can lay the foundation for the molecular research of colour formation in *P. lactiflora* still need to be further studied.

In this study, the full-length cDNAs of *PlANS* and *PlDFR* were isolated from *P. lactiflora*. They were both key enzymes in the downstream flavonoids metabolic pathways. The GUS staining results and the transcript levels analysis indicated that *PlANS* and *PlDFR* from flower can expressed in Arabidopsis leaves and tobacco flowers. Overexpression of *PlANS* and *PlDFR* in *Arabidopsis thaliana* (*A. thaliana*) and tobacco (K326) promoted the accumulation of flavonoids, and resulted in the colour changes of transgenic plants. All these results highlighted the importance of *PlANS* and *PlDFR* in anthocyanin biosynthesis that may impact both flower industry and molecular breeding technology. The aim of our study on identification and functional analysis of the two anthocyanin synthesis genes are to form novel colour for genetic improvement to provide more materials for new varieties.

Material and methods

Plant materials

The *P. lactiflora* plants with red petals, called 'Hongyanzhenghui' were cultivated in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32° 30′ N, 119° 25′ E) (Zhao et al. 2016). *A. thaliana* ecotype Columbia (*Col-0*) plants and tobacco (K326) were planted with the mixture of 3:1 (v/v) vermiculite and perlite and kept in a growth chamber (SANYO, MLR-359H, Japan).

Plant growth conditions

Three years old *P. lactiflora* plants were grown on the open-field cultivation conditions without additional fertilizer. The flower petals were picked off in May and used for cloning material. The growth chamber was controlled with a 16 h light (80–100 μmol m^{-2} s^{-1} illumination)/8 h darkness cycle and a day/night temperature 23/18 °C, with a relative humidity of 70%. The leaves of *A. thaliana* at bolting stage were used for qRT-PCR and HPLC detection, and tobacco flowers were used for flower colour identification.

RNA extraction and sequence analysis of two anthocyanin synthesis genes

The fresh petals collected from the flowers of 'Hongyanzhenghui' (red colour) at the same location were placed in liquid nitrogen immediately and then preserved at − 80 °C for RNA extraction. Total RNA was extracted from the petals with a Plant RNA kit (TaKaRa, Japan), following the manufacturer's protocol. Total leaf RNA (1 μg) was reverse-transcribed in a 10 μl reaction based on the superscript

first-strand synthesis system (Invitrogen, USA). Then the cDNAs were synthesized and 1 µl cDNA was used as PCR template. Using 5′ and 3′ end primers synthesized commercially (Genery, China) were designed based on the ORF sequence of the GenBank accession *ANS* (JQ070805.1), *DFR* (JQ070804.1). PCR reaction was as follows: one cycle of 94 °C (3 min); 35 cycles of 94 °C (30 s), 59 °C (30 s), 72 °C (1 min); and one cycle of 72 °C 10 min; 4 °C end. After testing by 1% (w/v) agarose gel electrophoresis, the PCR products were cloned into the pMD19-T Simple Vector (Takara, Japan) and sequenced. All of gene specific primers (Table 1) were designed through Primer Premier 6.0. Multiple alignment of deduced protein sequences of PlANS and PlDFR with those ones from other species were assembled with DNAMAN 7.0.2. A Neighbor–Joining phylogenetic tree was generated with MEGA-X, using the Poisson correction method and 1000 bootstraps.

Expression vector construction

Based on the ORF sequences of target genes *PlANS* and *PlDFR* which contend the SacI and SmaI restriction cut sites, the PCR products were digested by the restriction enzymes (Takara, Japan), and then purified. The purified products were combined to the expression vector pCAMBIA1301 (pB1301) through T4 DNA Ligase (Takara, Japan). The pB1301 was driven by the double Cauliflower mosaic virus (CaMV) 35S promoters followed by the GUS reporter gene. Primers (*PlANS* and *PlDFR*) used to get recombinant were shown in Table 1.

Transformation of two anthocyanin synthesis genes into *A. thaliana* and tobacco

The empty expression vector pB1301 and the pB1301-*ANS*, pB1301-*DFR* plasmids were used for the transformation of competent cells of *Agrobacterium tumefaciens* strain EHA105. Selected colonies were validated by PCR using primers (*ANS* and *DFR*; Table 1); ultrapure water as template was used as negative control. Arabidopsis *Col-0* plants were transformed using the floral-dip method (Clough and Bent 1998). The inflorescence of *A. thaliana* was soaked with 1/2 MS infection liquid with transformed *Agrobacterim* for 1 min, and then cultivated under dark condition for 12 h. The seeds collected were recorded as T_1 (the first generation) seeds. Transgenic seeds were all screened on MS medium containing 25 mg L^{-1} hygromycin (Hyg). Seeds were screened until the homozygous T_3 (the third generation) transgenic lines were obtained.

The tobacco plants were transformed by *Agrobacterium mediated* leaf disc method to infect tobacco (Grimberg et al. 2015). When the differentiated adventitious buds reached more than 2 cm, they were cut off and inserted into rooting medium. The rooting seedlings of tobacco (T_1) were transferred into green chamber. The flower colour was observed and recorded after flowering.

GUS staining

The homozygous T_3 generation of *A. thaliana* and transgenic tobacco callus were detected by histochemical GUS staining

Table 1 Primers used for PCR amplication in this study

Primers	Purpose	Primer sequence (5′–3′)	Annealing temperature (°C)	Fragment length (bp)
ANS-cloning	Clone	F: GAAAACATAGAGTAAACGTTGCAGC R: GCGGCCCAACTTAAAACTCAATTCT	59	1111
DFR-cloning	Clone	F: TCTTCTCATATCTTTCGACGTGGTT R: GCGGCCCAACTTAAAACTCAATTCT	59	1221
PlANS	Recombinant	F: TCCCCCGGGATGGTGAATTCAGTA (SmaI) R: CGAGCTCTCAATTCTTAAACTCTT (SacI)	60	1081
PlDFR	Recombinant	F: TCCCCCGGGATGGAAGCAGTGACTGA (Sma I) R: CGAGCTCTTAGATTGTGCCATTGACAT (SacI)	60	1111
ANS	PCR	F: AGATTAGAACAAGAAGTCGGTG R: CCAGGCTTTACACTTTATGC	51	842
DFR	PCR	F: GCACTTTCTCCAATCACAG R: CCAGGCTTTACACTTTATGC	51	830
qPlANS	qRT-PCR	F: AGATTAGAACAAGAAGTCGGTG R: GGAGGATGAAAGTGAGGG	52.5	139
qPlDFR	qRT-PCR	F: GCACTTTCTCCAATCACAG R: CAAATGTAGCGACCCTCT	52.5	134
Actin	qRT-PCR	F: TCTCCCGCTATGTATGTCGC R: TAAGGTCACGTCCAGCAAGG	52.5	172

method, and the nontransgenic plants of the same period was used as negative control. Both of them were placed in 20 µl X-Gluc staining solution and incubated overnight at 37 °C. After staining, the plant tissues were soaked with 70% ethanol for 15 min to remove chlorophyll and repeated decolourization for at least 3 times. The stained transgenic *A. thaliana* and tobacco callus were detected.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was conducted to investigate the expression levels of *PlANS* and *PlDFR* in the leaves of *A. thaliana.* A Trizol Kit (Aidlab, China) was used to extract total RNA from the leaves of *Col-0* and transgenic *A. thaliana*. A 1 µg sample of total RNA was treated with DNase to eliminate genomic DNA contamination. First strand cDNA was synthesized by using Oligo dT (TaKara, Japan), according to the manufacturer's instruction. The cDNAs were amplified with qRT-PCR primers (Table 1). The presence of DNA residues was tested using 2 µl of cDNA as the template in a 25 µl standard PCR reaction with *A. thaliana* Actin2 (AK230311.11). The qRT-PCR reaction was performed by denaturation at 94 °C for 30 s, followed by 35 cycles of 5 s denaturation at 94 °C, 30 s annealing at 52.2 °C, and 30 s extension at 72 °C, with a final extension at 72 °C for 5 min and 4 °C forever. qRT-PCR was performed with SYBR Green qPCR Mix (Takara, Japan) and the Bio-Rad CFX96 Real-Time PCR System (BIO-RAD, USA).

HPLC detection

The anthocyanins were extracted from the leaves of transgenic *A. thaliana* using extraction buffer and examined by high-performance liquid chromatography (HPLC) (Wu and Prior 2005; Ni et al. 2008). Each sample (1.0 g fresh weight) was ground in a mortar with liquid nitrogen. Then 6 ml of a mixture of extraction buffer (70:0.1:29.9; CH_3OH:HCl:H_2O) was added and vibrated at 4 °C for 24 h. The mixture was then centrifuged at 12,000 rpm for 10 min at 4 °C and each supernatant was filtered through a 0.22 µm Millipore ™ filter (Millipore Corp., Billerica, MA, USA).

Chromatographic analysis were carried out on a high-performance liquid chromatograph (Dionex, USA) equipped with a P680 HPLC Pump, Thermostatted Column Compartment TCC-100 and a Dionex Photodiode Array DAD-100 Detector (DAD). Anthocyanins were detected under the wavelength of 525 nm through HPLC column: TSK gel ODS-80Ts QA (4.6 mm × 250 mm^2) (Tosoh, Japan), the column temperature was 35 °C. Extracts were firstly passed through a 0.45 µm pore syringe filter (Anpel, China) and then a volume of 10 µL solution was injected for HPLC analysis. Flow rate was 0.8 mL min^{-1} and the mobile phase consisted of 10% formic acid water solution as solvent A (10:90; v/v, HCOOH: H_2O) and formic acid/acetonitrile/water (10:40:50; v/v/v, HCOOH: CH3CN: H_2O) as solvent B. The linear gradient profile was 10% B at 0 min, 20% B at 30 min, 30% B at 50 min, 40% B at 60 min, 50% B at 80 min, and then returned to10% B in 90 min. Data were analyzed with the Chromeleon software (Dionex, USA).

Results

Isolation and sequence analysis of two anthocyanin synthesis genes

PlANS (Genbank JQ070805.1) and *PlDFR* (Genbank JQ070804.1) were used for primers design during homologous-based cloning. PCR specific primers were designed according to both sequences above (*ANS*-cloning and *DFR*-cloning; Table 1). The cDNA from red flower peony 'Hongyanzhenghui' was used for PCR amplification. The full-length cDNA of cloned *ANS* and *DFR* were 1111 bp, 1221 bp containing 1065 bp, 1095 bp ORF with a stop codon and encoded a protein of 354, 364 amino acids, respectively. BLASTn analysis on NCBI showed that the cloned *PlANS* and *PlDFR* have 100% similarity with the published genes sequence.

Both PlANS and PlDFR amino acid sequences were aligned with sequences from different species which were downloaded from NCBI (Fig. 1). To further investigate the evolutionary relationships of *PlANS* and *PlDFR* with other plants, we constructed a phylogenetic tree using MEGA-X (Kumar et al. 2018) (Fig. 2). The amino acids sequence of two genes shared similarity at different extent with other species which have been shown to be involved in anthocyanin biosynthesis. From this, we inferred that *PlANS* and *PlDFR* may have a related function to other homologous genes.

The generation of over-expressors of *PlDFR* and *PlANS*

The full-length ORFs of *PlANS* and *PlDFR* were subcloned into the expression vector pB1301 and sequenced to be right (Fig. 3). The reconstructed expression vectors pB1301-*PlANS* and pB1301-*PlDFR* were also assessed by SacI and SmaI digestion, agarose gel electrophoresis proved that both strips size are correct. Finally, the correct expression vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 which was used for plant transgenic study.

Screening and assay of *PlDFR* and *PlANS* genes expression in *A. thaliana*

The *Agrobacterium tumefaciens* strain EHA105 carrying the pB1301-*PlANS* and pB1301-*PlDFR* and empty pB1301

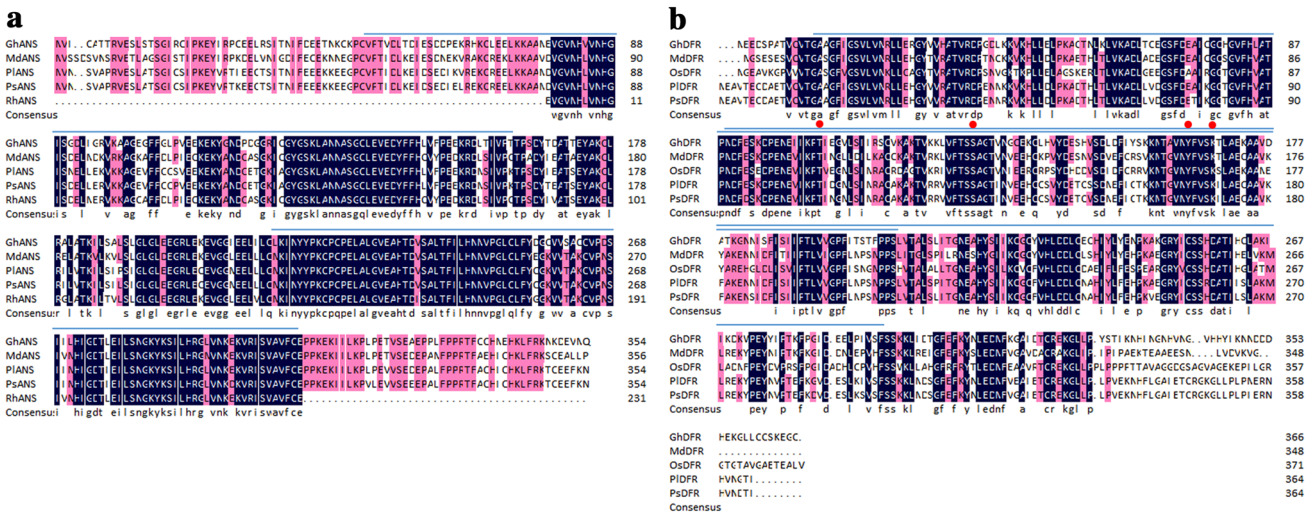

Fig. 1 Multiple alignment of deduced protein sequences of PlANS and PlDFR. **a** Multiple alignment of deduced protein sequence of PlANS with ANSs from other species. The blue bars highlight the conserved domains. *Gerbera hybrid* (AAY15743.2), *Malus domestica* (AAD26205.1), *Paeonia lactiflora* (AFI71900.1), *Paeonia suffruticosa* (AEN71543.1), *Rosa hybrid* (BAF96590.1). **b** Multiple alignment of deduced protein sequence of PlDFR with DFRs from other species. The blue bars highlight the NADP-binding site and substrate specificity domain, respectively; the filled red circles indicate the active sites of the proteins. *Gerbera hybrid* (CAA78930.1), *Malus domestica* (AAD26204.1), *Oryza sativa* (CAA69253.1), *Paeonia lactiflora* (AFI71899.1), *Paeonia suffruticosa* (AMW36065.1). (Color figure online)

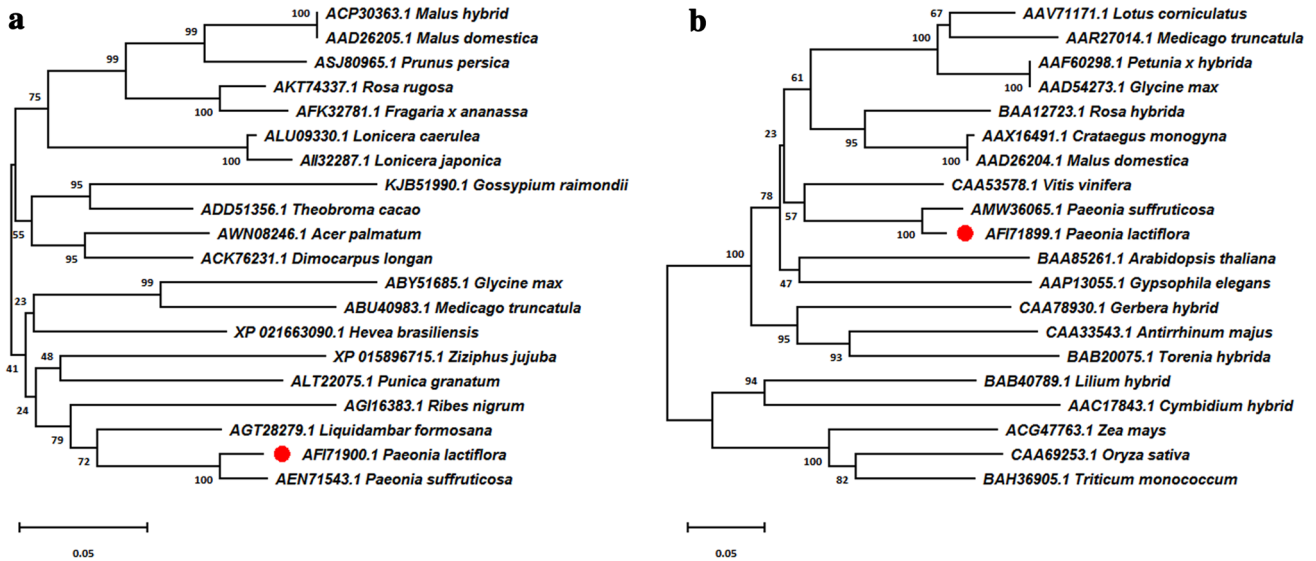

Fig. 2 Phylogenetic tree of PlANS and PlDFR proteins. **a** Phylogenetic tree of PlANS protein together with ANSs from other species. **b** Phylogenetic tree of PlDFR protein together with DFRs from other species

vectors were used for *A. thaliana* transformation. Transgenic lines of *A. thaliana* were selected on MS medium supplemented with Hygromycin (Hyg) 25 mg L^{-1} (Fig. 4a). The phenotype of the T_1 and T_2 lines was recorded. The resistance phenotype of T_2 seeds were stained by GUS. Histochemical staining of GUS activity was carried out to screen transgenic positive plants which were turned into blue (Fig. 4b). After sowing T_2 seedlings, T_3 seeds were collected as homozygous lines. Leaves of T_3 seedlings were used to extract genomic DNA for PCR detection with specific primers (*ANS* and *DFR*; Table 1). Both NPTII region and 35S promoter sequences were used to detect transgenic lines. Correct stripe lines can be recognized as positive one.

The transfected homozygous *A. thaliana* (pB1301) and *Col-0* were used as control groups and the results were shown in Fig. 4c. With SD treatment for 2 weeks, the

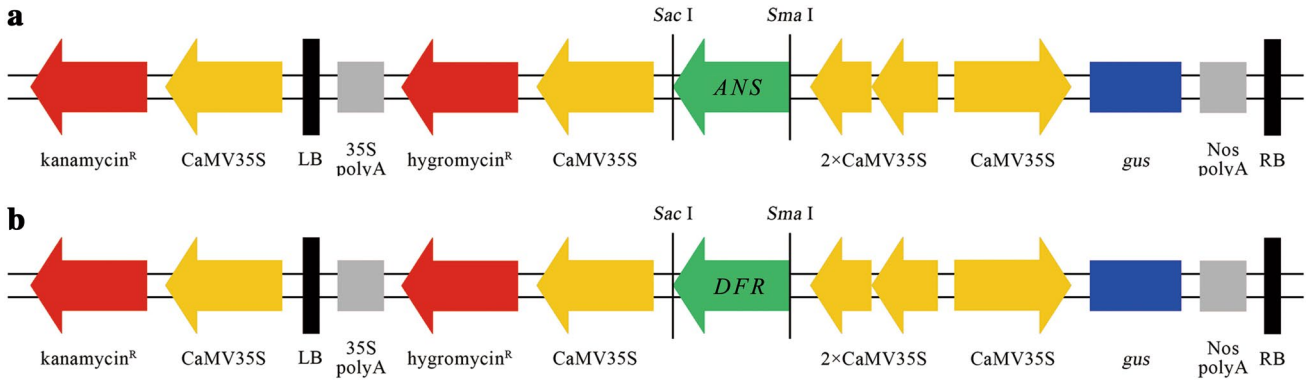

Fig. 3 Over-expressors of *PlANS* and *PlDFR* promoted by 2×CaMV35s promoter for Arabidopsis and tobacco transformation. **a** pB1301-*PlANS*, **b** pB1301-*PlDFR*. (Color figure online)

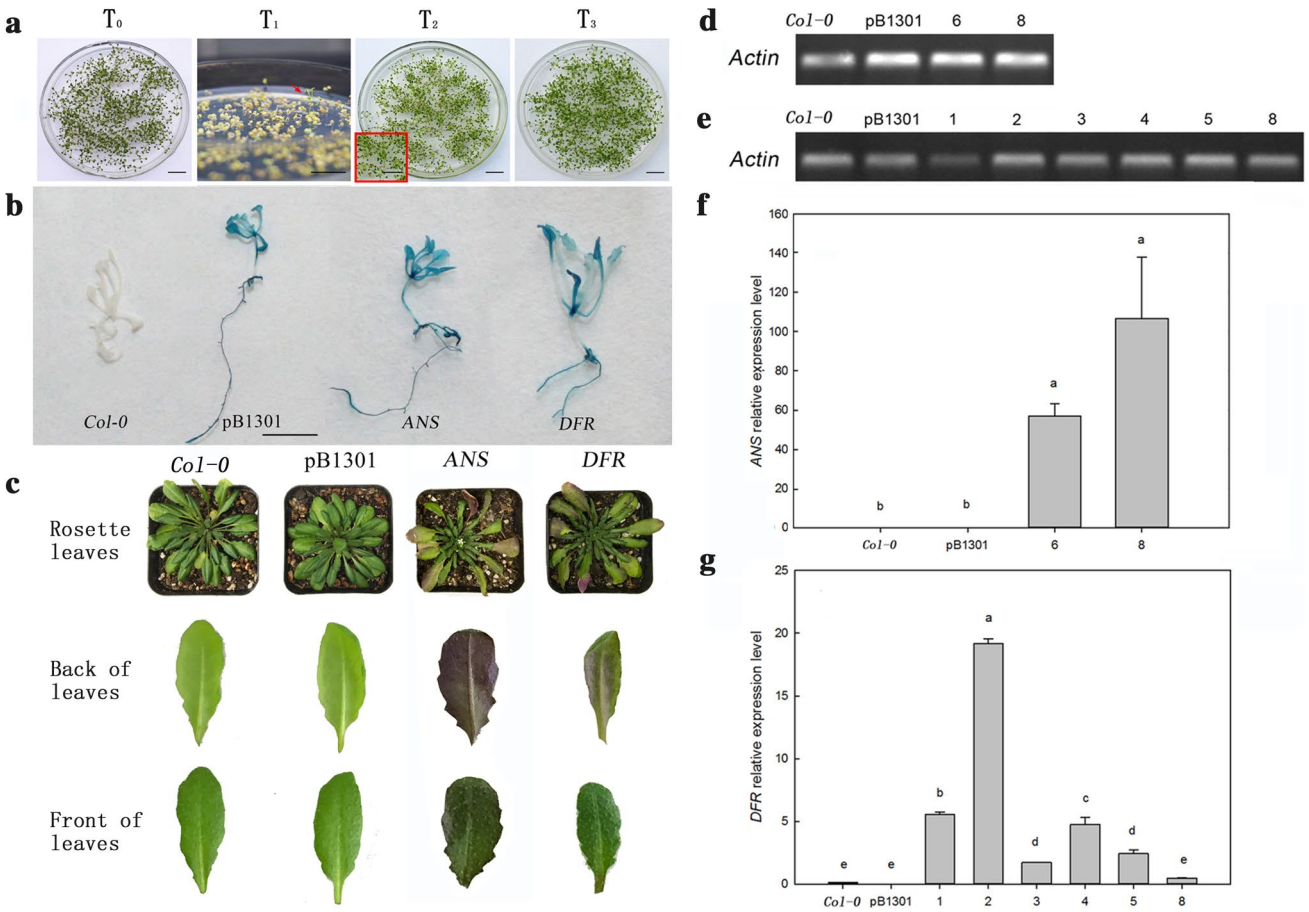

Fig. 4 Screening and identification of homozygous OX-*PlANS* and OX-*PlDFR* Arabidopsis. **a** The red arrow indicates the green T_1 seedings of successful transformation. The red outline indicates homozygous T_2 seedings. **b** Histochemical staining of GUS in transgenic Arabidopsis compared with it in *Col-0*. **c** Phenotypes of *Col-0*, the empty vector pB1301 and transgenic Arabidopsis overexpressing *PlANS* and *PlDFR*. **d**–**g** Indentification of overexpression lines by real-time quantitative PCR (qRT-PCR) of *PlANS* and *PlDFR* genes. Actin gene expression level in different plant of *Col-0*, *pB1301*, *PlANS* (**d**) and *PlDFR* (**e**) were amplified, respectively. **f**, **g** The values on the y-axis were relative expression level. Different letters above bars indicate statistical significance ($P<0.05$). (Color figure online)

leaves of both OX-*PlANS* and OX-*PlDFR* plants turned red at different degrees, OX-*PlANS* plants are redder than OX-*PlDFR* ones (Fig. 4c). In contrast, the colours of *Col-0* and pB1301 plants leaves were keeping green and not changed. qRT-PCR results (Fig. 4d–g) from T_3 homozygous were consistent with the phenotypes. The relative expression level of *ANS* in two *ANS*-transgenic homozygous lines was about 800–1600 times higher than that in the control groups, and the expression level of OX-*ANS* 8# was higher than OX-*ANS* 6#. The relative expression level of six *DFR*-transgenic homozygous lines was 10–250 times higher than control group, with the highest level in OX-*DFR* 2#.

To examine whether anthocyanins accumulated in *A. thaliana* leaves were consistent with the *PlANS* and *PlDFR* expression level, the anthocyanin content in leaves of transgenic plants were detected by HPLC (Fig. 5). The results revealed that their contents in the control and transgenic plants were quite different. At 525 nm, there were obvious peaks of anthocyanins in OX-*ANS* 8# (pink line) and OX-*DFR* 2# plants (blue line) compared with *Col-0* (black line).

Screening and assay of two anthocyanin synthesis genes expression in tobacco

The process of genetic transformation and regeneration of transgenic tobacco is shown in Fig. 6a–i. *PlANS* and *PlDFR* transgenic tobacco callus were regenerated through Hyg selection. GUS staining of transgenic tobacco callus turned into blue (Fig. 6d). During flowering stage, the flower colour of OX-*ANS* and OX-*DFR* plants became darker than control tobaccos, as shown in Fig. 6j. Royal Horticultural Society Colour Chart (RHSCC) measurement showed that the flowers colour of control, pB1301, OX-*PlANS* 8# and OX-*PlDFR* 2# were 65A, 73C, 68A, 64B, respectively.

Furthermore, Portable Imaging Spectrophotometer RM 200QC (X-Rite, USA) was used to detect the degree of flower colour change which was expressed through L*, a*, b* and C* values. L* represents lightness, a* represents the ratio of red/magenta and green, b* represents the ratio of yellow and blue and C* indicates chroma (saturation or purity of colour) (Zhao et al. 2011). The collected values from wild type (seven plants), pB1301 (seven plants), OX-*PlANS* (seven plants) and OX-*PlDFR* (seven plants) were analyzed through L*, a*, b* and C* comparison, respectively (Fig. 7). Statistic analysis showed that the L* and b*

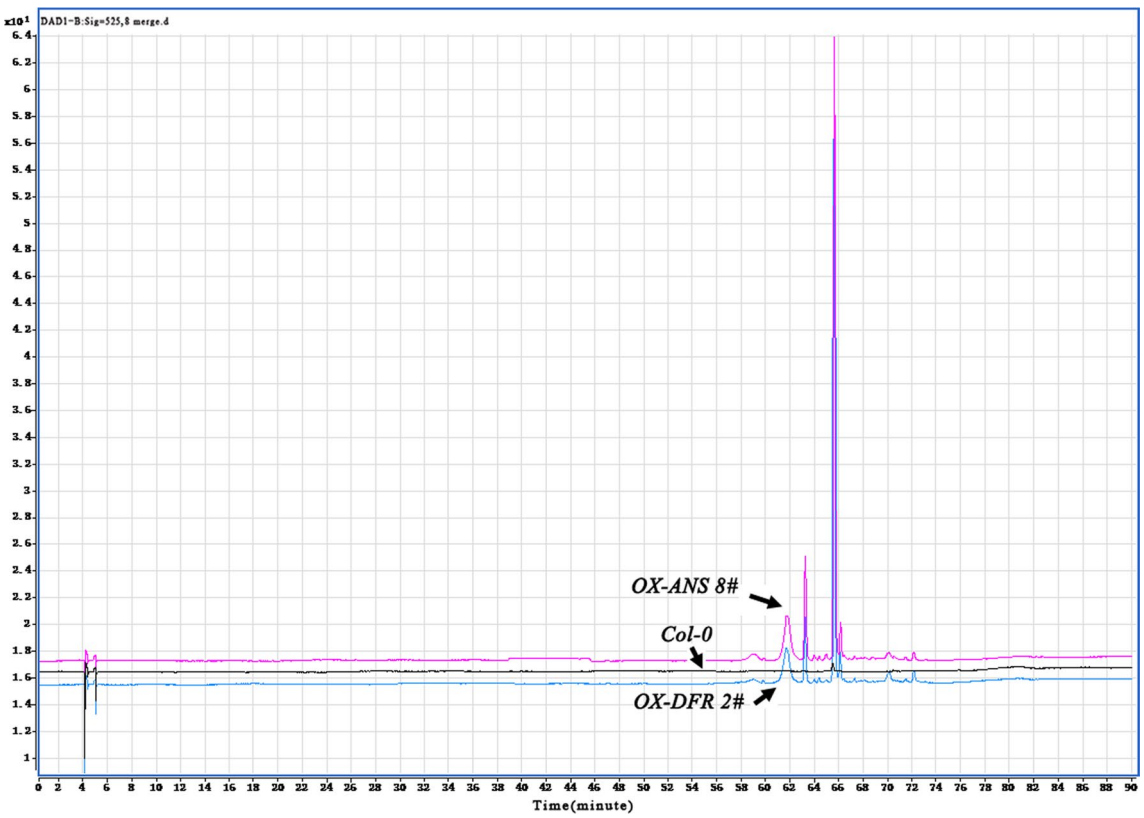

Fig. 5 HPLC chromatograms of anthocyanins (detected at 525 nm) in transgenic Arabidopsis leaves merge together. Anthocyanin content in the leaves of OX-*PlANS* (pink line), OX-*PlDFR* (blue line) Arabidopsis and *Col-0* (black line). (Color figure online)

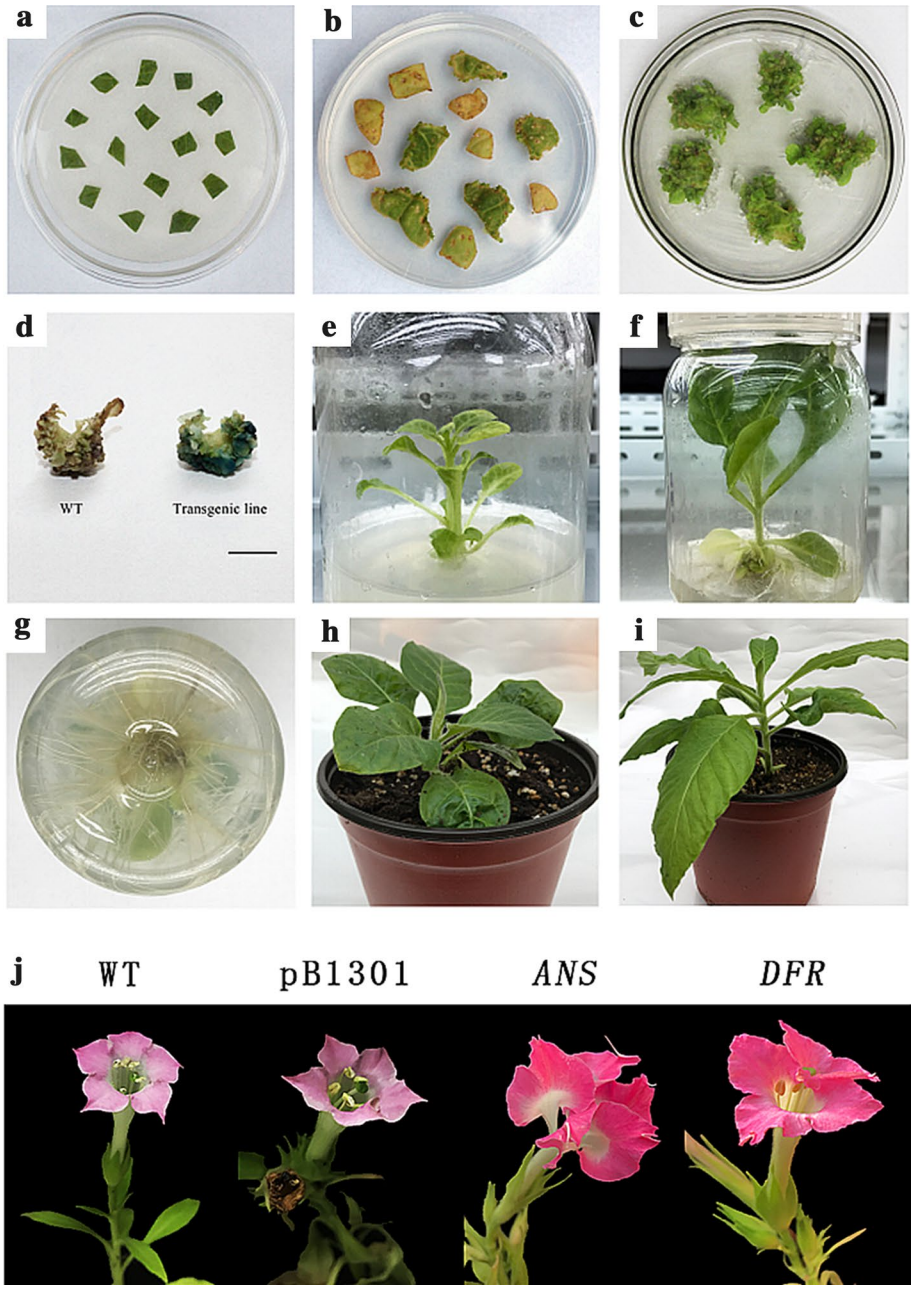

Fig. 6 The process of transformation and regeneration of the transgenic tobacco plants. **a** Co-culture, **b** Screening culture for 1–2 weeks, **c** Differentiation culture for 3–4 weeks, **d** GUS staining results of hygromycin-resistant callus tissue, **e** Re-culture of hygromycin-resistant buds, **f, g** Rooting culture, **h, i** Domestication and transplantation, **j** Phenotypes of flowers on the WT, the empty vector pB1301 and overexpressing *PlANS* and *PlDFR* tobacco

values of OX-*ANS* and OX-*DFR* were dropped obviously, however, their a* and C* values were 3–4 times higher than the wild types (Fig. 7b, d). This result revealed a redder and different degrees of saturation enhanced colour in *ANS* and *DFR* transgenic plants.

Discussion

Anthocyanin and anthoxanthin, especially the anthocyanin content was the main reason for giving *P. lactiflora* petal red, pink and white colours (Zhao et al. 2016). The peony petals showed white colour when the anthocyanin content was low; it will appear red when the anthocyanin content was high. Pn3G5G was reported as the major composition of anthocyanins in peony petals (Jia et al. 2008). Besides, there is a close correlation between *PlDFR* and *PlANS* transcript levels and anthocyanin contents in white and red peony cultivars, the transcription level of both genes were higher in red and pink cultivar than the white one (Zhao et al. 2016). In our previous study, we found the expression of *PlANS* and *PlDFR* genes were quite different between red and non-red cultivars which were speculated as a main reason for the formation of red colour (Zhao et al. 2012). However, it was

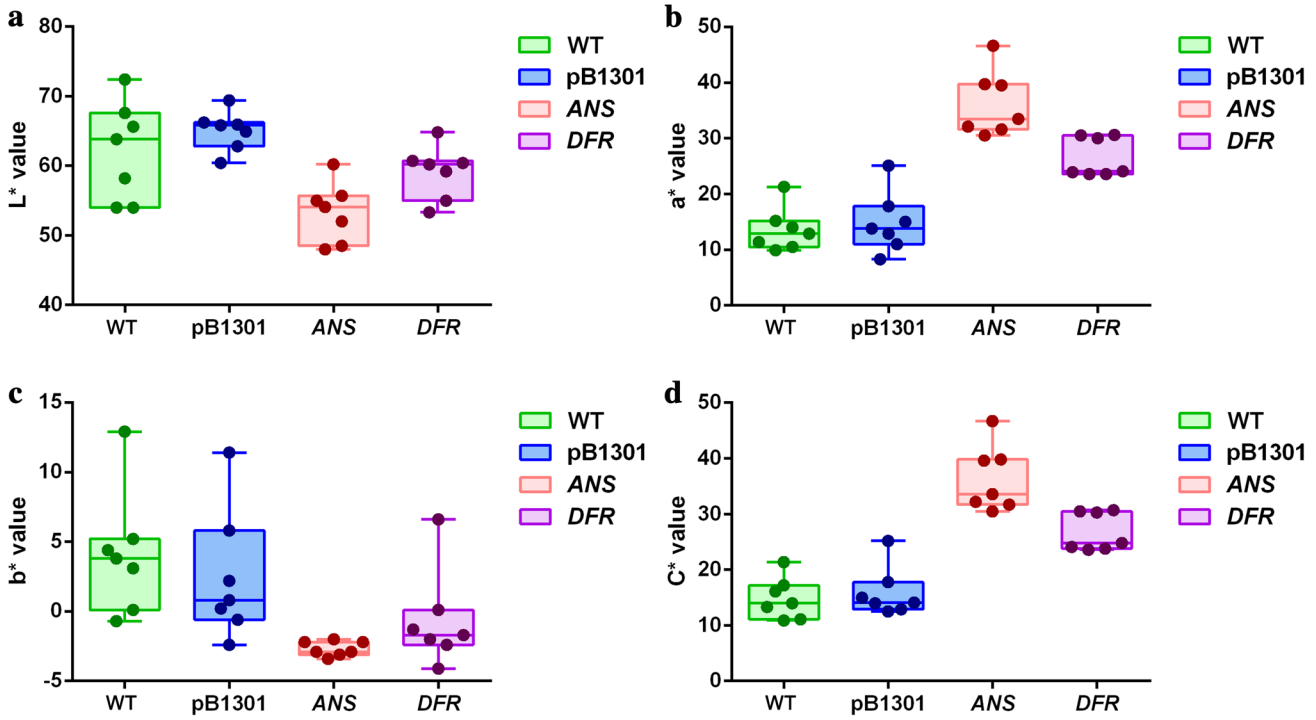

Fig. 7 Variation and distribution of colour parameters of four different types of tobacco flowers. **a** L*; **b** a*; **c** b*; **d** C*. (Color figure online)

unknown whether *PlDFR* or *PlANS* has independent function during the colour formation of peony petals.

So far, it was found that the mutation or RNAi of *DFR* gene could block the pathway of anthocyanin biosynthesis in tobacco and *Dendrobium*, respectively (Kazama et al. 2013; Ratanasut et al. 2015; Lim et al. 2016). This indicated that *DFR* gene is necessary for the flower colour formation in several species. The overexpression of the *PtrDFR* gene can obviously change tobacco petal colour and attend higher accumulation of anthocyanins (Huang et al. 2012). Furthermore, when introduced *DFR* gene of cranberry into tobacco, the corolla colour became dark pink (Polashock et al. 2002). In our research, after overexpression of *PlDFR*, the same significant phenotype was found in the floral organs of transgenetic tobacco. It indicated that *PlDFR* was positively correlated with the accumulation of anthocyanins, and played an important role in the flavonoid biosynthetic pathway. As for homologous genetic transformation, it was also found that the deletion or silencing of *ANS* gene can cause flower organ of *Calla Lily* and *Torenia fournieri* turned to white colour (Seitz et al. 2003; Aida et al. 2000). We cloned *PlANS* from red flower peony and used heterologous transformation system to clarify their potential functions. Compared to Qi's research, our transgenic leaves of *PlANS* transgenic Arobidopsis turned to be red instead of that stem of the transgenic *A. thaliana* with *MnANS* turned reddish (Qi et al. 2014). The *PlANS* gene also caused the deepening of tobacco petals which was similar to the transgenic tobacco of cocoa *TcANS* (Liu et al. 2013). These results revealed that both *PlDFR* and *PlANS* genes can affect biosynthesis of anthocyanins. However, which form of aglycones of anthocyanins was changed in transgenic plants still need further study.

As an ornamental plant, the genes related to pigments synthesis in *P. lactiflora* have different functions in the formation of flower colour (Tang et al. 2018; Wu et al. 2018a, b). Due to the lack of homologous transformation system in peony, it still stays at the level of chemical composition analysis and transcriptome sequencing and how to regulate the colour of peony is still unclear. In our study, it was demonstrated that separate transferring *PlDFR* and *PlANS* could also affect plant colour, and the phenotype of the transgenic *PlANS* lines are more pronounced than *PlDFR* transgenic lines. We speculated that it may be caused by the direct influence of *ANS* on the synthesis of anthocyanins which is located at downstream of the synthesis pathway. Although we did not transfer both *PlDFR* and *PlANS* genes together, Rosati's research work showed that forsythia displayed a novel bronze-orange petal colour when transferred *AmANS* and *MiDFR* together, which implies another application approach in the future (Rosati et al. 2003). Furthermore, our results provide a theoretical basis for further identification of flower colour-related gene function study in *P. lactiflora*.

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Author contributions JS and MS were responsible for conception and design of experiments. YW was responsible for data analysis, and drafting of the manuscript. JS, DQZ and JT revised the manuscript. All authors read and approved the final manuscript.

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