



Flower color modification through co-overexpression of the *VtF3'5'H* and *RhNHX* genes in *Rosa hybrida*

Junping Xu¹ · Ju Young Shin¹ · Pil Man Park¹ · Hye Ryun An¹ · Yae-Jin Kim¹ · Se Jin Kim¹ · Su Young Lee¹

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Abstract

Roses (*Rosa hybrida*) are a highly merchandised flower but lack blue varieties. Overexpression of the flavonoid 3',5'-hydroxylase (*F3'5'H*) gene can increase the accumulation of blue pigment (delphinidin anthocyanin). However, sometimes the effect of *F3'5'H* gene alone is inadequate for producing blue flowers. Furthermore, the internal environment of the cell, such as an increase in pH, can also help the conversion of anthocyanins to blue pigments. Nonetheless, genetic engineering methods can simultaneously introduce multiple genes at the same time to regulate the development of blue pigments to achieve the ultimate breeding goal of producing blue color in roses. In the present study, to simultaneously adjust the accumulation of delphinidin and vacuolar pH, we introduced the *Viola tricolor* flavonoid 3',5'-hydroxylase (*VtF3'5'H*) and *Rosa hybrida* Na⁺/H⁺ exchanger (*RhNHX*) genes into the white rose line “KR056002” using *Agrobacterium*-mediated transformation. The quantitative real time polymerase chain reaction (qRT-PCR) results showed that the heterologous genes in the transgenic lines were highly expressed in petals and leaves, and simultaneously promoted the expression of related anthocyanin synthesis structural genes. Obvious color changes were observed in both petals and young leaves, especially when petals changed from white to red-purple. The formation of delphinidin was not detected in the petals of control plants, whereas the petals of transgenic lines had higher delphinidin content (135–214 µg/l) and increased pH value (0.45–0.53) compared with those of control plants.

Key message

The introduction of heterologous *VtF3'5'H* and *RhNHX* genes into rose plants can affect the flower color by regulating the expression of anthocyanin synthesis pathway genes and internal cellular mechanisms.

Keywords Delphinidin · pH · Anthocyanin synthesis · Blue pigment

Abbreviations

NT	Non-transgenic	UPLC	Ultra Performance Liquid Chromatography
PCR	Polymerase chain reaction	PDA	Photometric diode array
RT-PCR	Reverse transcription polymerase chain reaction	SAS	Statistical Analysis System
qRT-PCR	quantitative real time polymerase chain reaction	<i>NHX</i> gene	Na ⁺ /H ⁺ exchanger gene
RHSCC	Royal Horticultural Society Color Chart	<i>CHS</i> gene	Chalcone synthase gene
		<i>CHI</i> gene	Chalcone flavanone isomerase gene
		<i>F3H</i> gene	Flavanone 3-hydroxylase gene
		<i>F3'5'H</i> gene	Flavonoid 3',5'-hydroxylase gene
		<i>DFR</i> gene	Dihydroflavonol 4-reductase gene
		<i>ANS</i> gene	Anthocyanidin synthase gene

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✉ Su Young Lee
lsy8542224@korea.kr

¹ Floriculture Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Wanju 55365, South Korea

Introduction

Flower color is a paramount feature of ornamental plants. Various colors have differential attractions to pollinators; for example, swallowtail butterflies prefer to visit red or orange

flowers, whereas moths prefer blue flowers (Kelber 1997; Hirota et al. 2012). From a public perspective, flower color preferences also differ. A survey of consumer preferences for roses showed that 60% of respondents rated that flower color as the most important feature, and their favorite rose colors were red (30%), purple (18%), and pink (15%) (Waliczek et al. 2018). Factors affecting flower color have been reported, including flavonoids, vacuole pH, co-pigmentation, light, and temperature (Asen et al. 1975; Zhao and Tao 2015). Anthocyanins, the largest group of flavonoids, have antioxidant effects and produce flowers of different colors (Del Valle et al. 2019). One of the main methods for breeders to develop desirable flower colors is to adjust the accumulation of various anthocyanins (Noda et al. 2013). There are over 20 types of anthocyanins, six of which are the most common: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (Zhang et al. 2022). In particular, delphinidin and malvidin are important anthocyanins for producing blue flowers (Fukui et al. 2003; Noda et al. 2013).

Roses are an important cut flower crop that ranks first in the international floriculture trade market (Darras 2021). Almost all colorful cut rose varieties are obtained through hybridization, spontaneous and induced mutations, and selections using traditional breeding methods (Datta 2018). Plant breeders attempt to breed blue colored roses, but because of the lack of intrinsic factors to produce delphinidin and the unsuitability of the intracellular environment for the development of blue flower colors, traditional breeding efforts are insufficient to achieve this goal (Datta 2018; Katsumoto et al. 2007). With advancements in science and technology, molecular breeding provides new feasible methods for breeding desired flower varieties (Katsumoto et al. 2007). Over the years, researchers have provided several methods for modifying flower color using genetic engineering through the study of anthocyanin synthesis pathway-related genes (Yin et al. 2021). *CHS*, *CHI*, *F3H*, *DFR*, *F3'H*, *F3'5'H*, and *ANS* are the key structural genes in anthocyanin biosynthesis, especially *F3'H* and *F3'5'H*, which play an imperative role in delphinidin anthocyanin synthesis (Ishiguro et al. 2012; He et al. 2013; Noda et al. 2013). For example, The bluing capability of the sepal color of hydrangea is positively correlated with the proportion of delphinidin in all anthocyanins compounds in sepals, which is regulated by the transcription ratio of *F3'5'H* to *F3'H* (Yuan et al. 2023). Roses lack blue and blue-purple flowers due to the absence of the endogenous *F3'5'H* gene encoding the key enzyme for delphinidin synthesis. The transgenic insertion of exogenous *F3'5'H* promoted the accumulation of delphinidin in roses, chrysanthemums, carnations, *Phalaenopsis* and verbena which produced the novel blue hues in the flowers (Togami et al. 2006; Tanaka and Brugliera 2013; Okitsu et al. 2018; Nakamura et al. 2020; Liang et al. 2020; Han et al. 2021). Furthermore, heterologous expression of

the *F3'5'H* gene results in the accumulation of 3',5'-hydroxylated anthocyanins (delphinidin, petunidin, and malvidin) in the petals of a red variety of petunia, and the flower color of some transgenic plants changed drastically, from red to deep red with deep purple sectors (Mori et al. 2004). In addition, the research group of Suntory (Suntory Ltd, Japan) has successfully obtained blue roses and commercial production by overexpressing the *viola F3'5'H* gene in roses (Katsumoto et al. 2007; Tanaka and Brugliera 2013). Sometimes, however, blue flowers cannot be achieved only through the overexpression of *F3'5'H* gene (He et al. 2013). By introducing several transgenes into plants, such as the *A3'5'GT* and *F3'5'H* genes, blue chrysanthemum was successfully produced through a two-step modification of the anthocyanin structure by B-ring hydroxylation and glycosylation (Noda et al. 2017).

Anthocyanins are related to vacuolar pH. The change in the pH value causes anthocyanins to produce various colors. Under strongly acidic, neutral, and alkaline conditions, anthocyanins are red, purple, and blue, respectively (Goto and Kondo 1991; Yoshida et al. 2003). The pH of blue flowers is higher than that of red flowers (Reuveni et al. 2001). When the accumulation rate of the anthocyanin compounds were same, adjusting the pH ranging from 2.9 to 6.8 resulted in red to blue flower color, which provided an evidence that the level of vacuolar pH is a significant factor in regulation of flower color (Liang et al. 2020). Changing the pH value of the vacuole is an important means of modifying flower color. Mutations in seven loci (*PHI–PH7* genes) in petunia reduced vacuolar acidity, resulting in blue color (De Vlaming et al. 1983; Faraco et al. 2017). For example, the *PH3* gene mutant causes the pH of petunia petal extracts to increase from 5.5 to 6, and the petal color changes from red to bluish/grayish (Faraco et al. 2014). A recent study suggested that *NHX1* may mediate the Na⁺/H⁺ exchange to regulate the vacuolar pH to promote blue coloration of *Phalaenopsis* petals (Xu et al. 2022). Virus-induced gene silencing of *peNHX1* reduced the petals coloration of *Phalaenopsis*, and the overexpression of *PeNHX1* caused the increase in pH resulting in the petals color change from red to blue (Xu et al. 2022). Moreover, mutations in the vacuolar Na⁺/H⁺ exchanger (*InNHX1*) gene of Japanese morning glory caused the vacuolar pH in the flower epidermis to drop by approximately 0.7, turning the blue flowers purple (Fukada-Tanaka et al. 2000).

In the present study, we introduced the *Rosa hybrida NHX* (*RhNHX*) and *Viola tricolor F3'5'H* (*VtF3'5'H*) genes into the *Rosa hybrida* line “KR056002” by using *Agrobacterium*-mediated transformation to determine whether the increase in pH and the overexpression of anthocyanin synthesis genes in petals and young leaves can improve anthocyanin biosynthesis. Furthermore, we observed the transcription level of

endogenous regulatory genes, especially the accumulation of delphinidin, which changes the flower color of white roses.

Materials and methods

Plant material

We used the *Rosa hybrida* line “KR056002” developed and cultivated by the Korean Rural Development Administration (RDA) Jeonju, Korea, to induce embryos (including embryogenic calluses) as transgenic materials on Schenk and Hildebrandt (SH) medium containing 2,4-dichlorophenoxyacetic acid (3 mg/l), L-proline (300 mg/l), and sucrose (30 g/l) by using in vitro roots, as described by Lee et al. (2008, 2010, 2013, 2022).

Plasmid construction

The *Agrobacterium tumefaciens* strain LBA4404, harboring the binary vector pPZP200 containing *RhNHX* cDNA from the petals of *Rosa hybrida* line “0R-40-31” cultivated by the RDA and *VtF3'5'H* cDNA from the petals of the *Viola tricolor* line “PS-12-34” cultivated by the Korea National College of Agriculture and Fisheries (KNCAF, Jeonju, Korea) was used in this study (Suppl. Fig. S1). The *RhNHX* and *VtF3'5'H* genes were driven by the cauliflower mosaic virus 35 S (CaMV 35 S) promoter. The *bar* gene, which confers

phosphinothricin (PPT) resistance, was used as a selection marker.

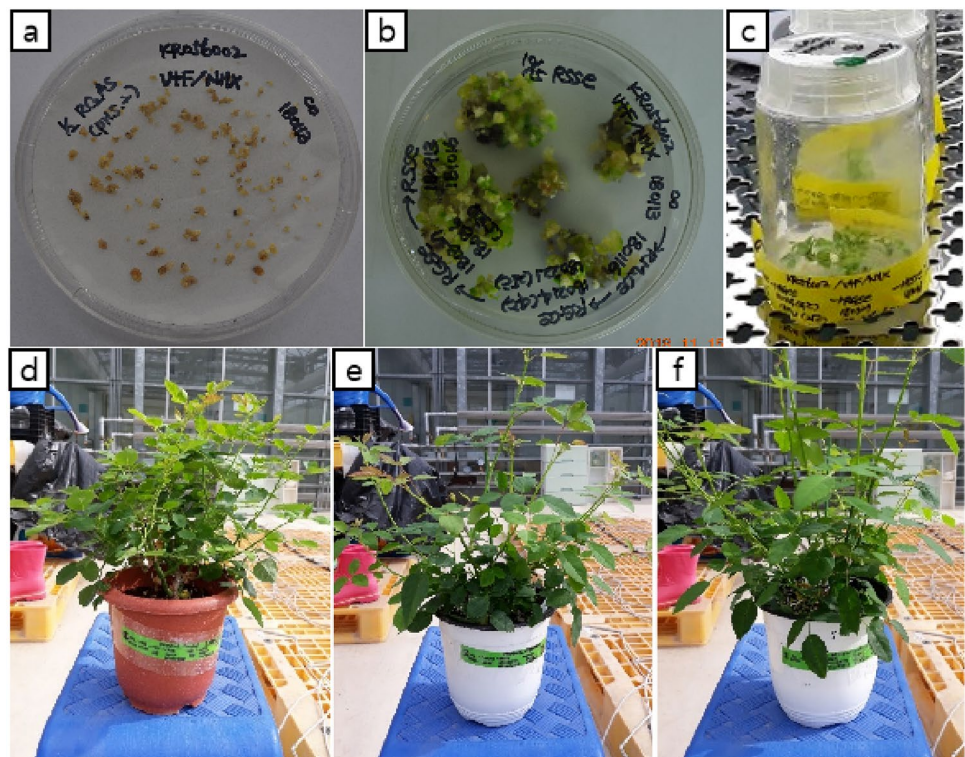
Genetic transformation

Genetic transformation was performed as described by Lee et al. (2013, 2020, 2022). Briefly, 3 ml somatic embryos (including embryogenic calluses) (Fig. 1a) derived from the in vitro roots of “KR056002” were incubated with the *Agrobacterium* suspension containing the vector LBA4404 harboring *RhNHX* and *VtF3'5'H* genes at 28 °C with shaking at 100 rpm for 30 min. Explants were co-cultivated in the dark for 3 days and then transferred to selection medium containing 2 mg/l PPT. Subculturing was performed every 4 weeks to obtain adventitious shoots (Fig. 1b).

DNA extraction and PCR analysis

Total genomic DNA was isolated from the leaves of different independent putative transgenic lines (2, 3, 4, 8, 9, and NT plants) using CTAB extraction solution (Biosesang, Korea), according to the manufacturer’s protocol. Plasmids based on pPZP200 containing *RhNHX* and *VtF3'5'H* genes were used as the positive controls. The primers and PCR conditions for the specific genes are shown in Suppl. Table S1. The reaction products were analyzed using electrophoresis

Fig. 1 Process of obtaining in vitro rose plants (c) through shoot formation (b) from somatic embryogenic callus (a), which was derived from in vitro root of breeding line KR056002, infected by *Agrobacterium tumefaciens* harboring *VtF3'5'H* and *RhNHX* genes. d–f Were non-transgenic plants, transgenic line 8 and 9, respectively, grown in the greenhouse after acclimatization. (Color figure online)



on a 2% (w/v) agarose gel stained with Dyne LoadingSTAR (DyneBio Inc., Korea).

Southern blot and northern blot analyses

Southern and Northern blot analyses were performed as described by Lee et al. (2013, 2020). Briefly, for Southern blot analysis, we first digested the leaf genomic DNA (60 µg) with the restriction enzyme EcoRI, then subjected it to electrophoresis in 0.9% (w/v) agarose gels and subsequently transferred it to a nylon membrane (Hybond-N, Amersham, UK) by capillary transfer following the manufacturer's instructions. The expression vector and NT were used as positive and negative controls, respectively. Hybridization was performed using standard protocols (Sambrook et al. 1989).

For northern blot analysis, 30 µg RNA from each line was extracted from petals using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA was electrophoretically separated on 6.3% (w/v) formaldehyde-containing 1% (w/v) agarose gel and then transferred to the membrane by capillary transfer. The 32P-labeled probes from the *RhNHX* gene of *Rosa hybrida* line "10R-40-31" and the *VtF3'5'H* gene of *Viola tricolor* line "10R-40-31" were used for subsequent analyses as described by Balestrazzi et al. (2009). The nylon membranes were autoradiographed and analyzed using a BAS-1800II Bio-Imaging Analyzer (Fujifilm, Tokyo, Japan).

Reverse transcription (RT-) and qRT-PCR analysis

RT-PCR and qRT-PCR were performed as previously described (Xu et al. 2018, 2021). In brief, total RNA was isolated from the in vitro leaves of transgenic lines (2, 3, 4, 8, and 9) and NT plants using TRIzol (Molecular Research Center, Cincinnati, OH), and cDNA was prepared using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The transcript levels of heterologous genes (*RhNHX*, *VtF3'5'H*) were analyzed using RT-PCR and qRT-PCR. The primers and PCR conditions for the specific genes are shown in Suppl. Table S2. The $2^{-\Delta\Delta C_t}$ method was used to calculate relative expression levels. The experiment was repeated thrice.

Plant acclimatization and phenotypic characterization

Two transgenic lines (8 and 9) and NT plants were successfully acclimated according to the method described by Xu et al. (2021) (Fig. 1d–f). Phenotypic characterizations of flowers were visually evaluated using the Royal Horticultural Society Colour Chart (RHSCC) (The Royal Horticultural Society, UK) and colorimeter (Konica Minolta, Osaka, Japan). These colors were expressed using

the CIE 1976 (L*a*b*) system (Gonnet 1995). The color coordinate L* represents lightness from black (0) to white (100), a* indicates red (positive) to green (negative), and b* indicates yellow (positive) to blue (negative). For each line, three fully open flowers were randomly selected for investigating color differences. The measurement positions were selected at six points on the upper, middle, and lower parts of the front and back of the petals, as shown in Suppl. Table S3. The scoring was repeated three times.

Analysis of the total anthocyanin content

To further confirm the changes in flower color of the transgenic plants, we measured the total anthocyanin content of the fully opened petals and first young leaves (Naing et al. 2018). A total of 0.15 g of fresh tissue was added to a 2 ml e-tube and ground evenly with a homogenizer. Then, 1.5 ml extraction solution (99:1 v/v methanol/HCl; Sigma, St. Louis, USA) was added and mixed well. After mixing, the solutions were maintained at 4 °C in dark for 24 h. Subsequently, they were centrifuged at 13,000 rpm at 4 °C for 20 min, and the supernatant was collected in a new 2 ml collection tube. The A530 and A657 values in the supernatant were measured using a QuickDrop spectrophotometer (Molecular Devices, USA). The anthocyanin content was calculated using the formula $(A530 - 0.25 \times A657)/\text{fresh weight}$. Three independent biological samples were used for each analysis.

Separation and determination of anthocyanins

For each line, 50 mg of petals was randomly ground into powder with liquid nitrogen, mixed with 5 ml of extraction solvent (acetonitrile:water:phosphoric acid = 20:80:0.2, v/v/v), and treated with an ultrasound homogenizer for 30 min. After centrifugation, 1 ml of supernatant was mixed with 100 µl 1 N HCl, and incubated at 100 °C for 60 min. The above solution was cooled at 4 °C for 5 min, filtered (0.22 µm polytetrafluoroethylene), and used for analysis. The anthocyanin content in petals was evaluated using UPLC. UPLC was used to identify six common anthocyanidins, namely, delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin. UPLC analysis was performed using a Waters ACQUITY UPLC H-Class System (Waters, Milford, MA, USA). An improved anthocyanin measurement method was used in this study. Briefly, 2 µl of each sample was injected onto an ACQUITY UPLC HSS T3 1.8 µm column (2.1 × 50 mm). The column was maintained at 40 °C. The following mobile phases (A–B) were used for the separation, namely, A: 0.3% phosphoric acid; B: 100% acetonitrile. The flow rate was maintained at

1 ml/min. Anthocyanins were detected using a photometric diode-array (PDA) detector at a wavelength of 525 nm. To establish a calibration curve, a series of standard solutions of six common anthocyanins were prepared.

Measurement of the plant tissue pH

The first young leaves and petals of each line (NT, NT 8, and NT 9) were collected separately. A total of 1 g of each sample was ground in liquid nitrogen using a mortar and pestle, and 10 ml of distilled water was added. The pH of the supernatant was immediately measured using a pH meter. The experimental methods were described by Manteau et al. (2003).

Transcript levels of anthocyanin pathway genes using RT-PCR and qRT-PCR

To compare the transcript levels of anthocyanin synthesis pathway genes (*RhCHS*, *RhCHI*, *RhF3H*, *RhF3'5'H*, *RhDFR*, *RhANS*) and heterologous genes (*RhNHX*, *ViF3'5'H*) in young leaves and petals of NT plants and lines 8 and 9, we used the same method as described above. The primers and PCR conditions are listed in Suppl. Table S2. The experiment was repeated thrice.

Statistical analysis

The Statistical Analysis System (SAS) statistical software package (version 9.4; SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. The data are expressed as the mean \pm standard error. Duncan's multi range test was used to determine the mean separation and significance at a 5% significance level.

Results

Molecular analysis of transgenic plants

To study the effects of the *RhNHX* and *ViF3'5'H* genes on the flower color, we introduced these two genes into the *Rosa hybrida* line "KR056002" using *Agrobacterium*-mediated genetic transformation. After genetic transformation, we obtained five lines that survived in selective media containing PPT. Subsequently, we performed molecular analyses of the five lines (2, 3, 4, 8, and 9). Using PCR analysis, we detected the presence of the *RhNHX* and *ViF3'5'H* genes in each transgenic line (Fig. 2a). The heterologous *RhNHX* was derived from *Rosa hybrida* cv. "10R-40-31." We speculate that the sequence of the *NHX*

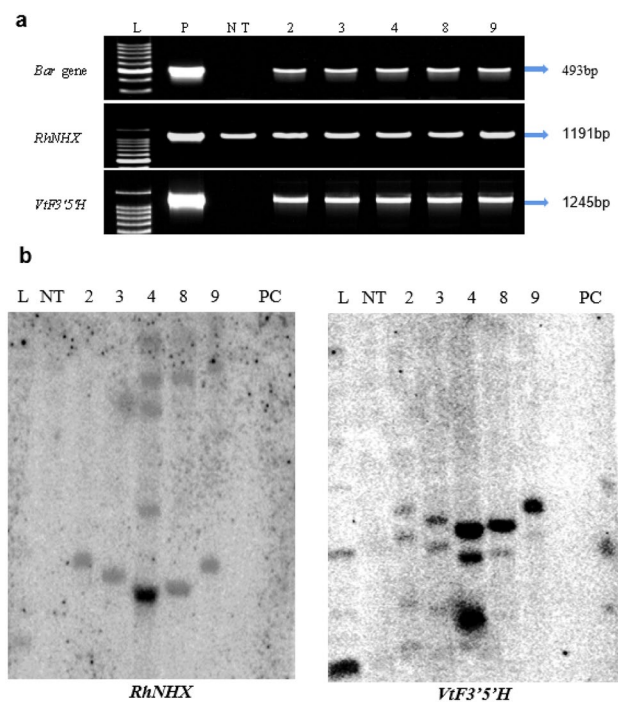


Fig. 2 PCR and Southern blot analyses in *RhNHX* and *ViF3'5'H*-transfected *Rosa hybrida* plants. **a** PCR analysis of the *bar*, *RhNHX*, and *ViF3'5'H* expression levels in transgenic lines (2, 3, 4, 8, and 9), P, and NT plants. **b** Southern blot analysis of *RhNHX* and *ViF3'5'H* for copy number identification in transgenic lines. L: 1 kb plus ladder marker; *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺ exchanger gene; *ViF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; P: plasmid; NT: non-transgenic plants; #2, 3, 4, 8, 9: transgenic lines; PC: positive control

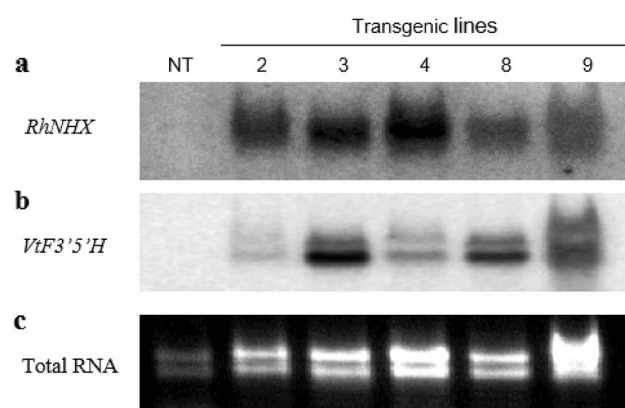


Fig. 3 Northern blot analysis of the expression of *RhNHX* and *ViF3'5'H* in *Rosa hybrida*. **a** Northern blot probed with *RhNHX*. **b** Northern blot probed with *ViF3'5'H*. **c** The total RNA used for northern blotting was derived from the transgenic lines (2, 3, 4, 8, and 9) and corresponding NT plants. *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺ exchanger gene; *ViF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants

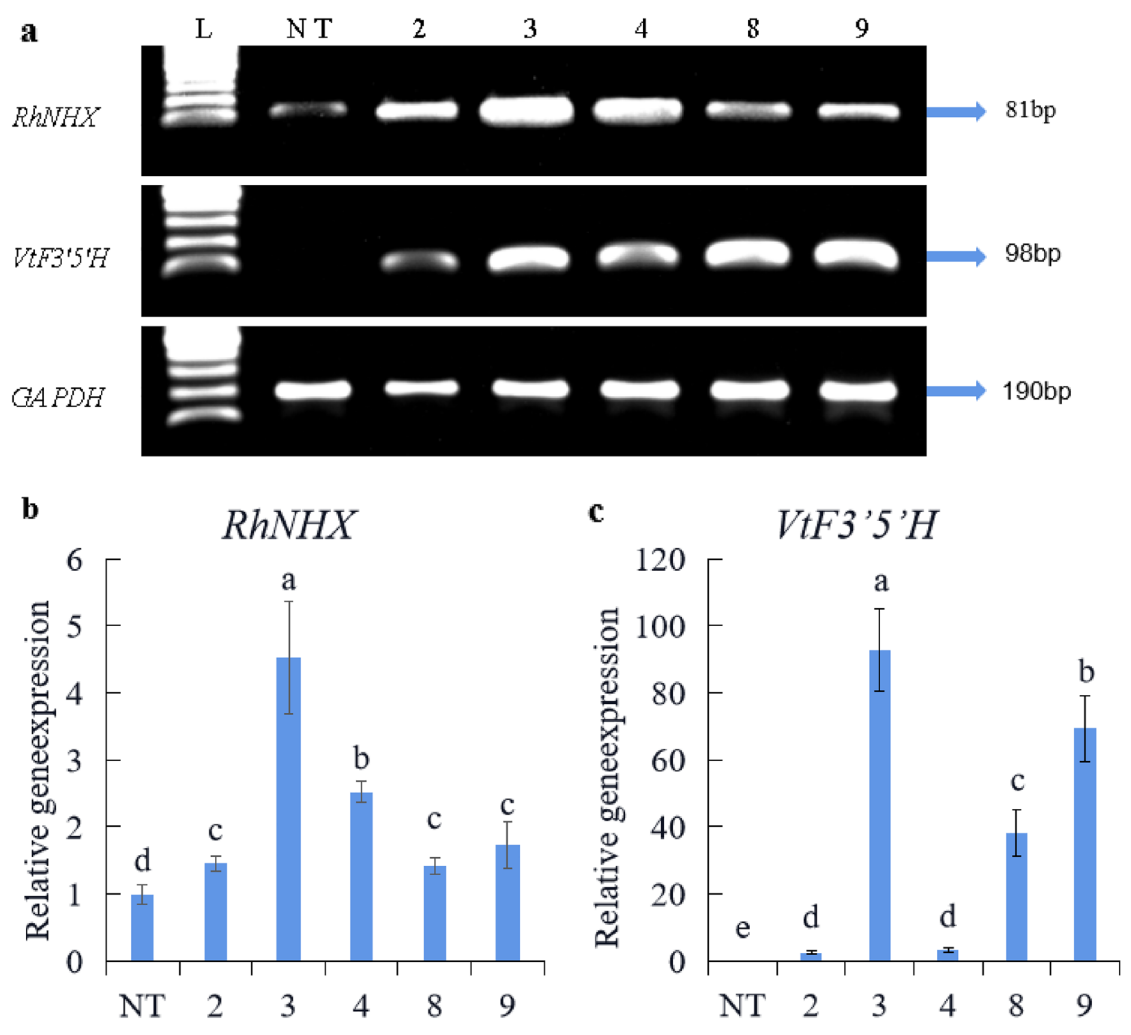


Fig. 4 PCR analysis of the expression levels of *RhNHX* and *VfF3'5'H*. **a** The expression levels of *RhNHX* and *VfF3'5'H* in the transgenic lines 2, 3, 4, 8, and 9 of *Rosa hybrida* compared to those of the corresponding NT plants. Comparison of the transcript levels of *RhNHX* (**b**) and *VfF3'5'H* (**c**) in transgenic lines. Error bars indicate the standard errors of the mean of three replicates. Expression levels are

shown relative to those of actin. Means with different letters are significantly different (Duncan's multiple range test, $p < 0.05$). *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺ exchanger gene; *VfF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants. (Color figure online)

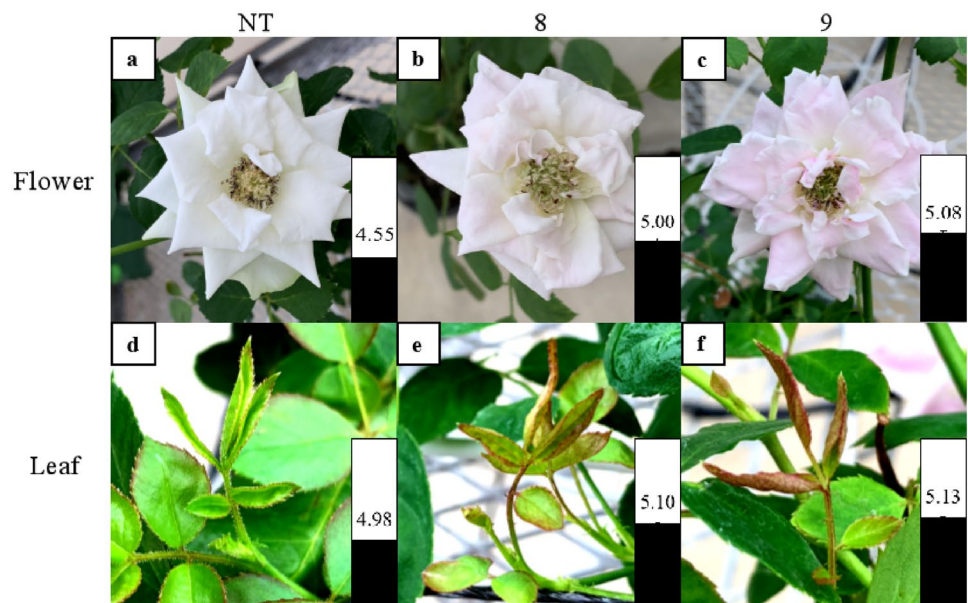
gene of the *Rosa hybrida* line "KR056002" is similar to that of the heterologous gene, so a similar band was also observed in non-transgenic (NT) plants (Fig. 2a). However, subsequent Southern blot, northern blot, and qRT-PCR analysis results confirmed that the heterologous gene *RhNHX* was successfully introduced into the five transgenic lines (Figs. 2b, 3, 4b).

The copy numbers of the *RhNHX* and *VfF3'5'H* genes were further detected using Southern blot hybridization using *RhNHX*- and *VfF3'5'H*-specific probes, respectively. Blotting results showed that the copy numbers of *RhNHX* and *VfF3'5'H* differed in the same transgenic lines. The five transgenic lines had one to five copies of the integrated *RhNHX* gene and one to three copies of the integrated

VfF3'5'H gene (Fig. 2b). DNA from the control NT plants did not generate hybridization bands (Fig. 2b).

The leaf RNA was isolated from the transgenic lines (2, 3, 4, 8, and 9), and NT plants were analyzed using northern blot with the specific *RhNHX* and *VfF3'5'H* probe (Fig. 3). *RhNHX* and *VfF3'5'H* mRNAs were detected in the five transgenic lines, but not in NT plants, indicating that the *RhNHX* and *VfF3'5'H* genes were expressed in the transgenic lines (Fig. 3). According to the different intensities of the bands, we found that the *RhNHX* gene expression level was higher in transgenic lines 3 and 4, while the *VfF3'5'H* gene expression level was higher in transgenic lines 3, 8, and 9 (Fig. 3). By comparing the results of the Southern blot and northern blot, we found

Fig. 5 pH of the flower petals (a–c) and young leaves (d–f) of NT plants and transgenic lines (8 and 9) co-expressing *RhNHX* and *VtF3'5'H* in *Rosa hybrida*. The bars represent the mean values of samples tested in triplicate (\pm) with standard error. *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺ exchanger gene; *VtF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants. (Color figure online)



that there was no correlation between copy number and gene expression levels (Figs. 2b, 3).

Transcriptional analysis of the *RhNHX* and *VtF3'5'H* genes in transgenic lines

RT-PCR was used to detect the mRNA expression levels of the *RhNHX* and *VtF3'5'H* genes in the transgenic lines (Fig. 4a). The *RhNHX* mRNA was also slightly expressed in the NT roses (Fig. 4a). To accurately measure the expression levels of *RhNHX* and *VtF3'5'H* among these lines, their transcription levels were quantified using qRT-PCR. As shown in Fig. 4b and c, the transcription levels of *RhNHX* and *VtF3'5'H* in the transgenic lines were significantly higher than those in the NT plants. Transgenic line 3 had the highest transcription level of *RhNHX*, followed by lines 4, 9, 2, 8, and NT plants (Fig. 4b), whereas the *VtF3'5'H* gene showed the highest transcription level in line 3, followed by lines 9, 8, 4, 2, while no expression of the *VtF3'5'H* gene was found in NT plants (Fig. 4c).

Phenotypic characteristics

To clearly observe the phenotypic characteristics of the transgenic lines, we successfully acclimatized transgenic lines 8, 9, and NT plants in a greenhouse. Unfortunately, due to the high expression of *RhNHX* and *VtF3'5'H* in line 3 rooting was inhibited and we could not get the plants in greenhouse (Li et al. 2018). The visual phenotypes of flower color and young leaves between the transgenic lines and NT plants differed significantly (Fig. 5). The young leaves of NT plants were green, whereas the young leaves of the two transgenic lines turned red (Fig. 5d–f). The color of the petals

was measured according to RHSCC, which showed that the petal color changed significantly from white (RHSCC white group) to red-purple (red-purple group) (Fig. 5a–c; Suppl. Table S3). The color of the petals was also measured using colorimetry, and we found that the values of the L* parameter (lightness) and b* parameter (yellowness) of the transgenic lines were lower than those of the NT plants, whereas the value of the a* parameter (redness) increased (Suppl. Table S3), indicating that the color became bluer.

Analysis of total anthocyanin accumulation and delphinidin content

When anthocyanins were extracted from the petals and young leaves of transgenic and NT plants, the petal extract of NT plants was light green and the leaves were dark green (Fig. 6c, d). The petal extract solutions of the two transgenic lines were light pink, and the leaves were dark red, with the line 9 extract solution being redder than the line 8 extract solution (Fig. 6c, d). Anthocyanin content analysis showed that the anthocyanin levels of transgenic plants were significantly higher than those of NT plants, while the anthocyanin levels in line 9 were higher than those in line 8 (Fig. 6a, b). We identified the content of six common anthocyanins using ultra-performance liquid chromatography (UPLC), but only delphinidin was detected in the transgenic lines (Fig. 7). Similar to the above results, the delphinidin content of transgenic line 9 was higher than that of line 8, whereas no delphinidin was detected in NT plants (Figs. 6, 7).

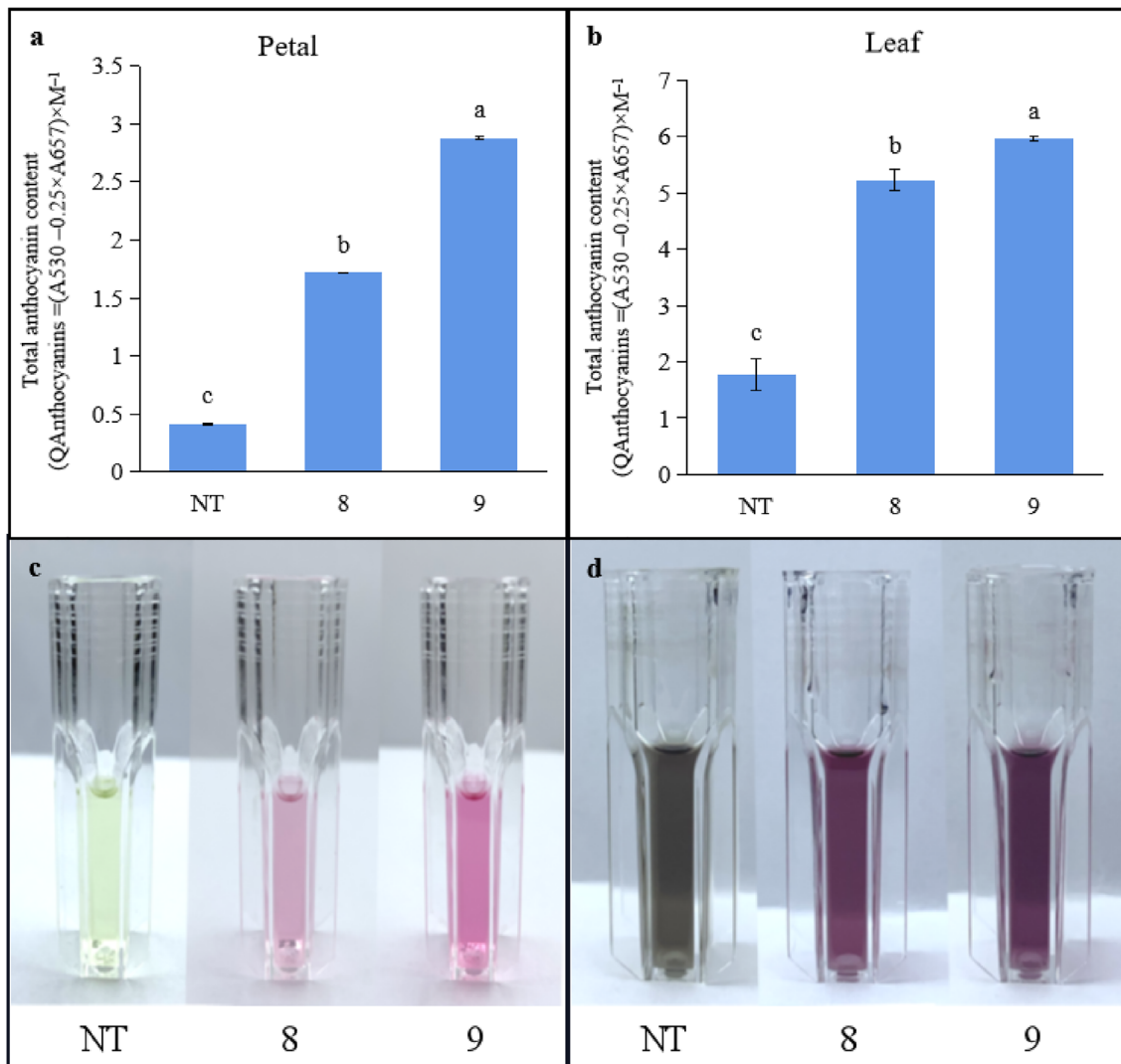


Fig. 6 Comparison of the total anthocyanin contents of NT plants and transgenic lines (8 and 9) co-expressing *RhNHX* and *ViF3'5'H* in *Rosa hybrida* (**a** petal; **b** leaf) and visual anthocyanin accumulation in vegetative tissues (**c** petal; **d** leaf). The bars represent the mean values

of samples tested in triplicate (\pm) with standard error. *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺ exchanger gene; *ViF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants. (Color figure online)

pH of petals and young leaves

The pH values of the extract solutions from the transgenic lines and NT plants were measured. We observed that the pH values of the petals and young leaves of the transgenic lines were higher than those of NT plants (Fig. 5). The pH of the petals of NT plants and transgenic lines 8 and 9 were 4.55, 5.0, and 5.08, respectively (Fig. 5a–c). The pH of young leaves was significantly higher than that of petals, and the pH values of the young leaves of NT plants and transgenic lines 8 and 9 were 4.98, 5.1, and 5.13, respectively (Fig. 5d–f).

Transcriptional analysis of anthocyanin pathway genes in transgenic lines

The expression of anthocyanin pathway genes (*RhCHS*, *RhCHI*, *RhF3H*, *RhF3'5'H*, *RhDFR*, and *RhANS*) in the young leaves and petals of transgenic lines and NT plants was detected using RT-PCR (Suppl. Fig. S2). We analyzed the expression levels of six anthocyanin biosynthesis genes in the petals and young leaves of the transgenic lines and NT plants (Fig. 8). The anthocyanin pathway gene expression levels in the young leaves and petals of the transgenic lines were significantly higher than those in NT plants. Except for the *RhF3'5'H* gene, the expression levels of the other genes in petals increased more than those in young leaves

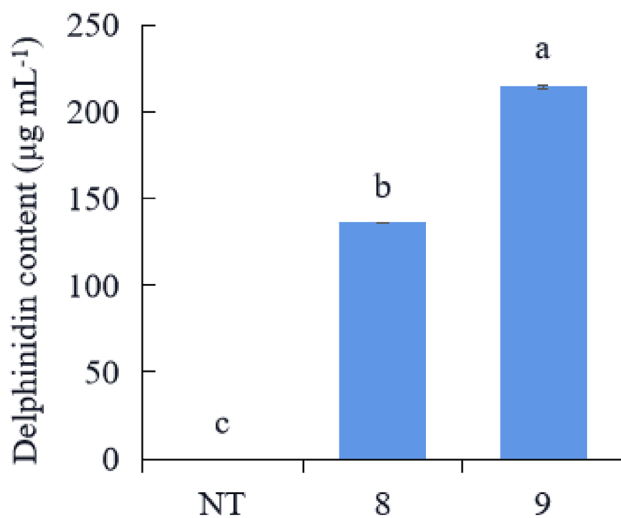


Fig. 7 Delphinidin contents in the petals of NT plants and transgenic lines (8 and 9) co-expressing *RhNHX* and *VtF3'5'H* in *Rosa hybrida*. The bars represent the mean values of samples tested in triplicate (\pm) with standard error. *RhNHX*: *Rosa hybrida* vacuolar Na⁺/H⁺-exchanger gene; *VtF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants. (Color figure online)

(Fig. 8a–f). This was also consistent with the results of the anthocyanin content measurements in young leaves and petals (Fig. 6). In brief, the anthocyanin content of NT plant petals was 0.41, whereas those in transgenic lines 8 and 9 were 1.72 and 2.88, respectively, which increased by 4.2-fold and 7-fold compared with those of NT plants (Fig. 6a). The anthocyanin content of NT plant leaves was 1.8, and those of transgenic lines 8 and 9 were 5.2 and 6.0, respectively, which increased 2.9-fold and 3.3-fold compared with those of NT plants (Fig. 6b). The expression levels of the endogenous gene *RhF3'5'H* of transgenic lines 8 and 9 increased 1.5-fold and 1.7-fold compared with that of NT plants in the petals, and 8.9-fold and 11.5-fold in young leaves (Fig. 8d). We speculate that the expression of the endogenous gene *RhF3'5'H* may be restricted in petals, whereas the heterologous gene *VtF3'5'H* is abundantly expressed in petals (Fig. 8d, h). The increase in the *RhNHX* gene expression levels was also higher in petals than in young leaves, which is consistent with the pH measurement results (Figs. 5, 8g).

Discussion

Although the *NHX* and *F3'5'H* genes can modify flower color independently, studies on repairing flower color by co-expressing *NHX* and *F3'5'H* have not been reported. In this study, we found that heterologous co-expression of *RhNHX* and *VtF3'5'H* in roses resulted in a significant increase in the anthocyanin content in young leaves and petals. Compared with NT plants, the phenotype of transgenic plants changed

significantly, and the petals of transgenic plants changed from white to red-purple. The young leaves of the NT plants remained green, whereas the transgenic lines showed obvious anthocyanin accumulation (Fig. 5; Suppl. Table S3). Previous studies have shown the modification of plant flower color using RNAi-mediated gene silencing or overexpression of the *F3'5'H* gene in plants, such as verbena (Togami et al. 2006), grapevines (Robinson et al. 2019), cyclamen (Boase et al. 2010), chrysanthemums (Noda et al. 2013; He et al. 2013), tobacco (Goto and Kondo 1991; Okinaka et al. 2003), and petunia (Mori et al. 2004; Ishiguro et al. 2012; Qi et al. 2013). Previous studies have shown that an increase in the vacuolar pH can increase the expression of blue color in flowers (Yoshida 1995; Mol et al. 1998; Ohnishi et al. 2005), such as the deep or strong reddish-purple buds of morning glory becoming moderate or light blue after flowering, and the pH of the epidermal tissue can also increase from approximately 6.5 to 7.5% (Asen et al. 1977). Our results also proved that the overexpression of the *RhNHX* gene caused a significant increase in the pH value of the petals and young leaves of transgenic lines (Fig. 5). Many studies have shown that *NHX* genes are closely related to salt stress tolerance in plants (Sahoo et al. 2016; Guo et al. 2020; Al-Harrasi et al. 2020). However, few studies have explored their role in changing flower color. It has been reported that *InNHX1* gene mutations in Japanese morning glory petals change their color from blue to purple, and that the vacuolar pH of purple flowers is significantly lower than that of blue flowers (Fukada-Tanaka et al. 2000; Yamaguchi et al. 2001).

In our study, we used RHSCC and colorimeters to measure the color phenotype of flowers and leaves, and we observed that the color became bluer, consistent with the results of previous studies (He et al. 2013). The varying shades found in blue flowers are attributed to delphinidin, and *F3'5'H* is the key gene for the synthesis of the anthocyanin delphinidin (Lou et al. 2014; Noda 2018). Our UPLC results showed that anthocyanins were not detected in NT petals, but delphinidin was detected in transgenic lines. *F3'5'H* may be involved in the biosynthesis of delphinidin, but it has little effect on the biosynthesis of cyanidin, pelargonidin and peonidin. However, the derivatives of delphinidin, petunidin and malvidin, have not been detected. This is consistent with the results of Nakamura et al., the heterologous expression of *F3'5'H* gene does not affect the content of petunidin and malvidin, which is useful for us to study the molecular function of *F3'5'H* in anthocyanin biosynthetic pathway (Nakamura et al. 2015). Wang et al. reported similar results, showing that the expression of the *CsF3'5'H* gene in tobacco plants produced delphinidin in the petals, but it was not detected in the wild type plants, and the petals changed from pale pink to magenta (Wang et al. 2014). The study by Katsumoto et al. in 2007, showed that the heterologous expression of the *viola F3'5'H* gene in roses

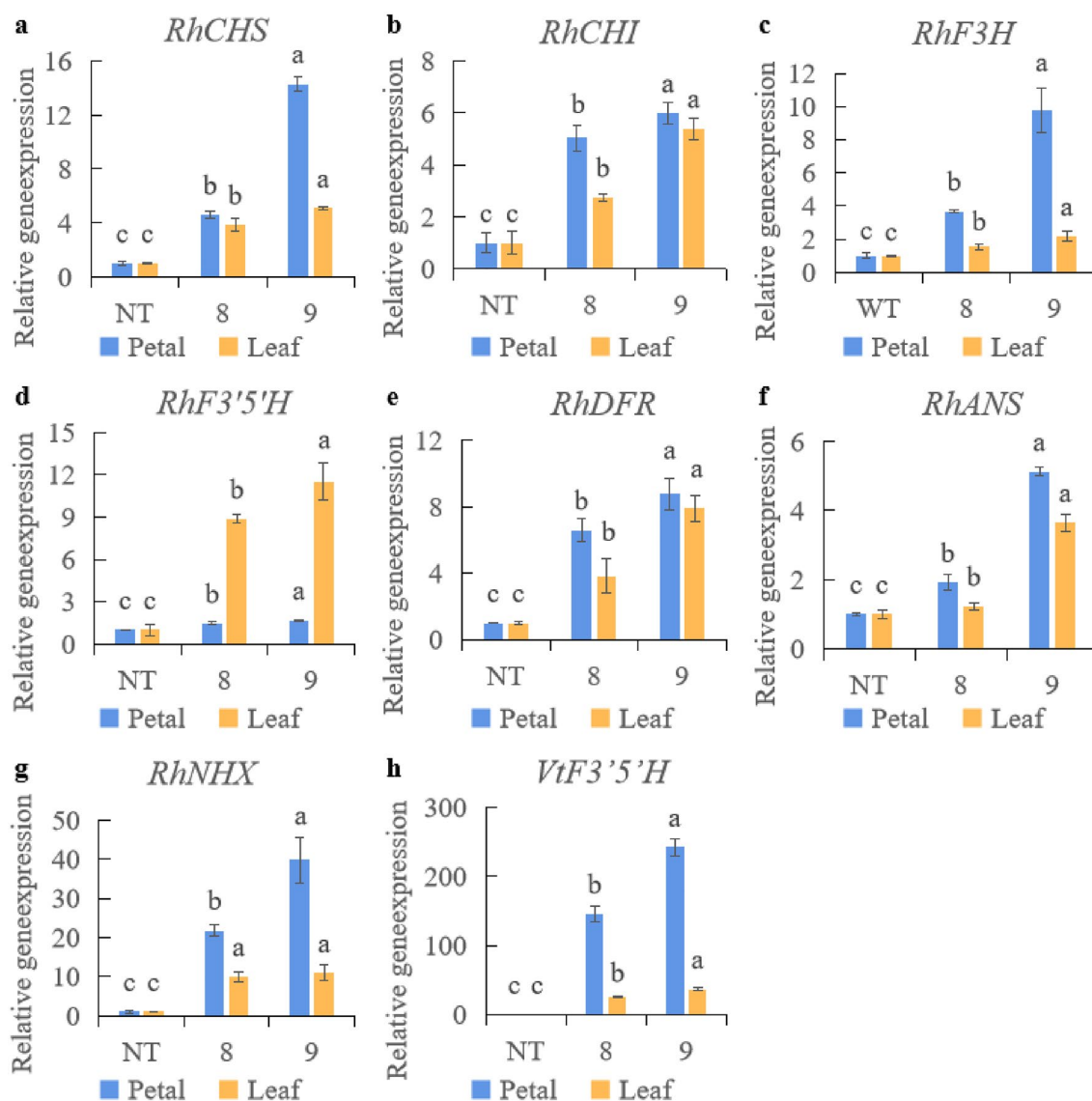


Fig. 8 Comparison of the transcript levels of the anthocyanin structural genes (a–f), *RhNHX* (g), and *VtF3'5'H* (h) expressed in NT plants and transgenic lines (8 and 9) co-overexpressing *RhNHX* and *VtF3'5'H* in *Rosa hybrida*. Error bars indicate the standard errors of the mean of three replicates. Expression levels are shown relative to actin expression. Means with different letters are significantly different (Duncan's multiple range test, $p < 0.05$). *RhCHS*, *Rosa hybrida*

chalcone synthase gene; *RhCHI*: *Rosa hybrida* Chalcone flavanone isomerase gene; *RhF3H*: *Rosa hybrida* flavanone 3-hydroxylase gene; *RhF3'5'H*: *Rosa hybrida* flavonoid 3',5'-hydroxylase gene; *RhDFR*: *Rosa hybrida* dihydroflavonol 4-reductase gene; *RhANS*: *Rosa hybrida* anthocyanin synthase gene; *RhNHX*: *Rosa hybrida* Na⁺/H⁺-exchanger gene; *VtF3'5'H*: *Viola tricolor* flavonoid 3',5'-hydroxylase gene; NT: non-transgenic plants. (Color figure online)

led to an increase in delphinidin accumulation in petals, and successfully produced blue roses (Katsumoto et al. 2007). Although we did not obtain blue roses as expected, this may be because the raw material “KR056002” we used was a white group and did not contain delphinidin. The transgenic line showed accumulation of a certain amount of delphinidin, but it was not enough to produce blue flowers.

Southern blot analysis showed that when the *RhNHX* and *VtF3'5'H* genes were transferred into plants in the same T-DNA vector, the integrated copy numbers of

the two genes were also different. These results suggest that when heterologous genes are inserted into the plant genome, the copy number and insertion sites are random and T-DNA may rearrange during chromosome integration (Beltrán et al. 2009). The expression of *RhNHX* and *VtF3'5'H* was detected in all transgenic lines using RT-PCR. Furthermore, the qRT-PCR results showed that the gene expression levels of *RhNHX* and *VtF3'5'H* were significantly different in each transgenic line. The expression level of *RhNHX* was highest in line 3, followed by lines

4, 9, 8, 2, and NT plants. In contrast, the highest expression level of the *VtF3'5'H* gene was detected in line 3, followed by lines 9, 8, 4, and 2, and NT plants. There was no relationship between the number of transgenic copies and their expression levels. These results are also consistent with previous findings (Beltrán et al. 2009). When the copy number is low (one or two), plants usually express the inserted heterologous gene at a high level, which is not absolute. If too many copies are present, it may lead to unstable expression or even gene silencing (Flavell 1994; Vaucheret et al. 1998). Our results also showed that line 3, with a low copy number, had higher expression levels than the other lines.

We found that the *RhNHX* gene expression levels in the transgenic lines were significantly higher in petals than in young leaves (Fig. 8g). In petals, line 8 and line 9 levels increased by 21.8-fold and 39.9-fold, respectively, whereas in young leaves, they increased 10-fold and 10.9-fold in lines 8 and 9, respectively. This is also consistent with our results for changes in the pH values of petals and young leaves (Fig. 5). Compared with NT plants, the pH value of the petals of line 8 increased by 0.45 (1.1-fold) and that of line 9 increased by 0.53 (1.12-fold), whereas the pH value of the young leaves of line 8 increased by 0.12 (1.02-fold) and that of line 9 increased by 0.15 (1.03-fold). However, the overexpression of *RhNHX* resulted in more obvious changes in the pH value of the petals. Sandhu et al. identified six *NHX* genes in *Medicago truncatula* using qRT-PCR and reported that gene expression in different organs was different (Sandhu et al. 2018). Our qRT-PCR results also showed that the heterologous gene *VtF3'5'H* was expressed at a high level in petals and young leaves, similar to the *RhNHX* gene (Fig. 8h). The expression level of the *VtF3'5'H* gene in petals was significantly higher than that in young leaves. These results are in accordance with those of previous reports showing that the expression level of *F3'5'H* gene was different in various tissues (Qi et al. 2013; Wang et al. 2014; Deng et al. 2018).

To better understand the effects of the heterologous genes *RhNHX* and *VtF3'5'H* on the anthocyanin synthesis pathway genes of roses, we used RT-PCR and qRT-PCR to detect the endogenous gene expression levels of *RhCHS*, *RhCHI*, *RhF3H*, *RhF3'5'H*, *RhDFR*, and *RhANS* in petals and young leaves, respectively (Fig. 8; Suppl. Fig. S2). Our RT-PCR results showed that *RhDFR* and *RhANS* were slightly expressed in the petals of NT plants (white flower), whereas the transgenic lines (red-purple flower) had significantly increased expression of *RhDFR* and *RhANS* (Suppl. Fig. S2). Our results are consistent with the findings of He et al., showing that pink, red, and purple chrysanthemums all express the different anthocyanin biosynthesis pathway genes (*CmCHS*, *CmCHI*, *CmF3H*, *CmF3'H*, *CmDFR*, *CmANS* and *Cm3GT*), while white flowers only expressed

CmCHS, *CmCHI*, *CmF3H*, and *CmF3'H* (He et al. 2013). Interestingly, in our study, the expression level of the endogenous *RhF3'5'H* gene in transgenic lines increased only 1.5- and 1.7-fold in petals, which was very different from that of the heterologous gene *VtF3'5'H* (Fig. 8d, h). Similar results have been previously reported showing that heterologous genes are overexpressed in transgenic lines, that the expression level of endogenous genes is not affected, and that their expression level is similar to that in NT plants (Mazarei et al. 2018). This may be because the introduction of heterologous genes may lead to different results (upregulation, suppression, and similarity) in the endogenous gene expression levels (Wang et al. 2006).

Conclusion

In this study, we introduced the two heterologous genes *VtF3'5'H* and *RhNHX* into the white *Rosa hybrida* line “KR056002” using *Agrobacterium*-mediated transformation. PCR, Southern blot, and northern blot analyses were used to determine the copy number and mRNA expression level of the heterologous genes in the transgenic lines. The qRT-PCR results showed that the introduction of heterologous genes increased the expression of anthocyanin pathway synthesis genes and increased the total anthocyanin accumulation and delphinidin content. The introduction of the *VtF3'5'H* gene promoted the synthesis of delphinidin anthocyanin, whereas the upregulation of the *RhNHX* gene resulted in an increase in the pH value of the petals and young leaves of transgenic lines. Finally, we achieved color modification, in which the petals of the transgenic plants turned from white to red-purple. Our study provides a new feasible scheme for color modification in rose plants.

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Author contributions SYL was the major investigator on this project. SYL and JX. planned and designed the experiment; JX wrote the manuscript. JX and JYS performed experiments and PMP, HRA, Y-JK. and SJK analyzed the data. All authors have read and agreed to the published version of the manuscript.

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Data availability The data supporting the findings of this study are available from the corresponding authors, upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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