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**Research Article**

# New Role of Rosea1 in Regulating Anthocyanin Biosynthetic Pathway in Hairy Root of Snapdragon (*Antirrhinum majus* L.)

[1, 2]An Zhang and [2]Min-Long Cui
[1]Graduate School, Chinese Academy of Sciences, Beijing 100039, China
[2]Group of Molecular Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, Liaoning 110016, China

**Abstract:** We investigated the transcriptional regulation of anthocyanin biosynthesis in hairy roots system by ectopically expressing *Rosea*1 and *Delila* and we found something different from previous research. The RT-PCR results revealed that *Rosea*1 could activate early and late biosynthetic genes tested, including *CHS*, *DFR* and *ANS*. *Delila* enhanced the expression of *CHS* weakly, but did not influence *DFR* or *ANS*. The two regulators, *Rosea*1 and *Delila*, failed to interplay each other. It was speculated that *Delila* would be ineffective in the absence of *Rosea*1, another MYB factor specifically controlling CHS may exist. This investigation provided a new way to increase anthocyanin content by over expressing a MYB factor, potentially to be used in the field of agriculture and food

**Keywords:** Anthocyanin biosynthesis, *Antirrhinum majus*, hairy root, snapdragon

## INTRODUCTION

Flavonoids are major plant secondary metabolites found through out the plant kingdom, including the model species Antirrhinum majus. These diphenylchroman compounds play important roles in plant growth and development by providing plants with gorgeous pigments (Winkel-Shirley, 2001), protecting against UV radiation (Veit and Pauli, 1999), attracting pollinators and other beneficial organisms (Buer *et al*., 2010). In addition, antioxidant properties of flavonoid compounds have nutritional value for human health (Liu *et al*., 2006; Winkel-Shirley, 2001). Biosynthesis of flavonoids, especially anthocyanins, which are responsible for purple coloration of leaves and flowers, is stimulated by abiotic and biotic stresses (Dixon and Paiva, 1995), including cold, high irradiance, excess sugar (Tsukaya *et al*., 1991), or deprivation of inorganic macronutrients like phosphorous and nitrogen (Morcuende *et al*., 2007; Scheible *et al*., 2004).

The flavonoid backbone is formed through the central flavonoid biosynthetic pathway (Winkel-Shirley, 2002). Transcriptional control of flavonoid biosynthesis has been intensively studied (Broun, 2005). The MYB/bHLH/ WD40 complex plays an important role in regulation of anthocyanin accumulation in plant species, such as Arabidopsis thaliana (Payne *et al*., 2000; Ramsay and Glover, 2005). The WD-repeat protein Transparent Testa Glabra1 (TTG1) (Walker *et al*., 1999) together with basic-helix-loop-helix (bHLH) transcription factors (Toledo-Ortiz *et al*., 2003), such as Glabra3 (GL3) (Payne *et al*., 2000), Transparent Testa8 (TT8) (Baudry *et al*., 2006; Nesi *et al*., 2000), or Enhancer of Glabra3 (EGL3) (Zhang *et al*., 2003) and R2R3-MYB transcriptional factors (MYB75, MYB90, MYB113, or MYB114) (Allan *et al*., 2008; Borevitz *et al*., 2000; Stracke *et al*., 2007, 2001; Zimmermann *et al*., 2004) to form the MYB/bHLH/ WD40 complex, which controls anthocyanin biosynthesis by upregulating the expression of Late Biosynthetic Genes (LBG), including DFR, ANS and UF3GT (Dooner *et al*., 1991; Gonzalez *et al*., 2008).

Antirrhinum majus is well established as a model system for molecular studies of anthocyanin biosynthesis, especially flower colour. Like other plant species, specific factors of R2R3-MYB and bHLH families interact to regulate genes in the anthocyanin biosynthetic pathway. In A. majus, a number of mutants that affect the activity of regulatory loci have been described, including delila, Eluta, rosea, Venosa and mutabilis (Martin and Gerats, 1993; Martin *et al*., 1991). Delila has been shown to encode a bHLH factor that is required for the activation of LBGs (Goodrich *et al*., 1992; Martin *et al*., 1991).

However, loss function of DELILA results in loss of pigmentation only in the corolla tube. *Mutabilis*, acts redundantly with *Delila* in activation of the LBGs in the lobe (Schwinn *et al*., 2006). R2R3-MYB transcription factors involved in the pigmentation of flowers have also been identified (Schwinn *et al*., 2006). The *Rosea* locus comprises two closely linked genes encoding R2R3-MYB proteins, Rosea1 and Rosea2. Rosea1 gives

**Corresponding Author:** An Zhang, Group of Molecular Ecology, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, Liaoning 110016, China

full-red corolla pigmentation in both the adaxial and abaxial epidermis. Rosea2 gives weak pigmentation, principally in the adaxial epidermis of the corolla lobes. A third gene, *Venosa*, encodes another R2R3-MYB protein that controls the anthocyanins in adaxial epidermal cells that overlie the veins of the corolla (venation), a phenotype visible only when *Rosea1* is inactive (Shang *et al*., 2011).

At the time of this writing, a significant body of work has accumulated implicating the importance of MYB and bHLH factors in the regulation in the flora color of snapdragon (Martin and Gerats, 1993; Shang *et al*., 2011). However, the number of reports showing the regulation of anthocyanin in other tissue and organ of this recalcitrant plant is surprisingly limited, especially with transgenic method. The objective of the study reported here was to investigate the transcriptional regulation mechanism of anthocyanin biosynthesis in hairy root of snapdragon overexpressing *Rosea*1 and *Delila* induced by *A. rhizogenes*. We found that Rosea1 could activate the expression of EBGs (early biosynthetic genes) (CHS) and LBGs (*DFR* and *ANS*); Delila could up regulate *CHS* weakly, but not LBGs. The expression profiles were at odds with early results in flora, especially the expression pattern of EBGs.

## MATERIALS AND METHODS

**Plant material:** Seeds of A. majus cv. JI7 were surface sterilized by briefly rinsing them first in 70% (v/v) ethanol and then in 2% (v/v) sodium hypochlorite for 5 min, after which they were washed with sterile, distilled water three times. The seeds were germinated on MS medium containing 2.5 g/L gelrite and 25 g/L sucrose at 25°C under a 16 h light/8 h dark photoperiod in a culture room with fluorescent light at an intensity of 5000 lux. Leaf and stem segments were isolated from 4-week-old plants as explants to induce hairy root.

**Preparation of A rhizogenes:** The pBI121 vector contains a Cauliflower Mosaic Virus (CaMV) 35S promoter::Rosea1 or Delila (full length cDNA) fusion sequence and the Neomycin Phospho Transferase gene (NPTII) as a selectable marker. This binary vector was electroporated into A.rhizogenes (AR1193). A. rhizogenes cultures were inoculated from glycerol stock and grown overnight at 28°C with shaking (180 rpm) in liquid Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) containing kanamycin (50 mg/L), to mid-log phase OD A600 0.5. Cells were collected by centrifugation for 10 min at 2000 rpm and resuspended in liquid inoculation medium (MS salts and vitamins containing 25 g/L sucrose). Cell density was adjusted to an OD A600 of 1.0 for inoculation.

**Inoculation and root culture:** Freshly grown A. rhizogenes (AR1193) in 3 mL LB medium containing 50 mg/L rifampicine and 100 mg/L kanamycin at 28°C for 24 h was used for inoculation. The bacterial suspension culture was diluted 1:50 with liquid MS medium. The leaf pieces and stem segments of 4-week-old plants after germination were cut and inoculated with diluted bacterial suspension for 5 min, followed by transfer to solidified MS co-cultivation medium supplemented with 25 g/L sucrose, 100 mM acetosyringone and 2.5 g/L gellan gum (pH 5.8). After 3 days of co-cultivation in darkness, the leaf pieces and stem segments were transferred to solid MS medium with 25 g/L sucrose and 300 mg/L cefotaxime. Root tips (about 1 cm) of induced adventitious roots including hairy roots were excised and transferred to the same medium. The culture was kept at 25°C under a 16/8 h photoperiod with fluorescent light (5000 lux). Axenic root cultures were established after two subcultures by transfers with 2-week intervals.

**Polymerase chain reaction analysis for Rosea1 and Delila gene:** Genomic DNA was extracted from hairy roots according to the modified CTAB method (Porebski *et al*., 1997). PCR was performed in a reaction mixture containing 100 ng of plant genome DNA, 100 mM of each dNTPs, 0.2 mM of each primer and 1U of Taq polymerase (TAKARA). Reactions were started with a denaturation at 94°C for 3 min, followed by 40 cycles of 93°C for 1 min, 55°C for 2 min and 72°C for 3 min; the program was terminated by an extension at 72°C for 10 min. Amplified DNA bands were analyzed by agarose gel electrophoresis at 100 V for 30 min followed by staining with ethidium bromide. Oligonucleotide primers are given in Table 1.

**Expression analyses:** Total RNA was extracted from hairy roots with the Plant RNeasy Mini kit (Qiagen). One microgram of total RNA was DNase I treated and used for cDNA synthesis with oligo (dT) primers and Superscript reverse transcriptase (Invitrogen). Semi-Quantitative RT-PCR was conducted for 30 cycles with the following thermal profiles: 94°C for 30 sec (3 min for the first cycle), 60°C for 30 sec and 72°C for 2 min 30 sec, with a 10 min terminal extension step at 72°C. Ubiquitin (UBI) was amplified as internal control.

Table 1: Primer sequences used in PCR analysis

| Gene name | Primer 5' | Primer 3' |
|---|---|---|
| *Delila* (for PCR) | ATGGCTACTGGTATCCAAAA | AAGTCTTCATAGTAACTTT |
| *Delila* (for RT-PCR) | ATGGCTATGCAACGCTCAT | CCCATTGATGCCTTCTGC |
| *Rosea*1 | ATGGAAAAGAATTGTCGTGGAG | TTAATTTCCAATTTGTTGGGCCTC |
| *CHS* | GCAGCAGCGGTTATAGTTG | CGCCGAAGACTTCCTCAT |
| *DFR* | GTGCGATTGACACTTGCC | CTGCCATCAGTATGATCGTTTG |
| *ANS* | GTACGCGAAGTGTCTTCGTT | TGTTGAGCAAAAGTCCGTGG |
| *UBI* | CCTCCTATGCTCCTGTCA | ATCGTCTTCTCCTTCCTCTA |

Amplified DNA bands were analyzed by agarose gel electrophoresis at 100 V for 30 min followed by staining with ethidium bromide. Oligonucleotide primers are given in Table 1.

## RESULTS

**Characteristics of hairy roots over expressing Rosea1 and Delila:** Snapdragon hairy roots were induced in leaf and stem explants inoculated with A. rhizogenes. Two weeks after infection with A. rhizogenes, the first adventitious roots appeared from the wounded leaf pieces and stem segments grown on MS medium containing 300 mg/L cefotaxime without plant growth regulators (data not shown). Six weeks after infection, more adventitious roots were obtained from explants, all the roots of which showed rapid growth, lateral brancing and plagiotropism (negatively geotropic) on solid hormone-free 1/2MS medium containing 300 mg/L cefotaxime.

Three categorys of hairy roots over expressing *Rosea1* were obtained: white (Fig. 1A), light red (Fig. 1B), deep red (Fig. 1C). Those hairy roots showed similar morphology besides color and we recognized the light red and deep red roots to be the transformants easily. But we could not confirm the white roots were transformants or not. So PCR was used to detect and verify the hairy roots. As the results shown in Fig. 1A, we knew that all the roots including white and red were transformants, because all the roots showing an objective fragment of 700 bp.

Hairy roots over expressing *Delila* showed similar characteristic compared to white roots over expressing *Rosea1*. PCR was also used to detect and verify the hairy roots whether they were transformants or not. At last we confirmed that almost of the roots tested were positive, as shown in Fig. 1B.

**Rosea1 enhances expression levels of EBGs and LBGs in hairy roots:** All of the hairy roots tested in Fig. 2 harbored Rosea1 cDNA, which was transferred by A. rhizogenes, but showed different color, 29 and 49 were white roots, 24 and 25 were light red roots, the others were deep red roots. In order to investigate the reason why those roots show different phenotype, Semi-Quantitative RT-PCR was recruited. We checked the expression levels of EBGs (CHS), LBGs (DFR and ANS). As the results shown in Fig. 3, we found that the coloration of roots and expression levels of EBGs and LBGs were related with the expression level of Rosea1. In the control and white transformants, expression level of CHS was hardly detected, expression levels of DFR, ANS and Rosea1 were not detectable; in light red roots, all the genes expression levels were higher than that in control and white roots; in the deep red roots, the expression levels were the topmost (Fig. 2). Those data nicely showed that EBGs and LBGs were upregulated by Rosea1.

We also measured the expression level of another regulator, Delila. No signal was detected in all the roots tested. So Rosea1 could not regulate the expression of Delila.

**Delila activates the expression of CHS, but not LBGs:** When roots overexpressed Delila, the roots showed white coloration (Fig. 1D). We said that Delila

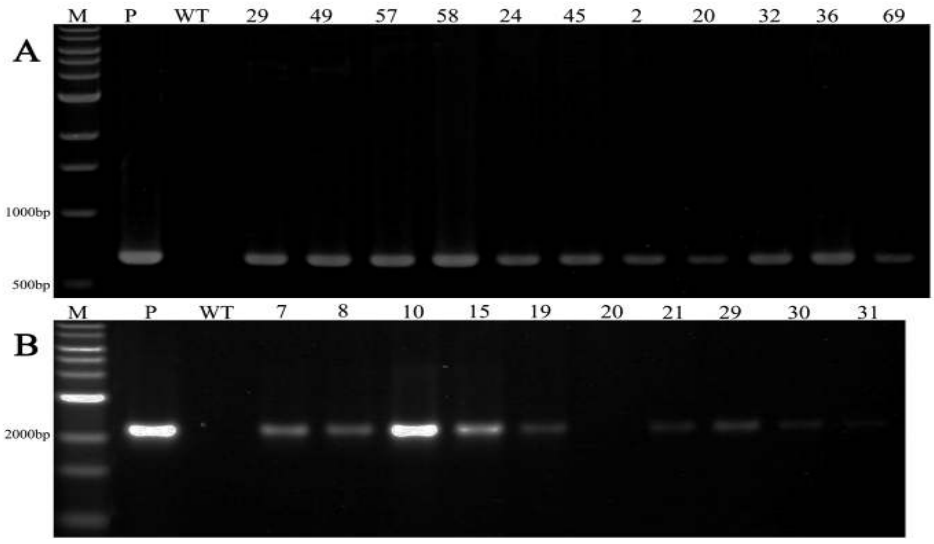


Fig. 1: PCR analysis of hairy roots overexpressing *Rosea1* and *Delila*. A, PCR results of hairy roots overexpressing *Rosea*1, the objective fragment is about 700bp; the number above on each lane indicates independent hairy root, 29,49,57,58 are white roots; 24 and 25 are light red roots; the others are deep red roots; B, PCR results of hairy roots overexpressing *Delila*, the objective fragment is about 2000bp; M, Marker, 1KB ladder; P, plasmid pBI121/*Rosea*1 or plasmid pBI121/*Delila* in A and B, respectively , positive control; Wt, JI7 wild type, negative control

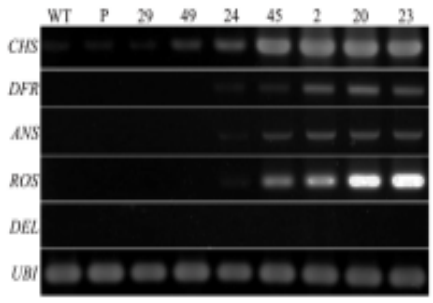


Fig. 2: Gene expression patterns of JI7 hairy roots overexpressing *Rosea1*. WT, JI7 wild type roots, negative control; P, hairy root expressing pBI121/*GUS*, negative control; the number above on each lane indicates independent hairy root, 29,49 are white roots; 24 and 25 are light red roots; the others are deep red roots; Take *UBI* of snapdragon as inner control

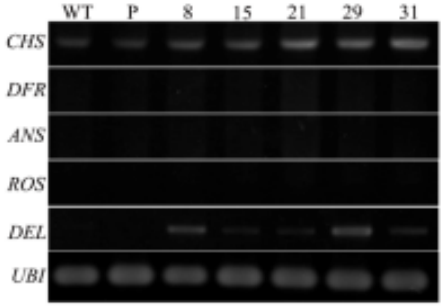


Fig. 3: Gene expression patterns of JI7 hairy roots overexpressing *Delila*. WT, JI7 wild type roots, negative control; P, hairy root expressing pBI121/*GUS*, negative control; the number above on each lane indicates independent hairy root; Take *UBI* of snapdragon as inner control

could not regulate the expression of EBGs or LBGs arbitrarily. Semi-Quantitative RT-PCR was also used to verify the role of Delila in hairy roots. From Fig. 3 we knew the expression level of CHS was related with the expression level of Delila. Other genes, such as DFR, ANS and Rosea1 were not detectable in all the roots. It was concluded that Delila could upregulate CHS weakly, but not LBGs (DFR, ANS) or Rosea1.

## DISCUSSION

The role of MYB and bHLH has been reported in a substantial body of literature (Baudry *et al*., 2006; Gonzalez *et al*., 2008; Goodrich *et al*., 1992; Schwinn *et al*., 2006). More often than not, MYB and bHLH factors involved in anthocyanin biosynthesis upregulated LBGs (DFR, ANS and 3GT) (Morcuende *et al*., 2007). In A.majus, MYB factor (Rosea1) and bHLH factor (Delila) were proved previously to control the expression levels of LBGs in flower. Here, we found something new with ectopic expression of Rosea1 and Delila in hairy roots system induced by A. rhizogenes.

Rosea1 determined the expression levels of EBGs and LBGs in hairy roots, thus the coloration. Another MYB factor specifically controlling EBGs may exist. In red hairy roots over expressing Rosea1, the RT-PCR results, which differed from previous reports in flower (Schwinn *et al*., 2006; Shang *et al*., 2011), indicated that Rosea1 regulates EBGs (CHS) and LBGs (DFR and ANS). Loss function of Rosea1 resulted in undetectable expression of DFR or ANS in floral petal of snapdragon, but CHS was not influenced, indicating that CHS was not controlled by Rosea1 in petal. In our hairy roots system, it was demonstrated that ectopic expression of Rosea1 led to high expression of CHS. So we speculated that another MYB factor specifically controlling EBGs may exist, which did not regulate LBGs, just as the mechanism in A. thaliana, in which EBGs were regulated by MYB11, MYB12 and MYB117 redundantly (Stracke *et al*., 2007). In the flower of snapdragon, Rosea1 and another MYB factor regulate expression of EBGs (CHS) redundantly. When one of them lost function, the expression levels of EBGs did not decrease. When Rosea1 over expressed, EBGs and LBGs expression levels increased. The genome information of snapdragon is unclear until now; the number of MYB family in snapdragon is unclear either. The MYB factor controlling EBGs specifically would be isolated and investigated in foreseeable future.

Delila regulated EBGs (CHS) weakly, not LBGs, in our hairy roots system. Previous studies demonstrated that Delila controlled LBGs in flower of snapdragon and other plant (Goodrich *et al*., 1992). TT8, belonging to bHLH family in A.thaliana, activated expression of DFR and BAN, depending on MYB-bHLH-WD40 complex (Nesi *et al*., 2000).

Although the importance of Rosea1 and Delila in regulation of flower coloration of A. majus has been confirmed abundantly (Dooner *et al*., 1991; Martin *et al*., 1991; Schwinn *et al*., 2006; Shang *et al*., 2011), the intricate relationship between the two factors was indistinct yet. Our results indicated that the two factors, Rosea1 and Delila, could not regulate each other by ectopically expressing either of them, unlike PAP1 and TT8 in Arabidopsis thaliana (Baudry *et al*., 2006; Zimmermann *et al*., 2004), which regulated reciprocally. Considering the mechanism in different tissue would differ vastly, further investigation in flower should be compared with hairy roots.

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