Chalcone Synthase-Encoding RdCHS1 Is Involved in Flavonoid Biosynthesis in Rhododendron Delavayi

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Article

Chalcone Synthase-Encoding *RdCHS1* Is Involved in Flavonoid Biosynthesis in *Rhododendron Delavayi*

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Abstract: Flower color is an important ornamental feature, often modulated by the contents of flavonoid. Chalcone synthase is the first key enzyme in the biosynthesis of flavonoid, but little is known about the role of *R. delavayi* CHS in flavonoid biosynthesis. In this paper, three *CHS* genes (*RdCHS1-3*) were successfully cloned from *R. delavayi* flowers. According to multiple sequence alignment and phylogenetic analysis, only RdCHS1 contained all the highly conserved and important residues, and classified into the cluster of bona fide CHSs. Thus RdCHS1 was then subjected for further functional analysis. Real-Time PCR analysis revealed that transcripts of *RdCHS1* was the highest in leaves and lowest in root, did not match the anthocyanin accumulation patterns during flower development. Biochemical characterization displayed that RdCHS1 could catalyze *p*-coumaroyl-CoA and malonyl-CoA molecules to produce naringenin chalcone. Physiological function of *RdCHS1* was checked in *Arabidopsis* mutant and tobacco, the results showed *RdCHS1* transgenes could recover the color phenotypes of *tt4* mutant and made tobacco flower color from pink to dark pink through modulating the expression of endogenous structural and regulatory genes in tobacco. All these results demonstrated that RdCHS1 is a bona fide CHS and contribute to flavonoid biosynthesis in *R. delavayi*.

Keywords: flower color; flavonoid; chalcone synthase; enzyme activity; *Rhododendron delavayi*

1. Introduction

Flower color, a vital trait of ornamental plants, is mainly determined by flavonoid [1,2]. Flavonoids are a large group of plant natural pigments which comprise of chalcones, aurones, flavones, isoflavones, flavandiols, flavonols, proanthocyanins, anthocyanins and so on [3]. In addition to tissue pigmentation, flavonoids also fulfill significant roles in multiple physiological processes, such as UV protection, auxin transport, phytopathogens and herbivores defense, signaling between plants and microbes as well as pollen development [4,5]. Importantly, flavonoids have a wide variety of health benefits to human, including delay the aging of immune organs and nervous system, eyesight improvement, prevention of the cancer, Alzheimer's diseasea and cardiovascular disease [6,7]. Extensive studies about flavonoid biosynthetic pathway have been finished through using *Petunia hybrida* (petunia) [8], *Antirrhinum majus* (snapdragon) [9] and *Arabidopsis thaliana* (Arabidopsis) [10] as models, and a great deal enzymes that taken part in flavonoid biosynthesis have been characterized [11].

Chalcone synthase (CHS), a polyketide synthase, is crucial in biosynthetic pathway of flavonoid and serves as the gatekeeper to regulate its biosynthesis [12]. CHS catalyzes a three-step condensative reaction from trimolecular malonyl-CoA and monomolecular *p*-coumaroyl-CoA to produce naringenin chalcone [13]. The first step, coumaroyl moiety is loaded to the site of active cysteine (Cys164). Next, condensation reactions occur based on the decarboxylation of malonyl-CoA to produce nucleophile for chain elongation. Finally, the reaction generate thioester-linked tetraketide

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which cyclizes into a hydroxylated aromatic ring to yield chalcone [14]. Due to the important roles on starting flavonoid biosynthesis, CHSs have been intensively researched in many higher plants, including petunia [8], *Arabidopsis* [10], snapdragon [9], *Oncidium orchid* [15], apple [16], grape [17], *Gerbera hybrida* [18], *Dianthus chinensis* [19] and so on. In *Antirrhinum majus*, the first *CHS* mutant called white nivea was described, which was generated by deleting its single *CHS* gene [20]. For petunia, there are more than 8 copies of *CHS* genes in its genome, but only *CHS-A* and *CHS-J* transcript in floral tissues and conspicuously down-regulated in the white parts of 'Red Star' [21,22]. In addition, the spatial suppression of *CHSA* also caused the natural bicolor floral phenotype as well as inability to generate functional pollen tubes [23]. In contrast to petunia, the maize *CHS* mutants can still initiate pollen tube growth, but is incapable of sustaining its growth exceed 12 h, ultimately leading to male sterile [24]. The *Arabidopsis tt4* mutant which lacks the brown tannins in seed and anthocyanin in the cotyledons and hypocotyls are demonstrated causing by a mutation in the *CHS* gene, and other phenotypic effects such as change in pollen fluorescence and root morphology were also reported later [25,26]. In *Gerbera hybrida*, three like genes of *CHS* are expressed during its flower development, whereas only *CHS1* transcript corresponds with the synthesis of anthocyanins and flavanols [27]. As for Asiatic hybrid lily, also three *CHS* genes (*CHSA-CHSC*) are active in colored tepals, but transcript patterns of them are diverse [28,29]. Therefore, all the mentioned results indicate that CHS plays indispensable roles during plant development and is crucial for petal color formation in some plants.

Rhododendron delavayi (*R. delavayi*) belonging to Ericaceae family is one of the significant ornamental plant species. Considering its vivid flowers and high horticultural values, *R. delavayi* has been becoming increasingly popular in the world [30]. However, the first rate-limiting enzyme, chalcone synthase, which is vital for petal coloration has not been isolated and analyzed from *R. delavayi*. Here a *CHS* gene (named as *RdCHS1*) was isolated from petals of *R. delavayi* and its functional roles on flavonoid biosynthesis had been comprehensively demonstrated. Expression analysis of *RdCHS1* was conducted showing that it might participate in the biosynthesis of flavonoid (not only anthocyanin) in all detected tissues. Subsequently, catalytic property of RdCHS1 was confirmed and its function in planta was verified by transferring into *Arabidopsis tt4* mutant. At the same time, *RdCHS1* was also ectopicly expressed in tobacco, the data displayed that it could strengthen the pink color of the corolla from pale pink to dark pink. In this paper, we comprehensively identify the function of chalcone synthase in *R. delavayi*, and prove *RdCHS1*is a *CHS* gene working on flavonoid biosynthesis in *R. delavayi*.

2. Results

2.1. Cloning and sequence analysis of RdCHSs

According to transcriptome data of *R. delavayi*, three *CHS* genes were identified through blastn alignment with reference genes from *Arabidopsis* and proximal species. The cDNA sequences of *RdCHS1* (SUB13889370), *RdCHS2* and *RdCHS3* were cloned and found to have the ORFs of 1182 bp, 762 bp and 1170 bp encoding 393, 253 and 389-amino acids protein. Protein sequence alignment for RdCHSs protein in comparison to other plant CHSs was made, the results disclosed that RdCHS1, RdCHS2 and RdCHS3 all contained three highly conserved residues (marked with yellow box) which form the active site of CHS, but the second important Phe residues (marked with blue box) determining the catalytic specificity of CHS was replaced by Val in RdCHS2 and RdCHS3. Meanwhile, the first five residues (marked with black triangle) which help in forming the coumaroly-CoA binding and cyclization pocket was absent in RdCHS2. In two signature motifs (marked with green box), only RdCHS2 and RdCHS3 contain different amino acid residues compared to other CHSs whose function had been identified (Figure 1). Moreover, phylogenetic analysis also revealed that RdCHS1 was grouped into the cluster of bona fide CHSs and evolutionarily closest to the CHS from *Vitis vinifera*, while RdCHS2 and RdCHS3 were classified into Non-CHS Type III PKS cluster, thus, RdCHS1 was subjected for further functional analysis (Figure 2).

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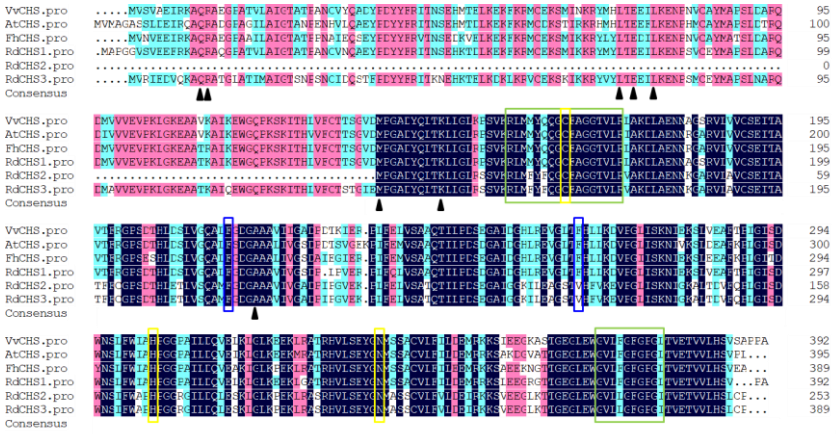

Figure 1. Amino acid sequences alignment of CHS protein in *R. delavayi* with proteins from other species. The yellow box represents three conserved catalytic residues in CHS. The blue frame amino acid determines the specificity of CHS substrate. The green rectangular box indicates the highly conserved domains of CHS. The black triangles represent important residues in binding to coumarinyl coenzyme A and residues specific to the cyclic reaction of CHS.

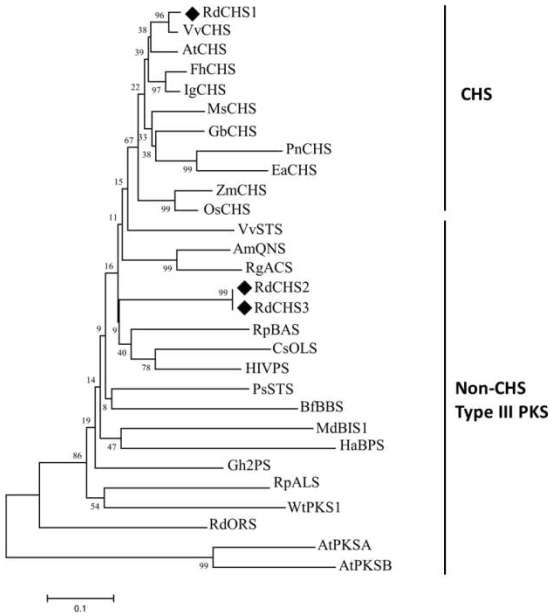

Figure 2. Phylogenetic analyses of RdCHSs. Plant species and GenBank accession numbers are as follows: FhCHS (Freesia hybrida, AEO45114.1), IgCHS (Iris germanica, BAE53636.1), VvCHS (Vitis vinifera, BAA31259.1), AtCHS (Arabidopsis thaliana, AAA32771.1), ZmCHS (Zea mays, CAA42763.1), OsCHS (Oryza sativa, BAA19186.2), GbCHS (Ginkgo biloba, AAT68477.1), MsCHS2 (Medicago sativa, P30074.1), PnCHS (Psilotum rudum, BAA87922), EaCHS (Equisetum arvense, Q9MBB1.1), AmQNS (Aegle marmelos, AGE44110), RgACS (Ruta graveolens, CAC14058.1), RpBAS (Rheuam palmatum, AAK82824.1), VvSTS (Vitis vinifera, ABV82966.1), PsSTS (Pinus sylvestris, CAA43165), Gh2PS (Gerbera hybrida, P48391.2), RpALS (Rheum palmatum, AAS87170), CsOLS (Cannabis sativa, B1Q2B6), HIVPS (Huamulus lupulus, ACD69659.1), HaBPS (Hypericum androsaemum, AAL79808.1), MdBIS1 (Malus domestica, NP001315967), BfBBS (Bromheadia finiaysoniana, CAA10514.1), WtPKS1 (Wachendorfia thyrsiflora, AAW50921), RdORS (Rhododendron dauricum, BAV83003), AtPKSA (Arabidopsis thaliana, O23674), and AtPKSB (Arabidopsis thaliana,Q8LDM2). QNS, OLS, ALS, BIS, and ORS stand for quinolone synthase, olivetol synthase, aloesone synthase, 3, 5-dihydroxybiphenol synthase, and orcinol synthase, respectively.

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2.2. RdCHS1 expression patterns in developing flowers and different tissues

Transcript patterns of RdCHS1 were checked in R.delavayi using Real-Time PCR. RdCHS1 was expressed globally in all tissues and varied according to tissue type, with the highest transcript abundance detected in leaves and the lowest in roots. Furthermore, similar levels of RdCHS1 mRNA were observed in flower organs including petals, toruses, scapes, pistils and stamens (Figure 3A). Then, more detailed transcript patterns of RdCHS1 were examined during flower different developmental stages. As shown in Figure 3B, expression profile of RdCHS1 increased steadily at early two stages, decreased in later two stages, and achieved its highest expression level at the last stage which was inconsistent with the anthocyanin synthesis patterns during R. delavayi flower development [31]. Therefore, the up mentioned findings suggest that RdCHS1 may be involved in the biosynthesis of not only anthocyanin but also other flavonoid in all detected tissues.

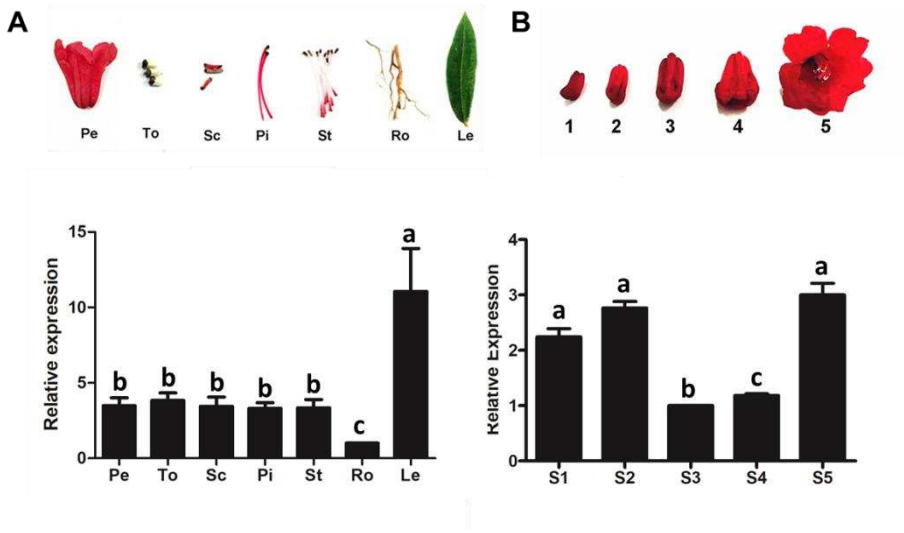

Figure 3. Expression profiles of RdCHS1 in R. delavayi. (A) Relative expression levels of RdCHS1 gene in different tissues; Pe, petals; To, toruses; Sc, scapes; Pi, pistils; St, stamens; Ro, roots; Le, leaves. (B) Relative expression levels of RdCHS1 at five flower developmental stages; S1, flower buds about 1 cm; S2, flower buds about 1.5 cm; S3, flower buds about 2 cm; S4, freshly opened flowers; S5, blooming flowers. Results represent means ± SE from three biological replicates.

2.3. Biochemical characterization of RdCHS1

As a crucial step to confirm the biological function of RdCHS1, it was heterologously expressed as a thioredoxin-fusion protein along with His-tag in *E. coli*. The recombinant purified RdCHS1 displayed a single protein band on SDS-PAGE (Figure 4A). Then, *in vitro* enzymatic assays were performed to elucidate catalytic activity of the recombinant RdCHS1 in the presence of malonyl-CoA and p-coumaroyl-CoA. As reported previously, naringenin chalcone (NC), the catalytic product of CHS, can spontaneously convert to naringenin in aqueous solutions [36]. Therefore, both naringenin chalcone and naringenin were regarded as the products of recombinant RdCHS1 in HPLC analysis. Compared to the control, naringenin chalcone and naringenin product were detected in reaction with RdCHS1 protein which matched the authentic samples (Figure 4B-D). This result indicates that RdCHS1 encodes a biochemically functional CHS protein catalyzing the synthesis of naringenin chalcone from malonyl-CoA and p-coumaroyl-CoA.

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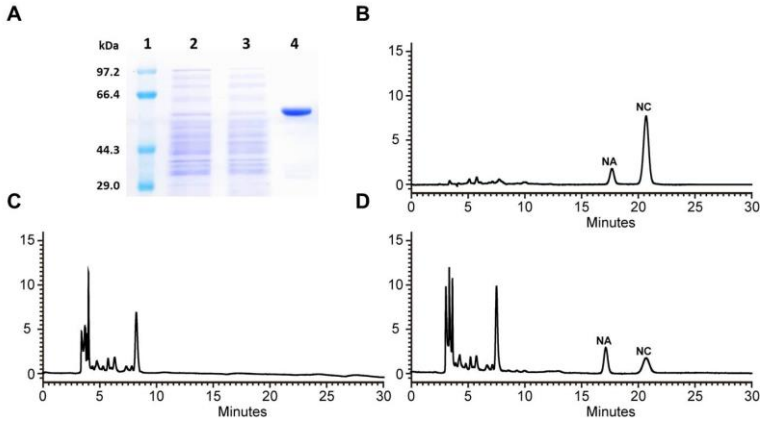

Figure 4. Biochemical assays of recombinant RdCHS1. (A) Expression of RdCHS1 in *E. coli*. (1) Maker (2) Total soluble protein from *E. coli* expressing pET-32a (+) vector (3) Total soluble protein from *E. coli* expressing *RdCHS1* prior to induction by IPTG (4) Purified RdCHS1. (B) Standard of naringin chalcone. (C) The control (empty pET-32a vector). (D) HPLC profiles of the reaction products of RdCHS1.

2.4. Complementation of the tt4 mutant with RdCHS1

To determine the effect of *RdCHS1* on anthocyanin biosynthesis, it was transformed into *Arabidopsis tt4* mutant, and more than ten independent lines were obtained. In comparison, wild type Arabidopsis accumulated anthocyanins in their hypocotyls and tannins in the seed coats, but *tt4* mutant could not. As present in Figure 5A, transformation with *RdCHS1* could successfully recover the pigmentation phenotype of *tt4* mutant. Meanwhile, RT-PCR was also carried out to affirm the expressions of *RdCHS1*, amplicons absent in Col-0 and tt4 mutant were observed in transgenic seedlings (Figure 5B). Additionally, for determining the change of anthocyanins in details, HPLC analysis was conducted. The results in Figure 6 showed that anthocyanins in wild type *Arabidopsis* (peak 1-4) were all absent in *tt4* mutant, while the seedlings carrying *RdCHS1* could successfully complement the biosynthesis of cyanindin and pelargonidin which coincided with the quantification of total anthocyanin (Table S2, Figure 5C). Therefore, these above results imply that RdCHS1 possesses the similar activity to AtCHS, a chalcone synthase involved in flavonoids biosynthesis *in vivo*.

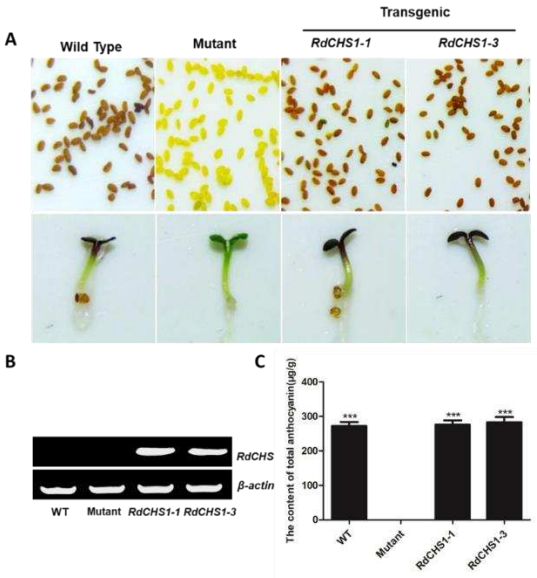

Figure 5. Complementation of RdCHS1 function in Arabidopsis tt4 mutant. (A) Phenotype of the wild type, mutant (tt4) and T2 transgenic lines. (B) Expressional analysis of RdCHS1 in wild-type, mutant

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and transgenic lines. (C) Total contents of anthocyanins in Arabidopsis seedlings. Results correspond to means from three biological replicates. Asterisks indicate significant differences between means of wild-type and transgenic plants calculated by Tukey HSD test (***P<0.001).

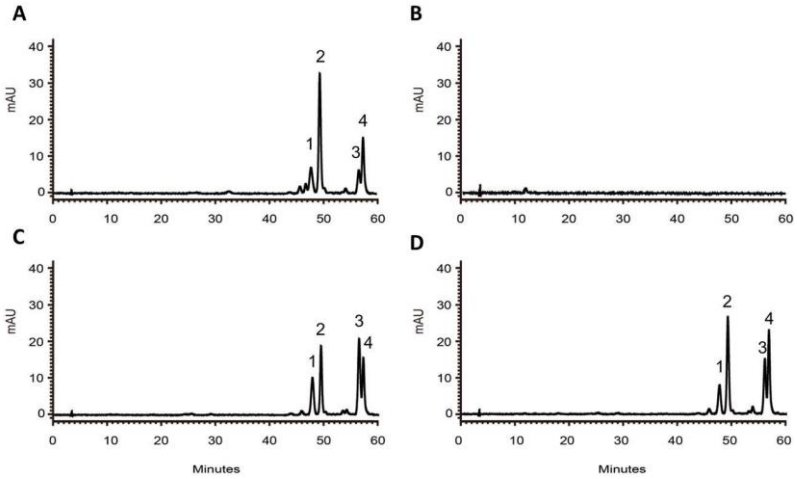

Figure 6. HPLC analyses of anthocyanins in Arabidopsis seedlings. HPLC chromatograms of the samples from seedlings of wild-type (A), mutant (B), RdCHS1-1 (C) and RdCHS1-3 (D) .

2.5. Overexpression of RdCHS1 in tobacco

In order to verify the function of *RdCHS1* on biosynthesis of anthocyanin in flowers, it was synchronously transformed into tobacco plants. After screening, 15 independent transgenic lines were generated, and two independent lines exhibiting significant strengthened flower color were selected for further analysis (Figure 7A). The presence of *RdCHS1* on molecular levels was examined through using RT-PCR, and the contents of anthocyanin in tobacco corollas were also determined by HPLC (Figure 7B, D). Quantitative results displayed that the amount of anthocyanin in transgenic flowers was higher than that in non-transformed flowers which accounted for 131.7-184.2% of the total anthocyanin in control (Figure 7C). Thus, it becomes clear that ectopic expression of *RdCHS1* can increase anthocyanin accumulation in tobacco flowers.

Increased anthocyanin in transgenic tobacco flowers implied that coordinate interaction might existed between *RdCHS1* and endogenous enzymes that involved in the biosynthesis anthocyanin. Hence, Real-Time PCR analysis was carried out to investigate the effect of *RdCHS1* over-expressions on anthocyanins pathway in transgenic flowers. As present in Figure 8, all the investigated genes except *NtF3′5′H* were consistently up-regulated compared to wild-type tobacco. Moreover, ectopic expression of *RdCHS1* in tobacco strongly influenced the transcript levels of *NtCHS* and *NtAN2*, and their transcript abundance were 2.4 to 5.51-fold higher in both transgenic lines. So these findings reveal that *RdCHS1* overexpression can modulate the expressions of endogenous anthocyanin pathway genes in tobacco.

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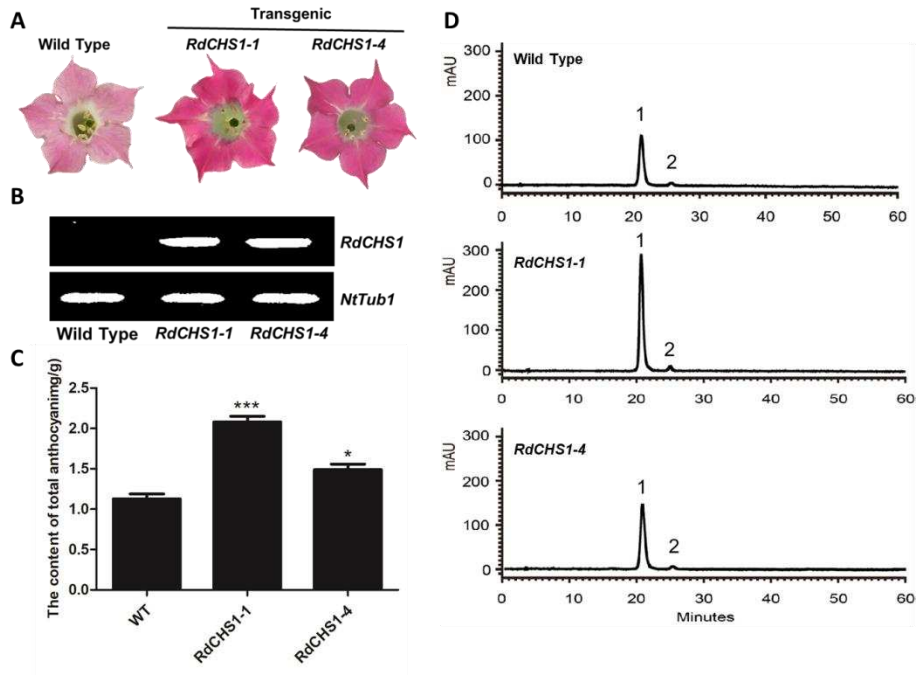

Figure 7. Effect of *RdCHS1* on anthocyanin accumulation in transgenic tobacco flowers. (A) Tobacco flowers of wild-type and transgenic lines. (B) Expression profiles of *RdCHS1* in flowers of transgenic tobacco. (C) Quantitation of anthocyanin accumulation levels in transgenic tobacco flowers with HPLC. (D) HPLC chromatograms of the samples from flowers of wild-type and transgenic tobacco. Results correspond to means from three biological replicates. Asterisks indicate significant differences between means of wild-type and transgenic plants calculated by Tukey HSD test (**P<0.01; *P<0.05).

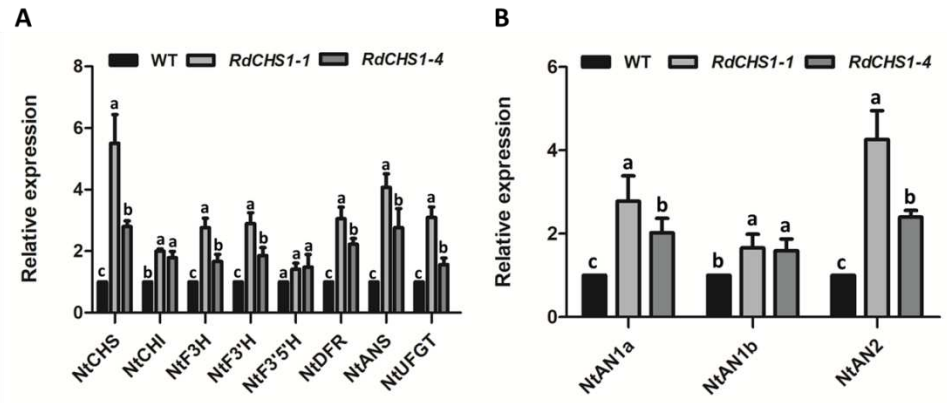

Figure 8. Expression analyses of endogenous anthocyanin biosynthetic genes in corollas of transgenic tobacco. (A) Expression profiles of structure genes in corollas of transgenic tobacco. (B) Expression profiles of regulatory genes in corollas of transgenic tobacco. Results represent means ± SE from three biological replicates. Different letters above the bars indicate significant difference between the samples judged by Tukey HSD test (P<0.01).

3. Discussion

The CHS enzyme family is crucial for plant growth and development. They are ubiquitous in different plant species and encoded by multiple genes. For example, 3, 4, 12 and 14 *CHS* genes have been cloned and identified from *Lilium* spp. [28,29], *Dahlia variabilis* [37], *Zea mays* [38] and *Petunia hybrida* [39], several of them are the true CHSs while others involve in various metabolic pathway [40]. *Arabidopsis* has four *CHS* genes, of which one is the true CHS and takes part in flavonoid biosynthesis [41]. Of RdCHSs, Protein sequence alignment and phylogenetic analysis showed that

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RdCHS1 was closely related to the bona fide CHSs indicating its ability to produce naringenin chalcone (Figure 1, 2).

CHSs of plant share highly similarity in their amino acid sequence. RdCHS1 amino acid are 82.6-91.4% identical with *Arabidopsis* CHS and *Vitis vinifera* CHS (Figure 1). An increasing number of PKSs (except for CHS), such as stilbene synthase (STS), acridone synthase (ACS) and 2-pyrone synthase (2PS) have been demonstrated possess the identical catalytic mechanism with CHS but are different in the intramolecular cyclization patterns and predilection for starter substrate [42,43]. The above enzymes are also quite similar at amino acid level with CHS. Thus, plentiful CHS sequences in public databases, identified only through their sequence similarity, may encode other related enzymes in fact [44]. Alternatively, the kinds of amino acid in protein sequence could help to define the CHS. The Phe residues (Phe215 and Phe265) are two gatekeepers in CHSs that facilitate substrate loading and appropriate folding during cyclization process [14,42]. Phe265, a critical residue for the substrate selectivity, is conserved in the sequence of CHSs but changes in other plant PKSs [43,45]. In OsCHS9, Phe265 is substituted with Gly which displays undetectable CHS activity, instead encodes GUS catalyzing the production of bisdemethoxycurcumin [46]. RdCHS1 contains both Phe residues in amino acid sequence implying it was functional CHSs in *R. delavayi* and exhibits CHS activity. While in RdCHS2 and RdCHS3, Phe265 is replaced by Val indicating their function as other plant PKSs (Figure 1), thus RdCHS1 was selected for further analysis.

Gene expression patterns are correlated with their functions, differential analysis of gene expression can provide key information for the study of gene features, regulations as well as origin [47,48]. The expression patterns of *RdCHS1* are spatially regulated. Its highest mRNA level was detected in leaves followed by flower tissues, and least in roots. Meanwhile, *RdCHS1* expression in flowers is also developmentally regulated, but not associated with accumulation of total anthocyanins (Figure 3). These are different from the results in *Petunia hybrida* and *Gerbera hybrida*, in which CHS expression is excessive in flower and coupled with anthocyanin pigmentation [21,27]. Together with the high expression in leaves, *RdCHS1* expression profiling in this research suggest that it might not be the dominant CHS enzyme in *R. delavayi* petals.

In vitro enzymatic assays showed RdCHS1 was an authentic CHS similar to CHS enzymes in *Gerbera hybrida* (GCHS1 and GCHS3) that could convert malonyl-CoA and *p*-coumaroyl-CoA molecules into naringenin chalcone (Figure 4) [18]. Although RdCHS1 performed typical CHS function, it does not seem to be a major functional CHS in petal pigmentation according to its highest expression in leaf and lower catalytic efficiency towards malonyl-CoA and *p*-coumaroyl-CoA. Recent studies have reported that CHS gene in *Physcomitrella patens* can accept dihydro-p-coumaroyl-CoA and cinnamoyl-CoA to produce relevant chalcones [49]. Similarly, *CHS* gene from *Scutellaria baicalensis* also has the ability to convert isovaleryl-CoA, phenylacetyl-CoA, isobutyryl-CoA and benzoyl-CoA into a variety of products containing the aromatic polyketide which is unnatural [50]. Therefore, CHS is generally a promiscuous enzyme in the light of substrate specificity which suggests its functional diversification during the process of evolution. In view of above discoveries, further researches are needed to explore the catalytic properties of RdCHS1 so as to determine its function in plants and also lay the foundation for functional divergence study of *CHS* gene family in *R. delavayi*.

A few studies have observed the influence of reducing CHS activity in various plants. Such as in *Arabidopsis*, *tt4* was the CHS gene mutant, which displayed a deficiency in the synthesis of anthocyanin, and also exhibited an absence on the pigment of seed coat [51]. Thus, *tt4* mutant is a suitable model for verifying whether *RdCHS1* is taken part in anthocyanin and proanthocyanidin biosynthesis. As expected, *RdCHS1* completely restored the purple coloration of *tt4* in hypocotyls and cotyledons and the pigment in seed coats, which confirmed the function of RdCHS1 as CHS *in vivo* (Table S2, Figure 5). These results are similar to the case of maize, its *C2* gene encoding CHS was also overexpressed in *tt4* mutant and exhibited alike pigmentation phenotype as well as accumulation patterns of flavonoid [52]. Meanwhile, complementation of *Arabidopsis* flavonoid mutants were also conducted by other enzymes involved in flavonoid biosynthesis such as chalcone isomerase from *Ophiorrhiza japonica* and dihydroflavonol 4-reductase from *Dryopteris erythrosora*, all these findings

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demonstrated that the function of enzymes participated in flavonoid biosynthesis are exchangeable among different plant species [53,54].

Comparing to wild-type tobacco, overexpression of *RdCHS1* gene resulted in dark-pink flowers and increased cyanidin-type anthocyanins in transgenic lines (Table S4, Figure 7), and this similar phenomenon had also been looked at in *Solanum lycopersicum* [55]. Interestingly, the enhanced biosynthesis of anthocyanins in *RdCHS1* transgenic tobacco is due to the increased expression of endogenous structure genes (*NtCHS*, *NtCHI*, *NtF3H*, *NtF3'H*, *NtANS* and *NtUFGT*) and regulatory factors (*NtAN1a*, *NtAN1b*, *NtAN2*) (Figure 8). Up-regulatuon of these endogenous gene expressions may be due to the positive feedback regulation of flavonoid biosynthetic genes through pathway intermediates, or that RdCHS1 protein may directly interact with transcriptional regulation proteins to increase anthocyanin accumulation [56]. *NtAN1a*/*NtAN1b* (two bHLH transcription factors) and *NtAN2* (a R2R3-Myb transcription factor) had been demonstrated regulating anthocyanin synthesis in tobacco, thus their massive expressions promote the total metabolic flux in transgenic tobacco [57,58].Unexpectedly, the transcript level of *NtF3'5'H* remained constant, and this may be because the host tobacco is incapable to produce delphinidin-type anthocyanins on account of dihydromyricetin deficiency [59]. Overall, the above results prove that *RdCHS1* is an anthocyanin-related gene and involved in flower color formation in *R. delavayi.*

4. Materials and Methods

4.1. Plant materials

R. delavayi was grown in the experimental field of Gui Zhou Normal University. At anthesis, scapes, petals, pistils, toruses, roots, stamens, leaves, and flowers at different stage (stage 1-5) were sampled. *Arabidopsis* T-DNA insertion mutant (*tt4*, SALK020583) and wild-type in Columbia ecotype background were gained from NASC (the Nottingham Arabidopsis Stock Center) and cultivated in the glasshouse. T2 transgenic *Arabidopsis* seedlings cultured for seven days were harvested from anthocyanin inductive medium (1/2 MS adding 3% sucrose) and used for RT-PCR and anthocyanin analysis. Tobacco (K326) plants applied to transformation were kept in the condition with 12 h light at 22 °C. Blooming flowers of T1 transgenic tobacco were collected. All above plant samples were quick-frozen immediately, and saved at -80 °C for later analysis.

4.2. Chemicals

Malonyl-CoA, *p*-CoumaroylCoA, and Naringin chalcone used in enzyme activity analysis were got from Sigma-Aldrich (USA). Cyanidin 3-*O*-glucoside was purchased from Phytolab (Germany).

4.3. Full-length cDNA cloning of RdCHS1

Extracted RNA from the flowers of *R.delavayi* was used for the synthesis of cDNA through using Takara M-MLV reverse transcriptase. Based on assembled transcriptomic information (SRR26283938), specific primers for *RdCHS* CDS (coding sequence) cloning were designed (Table S1). After amplifying, products of PCR were inserted into pMD18-T vectors (Takara, Japan) and transformed into the competent cells of JM109. After enzyme digestion verification, multiple positive clones were selected and subjected to sequence for verifying the accurate nucleic acid order of RdCHSs.

4.4. Sequence alignment and phylogenetic analysis

Multi-alignment of different CHS sequences was performed employing DNAMAN 5.0. Phylogenetic tree was built using CHS protein from diverse plants by MEGA 6.0 with 2000 bootstrap replicates for estimating the confidence of tree clade.

4.5. Gene expression analysis

Total RNAs were extracted from *R.delavayi* and tobacco samples. BioRad CFX96 Real-Time PCR System was selected for gene expression analysis with gene-specific primers (Table S1). *RdActin* and

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NtTublin were selected as an internal control for *R.delavayi* and tobacco samples respectively. PCR conditions were the same as reported in previous paper [31]. Each sample was run in threes, and $2^{-\Delta\Delta CT}$ method was performed for calculating the expression values. For confirming the specific amplification, agarose gel electrophoresis as well as melting curve analysis were done.

4.6. Soluble protein extraction and CHS enzyme assay

The complete ORF of *RdCHS1* was amplified through PCR method. Resulting fragments were introduced into the pET-32a vector and transformed into *Escherichia. coli* strain BL-21 to express the protein with an N-terminal His6 tag. Protein expression and purification were conducted as reported previously [32]. Briefly, bacterial fluid containing corresponding plasmid was induced for 48 h at 15 ℃ after adding IPTG. Recombinant RdCHS1 protein with His tag was purified using TransGen purification kit following the instructions. Protein purity was then analyzed through SDS-PAGE.

Chalcone synthase activity for generating naringenin chalcone was checked in reaction mixtures consisted of 160 μM malonyl-CoA, 80 μM *p*-coumaroyl-CoA, 100 mM potassium phosphate (pH 7.2) and 30 μg purified recombinant protein. After incubation at 30 ℃ for 60 min, the assay mixtures were terminated via extracting twice with 100 μL ethyl acetate followed by centrifugation for 20 min. The formed products were detected by high-performance liquid chromatography (HPLC) using C18 column through monitoring the absorbance at 304 nm. The mobile phase was the miscible liquids of 3% acetic acid, 47% water and 50% methanol.

4.7. Expression vector construction and transformation of Arabidopsis and tobacco

The pBI121 which contain CaMV35S promoter and NPTII was used to construct a binary vector for *Arabidopsis* and tobacco transformation. After construction, over-expression cassette was sequenced for confirming the correct insertions of *RdCHS1* and then immobilized into *Agrobacterium tumefaciens* strain GV3101 via freeze-thaw method. *Arabidopsis* transformation was conducted according to the method employed in previous reports [33]. After sterilization, transgenic *Arabidopsis* seeds selection was carried out on 1/2 MS medium supplemented with kanamycin (50 mg L^{-1}). Following 1 week growth, T2 *Arabidopsis* transgenic seedlings with purple cotyledons were sampled and used for later analysis. Meanwhile, tobacco transformation was also carried out following a previously reported protocol [34]. Transgenic tobacco seedlings were selected and grew in a green house. Just fully expanded flowers were harvested and used for further analysis. In order to confirm the over-expressions of *RdCHS1*, RT-PCR analysis was performed in *Arabidopsis* and tobacco using *β-actin* and *NtTublin* as control.

4.8. Anthocyanin analysis of transgenic Arabidopsis seedlings and tobacco flowers

For anthocyanin analysis, 0.2 g freeze-dried sample from transgenic *Arabidopsis* seedlings and tobacco flowers were powdered and extracted using 1 mL extracting solutions (H_2O: MeOH: HCl =75/24/1) for 14 h at 4 °C . The extract solution was centrifuged and filtered through 0.22 μm microporous filters. Then the extracted compounds were detected by HPLC using C18 column. Two eluent, 5% formic acid (A) and 1.5% methanol (B), were used for elution. Gradient elution was conducted at a 1.0 mL min^{-1} flow rate: 0-10 min, 86-83% A; 10-35 min, 83-77% A; 35-60min, 77-53% A; 60-67 min, 53-86% A; 67-70 min, 86% A. Flow rate was 1 ml min^{-1}, monitored wavelength was 520 nm, and the column temperature was 35 °C. Anthocyanin concentrations were estimated through using 3-*O*-glucoside standards basing on the method described by Fanali [35].

5. Conclusions

Here, we cloned and characterized *RdCHS1* gene from *R. delavayi* and demonstrated its role *in vitro* and in plants. We found that *RdCHS1* was expressed globally in all tissues and not associated with anthocyanin synthesis during flower development, which revealed that *RdCHS1* may be participated in the biosynthesis of not only anthocyanin but also other flavonoid in *R. delavayi*. Meanwhile, enzyme activity assay was conducted and found RdCHS1 had the CHS activity which

converts malonyl-CoA and *p*-coumaroyl-CoA substrates into naringenin chalcone. Furthermore, physiological role of *RdCHS1* was studied in *Arabidopsis tt4* mutant and tobacco, the results displayed that *RdCHS1* recovered *tt4* mutant phenotypes and led to dark-pink tobacco flowers suggesting manipulation of RdCHS1 would contribute to modifying the color of other ornamental plants.

Supplementary Materials: Table S1. List of primers used in this study; Table S2. The anthocyanin profiles in acidic MeOH-H_2O extracts of the *RdCHS1* transgenic *Arabidopsis*; Table S3. HPLC-DAD and HPLC-ESI-MS analysis of anthocyanin in acidic MeOH-H_2O extracts of the wild-type *Arabidopsis* and *RdCHS1* over-expressing lines; Table S4. HPLC-ESI-MS analysis of anthocyanin extracts of *RdCHS1* over-expressing transgenic tobacco flowers.

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