Characterization of Three Chalcone Synthase-like Genes in *Dianthus Chinensis*

Jia Liu

Inner Mongolia Agriculture University: Inner Mongolia Agricultural University

Xi-Long Hao

Inner Mongolia Agriculture University: Inner Mongolia Agricultural University

Xue-Qin He (✉ xueqinhe@imau.edu.cn)

Inner Mongolia Agriculture University: Inner Mongolia Agricultural University

---

Original Article

Keywords: *Dianthus chinensis*, chalcone synthase, Tobacco rattle virus, virus-induced gene silencing, flower color

Posted Date: February 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-159109/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 13th, 2021. See the published version at <https://doi.org/10.1007/s11240-021-02081-8>.

Abstract

Being an important garden plant, *Dianthus chinensis* flower has a great variety of colors and color patterns. Chalcone synthase (CHS) is the key enzyme in the anthocyanin biosynthetic pathway. Although CHS genes have been isolated and characterized in ornamental plants, the CHS gene is still unknown in *D. chinensis*. In our study, three CHS genes, *DchCHS1* (KX893854), *DchCHS2* (MK404175) and *DchCHS3* (MK416198) were isolated in *D. chinensis*. Their deduced amino acid sequences show high homology with the known CHS sequences in *Caryophyllaceae*. The phylogenetic tree suggests that the *DchCHS1* and the *DchCHS3* have a close relation with the known CHS sequences in *Caryophyllaceae* and the *DchCHS2* is different from them. The *DchCHSs* were characterized by the *Tobacco Rattle Virus* (TRV)-based virus-induced gene silencing (VIGS) system. We obtained white or pale purple flowers in the *DchCHS1*-silenced flowers and reducing purple flowers in the *DchCHS2*-silenced and the *DchCHS3*-silenced flowers. The anthocyanin content and the transcript level of the silenced *DchCHS* were significantly reduced in accordance with the silencing phenotypes. The *DchCHSs* showed different expression patterns during floral bud developments, among flower colors and in organs. Their expression levels in the purple flower were greatly higher than those in the white flower. Compared with *DchCHS2* and *DchCHS3*, *DchCHS1* was abundantly expressed at each floral bud stage, in each flower color and in the flower organ. In conclusion, the three *DchCHSs* are all involved in the anthocyanin synthesis and the flower coloration, and *DchCHS1* probably plays a major role in *D. chinensis* flowers.

Introduction

Flower color is a commercially important characteristic in ornamental plants. Anthocyanin is the largest class of flavonoids and responsible for pink, red, violet, blue and purple colors of flowers and other tissues. Chalcone synthase (CHS) is the first key enzyme in the anthocyanin biosynthesis pathway, which has been extensively described. The reduction of *CHS* transcript inhibits flower pigmentation and leads to flower phenotypes ranging from small streaks of white through to completely white flowers (Deroles et al. 1998; Chen et al. 2004; Fukusaki et al. 2004; Koseki et al. 2005; Ohno et al. 2018; Gu et al. 2019; Nabavi et al. 2019). Numerous CHS genes have been isolated and characterized in ornamental plants. Out of the 12 copies of *CHSs* identified in *petunia hybrid*, the expressions of *CHSA* (AF233638) and *CHSJ* (X14597) are mainly restricted to floral tissues (Koes et al. 1989) and are dramatically reduced in the white sectors of *Petunia hybrida* 'Red Star' flowers (Koseki et al. 2005). The natural bicolor floral phenotype of *Petunia hybrida* 'Picotee' and 'Star' is caused by the spatial repression of *CHSA* (Morita et al. 2012). Three *CHS*-like genes are active during the corolla development in *Gerbera hybrida*. Among them, *CHS1* (Z38096) and *CHS3* (Z38098) are temporally expressed and correlated with the flavanol and anthocyanin synthesis (*CHS1*) (Helariutta et al. 1995). Dahlias have redundant *CHSs* derived from their high polyploidy. In pure white flowers of the octoploid dahlia, *CHS1* (AB576660) and *CHS2* (AB591825) are simultaneously silenced by post-transcriptional gene silencing (Ohno et al. 2011). *CHSA* (AB058638), *CHSB* (AB058639) and *CHSC* (AB058640) in Asiatic hybrid lily are expressed in anthocyanin-pigmented tepals, but the expression pattern of *CHSB* is different from those of *CHSA* and *CHSC* (Nakatsuka et al. 2003; Suzuki et

al. 2016). The CHS genes are known to diversify into a super-gene family in plants. The studies suggest that the number of *CHS* super-gene family members is different in plant species and in controlling flower colors.

Dianthus chinensis is an important ornamental species in the genus *Dianthus*, *Caryophyllaceae*. Except for yellow, it exhibits a wide variation of flower colors, such as pink, purple, red, white, ivory white, etc. It is also rich in color patterns of flowers, including the occurrence of several types of marking in the center and bicolor phenotypes, in which two distinct colors occur on individual petals. Gaining and characterizing CHS genes involving in the anthocyanin biosynthesis pathway and the flower coloration is important to illustrate a diversity of flower colors in *D. chinensis*. Virus-induced gene silencing (VIGS) has been widely used in various plant species to explore the gene functions (Chen et al. 2004; Quadrana et al. 2011; Singh et al. 2012; Manmathan et al. 2013; Zhong et al. 2014; Dobnik et al. 2016). In this paper, three CHS genes were isolated in *D. chinensis* and their functions were studied using VIGS. Meanwhile, their expression profiles were studied during floral bud developments, among flower colors and in organs. The results demonstrate that the three CHS genes are associated with the anthocyanin synthesis and the flower coloration, and *DchCHS1* is a major CHS gene on the controlling flower color in *D. chinensis*.

Materials And Methods

Plant materials

D. chinensis seeds (www.ebay.co.uk/) were planted in plastic pots with Pindstrup growing media (Pindstrup Substrate, Latvia) and kept in a growth chamber with a cycle of 16 h light with 22 °C and 8 h dark with 20 °C. The light intensity was 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The seedlings were transplanted in the greenhouse at Inner Mongolia Agricultural University, Hohhot, China.

Floral buds were classified into three development stages (Fig. 1A). Buds at stage 1 and stage 2 were about 1 cm and 1.5 cm in length, respectively. Both had no pigment in petals. Buds at stage 3 were about 2.0 cm in length with pigments in petals.

Flowering of detached floral buds

Floral buds with stems were collected from the greenhouse and placed immediately in water. They were delivered to the laboratory within 20 min of harvest. The stems were recut to 2 cm under water. The buds were inserted into plastic tubes containing nutrition buffers and placed in the growth chamber as mentioned above. The nutrition buffer was prepared according to Shang et al. (2007). The flowering percentage of the detached floral buds was calculated.

Isolation of *DchCHSs*

Total RNA was extracted with Trizol reagent (Invitrogen) from leaves. The first-strand cDNA was synthesized from 1 μg of total RNA and used as the template. Based on the *CHS* sequence of *D.*

caryophyllus (Z67982.1), DchCHS1-F (5'-GTCGCTTCATGCTCTACCAAC-3') and DchCHS1-R (5'-GCTAGGACTGAACGCATCCTC-3') were designed to amplify a 407 bp fragment of *DchCHS1* (KX893854). The PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced (Takara Biotechnology Co., Ltd). The 3-end cDNA sequence of *DchCHS1* was amplified with the gene-specific primer (5'-GGCTCACTTTTCACCTGCTC-3') and the 3'RACE universal primer by the RACE technique. Based on in-house unpublished RNA-seq data of *D. chinensis*, two unigenes annotated as chalcone synthases, c22817 and c33072 were selected.

Sequence analysis

Homologous sequences of the *DchCHSs* were identified using the BLAST program on the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Amino acid sequence alignment was generated using DNAMAN 5.0. Phylogenetic analysis was performed using MEGA 6.0 with a neighbor-joining method.

Plasmid construction

The first-strand cDNA was used as the template. For *DchCHS1* silencing, a 407 bp fragment (1 -407 bp) was amplified with DchCHS1-F and DchCHS1-R. For *DchCHS2* silencing, a 446 bp fragment (126-571 bp) was amplified with 5'-ACCCTCCCAACCAAATGACC-3' and 5'-TAGTCGCAAACCGTACCCC-3'. For *DchCHS3* silencing, a 474 bp fragment (884-1357 bp) was amplified with 5'-TCCAGACCTGACAATTGAGC-3' and 5'-TGAAGCACACGGTCTCAAC-3'. The amplified fragments were inserted into pGEM-T Easy vectors and sequenced. The inserted fragments were excised from the plasmids by *EcoRI* restriction enzyme and sub-cloned into pTRV2 vectors to generate pTRV2-*DchCHS1*, pTRV2-*DchCHS2* and pTRV2-*DchCHS3*. The resulting plasmids were sequenced to verify correct insertions of the fragments.

Preparation of *Agrobacterium*

Electrocompetent cells of *Agrobacterium tumefaciens* strain GV3101 were transformed with pTRV1, pTRV2, pTRV2-*DchCHS1*, pTRV2-*DchCHS2* and pTRV2-*DchCHS3*, respectively. The transformed cells were selected on LB plates containing 50 mg L⁻¹ kanamycin with 50 mg L⁻¹ rifampicin. The positive colonies of pTRV1 and pTRV2 were verified using pTRV1 primers (5'-TTACAGGTTATTTGGGCTAG-3' and 5'-CCGGGTTCAATTCCTTATC-3') and pTRV2 primers (5'-ACGGACGAGTGGACTTAGATTTC-3' and 5'-GTTTAATGTCTTCGGGACATGC-3'), respectively. The positive colonies of pTRV2-*DchCHS1*, pTRV2-*DchCHS2* and pTRV2-*DchCHS3* were confirmed with pTRV2 primers. The transformed *Agrobacterium* cells were cultured overnight at 28 °C in LB medium containing appropriated antibiotics. After centrifugation, the *Agrobacterium* cells were suspended and incubated in the infiltration buffer (10 mM MES, 150 µM acetosyringone and 10 mM MgCl₂) to a final OD₆₀₀ of 3.0 at 25°C.

Vacuum infiltration of detached floral buds

According to the flowering percentage of detached floral buds in the nutrition buffer, we collected the buds at stage 2 and stage 3. The buds at the same stage from the same plant were divided into two parts. One part was submerged in the infiltration buffer containing a 1:1 ratio mixture of pTRV1 with pTRV2 (control), and the other part in the infiltration buffer containing a 1:1 ratio mixture of pTRV1 with pTRV2 constructs containing different *DchCHS* fragments. After infiltration in a -100 kPa vacuum chamber for 20 min, the buds were inserted into plastic tubes containing the nutrition buffer and put in the growth chamber as mentioned above.

Analysis of anthocyanin contents and *DchCHS*s expressions in the silenced flowers

The flowers of the control and the flowers with silencing phenotypes were collected and ground to a fine powder under liquid nitrogen.

Pigments were extracted from 0.1 g of the ground powder. The ground powder was added to 600 μ L of 1% HCl in methanol (v/v) and incubated overnight at 4 °C with gentle shaking. The extract was mixed with 400 μ L of water and 400 μ L of chloroform. After centrifugation, the absorbance of the supernatant was measured at 530 and 657 nm. The anthocyanin content was calculated using $A_{530} \times 0.25 A_{657}$ (Rabino and Mancinelli 1986).

Total RNA was extracted from 0.1 g of the ground powder and used to synthesize the first-strand cDNA, which was used as the template for qRT-PCR. QRT-PCR was performed using Roche LightCycler 480 II (Switzerland). Reactions contained 10 μ L 2 x SYBR Advantage qPCR Premix (Takara), 0.2 μ L cDNA template and 0.6 μ M of each gene-specific primer in a final volume of 20 μ L. The gene-specific primers were 5'-TGTTGAGCGACTTTGGGAAC-3' with 5'-CCCTTCACCTGTTGTGGTTG-3' for *DchCHS1*, 5'-CCGCAAATAGCATACAAAAGC-3' with 5'-CCTCCTGGGTGAACCACATAAA-3' for *DchCHS2* and 5'-CGCCGATTACCAGCTCACC-3' with 5'-CCGTGCCTCCAGCAAAGC-3' for *DchCHS3*. These primers were designed outside the region used for VIGS to avoid amplification of RNA from the silencing vectors. *DchACTIN* (KX664102) from in-house unpublished RNA-seq data was used as an internal control and its primers were 5'-ATGCCCCCGCTATGTATGT-3' and 5'-GCCAAATCAAGACGCAAGAT-3'. The thermal program was as follows: 1 cycle of 10 min at 95 °C; 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Three biological replicates were made. The relative expression level of the gene was presented by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Analysis of expression levels of the *DchCHS*s

Total RNA was extracted from petals of floral buds at each stage (Fig. 1A), flowers of three kinds of flower colors (Fig. 2A) and organs of purple flowering plants (Fig. 3A), and was used to synthesize the first-strand cDNA. The organs included roots, lower-stems (with pigment in nodes and internodes), upper-stems (with no pigment), leaves and flowers. The procedure of qRT-PCR, the gene-specific primers for amplifying *DchCHS*s transcripts and the internal control were the same as mentioned above.

Results

Sequence analysis of the DchCHS genes

The full-length *DchCHS1* cDNA of 898 bp (KX893854) includes a predicted open reading frame (ORF) of 705 bp. Two unigenes encoding putative chalcone synthases were identified in an in-house *D. chinensis* transcriptome database and re-named as *DchCHS2* (MK404175) and *DchCHS3* (MK416198). The full-length *DchCHS2* cDNA of 1332 bp includes an ORF of 1197 bp and the full-length *DchCHS3* cDNA of 1690 bp includes an ORF of 1176 bp. Their deduced amino acid sequences share high homology with the predicted CHSs in *Caryophyllaceae*, including the DcaCHS (Z67982) in *Dianthus caryophyllus*, the DmoCHS (AF267173) in *Dianthus monspessulanus*, the GpaCHS (AY309966) in *Gypsophila paniculate* and the SliCHS (KT954903) in *Silene littorea* (Fig. 4).

The evolutionary relationships of the DchCHSs, the four CHSs in *Caryophyllaceae* and nine CHSs from other ornamental plants were studied (Fig. 5). The nine CHSs, including two of *Petunia hybrida* in *Solanaceae* (Koes et al. 1989; Koseki et al. 2005), two of *Dahlia variabilis* (Ohno et al. 2011) and two of *Gerberahybrida* in *Asteraceae* (Helariutta et al. 1995), and three of *Lily hybrida* in *Lilium* (Nakatsuka et al. 2003; Suzuki et al. 2016), are related with flower pigments. The DchCHS1, the DchCHS3 and the four CHSs belong to the *Caryophyllaceae* family and cluster together in the phylogenetic tree (Fig. 5). It might be that they diverge from a recent common ancestor. The DchCHS2 in a separate group shows that it has a remote relation not only with the CHSs from other ornamental plants, but also with the CHSs in *Caryophyllaceae* (Fig. 5). The result suggests that the *DchCHSs* encoding proteins might be different in enzymatic activities.

Effect of silencing *DchCHSs* in flowers

Half of the detached flower buds at stage 1 and 100% at stage 2 and 3 were opened in the nutrition buffer (Fig. 1A). Therefore, the buds at stage 2 and 3 were used as explants for infiltration. Four days after infiltration, only the flowers from the detached floral buds at stage 2 showed silencing phenotypes (Fig. 6Aa, Ba, Ca).

One hundred detached floral buds were treated with pTRV2-*DchCHS1*. Nineteen flowers showed white or pale purple colors (Fig. 6Aa). The anthocyanin content in the silenced flower (8.16 ± 1.41 per 100 mg fresh petals) was dramatically lower than that in the TRV control flowers (28.54 ± 0.72 per 100 mg in fresh petals) (Fig. 6Ab). Meanwhile, *DchCHS1* expression was significantly down-regulated (Fig. 6Ac).

Eleven flowers appeared pale purple when pTRV2-*DchCHS2* was delivered into 100 detached floral buds (Fig. 6Ba). The silencing phenotype was associated with a significant reduction of the anthocyanin content (17.54 ± 4.56 per 100 mg fresh petals versus 27.80 ± 3.06 per 100 mg fresh petals) (Fig. 6Bb) and of *DchCHS2* expression (0.45 ± 0.31 versus 1.00 ± 0.55) (Fig. 6Bc).

Fifteen percent of the detached floral buds treated with pTRV2-*DchCHS3* showed reducing purple colors in the flowers (Fig. 6Ca). Compared with the TRV control, the anthocyanin content (Fig. 6Cb) and *DchCHS3* expression greatly decreased in the silenced flower (Fig. 6Cc).

Expression patterns of the *DchCHSs*

The floral buds at stage 1 and 2 with un-pigmented petals and those at stage 3 with pigmented petals were collected to study expression profiles of the *DchCHSs* in *D. chinensis* (Fig. 1). The expressions of *DchCHS1* and *DchCHS2* were sharply down-regulated from stage 1 to stage 2 and kept stable from stage 2 to stage 3 (Fig. 1B). No obvious difference in *DchCHS3* expression was observed during the floral bud development (Fig. 1B). *DchCHS1* expression at each stage was significantly higher than those of *DchCHS2* and *DchCHS3*, and *DchCHS3* expressions at stage 2 and 3 were significantly higher than the *DchCHS2* (Fig. 1C).

We chose three kinds of flower colors to investigate expression patterns of the *DchCHSs* (Fig. 2). The expressions of *DchCHS1* and *DchCHS3* in purple flowers were higher than those in white with purple center (W+P) flowers, and those in W+P flowers were dramatically higher than those in white flowers (Fig. 2B). *DchCHS2* expression in the purple was 20.93 times higher than that in the white and 40.25 times higher than that in the W+P (Fig. 2B). In each flower color, *DchCHS1* expression was substantially higher than the *DchCHS3*, and the *DchCHS3* was substantially higher than the *DchCHS2* (Fig. 2C).

We also evaluated expression levels of the *DchCHSs* in organs (Fig. 3). The expression levels of *DchCHS1* in flowers were 2.82 times higher than those in leaves, 4.78 times higher than those in lower-stems, 5.73 times higher than those in roots and 44.08 times higher than those in upper-stems (Fig. 3B). *DchCHS2* expressions in leaves were dramatically higher than those in the other organs, and there is no difference among those in the other organs. No obvious difference in *DchCHS3* expression was observed among the organs except for up-stems (Fig. 3B).

Discussion

CHS has been shown to be encoded by a multigene family in ornamental plants. Twelve CHS genes have been isolated in *Petunia hybrida* (Koes et al. 1989). Three *CHS*-like genes isolated in *Gerbera hybrida* are specifically expressed in the corolla (Helariutta et al. 1995). Four and three CHS genes have been obtained in *Dahlia variabilis* (Ohno et al. 2011) and in Asiatic hybrid lily (Nakatsuka et al. 2003; Suzuki et al. 2016), respectively. *D. chinensis* exhibits a wide variation in flower colors. In our paper, we got three *DchCHSs* in *D. chinensis*. The *DchCHS1*, the *DchCHS3* and the four CHSs from *Caryophyllaceae* in one cluster show that they might diverge from a recent common ancestor (Fig. 5). The *DchCHS2* is different. It is clustered into a separate group from the CHSs in *Caryophyllaceae* and the CHSs from other plant species (Fig. 5). Low homology at the protein level between expressed CHS genes indicates that these genes encoding proteins probably have slightly different enzymatic activities (Koes et al. 1989).

The Tobacco rattle virus (TRV)-based VIGS system has been developed and used for verifying gene functions in diverse plants (Chen et al. 2004; Singh et al. 2012; Zhong et al. 2014; Dobnik et al. 2016). The infection of virus vector carrying sequences of plant genes is replicated and produces double-stranded RNA molecules in infected plants. These double-stranded RNA molecules will be cut into small oligonucleotides (siRNA). The siRNA acts as a guide to target the degradation of endogenous mRNA

homologous and the symptoms would appear in the infected plant as the loss of the function of the target protein (Kalantidis et al. 2008; Demircan and Akkaya 2010; Senthil-Kumar and Mysore 2011). CHS is the first key enzyme in the anthocyanin synthesis. Suppression of the CHS gene will obtain white or light color flowers (Suzuki et al. 2000; Aida et al. 2000). The TRV-VIGS approach was used to silence the endogenous CHS gene in *D. chinensis*. Silencing *DchCHS1* produced white or attenuated purple flowers (Fig. 6Aa). Silencing *DchCHS2* (Fig. 6Ba) or *DchCHS3* (Fig. 6Ca) resulted in reducing purple flowers, but no white sector was appeared in the flowers. The silencing phenotypes were associated with substantial reductions of anthocyanin contents and transcript levels of the silenced *DchCHS* (Fig. 6Ab-c, 6Bb-c, 6Cb-c). The silencing rate of *DchCHS1* was 19% and higher than those of *DchCHS2* and *DchCHS3*. The results show that the three *DchCHSs* are involved in the anthocyanin biosynthesis and in the controlling flower color in *D. chinensis*.

The CHS genes, which are expressed in floral tissues and involved in anthocyanin synthesis, have different expression levels and expression patterns in ornamental plants. In *Petunia hybrida*, the expression level of *CHSA* is far higher than that of *CHSJ* in floral tissues (Koes et al. 1989), and probably encodes a major CHS protein working in the anthocyanin biosynthesis (Koseki et al. 2005; Morita et al. 2012). Two of three *CHS*-like genes are specifically expressed in the corolla and their encoding enzymes have different catalytic properties during the corolla development in *Gerbera hybrida* (Helariutta et al. 1995). The expressions of *CHS1* and *CHS2* in the colored areas of bicolor flower petals are stronger than those in the pure white areas and are unrelated with the petal developmental stage except for the early stage in *Dahlia variabilis*. Cosuppression of them produces pure white parts of petals (Ohno et al. 2011). Recent research shows that *CHS2* is the key gene involved in bicolor formation of dahlia (Ohno et al. 2018). Three CHS genes in Asiatic hybrid lily are expressed in anthocyanin-pigmented tepals, but their expression patterns are different (Nakatsuka et al. 2003; Suzuki et al. 2016). Our study showed that the expression patterns of the *DchCHSs* were different during floral bud developments (Fig. 1B), among flower colors (Fig. 2B) and in organs (Fig. 3B). Their expression levels were inconsistent with the pigment accumulation in the petals of floral buds (Fig. 1A-B) and the size of pigments in the flowers (Fig. 2A-B). *DchCHS1* expression remained the highest level and *DchCHS2* expression remained the lowest level at each floral bud stage (Fig. 1C) and in each flower color (Fig. 2C). The expression of *DchCHS1* in flowers and the expression of *DchCHS2* in leaves were far higher than those in other organs (Fig. 3B). *DchCHS3* expression had no difference during floral bud development (Fig. 1B), between the purple and the white with purple center (Fig. 2B) and among organs except for up-stems (Fig. 3B). The expression levels of *DchCHS1*, *DchCHS2* and *DchCHS3* increased 9.25-fold, 20.93-fold and 24.92-fold between purple flowers and white flowers, respectively (Fig. 2C). It further shows that their encoding proteins might be involved in the controlling flower color. At least three *DchCHSs* are anthocyanin-related genes in *D. chinensis*, which may be the reason that it is rich in flower colors and color patterns of flowers. However, how they function on the anthocyanin biosynthesis and the flower coloration is still unknown.

Conclusion

The three *DchCHS*s obtained in *D. chinensis* are involved in the anthocyanin biosynthesis and in controlling flower color, but their functions might be a slightly different. *DchCHS1* is the major *CHS* gene expressed at each floral bud stage, in each flower color and in flower organs, and probably encodes a major CHS protein working in the anthocyanin synthesis in *D. chinensis* flowers.

Declarations

Acknowledgments This study was supported by the National Natural Science Foundation of China (31960611).

Funding This study was supported by the National Natural Science Foundation of China (31960611).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable for that section.

Compliance with ethical standards

Competing interest The authors declare that they have no competing interests.

Authors' contributions JL conducted the experiments and acquired the results. XLH carried out the data analysis. XQH designed the research and wrote the manuscript. All authors read and approved the final manuscript.

References

1. Aida R, Kishimoto S, Tanaka Y, Shibata M (2000) Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. *Plant Sci* 153:33–42. [https://doi.org/10.1016/S0168-9452\(99\)00239-3](https://doi.org/10.1016/S0168-9452(99)00239-3)
2. Chen JC, Jiang CZ, Gookin TE, Hunter DA, Clark DG, Reid MS (2004) Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. *Plant Mol Biol* 55:521–530. <https://doi.org/10.1007/s11103-004-0590-7>
3. Demircan T, Akkaya MS (2010) Virus induced gene silencing in *Brachypodium distachyon*, a model organism for cereals. *Plant Cell Tissue and Organ Culture* 100(1): 91–96. <https://doi.org/10.1007/s11240-009-9623-x>
4. Deroles SC, Bradley JM, Schwinn KE, Markham KR, Bloor S, Manson DG, Davie KM (1998) An antisense chalcone synthase cDNA leads to novel colour patterns in lisianthus (*Eustoma grandiflorum*) flowers. *Mol Breeding* 4 (1):59–66.
5. Dobnik D, Lazar A, Stare T, Gruden K, Vleeshouwers VGAA, Žel J (2016) *Solanum venturii*, a suitable model system for virus-induced gene silencing studies in potato reveals StMCK6 as an important player in plant immunity. *Plant Methods* 12:29–40. <https://doi.org/10.1186/s13007-016-0129-3>

6. Fukusaki EI, Kawasaki K, Kajiyama S, Ana Ch-II, Suzuki K, Tanaka Y, Kobayashi A (2004) Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference. J Biotechnol 111: 229-240. [https://doi.org/ 10.1016/j.jbiotec.2004.02.019](https://doi.org/10.1016/j.jbiotec.2004.02.019)
7. Gu ZY, Men SQ, Zhu J, Hao Q, Tong NN, Liu ZA, Zhang HC, Shu Q, Wang LS (2019) Chalcone synthase is ubiquitinated and degraded via interactions with a RING-H2 protein in petals of *Paeonia*'He Xie'. J Exp Bot 70 (18): 4749–4762. [https://doi.org/ 10.1093/jxb/erz245](https://doi.org/10.1093/jxb/erz245)
8. Helariutta Y, Elomaa P, Kotilainen M, Griesbach RJ, Schröder J, Teeri TH (1995) Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae). Plant Mol Biol 28(1): 47-60. [https://doi.org/ 10.1007/BF00042037](https://doi.org/10.1007/BF00042037)
9. Kalantidis K, Schumacher HT, Alexiadis T, Helm JM (2008) RNA silencing movement in plants. Biol Cell 100(1):13–26. [https://doi.org/ 10.1042/BC20070079](https://doi.org/10.1042/BC20070079)
10. Koes RE, Spelt CE, Mol JNM (1989) The chalcone synthase multigene family of *Petunia hybrid* (V30): differential, light-regulated expression during flower development and UV light induction. Plant Mol Biol 12(2):213-225. [https://doi.org/ 10.1007/BF00020506](https://doi.org/10.1007/BF00020506)
11. Koseki M, Goto K, Masuta C, Kanazawa A (2005) The star-type color pattern in *Petunia hybrida* 'red Star' flowers is induced by the sequence-specific degradation of the *chalcone synthase* RNA. Plant Cell Physiol 46(11):1879-1883. <https://doi.org/10.1093/pcp/pci192>
12. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25(4):402–408. [https://doi.org/ 10.1006/meth.2001.1262](https://doi.org/10.1006/meth.2001.1262)
13. Manmathan H, Shaner D, Snelling J, Tisserat N, Lapitan N (2013) Virus-induced gene silencing of *Arabidopsis thaliana* gene homologues in wheat identifies genes conferring improved drought tolerance. J Exp Bot 64 (5):1381–1392. [https://doi.org/ 10.1093/jxb/ert003](https://doi.org/10.1093/jxb/ert003)
14. Morita Y, Saito R, Ban Y, Tanikawa N, Kuchitsu K, Ando T, Yoshikawa M, Habu Y, Ozeki Y, Nakayama M (2012) Tandemly arranged chalcone synthase A genes contribute to the spatially regulated expression of siRNA and the natural bicolor floral phenotype in *Petunia hybrida*. Plant J 70: 739-749. [https://doi.org/ 10.1111/j.1365-313X.2012.04908.x](https://doi.org/10.1111/j.1365-313X.2012.04908.x)
15. Nakatsuka A, Izumi Y, Yamagishi M (2003) Spatial and temporal expression of chalcone synthase and dihydroflavonol 4-reductase genes in the Asiatic hybrid lily. Plant Sci 165(4):759-767. [https://doi.org/ 10.1016/S0168-9452\(03\)00254-1](https://doi.org/10.1016/S0168-9452(03)00254-1)
16. Nabavi SM, Šamec D, Tomczyk M, Milella L, Russo D, Habtemariam S, Sunter I, Rastrelli L, Daglia M, Xiao J, Giampieri F, Battino M, Sobarzo-Sanchez E, Nabavi SF, Yousefi B, Jeandet P, Xu S, Shirooie S (2020) Flavonoid biosynthetic pathways in plants: versatile targets for metabolic engineering. Biotechnol Adv 38. <https://doi.org/10.1016/j.biotechadv.2018.11.005>
17. Ohno S, Hori W, Hosokawa M, Tatsuzawa F, Doi M (2018) Post-transcriptional silencing of chalcone synthase is involved in phenotypic lability in petals and leaves of bicolor dahlia (*Dahlia variabilis*) 'Yuino'. Planta 247(2):413-428. [https://doi.org/ 10.1007/s00425-017-2796-3](https://doi.org/10.1007/s00425-017-2796-3)

18. Ohno S, Hosokawa M, Kojima M, Kitamura Y, Hoshino A, Tatsuzawa F, Doi M, Yazawa S (2011) Simultaneous post-transcriptional gene silencing of two different chalcone synthase genes resulting in pure white flowers in the octoploid dahlia. *Planta* 234(5): 945-958. <https://repository.kulib.kyoto-u.ac.jp/dspace/bitstream/2433/189856/1/s00425-011-1456-2.pdf>
19. Quadrana L, Rodriguez MC, López M, Bermúdez L, Nunes-Nesi A, Fernie AR, Descalzo A, Asis R, Rossi M, Asurmendi S, Carrari F (2011) Coupling virus-induced gene silencing to exogenous green fluorescence protein expression provides a highly efficient system for functional genomics in *Arabidopsis* and across all stages of tomato fruit development. *Plant Physiol* 156(3): 1278–1291. <https://doi.org/10.1104/pp.111.177345>
20. Rabino I, Mancinelli AL (1986) Light, temperature, and anthocyanin production. *Plant Physiol*, 81, 922–924.
21. Senthil-Kumar M, Mysore KS (2011) New dimensions for VIGS in plant functional genomics. *Trends Plant Sci*, 16(12), 656–665. <https://doi.org/10.1016/j.tplants.2011.08.006>
22. Shang YJ, Schwinn KE, Bennett MJ, Hunter DA, Waugh TL, Pathirana NN, Brummell DA, Jameson PE, Davies KM (2007) Methods for transient assay of gene function in floral tissues. *Plant Methods* 3(1):1-12. <https://doi.org/10.1186/1746-4811-3-1>
23. Singh A, Liang YC, Kumar P, Jiang CZ, Reid MS (2012) Co-silencing of the *Mirabilis* antiviral protein (MAP) permits virus induced gene silencing (VIGS) of other genes in Four O’Clock plants (*Mirabilis jalapa*). *J Hortic Sci Biotech* 87 (4):334–340. <https://doi.org/10.1080/14620316.2012.11512873>
24. Suzuki K, Suzuki T, Nakatsuka T, Dohra H, Yamagishi M, Matsuyama K, Matsuura H (2016) RNA-seq-based evaluation of bicolor tepal pigmentation in Asiatic hybrid lilies (*Lilium spp.*). *BMC Genomics* 17(1): 611-629. <https://doi.org/10.1186/s12864-016-2995-5>
25. Suzuki KI, Xue HM, Tanaka Y, Fukui Y, Fukuchi-Mizutani M, Murakami Y, Katsumoto Y, Tsuda S, Kusumi T (2000) Flower color modifications of *Torenia hybrida* by cosuppression of anthocyanin biosynthesis genes. *Mol Breeding*, 6, 239–246. <https://doi.org/10.1023/A:1009678514695>
26. Zhong XH, Yuan X, Wu Z, Khan MA, Chen J, Li XX, Gong BH, Zhao Y, Wu J, Wu CY, Yi MF (2014) Virus-induced gene silencing for comparative functional studies in *Gladiolus hybridus*. *Plant Cell Rep* 33(2): 301-312. <https://doi.org/10.1007/s00299-013-1530-2>

Figures

Figure 1

Expressions of *DchCHS*s at 3 stages of floral buds in *D. chinensis*. A The floral buds and its own petals in the floral buds; B qRT-PCR analysis of three *DchCHS*s at 3 stages compared with their respective expressions at stage1; C qRT-PCR analysis of three *DchCHS*s at 3 stages compared with *DchCHS1* expression at stage 1.

Figure 2

Expression of *DchCHS*s in three kinds of flower colors of *D. chinensis*. A Three kinds of flower colors; B qRT-PCR analysis of three *DchCHS*s compared with their respective expressions in the white; C qRT-PCR analysis of three *DchCHS*s compared with *DchCHS1* expression in the white.

Figure 3

Expressions of DchCHSs in organs of *D. chinensis*. A The purple flowering plant; B qRT-PCR analysis of three DchCHSs in different organs compared with their respective expressions in roots

DcaCHS	...MASIEEIRQA ⁸⁶ PRADGPATILAIGTATPPNAIQADYPDYFVRVTKS.EHMTLKEKFRMCDKSMIKKRYMYLTEEILKENPNLCEY	86
DmoCHS	...MASIEEIRQA ⁸⁶ QRADGPATILAIGTATPPNAIQADYPDYFVRVTKS.EHMTLKEKFRMCDKSMIKKRYMYLTEEILKENPNLCEY	86
GpaCHS	.....RMCDKSMIKKRYMHLTEEDILKEKPNLCEY	29
SliCHS	...MASIEEIRKA ⁸⁶ QRADGPATILAIGTATPPNCVYQADYPDYFVRVTKS.EHMTDLKEKFRMCDKSMIKKRYMHLTEEILKEKPNLCEY	86
DchCHS1	.....	0
DchCHS2	...MGSITEYLEKANSY.KATILSIGTANPPNQMTQEEFVNIFYFRVTNLGDDITQFKDKFKLICEKTGIRKRYMHLTEDFVKENPNIIDQ	86
DchCHS3	...MASIEDIRKA ⁸⁶ QRAEGPATILAIGTATPPNCVYQADYPDYFVRVTKS.EHMTGLKEKFRMCDKSMIKKRYMFLTEEDILKEKTNLCEY	86
Consensus		
DcaCHS	MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQITKLLGLRPSVRREMLYQQGCFAGGTVLR ¹⁷⁶ LAKD	176
DmoCHS	MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQITKLLGLRPSVRREMLYQQGCFAGGTVLR ¹⁷⁶ LAKD	176
GpaCHS	MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQITKLLGLRPSVRREMLYQQGCFAGGTVLR ¹¹⁹ LAKD	119
SliCHS	MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQITKLLGLRPSVRREMLYQQGCFAGGTVLR ¹⁷⁶ LAKD	176
DchCHS1	.....MLYQQGCFAGGTVLR ¹⁹ LAKD	19
DchCHS2	NASSFNIRQDILVEEVPRLGHEASLKAIEEWGQPKSNITHIIFCTVSGIAMPGCDYELLKLLDLQFTVQREMLYQQGCFAGGTVLR ¹⁷⁶ LAKY	176
DchCHS3	MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQITKLLGLRPSVRREMLYQQGCFAGGTVLR ¹⁷⁶ LAKD	176
Consensus	mlyqqgc ggtvrlrak	
DcaCHS	LAENNKDARVIVVCSEITAICFRGPTAAALDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMAWAGQTILPDSGAIDGHLRE ²⁶⁵ VGLTF	265
DmoCHS	LAENNKGARVIVVCSEITAICFRGPTAAALDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMAWAGQTILPDSGAIDGHLRE ²⁶⁵ VGLTF	265
GpaCHS	LAENNRGARVIVVCSEITAICFRGPTETHLDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMVWAAQTILPDSGAIDGHLRE ²⁰⁸ VGLTF	208
SliCHS	LAENNRGARVIVVCSEITAICFRGPTDTHLDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMVWAAQTILPDSGAIDGHLRE ²⁶⁵ VGLTF	265
DchCHS1	LAENNKGARVIVVCSEITAICFRGPTAAALDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMAWAGQTILPDSGAIDGHLRE ¹⁰⁸ VGLTF	108
DchCHS2	IVDSNP ²⁶⁶ PHARVIVVCSEFSTICFRQSNSTQDMSMVGQALFTDGDAPMIIIGANPDESIGERFIIFEIVSAVQSLIPDTKWVGGLRETGLNF	266
DchCHS3	LAENNRGARVIVVCSEITAICFRGPTETHLDSMVGQALFGDGAGALIVGSDPDLST.ERELFQMAWAAQTILPDSGAIDGHLRE ²⁶⁵ VGLTF	265
Consensus	n arvl vcse icfr dsmvgqalf dg a i g pd i exp f a q l pd g lre gl f	
DcaCHS	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ³⁵⁵ LDEMREKSLR	355
DmoCHS	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ³⁵⁵ LDEMREKSLR	355
GpaCHS	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ²⁹⁸ LDEMREKSLR	298
SliCHS	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ³⁵⁵ LDEMREKSLR	355
DchCHS1	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ¹⁹⁸ LDEMREKSLR	198
DchCHS2	YLSRKLEITANSIQKEIHNALALCIYDINSIFYVVEPGGLTIINKMEDKLGLEKIDTSRYVLSEFGNMWSATVHIVLDEMREK ³⁵⁶ SWK	356
DchCHS3	HLLKDVFGIISKNIITNPEEAFSPICVSDWNLFWIAHPGGPAILDQVEAKLGLKEEKIAATRNVLSDFGNMSSACVFI ³⁵⁵ LDEMREKSLK	355
Consensus	l p i l a g d n f pgg il e klgl e kl r vls gnm sa v f ldemr ks	
DcaCHS	DGATTGEGLDWGVLFGEFGGLTIVETVVLSVPLNC... ³⁹¹	391
DmoCHS	DGATTGEGLDWGVLFGEFGGLTIVETVVLSVPLNC... ³⁹¹	391
GpaCHS	DGATTGEGLDWGVLFGEFGGLTIVETVVLSVPEVNC... ³³⁴	334
SliCHS	DGATTGEGLDWGVLFGEFGGLTIVETVVLSVPLTN... ³⁹¹	391
DchCHS1	DGATTGEGLDWGVLLGEFGGLTIVETVVLSVPLNC... ²³⁴	234
DchCHS2	ECKSTTGEGLKYGVLICVGGVTHETVVLSFAINAAA... ³⁹⁴	394
DchCHS3	ECCATTGEGLDWGVLFGEFGGLTIVETVVLSVPEVNN... ³⁹¹	391
Consensus	g ttgegl gvl g gp t etvvl s	

Figure 4

Alignment of the putative protein sequence of DchCHSs with its homologues from caryophyllus family. The accession numbers of the used amino acid sequences are as follows: DcaCHS (Z67982) in *Dianthus caryophyllus*, DmoCHS (AF267173) in *Dianthus monspessulanus*, GpaCHS (AY309966) in *Gypsophila paniculate*, SliCHS (KT954903) in *Silene littorea*.

Figure 5

Phylogenetic tree based on the amino acid sequences of DchCHSs with its homologues from other species. The tree was constructed using MEGA 6.0 and a neighborjoining method, with 1 000 bootstrap replications. The proteins of used genes (with GenBank accession number) in this analysis were as follows:: DcaCHS (Z67982) in *Dianthus caryophyllus*, DmoCHS (AF267173) in *Dianthus monspessulanus*, GpaCHS (AY309966) in *Gypsophila paniculate*, SliCHS (KT954903) in *Silene littorea*, DvaCHS1 (AB576660) and DvaCHS2 (AB591825) in *Dahlia variabilis*, GhyCHS1 (Z38096) and GhyCHS3 (Z38098) in *Gerbera hybrida*, LhyCHSA (BAB40786), LhyCHSB (AB058639) and LhyCHSC (AB058640) in *Lilium hybrid*, PhyCHSA (AF233638) and PhyCHSJ (X14597) in *Petunia x hybrida*.

Figure 6

VIGS of DchCHSs using the TRV vector in *D. chinensis*. A Floral buds were infiltrated with empty vector (left) and with pTRV2-DchCHS1 (right). a Silencing phenotype. b Anthocyanin content. c qRT-PCR analysis; B Floral buds were infiltrated with empty vector (left) and with pTRV2-DchCHS2 (right). a Silencing phenotype. b Anthocyanin content. c qRT-PCR analysis; C Floral buds were infiltrated with empty vector (left) and with pTRV2-DchCHS3 (right). a Silencing phenotype. b Anthocyanin content. c qRT-PCR analysis. The * symbol represents significant difference ($P < 0.05$) according to Student's t-test.