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ORIGINAL ARTICLE

Characterization of three chalcone synthase-like genes in *Dianthus chinensis*

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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 significantly 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.

Key message
At least three *DchCHSs* are involved in the anthocyanin synthesis and the flower coloration in *D. chinensis*. It may be the reason for its richness in flower colors and color patterns.

Keywords *Dianthus chinensis* · Chalcone synthase · Tobacco rattle virus · Virus-induced gene silencing · Flower color

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. 2020). Numerous CHS genes have been isolated and characterized in ornamental plants. Out of the 12 copies of *CHSs* identified

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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 involved 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 working on the flower color in *D. chinensis.*

Materials and methods

Plant materials

Dianthus 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 μmol m^{-2} 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′-GGCTCACTTTTCACC TGCTC-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)

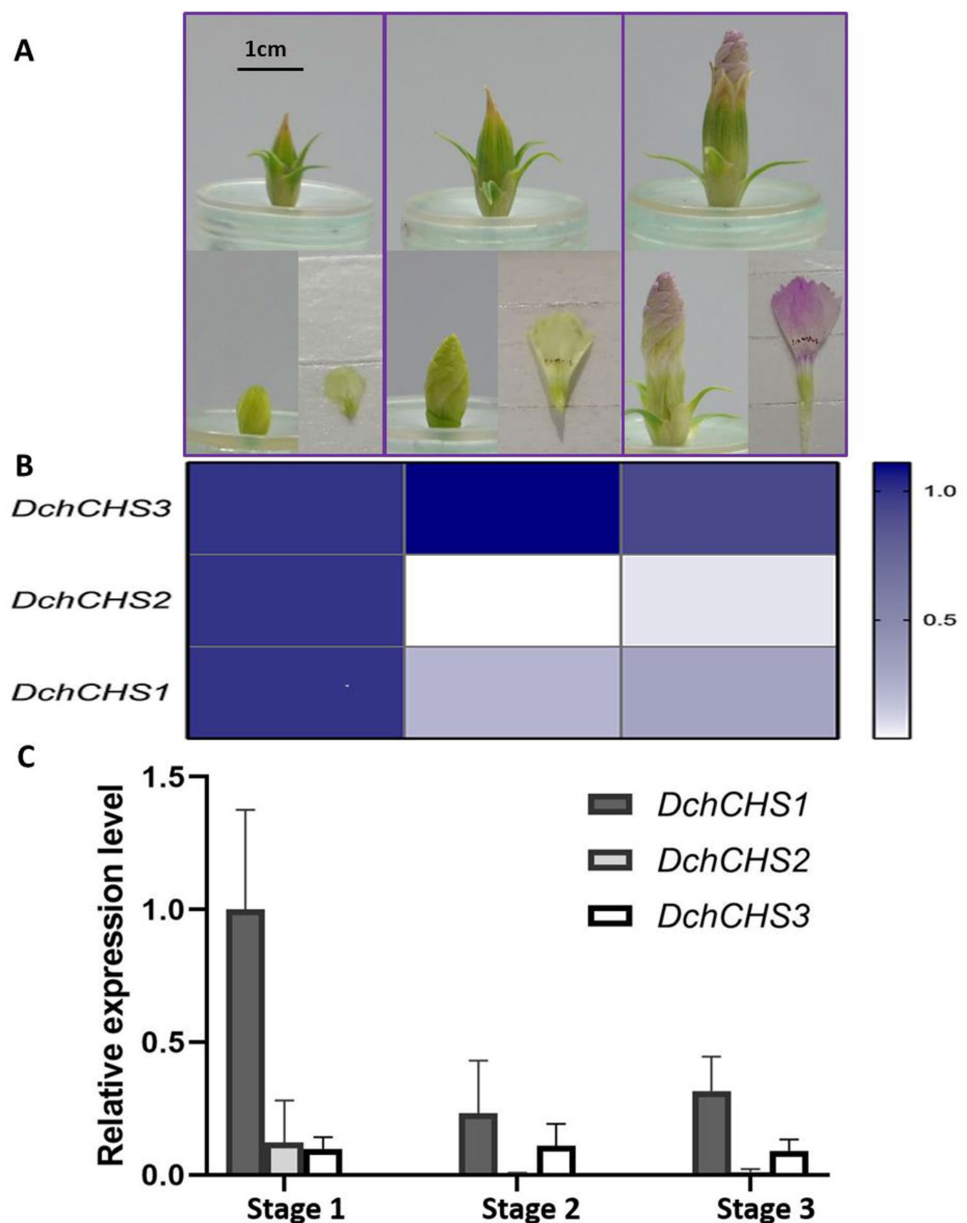

Fig. 1 Expressions of *DchCHSs* at 3 stages of floral buds in *D. chinensis*. **a** The floral buds and its own petals in the floral buds; **b** qRT-PCR analysis of three *DchCHSs* at 3 stages compared with their respective expressions at stage1; **c** qRT-PCR analysis of three *DchCHSs* at 3 stages compared with *DchCHS1* expression at stage 1

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′-TAGTCGCAAAACCGTACCCC-3′. For *DchCHS3* silencing, a 474 bp fragment (884–1357 bp) was amplified with 5′-TCCAGACCTGACAATTGAGC-3′ and 5′-TGA AGCACAACGGTCTCAAC-3′. The amplified fragments were inserted into pGEM-T Easy vectors and sequenced. The inserted fragments were excised from the plasmids by *Eco*RI 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′-TTACAGGTTATT TGGGCTAG-3′ and 5′-CCGGGTTCAATTCCTTATC-3′) and pTRV2 primers (5′-ACGGACGAGTGGACTTAG ATTC-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 $MgCl_2$) to a final OD_{600} 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 *DchCHSs* 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 μL of 1% HCl in methanol (v/v) and incubated overnight at 4 °C with gentle shaking. The extract was mixed with 400 μL of water and 400 μL of chloroform. After centrifugation, the absorbance of the supernatant was measured at 530 and 657 nm. The anthocyanin content was calculated using $A_{530}–0.25A_{657}$ (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 μL 2 × SYBR Advantage qPCR Premix (Takara), 0.2 μL cDNA template and 0.6 μM of each gene-specific primer in a final volume of 20 μL. The gene-specific primers were 5′-TGTTGAGCGACTTTGGGAAC-3′ with 5′-CCCTTCACCTGTTGTGGTTG-3′ for *DchCHS1*, 5′-CCGCAAATAGCATACAAAAGC-3′ with 5′-CCTCCT GGGTGAACCACATAAA-3′ for *DchCHS2* and 5′-CGC CGATTACCAGCTCACC-3′ with 5′-CCGTGCCTCCAG CAAAGC-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′-ATGCCCCCGCTA TGTATGT-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 *DchCHSs*

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 *DchCHSs* 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 *Gerbera hybrida* 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

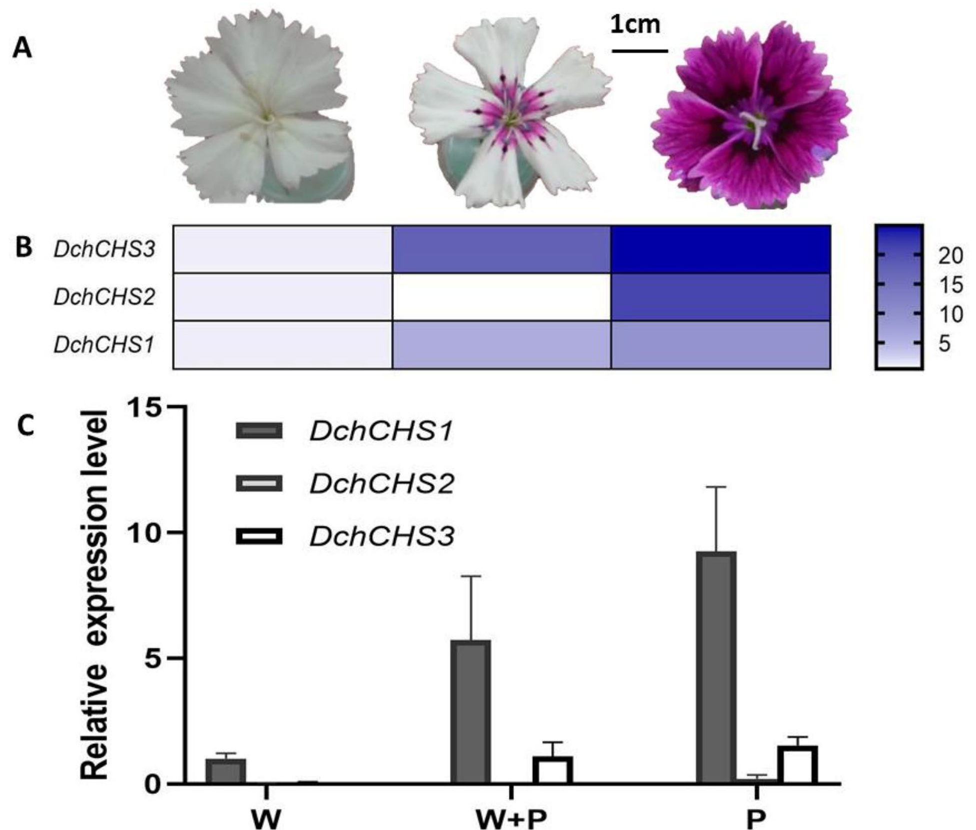

Fig. 2 Expression of *DchCHSs* in three kinds of flower colors of *D. chinensis*. **a** Three kinds of flower colors; **b** qRT-PCR analysis of three *DchCHSs* compared with their respective expressions in the white; **c** qRT-PCR analysis of three *DchCHSs* compared with *DchCHS1* expression in the white

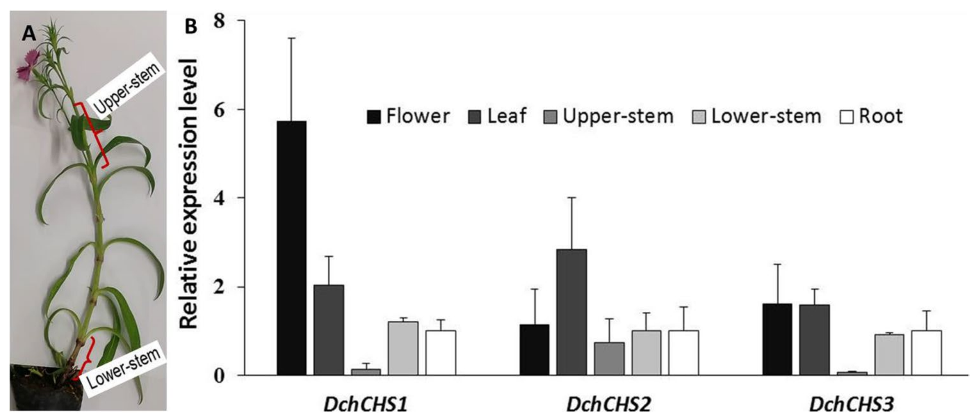

Fig. 3 Expressions of *DchCHSs* in organs of *D. chinensis*. **a** The purple flowering plant; **b** qRT-PCR analysis of three *DchCHSs* in different organs compared with their respective expressions in roots

DchCHSs encoding proteins might be different in enzymatic activities.

Effect of silencing *DchCHSs* in flowers

Fifty percent of the detached flower buds were opened at stage 1, and 100% were opened at stage 2 and stage 3 in the nutrition buffer (Fig. 1a). Therefore, the buds at stage 2 and stage 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

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DcaCHS     ...MASIEEIRQAPRADGPATILAIGTATPPNAIYQADYPDYYFRVTKS.EHMTELKEKFRRMCDKSMIKKRYMYLTEEILKENPNLCEY   86
DmoCHS     ...MASIEEIRQAQRADGPATILAIGTATPPNAIYQADYPDYYFRVTKS.EHMTELKEKFRRMCDKSMIKKRYMYLTEEILKENPNLCEY   86
GpaCHS     .............................................................RMCDKSMIKKRYMHLTEDILKEKPNLCEY   29
SliCHS     ...MASIEEIRKAQRADGPATILAIGTATPPNCVYQADYPDYYFRVTKS.EHMTDLKEKFRRMCDKSMIKKRYMHLTEEILKEKPNLCEY   86
DchCHS1    ..........................................................................................    0
DchCHS2    ...MGSITEYLEKANSY.KATILSIGTANPPNQMTQEEFVNFYFRVTNLGDDITQFKDKFKLICEKTGIRKRYMHLTEDFVKENPNIIDQ   86
DchCHS3    ...MASIEDIRKAQRAEGPATILAIGTATPPNCVYQADYPDFYFRVTKS.EHMTGLKEKFKRMCDKSMIKKRYMFLTEDILKEKTNLCEY   86
Consensus

DcaCHS     MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFMLYQQGCFAGGTVLRLAKD  176
DmoCHS     MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFMLYQQGCFAGGTVLRLAKD  176
GpaCHS     MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFMLYQQGCFAGGTVLRLAKD  119
SliCHS     MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFMLYQQGCFAGGTVLRLAKD  176
DchCHS1    .......................................................................MLYQQGCFAGGTVLRLAKD   19
DchCHS2    NASSFNIRQDILVEEVPKLGHEASLKAIEEWGQPKSNITHIIFCTVSGIAMPGCDYELLKLLDLQPTVQRFMLYQQGCYGGGTVLRLAKY  176
DchCHS3    MGSSLDTRQDMVVSEVPRLGKEAAVKAIKEWGQPKSKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFMLYQQGCFAGGTVLRLAKD  176
Consensus                                                                         mlyqqgc  ggtvlrlak

DcaCHS     LAENNKDARVLVVCSEITAICFRGPTEAALDSMVGQALFGDGAGALIVGSDPDLSI.ERPLFQMAWAGQTLLPDSDGAIDGHLREVGLTF  265
DmoCHS     LAENNKGARVLVVCSEITAICFRGPTEAALDSMVGQALFGDGAGALIVGSDPDLSI.ERPLFQMAWAGQTLLPDSEGAIDGHLREVGLTF  265
GpaCHS     LAENNRGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGAGAVIVGSDPDLSI.ERPLFQMVWAAQTLLPDSEGAIDGHLREVGLTF  208
SliCHS     LAENNRGARVLVVCSEITAICFRGPTDTHLDSMVGQALFGDGAGALIVGSDPDLSI.ERPLFQMVWAAQTLLPDSEGAIDGHLREVGLTF  265
DchCHS1    LAENNKGARVLVVCSEITAICFRGPTEAALDSMVGQALFGDGAGALIVGSDPDLSI.ERPLFQMAWAGQTLLPDSEGAIDGHLREVGLTF  108
DchCHS2    IVDSNPHARVLAVCSEFSTICFRQSNSTQMDSMVGQALFTDGDAAMIIGANPDESIGERPIFEIVSAVQSLIPDTKWGVGGQLRETGLNF  266
DchCHS3    LAENNKGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGAGALIVGSDPDLTI.ERPLFQMAWAAQTLLPDSEGAIDGHLREVGLTF  265
Consensus      n  arvl vcse   icfr       dsmvgqalf dg  a i g  pd  i erp f    a q l pd      g lre gl f

DcaCHS     HLLKDVPGIISKNITNALEDAFSPIGVSDWNNLFWIAHPGGPAILDQVEAKLGLKEEKLAATRNVLSDFGNMSSACVLFILDEMRKKSLR  355
DmoCHS     HLLKDVPGIISKNITNALEEAFSPIGVSDWNNLFWIAHPGGPAILDQVEAKLGLKEEKLAATRNVLSDFGNMSSACVLFILDEMRKKSLR  355
GpaCHS     HLLKDVPGLISKNINKALDEAFSPLGISDWNSLFWIAHPGGPAILDQVEAKLGLKEDKLTATRNVLSDYGNMSSACVLFILDEMRNKSLR  298
SliCHS     HLLKDVPGLISKNINKALEEAFNPLGISDWNSLFWIAHPGGPAILDQVEAKLGLKEEKLAATRNVLSDFGNMSSACVLFILDEMRKKSLR  355
DchCHS1    HLLKDVPGIISKNITNALEEAFSPIGVSDWNNLFWIAPPGGPAILDQVEAKLGLKEEKLAAPRNVLSDFGNMWSACVLFILDEMRKKSLR  198
DchCHS2    YLSRKLPEITANSIQKPLHNALAALGIYDLNSIFYVVHPGGLTILNKMEDKLGLHEQKLDTSRYVLSEFGNMWSATVIFVLDEMRKKSWK  356
DchCHS3    HLLKDVPGLISKNINKALEEAFTPLGISDWNSIFWIAHPGGPAILDQVEAKLGLKEEKLTATRNVLSEFGNMSSACVLFILDEMRKKSLK  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     DGATTTGEGLDWGVLFGFGPSLTVETVVLHSVPLNC...                                                    391
DmoCHS     DGATTTGEGLDWGVLFGFGPGLTVETVVLHSVPLNC...                                                    391
GpaCHS     DGAATTGEGLDWGVLFGFGPGLTVETVVLHSVPVNC...                                                    334
SliCHS     DGATTTGEGLDWGVLFGFGPGLTVETVVLHSVPLTN...                                                    391
DchCHS1    DGATTTGEGLDWGVLLGFGPGLTVETVVLHSVPLNC...                                                    234
DchCHS2    EGKSTTGEGLKYGVLLGVGPGVTIETVVLRSFAINAAA.                                                    394
DchCHS3    EGCATTGEGLDWGVLFGFGPGLTVETVVLHSVPVNN...                                                    391
Consensus   g  ttgegl  gvl g gp  t etvvl s
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Fig. 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*

(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 stage 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 *DchCHS*1 and *DchCHS*3 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). *DchCHS*2 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

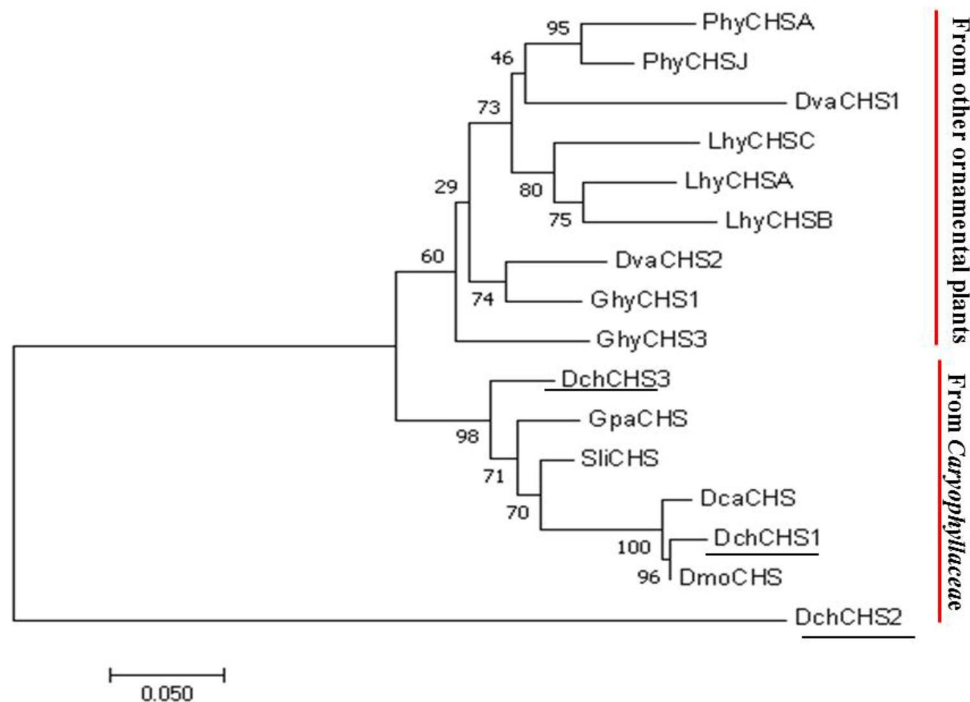

Fig. 5 Phylogenetic tree based on the amino acid sequences of *DchCHSs* with its homologues from other species. The tree was constructed using MEGA 6.0 and a neighborjoining method, with 1 000 bootstrap replications. The proteins of used genes (with GenBank accession number) in this analysis were as 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 *LyhCHSC* (AB058640) in *Lilium hybrid*, *PhyCHSA* (AF233638) and *PhyCHSJ* (X14597) in *Petunia x hybrida*

each flower color, *DchCHS*1 expression was substantially higher than the *DchCHS*3, and the *DchCHS*3 was substantially higher than the *DchCHS*2 (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 upper-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

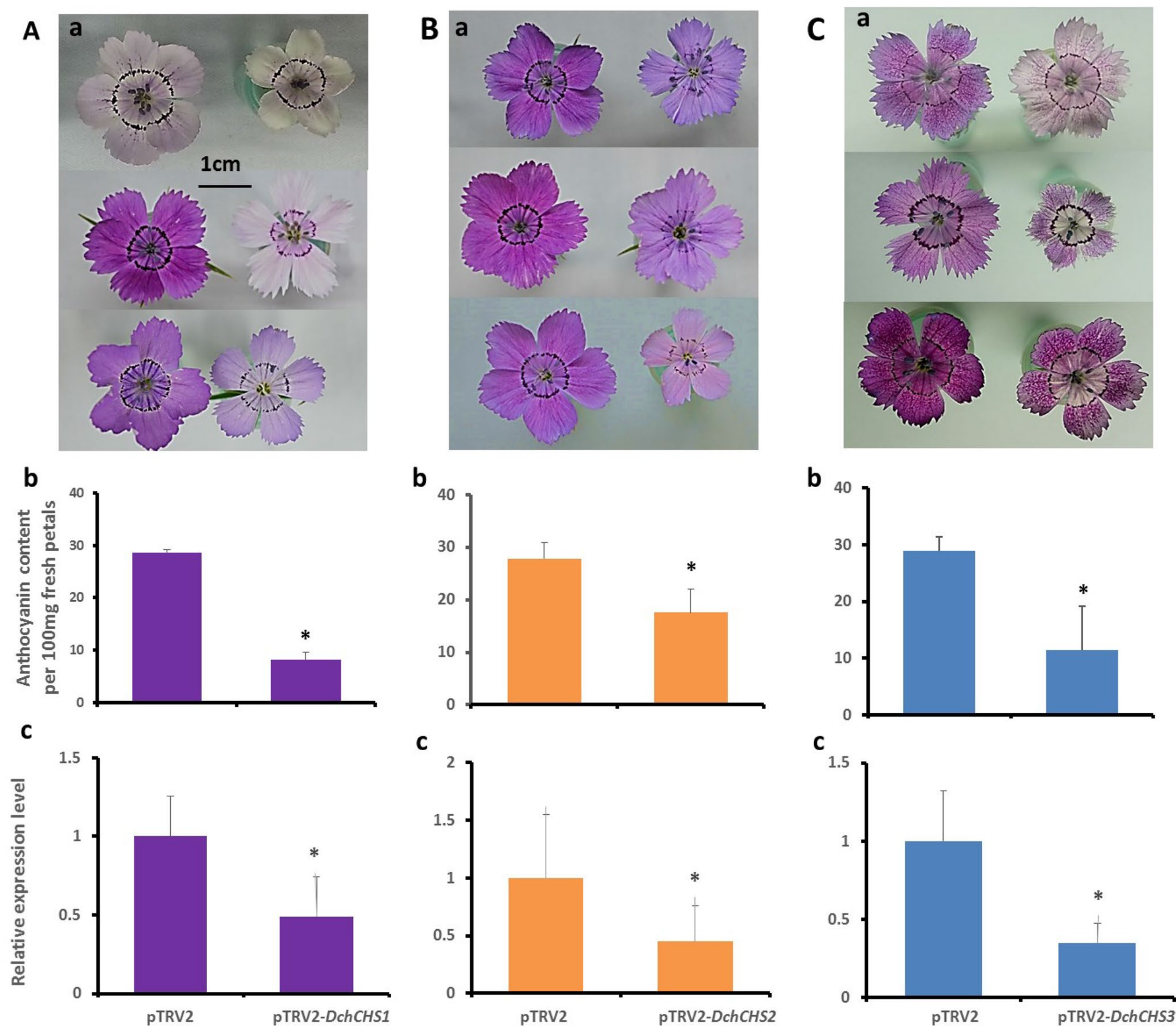

Fig. 6 VIGS of *DchCHSs* using the TRV vector in *D. chinensis*. **A** Floral buds were infiltrated with empty vector (left) and with pTRV2-*DchCHS1* (right). **a** Silencing phenotype. **b** Anthocyanin content. **c** qRT-PCR analysis; **B** Floral buds were infiltrated with empty vector (left) and 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

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 in the flowers. The silencing phenotypes were associated with substantial reductions of anthocyanin contents and transcript levels of the silenced *DchCHS* (Fig. 6Aa, Ab, Ac, Bb, Bc, Cb, Cc). 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 are related to the 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*. Simultaneous post-transcriptional gene silencing 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 upper-stems (Fig. 3b). The expression levels of *DchCHS*1, *DchCHS*2 and *DchCHS*3 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 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 *DchCHSs* obtained in *D. chinensis* are involved in the anthocyanin biosynthesis and in flower color, but their functions might be 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.

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Author 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.

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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.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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