Running title: RNA silencing of *CHS* in non-transgenic petunia

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Short Communication

The star-type color pattern in *Petunia hybrida* 'Red Star' flowers is induced by the sequence-specific degradation of the *chalcone synthase* RNA

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Petunia hybrida ‘Red Star’ is a variety whose flowers exhibit a star-type red and white bicolor pattern. We analyzed the mRNA levels of six genes involved in anthocyanin biosynthesis. Only the level of *chalcone synthase* (*CHS*) mRNA was depressed in the unpigmented flower sectors. Both transcriptional activity and the accumulation of short interfering RNA of *CHS* in the unpigmented sectors were detected. Viral infection blocked the generation of *CHS*-silenced sectors. These results indicate that sequence-specific degradation of the *CHS* RNA is the primary cause of the formation of white sectors in the ‘Red Star’ flowers.

Keywords: chalcone synthase - *Cucumber mosaic virus* - *Petunia hybrida* - post-transcriptional gene silencing - RNA silencing - short interfering RNA

RNA silencing refers to the suppression of specific genes that is induced by the presence of homologous double-stranded RNA (dsRNA) sequences. Plants display two types of RNA silencing: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS; for reviews, see Matzke et al. 2001; Baulcombe 2004). PTGS involves sequence-specific degradation of RNA in the cytoplasm and is mediated by short interfering RNAs (siRNAs) produced by the dsRNA-processing enzyme Dicer. The siRNAs serve as guides for the ribonuclease complex, RNA-induced silencing complex (RISC), which cleaves the homologous mRNAs. This pathway is also referred to as quelling in *Neurospora* and as RNA interference (RNAi) in various other organisms (for a review, see Mello and Conte Jr. 2004). TGS is the repression or inactivation of transcription and is associated with an increased level of DNA methylation in the affected promoters, as well as changes in histone modification. The siRNAs may also direct DNA methylation (for a review, see Matzke et al. 2004). Like siRNAs, micro RNAs (miRNAs) derive from the Dicer cleavage of a precursor single-stranded RNA folded into a hairpin structure. MiRNAs negatively regulate the expression of endogenous genes either through RNA cleavage or arrest of translation, which is another pathway of RNA silencing (reviewed by Baulcombe 2004).

The understanding of RNA silencing was improved using transgenic petunia plants in which both endogenous and introduced *chalcone synthase-A* (*CHS-A*) genes were inactivated by the overexpression of the transgene, a type of co-suppression (Napoli et al. 1990; van der Krol et al. 1990a). Because *CHS-A* catalyzes an essential step in the biosynthesis of anthocyanins, *CHS-A* gene silencing is recognized as the inhibition of the wild-type pigmentation in flowers (Napoli et al. 1990; van der Krol et al. 1990a).

Twelve copies of *CHS* gene have been identified in petunia (Koes et al. 1989). *CHS-A* is the major *CHS* gene expressed during flower development, and probably encodes a major CHS protein working in the anthocyanin biosynthesis. *CHS-J* is also expressed in flower. These two genes show similar patterns of expression in response to

light and developmental stimuli. However, the mRNA level of *CHS-J* is approximately 10 times lower than that of *CHS-A* (Koes et al. 1989). Whether *CHS-J* has a distinct role is unknown.

The silencing of *CHS* in transgenic petunias produces both white flowers and an array of flower color patterns (reviewed by Jorgensen 1995). By 1838, early plant hybridizers, who created horticultural petunias from crosses among wild species, had already produced flower color patterns similar to those of *CHS-A* transgenic petunias (Jorgensen 1995, and references therein). Petunia plants that produce 'Red Star'-type flowers are included in these early varieties. The red petals lack pigmentation along the vein tissue, resulting in a star-type color pattern.

In 'Red Star,' the *CHS* mRNA level is lower in the white sectors than in the red sectors (Koes et al. 1989; van der Krol et al. 1990b). Metzlaff et al. (1997) found that a 3'-end specific RNA fragment of *CHS-A* is generated by a cleavage of mRNA and is more resistant to degradation than a 5'-end RNA, just as it is in the white flowers of transgenic petunias exhibiting PTGS of *CHS-A*. They proposed a model of auto-regulatory cycles of RNA paring and cleavage via complementarity between the 3' end of coding region and the 3' untranslated region of *CHS-A*. Teycheney and Tepfer (2001) showed that viral infection of petunias with star-type flowers caused a partial restoration of anthocyanin pigmentation, which correlated to a local increase in the *CHS-A* mRNA level in the flowers, probably because the virus suppresses sequence-specific RNA degradation. These reports suggest that a reduction of the *CHS-A* mRNA level in certain petal cells causes the star-type flower phenotype and that the mRNA reduction most likely occurs at a post-transcriptional level. However, the status in the 'Red Star' petunia of the mRNA levels of other genes involved in anthocyanin biosynthesis is not known. Moreover, there is no direct evidence for the occurrence of sequence-specific RNA degradation in this plant, e.g., transcriptional activity of the causal gene(s) as well as the production of siRNA. In this study, we compared the mRNA levels of the genes involved in

anthocyanin biosynthesis in the white and red sectors of ‘Red Star’ flowers. We also conducted experiments to determine whether the generation of white sectors in ‘Red Star’ flowers involves post-transcriptional degradation of mRNA.

We compared the mRNA level of genes involved in the anthocyanin biosynthesis pathway (Holton and Cornish 1995) in the red and white sectors of the petals using reverse transcription-mediated real-time PCR (real-time RT-PCR) analysis. We analyzed the mRNA levels of six genes, i.e., *CHS-A*, chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*), and flavonoid 3-glucosyltransferase (*3GT*), at the stage of flower development when the mRNA level of *CHS-A* is highest (Koes et al. 1989; Fig 1). The mRNA levels of these genes were quantified relative to the mRNA level of *α-tubulin*, and the values of the white and red flower sectors were compared. Among the genes we analyzed, only *CHS-A* mRNA showed a depressed level of accumulation in the white sectors of the flowers. The mRNA of the other genes accumulated in both the white and red sectors, although we observed slight differences in the mRNA levels between these tissues. Thus, the lack of pigmentation was associated with a reduced *CHS-A* mRNA level, as reported previously, but was not accompanied by reduced mRNA levels of the other genes in the biosynthetic pathway. We do not know whether the different levels of mRNA from the other genes in the white and red sectors reflected differences in gene expression between different parts of the petals or whether the difference is associated with the active or inactive state of anthocyanin biosynthesis.

We examined whether the *CHS-A* gene is transcribed. We performed a run-on transcription assay using nuclei isolated from the white sectors or red sectors of the flower. The transcription of the *CHS-A* gene was detected in the white sectors of the flower as well as in the red sectors (Fig. 2A).

Since the production of siRNA is a hallmark of the occurrence of sequence-specific degradation of RNA, we next looked for the presence or absence of

CHS-A siRNAs. A Northern blot analysis of the small RNA fraction revealed that *CHS-A* siRNAs accumulated in the white, but not the red, sectors of the flower (Fig. 2B). The siRNAs may be the products of the ongoing cycling reaction of sequence-specific RNA degradation in the white sectors rather than systemic and diffusible signals of the reaction.

In addition, we examined whether infection by a virus carrying a suppressor of sequence-specific RNA degradation may affect the flower-color pattern as well as the *CHS-A* mRNA level. We used *Cucumber mosaic virus* O strain (CMV-O) for this assay. CMV encodes 2b protein, which suppresses sequence-specific RNA degradation through preventing translocation of silencing signal (for a review, see Baulcombe 2002). When the 'Red Star' plants were infected with CMV-O, all white sectors of the flowers turned red (Fig. 3A). Real-time RT-PCR analysis showed that the *CHS-A* mRNA level increased significantly in the floral areas that corresponded to the white sectors (Fig. 3B). This clearly indicates that the reversion to pigmentation is correlated with the increase in the *CHS-A* mRNA level. Teycheney and Tepfer (2001) showed that three viruses cause either patchy pigmentation or a decrease in the area of white sectors in star-type petunia flowers. We observed an almost complete loss of the star-type flower pattern. Thus, the occurrence of silencing in the flowers can be suppressed in almost all cells by a viral suppressor protein. We detected a very similar change induced by other isolates, such as CMV-L and CM95 (data not shown).

We also analyzed the mRNA level of *CHS-J*. Real-time RT-PCR analysis showed that the *CHS-J* mRNA level in the white sectors was reduced to 13.3 ± 5.9 % of the *CHS-J* mRNA level in the red sectors. At present it is not certain whether changes in the *CHS-J* mRNA level affects flower color because *CHS-J* mRNA shares only a small portion in the *CHS* mRNA pool in flower cells of petunia. In any case, our results indicate that sequence-specific degradation of the *CHS* RNA occurred and it resulted in the reduction of the *CHS* mRNA level in the white sectors of flower.

Some questions remain. How is sequence-specific RNA degradation induced? Why is the flower pattern a star type? Why is there a distinct boundary between the white and red sectors of the flower? The occurrence of PTGS is sometimes associated with the status of gene(s) in the genome. Two studies have reported the occurrence of PTGS in non-transgenic plants: a rice mutant, *Low glutelin content1*, which was generated by gamma-irradiation (Kusaba et al. 2003), and commercial soybeans that lost pigmentation in their seed coats (Senda et al. 2004). In both cases, the occurrence of PTGS was associated with the locations of homologous genes that form an inverted-repeat structure, which may produce dsRNA molecule by read-through transcription, a potent inducer of RNA silencing. An analysis of the genomic structure of the *CHS* genes may shed light on whether the status of the *CHS* genes in the 'Red Star' genome is responsible for sequence-specific RNA degradation.

The white sector of 'Red Star' flowers forms along the veins in the center of each petal. This may indicate the movement of diffusible silencing signal molecules or the occurrence of a reaction caused by such signals. The apparent sequence specificity of RNA degradation indicates either that the signaling molecule is RNA or that it has an RNA component. Alternatively, the induction of sequence-specific RNA degradation may be ascribed to a difference in the rate of gene expression in the corresponding cells. We found that mRNA levels of several genes in the anthocyanin pathway were lower in the red than in the white sectors of 'Red Star' (see Fig. 1). The cells in proximity to the vein tissue may have slightly higher level of transcriptional activity or may temporarily accumulate the *CHS* mRNA. Given that a gradient in the *CHS* transcription rate (or its temporary accumulation level or both) depends on the distance from the vein, one plausible explanation for the formation of the distinct boundary between the white and red sectors is the existence of a threshold in the temporary RNA level that determines whether sequence-specific RNA degradation is triggered (Smith et al. 1994) and a cycling of the reaction that reinforces its continuity once it has begun (see Matzke et al. 2001).

The notion that a slight difference in the transcription rate may affect induction of sequence-specific RNA degradation is consistent with the finding that a color phenotype induced by PTGS is highly sensitive to small quantitative changes in gene dosage and gene expression (Jorgensen 1995).

Materials and Methods

We used the *Petunia hybrida* Vilm. variety 'F1 Carnival Red Star' (Takii Seed Co., Japan). Total RNA was isolated from plants as described by Napoli et al. (1990), except that we removed genomic DNA from the RNA fraction using DNaseI (Takara). One µg of the total RNA was used as the template for cDNA synthesis. The cDNA synthesis reaction mixture was prepared by mixing 4 µl 5X reaction buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 2 µl 0.1 M DTT, 0.5 µl RNaseOUT inhibitor (Invitrogen), the total RNA solution, and water to a final volume of 19 µl. The mixture was heated at 65°C for 5 min and rapidly cooled on ice. After the addition of 1 µl of reverse transcriptase (M-MLV, Invitrogen), the cDNA synthesis was performed at 42°C for 1 hr. The reverse transcriptase was inactivated by heating the sample at 99°C for 1 min. Real-time RT-PCR was carried out using a 1-µl aliquot of the reaction mixture and DyNAmo SYBR Green qPCR Kit (FINNZYMES) with a DNA Engine Opticon 2 System (MJ research). The PCR cycle was 94°C for 30 sec, 61°C for 30 sec, 72°C for 1 min, and 78°C for 2 sec. This cycle was repeated 40 times. Fluorescence quantification was carried out before and after the incubation at 78°C to monitor for the formation of primer-dimers. A reaction mixture without reverse transcriptase was used as a control to confirm that no amplification occurred from genomic DNA contaminates in the RNA sample. Primers for PCR were designed based on nucleotide sequences deposited on database. The database accession numbers are as follows: *CHS-A*, AF233638; *CHI*, AF233637; *F3H*, AF022142; *DFR*, AF233639; *ANS*, X70786; *3GT*, AB027454; *CHS-J*, X14597. Primers for petunia *α-tubulin* gene were designed based on a partial cDNA sequence of this gene (our

unpublished data). Primers for PCR are as follows: *CHS-A*, 5'-GGCGCGATCATTATAGGTTC-3' and 5'-TTTGAGATCAGCCCAGGAAC-3'; *CHI*, 5'-TACGGCGATAGGTGTGTATC-3' and 5'-GGCAAGATCGTAGTAACTCG-3'; *F3H*, 5'-ACTTGGATCACTGTTCAGCC-3' and 5'-ATACACTATCGCCTCTGGTG-3'; *DFR*, 5'-GCTATCATCTACGATGTGGC-3' and 5'-TGTCGACAAGTATCGATGGC-3'; *ANS*, 5'-TACCTGAGACTGTCACTGAG-3' and 5'-GCAGTATCCAGTTCATCCTC-3'; *3GT*, 5'-GCAGTGGCAGAAGCATTAGA-3' and 5'-CACATGATATGCCCTCCAAA-3'; *CHS-J*, 5'-AAAGTTTAGTGGAGGCATTCC-3' and 5'-TCCATACTCACTCAAGACATG-3'; *α-tubulin*, 5'-GCCACCATCAAGACCAAGC-3' and 5'-ACCTCAGCAACACTGGTTGA-3'. In all PCR experiments, amplification of a single DNA species was confirmed by both melting curve analysis of real-time PCR and gel electrophoresis of PCR products. The nuclear run-on transcription assay was performed according to the method of Kanazawa et al. (2000), except that Kirby solution was not used. *CHS-A* siRNA was detected by a Northern blot analysis of the small RNA fraction using a digoxigenin-labeled probe as described by Goto et al. (2003). For the viral infection, the leaves of young petunia plants were dusted with carborundum and rub-inoculated with the purified virus at 50 µg/ml. We used CMV strains maintained at Hokkaido University, Japan. The systemic infection of the virus in the petunias was confirmed by RT-PCR of the viral RNA. To analyze changes in the *CHS-A* mRNA level, RNA was isolated from flowers 3-4 weeks after inoculation with the virus.

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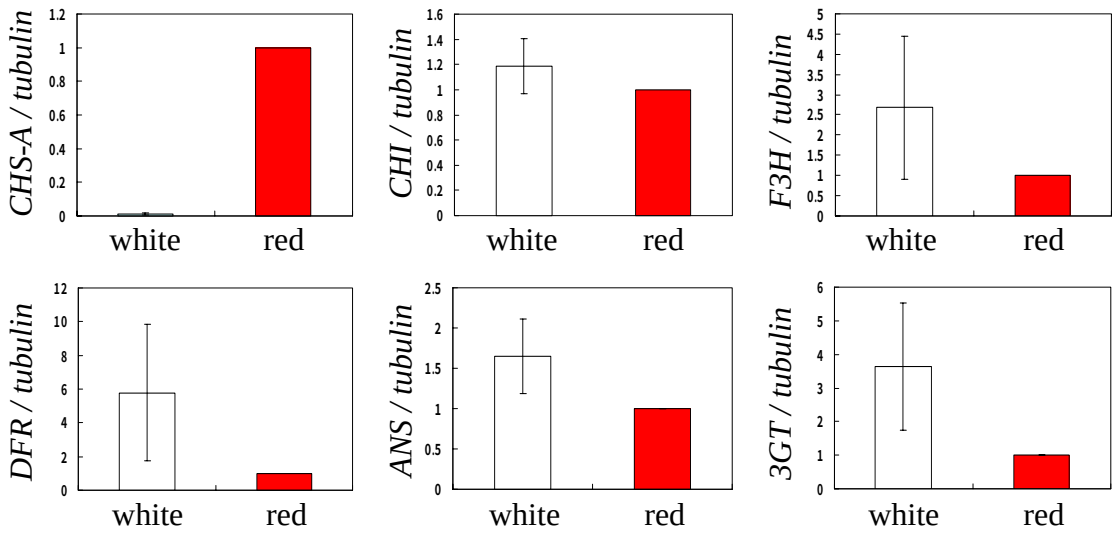
Fig. 1 Comparison by real-time PCR analysis of the mRNA levels of genes involved in the anthocyanin biosynthetic pathway in the red and white sectors of ‘Red Star’ petunia flowers. The mRNA levels of the genes *CHS-A*, *CHI, F3H, DFR, ANS*, and *3GT* are shown. The mRNA levels were quantified relative to the mRNA level of *α-tubulin*, and the relative values of the white and red flower sectors were compared. The red sector value was set at 1. The data represent the mean and standard errors obtained from three replicates of all processes of the analysis.

Fig. 2 Sequence-specific degradation of the *CHS-A* RNA occurred in the white sectors of the flowers. (A) Run-on transcription assay using isolated nuclei from the red and white sectors of ‘Red Star’ flowers. Nuclei isolated from the flowers were used to synthesize radio-labeled run-on transcripts. The labeled RNA was hybridized to DNA blotted on a filter containing pBluescript plasmid (for a negative control), a *Nco*I-*Hin*dIII fragment that contained exon 1 and a portion of intron 1 of the petunia *CHS-A* gene, and the *Antirrhinum majus* ubiquitin gene (EMBL accession number AMUBIMRP X67957; for a positive control). (B) Northern blot analysis of low-molecular-weight RNA from the red and white ‘Red Star’ flower sectors. Lane ‘M’, a 20-mer *CHS-A* DNA primer for a size control; lane ‘red’, small RNA fraction from the red flower sector; lane ‘white’, small RNA fraction from the white flower sector. Ethidium-bromide-stained 5S RNA and tRNA bands are shown below the panel to demonstrate that an equal amount of the small RNA fraction was loaded.

Fig. 3 Changes in the flower color pattern and the *CHS-A* mRNA level due to virus infection. (A) Virus-induced changes in the flower phenotype. Left, a ‘Red Star’ flower; right, a flower produced by ‘Red Star’ plants 10 days after inoculation with CMV-O. (B) Virus-induced changes in the mRNA level of *CHS-A*. The *CHS-A* mRNA level relative to the *α-tubulin* mRNA level was assessed for the following tissues: from left to right, white

sectors of ‘Red Star’ flowers; red sectors of ‘Red Star’ flowers; sectors of CMV-O-infected ‘Red Star’ flowers corresponding to the white sectors of uninfected flowers; sectors of CMV-O-infected ‘Red Star’ flowers corresponding to the red sectors of uninfected flowers. The RNA was isolated from virus-infected plants 3-4 weeks after inoculation. The *CHS-A* mRNA level relative to the *α-tubulin* mRNA level in the red sectors of uninfected ‘Red Star’ flowers was given a value of 1. Data represent the mean and standard errors obtained from three replicates of all analyses, including RNA extraction, cDNA synthesis, and real-time PCR analysis.

Figure 1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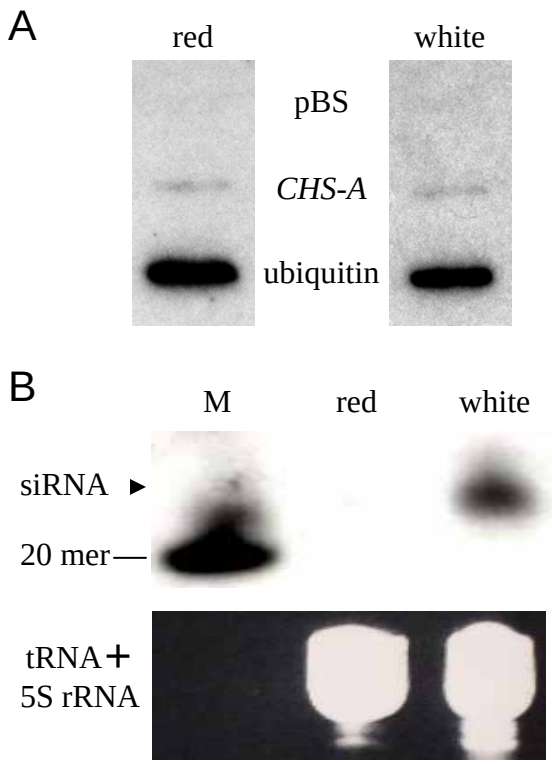
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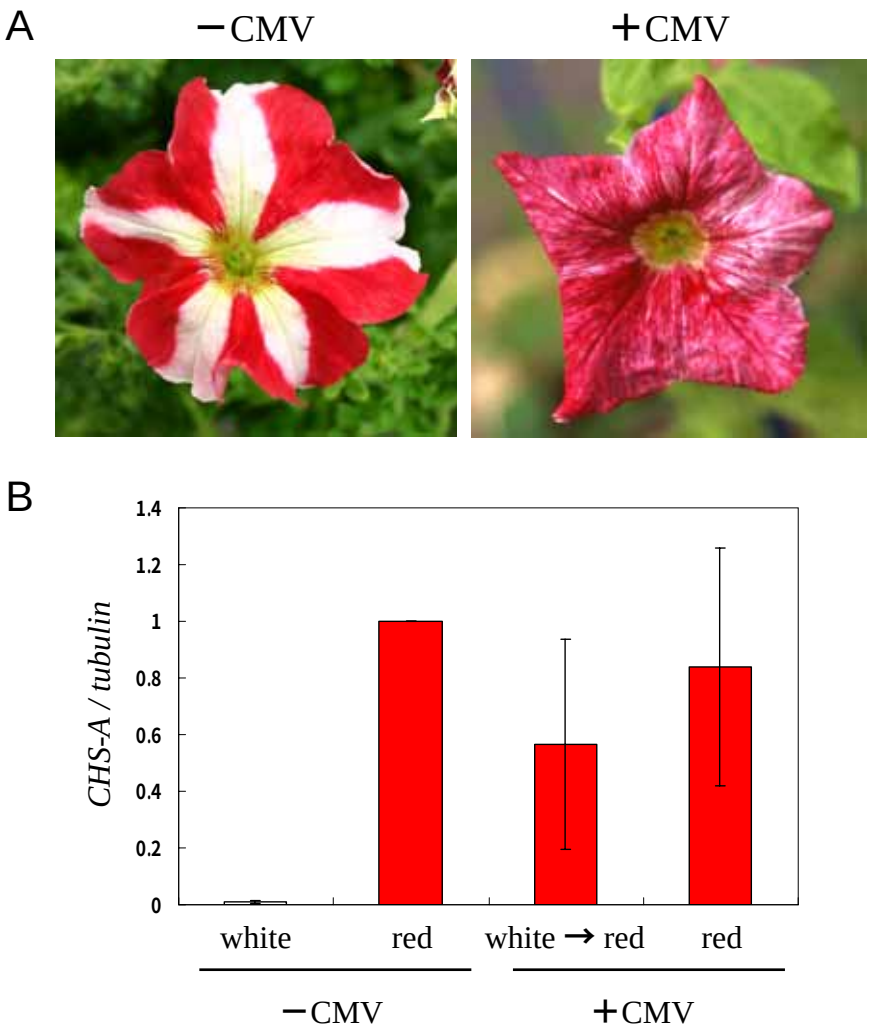
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