Gene Expression in the *Star* Mutation of *Petunia* × *hybrida* Vilm.

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ABSTRACT. Gene silencing is one of the ways in which gene expression is controlled. The authors have developed a model system to study anthocyanin gene silencing using a recessive mutation in *Petunia* Juss. (*Star* mutation) and the ability of certain viruses to reverse the gene silencing mutation. In healthy plants, the star pattern was enhanced (increase in level of gene silencing) under high temperature or light growing conditions. Virus infection did not significantly influence the star pattern when plants were grown under either low-light or low-temperature conditions. Under high-light and -temperature conditions, virus infection reverses silencing, leading to a change in the star pattern. These changes in the star pattern corresponded to changes in gene expression. Viral infection had a greater affect on regulatory gene (*Wd40*, *Myc*, and *Myb*) expression than on structural gene expression (*Chs* and *Ans*).

The *Star* mutation in *Petunia* × *hybrida* results in flowers expressing a white star pattern on a pigmented background. This mutation was first described in 1838 as *P. vittata* Vilm. (Ewart, 1984). A major breakthrough in breeding occurred when A. Howard released the cultivar ‘Howard’s Star’ in the late 1800s, which is in the genetic background of nearly every modern star cultivar.

Anthocyanin biosynthetic gene expression within the white and colored tissues of the *Star* mutation has been extensively studied. There is an absence of chalcone synthase enzyme (CHS) activity in the white tissue compared with the colored tissue (Mol et al., 1983). A more complete analysis of structural gene expression in the *Star* mutant demonstrated that the level of expression of chalcone isomerase gene (*Chi*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol reductase (*Dfr*), flavonoid 3-glucosyltransferase (*3Gt*), and anthocyanin synthase (*Ans*) was similar in white and red tissues (Koseki et al., 2005). Chalcone synthase gene (*Chs*) was the only anthocyanin biosynthetic gene with differential expression.

The downregulation of *Chs* appears to be mediated by post-transcriptional gene silencing (PTGS). Plants showing the *Star* floral phenotype occurred in a population of transgenic plants expressing an extra copy of *Chs* (Napoli et al., 1990; van der Krol et al., 1990). This cosuppression phenomenon was shown to be the result of *Chs* messenger RNA (mRNA) degradation (Metzlaff et al., 1997; van Blokland et al., 1994). In addition, virus infection can restore anthocyanin production in white *Star* tissue by inhibiting PTGS (Teycheney and Tepfer, 2001).

There is no information on regulatory gene expression in the *Star* mutation. Anthocyanin structural gene transcription requires the expression of at least one of each of three distinct transcription factor families: MYC, MYB, and WD40 (Griesbach, 2005). The WD40 family encodes proteins characteristic of the β -propeller group. WD40 is defined by a 40-residue core region delineated by a glycine–histidine dipeptide and a tryptophan–aspartic acid (WD) dipeptide. This motif is repeated in tandem 4 to 16 times (Smith, 1999). In *Petunia*, *An11* encodes an anthocyanin-specific WD40 transcription factor (de Vetten et al., 1997). In this paper, *An11* is denoted *WD_{An11}*.

The *Myc* gene family encodes proteins characteristic of human MYC oncoprotein transcription factors (Buck and Atchley, 2003). MYC is defined by an N-terminal end of ≈ 20 hydrophilic basic amino acids and a C-terminal end of ≈ 40 hydrophobic amino acids that form a basic helix–loop–helix (bHLH) structure. In *Petunia*, *An1* encodes an anthocyanin-specific MYC transcription factor (Spelt et al., 2000, 2002). In this paper, *An1* is denoted *Myc_{An1}*.

The *Myb* gene family encodes proteins characteristic of human MYB oncoprotein transcription factors (Borevitz et al., 2000; Stracke et al., 2001). MYB is defined by an N-terminal end rich in basic amino acids and a C-terminal end rich in acidic amino acids. MYBs are classified into three subfamilies based upon the number of adjacent repeats in the MYB domain: MYB1R (one repeat), R2R3-MYB (two repeats), and MYB3R (three repeats). The R2R3 class is involved in anthocyanin expression. In *Petunia*, *An2* encodes an anthocyanin-specific MYB transcription factor (Quattrocchio et al., 1998, 1999). In this paper, *An2* is denoted *Myb_{An2}*.

The three transcription factors MYC, MYB, and WD40 form a complex that binds to structural gene promoters. The following description of this complex was compiled from several sources (Baudry et al., 2006; Hartman et al., 2005; Hernandez et al., 2004; Ramsay and Glover, 2005; Zimmermann et al.,

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Fig. 1. (A–E) *Petunia xhybrida* clones expressing different flower color patterns. (A) 'Primetime Burgundy Star' parent. (B) 'Celebrity Burgundy Frost' parent. (C) 'Star-Minus' seedling. (D) 'Star' seedling. (E) 'Star-Plus' seedling.

Fig. 2. (A–D) The effects of photoperiod and light intensity on the expression of the *Star* flower color pattern in *Petunia xhybrida* 'Star'. (A) Ten hours of 215 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. (B) Sixteen hours of 215 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. (C) Ten hours of 435 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. (D) Sixteen hours of 435 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light.

2004). MYC associates with WD40. Either before or after its association with WD40, MYC binds to the amino acid sequence (DE)LX₂(RK)_xLX₆LX₃R on helices 1 and 2 of the R3 repeat in MYB. Once associated, MYB binds to the structural gene promoter's MYB recognition element containing the nucleotide consensus sequence AACCTA. MYC binds to the pro-

moter's E-box containing the nucleotide consensus sequence CAGCTG.

Both the *Myc* and *Myb* gene families contain members that have arisen by gene duplication (Hansen et al., 2000; Zhang et al., 2000). Structural gene regulation is defined by the diversity among the *Myc* and *Myb* alleles, each of which regulates expression in a different manner. For example *Myb_{Ros}* from *Antirrhinum majus* L. increases the anthocyanin concentration of vegetative tissue when expressed in *P. xhybrida* and of floral tissue when expressed in *Eustoma grandiflorum* Grise. (Schwinn et al., 2001). In *Petunia*, the combination *Myc_{An1}/Myb_{An2}* induces anthocyanin pigmentation in the flower limb, whereas the *Myc_{An1}/Myb_{An4}* combination induces anthocyanin pigmentation in the anthers, and the *Myc_{An1}/Myb_{Ph4}* combination induces vacuolar acidification (Quattrocchio et al., 2006).

Besides controlling structural gene expression, these regulatory genes can also control their own expression. For example in *Arabidopsis thaliana* (L.) Heynh. seeds, the transcription of flavonoid structural genes is controlled by an MYC_{TT8}, MYB_{TT2}, and WD40_{TTG1} complex (Baudry et al., 2006). Interestingly, this same regulatory gene complex also controls the transcription of one of its members (*Myc_{TT8}*). In humans, MYC can simultaneously activate transcription and limit translation of the same gene through micro RNA (miRNA) (O'Donnell et al., 2005). In human cells, MYC binds to the E-box in the promoters of several regulatory genes, as well as the E-box in promoters of miRNAs specific to those genes. MYC might also regulate genes in plants through a similar miRNA route.

miRNAs control translation by degrading mRNA having a homologous sequence. The following description of the miRNA pathway was compiled from several sources (Bartel, 2004; Kidner and Marteinsen, 2005; Jones-Rhoades et al., 2006). In the miRNA pathway, the miRNA loci encode transcripts called pri-miRNAs that are ≈ 1 kb and have both a cap and poly-A tail. The pri-miRNA is cleaved in the nucleus by RNase III-type Dicer enzymes (DCL) to form a 60 to 70-nt, hairpin folded pre-miRNA. The pre-miRNA has a 2-nt 3' overhang containing a 5'-monophosphate and 3'-OH groups. The pre-miRNA is further processed by Hua Enhancer 1 (HEN1), Hyponastic Leaves 1 (HYL1), and DCL into a 21 to 23-nt miRNA. Hua Enhancer 1 methylates the 2'-OH of the 3'-terminal nucleotide. The miRNA then forms an imperfect double-stranded RNA (dsRNA) that is transported out of the nucleus by the Exportin-5 protein (HST). The dsRNA is rapidly unwound in the cytoplasm and is transferred to another enzyme complex called RISC (RNA-induced silencing complex). The RISC includes an Argonaute protein (AGO). The 3' end of the miRNA binds to the PAZ domain of AGO and aligns it with the target mRNA, which binds to the PIWI domain of AGO. The mRNA is then cleaved between the 10th and 11th

bases from the 5' end of miRNA match. The cleaved mRNA is further degraded by Exoribonuclease 4 (XRN4).

Another class of small RNAs called silencing RNAs (siRNAs) behave in the same manner as miRNAs. The difference between the siRNA and miRNA pathways is the source of the dsRNA. In the siRNA pathway, an aberrant RNA is the template for a dsRNA (Baulcombe, 2004).

The original *Star* mutation was influenced by both light intensity and temperature (Schröder, 1934). Under high light intensity or high temperature, the white star pattern was enhanced. This paper describes research to develop *P. ×hybrida* lines that differentially express the *Star* mutation under different temperature and light conditions. These lines were used to study regulatory gene expression in white and colored tissue of the *Star* mutation in both virus-free and virus-infected plants.

Materials and Methods

PLANT MATERIAL AND GROWING CONDITIONS. Seeds of *P. ×hybrida* ‘Primetime Burgundy Star’ and *P. ×hybrida* ‘Celebrity

Burgundy Frost’ were obtained from Stokes Seed (Buffalo, NY) and germinated, and the resulting plants grown under standard greenhouse conditions that varied throughout the year.

From the F₂ population of the cross of ‘Primetime Burgundy Star’ by ‘Celebrity Burgundy Frost’, plants with various degrees of *Star* expression (i.e., percentage of white tissue) were selected. Selected plants were then vegetatively propagated through cuttings and grown under controlled growth chamber conditions. Conditions included high, medium, or low temperature (30 °C day/25 °C night, 23 °C day/18 °C night, or 20 °C day/15 °C night), high or low light intensity (215 or 435 μmol·m⁻²·s⁻¹), and long or short days (16 or 10 h) in all possible combinations. Flowers were harvested after 3 to 4 months of growth under defined conditions.

The effects of virus infection on the expression of the *Star* mutation were studied. Standard leaf inoculation procedures (Dijkstra and Jager, 1998) were used to infect plants with a potyvirus: tobacco etch virus (TEV), pepper mottle virus (PepMoV), or potato virus Y (PVY). Flowers were harvested after 3 to 4 months of growth under defined conditions. Comparisons between virus-free and infected plants were made.

Expression data were imported into SigmaStat 3.1 (Systat Software, San Jose, CA) and analysis of variance (ANOVA) performed. Each experiment was replicated five times. In those experiments that showed a statistically significant difference, pairwise multiple comparisons between the means were made using Tukey’s test for multiple comparisons. Data are reported as the average.

COLOR AND pH DETERMINATION. Flower color was determined using Munsell notation. The Munsell Book of Color (Munsell Color Services, New Windsor, NJ) was used instead of the Royal Horticultural Society’s Color Charts because it is not possible to interpolate between color chips using the Royal Horticultural Society’s Color Charts (Griesbach and Austin, 2005).

The extent of the star pattern was measured through digital analysis. Digital photographs were taken of flowers and analyzed with WinCam 2004a (Regent Instruments, Quebec City, Canada). Three different color classes were used to measure the area of red tissue. Similarly, three different color classes were used to measure the area of white tissue. The measurements of each color class were summed to obtained the percentage of white tissue. Measurements from either three or five different flowers per experiment were averaged and compared across treatments.

For the pH determinations, the upper epidermis of single flowers was peeled and the epidermal strips from a single tissue (red or white) were combined. Strips were then ground into a suspension with distilled water, and the pH of the suspension

Fig. 3. (A–D) The effects of photoperiod and light intensity on the expression of the *Star* flower color pattern in *Petunia ×hybrida* ‘Star-Plus’. (A) Ten hours of 215 μmol·m⁻²·s⁻¹ light. (B) Sixteen hours of 215 μmol·m⁻²·s⁻¹ light. (C) Ten hours of 435 μmol·m⁻²·s⁻¹ light. (D) Sixteen hours of 435 μmol·m⁻²·s⁻¹ light.

Table 1. The effect of light exposure on the expression of the *Star* pattern in *Petunia ×hybrida* ‘Star’ and ‘Star-Plus’ at 23 °C day/18 °C night temperature.

Plant	White tissue [mean ± SE (%)]			
	Day length (10 h)		Day length (16 h)	
	Light intensity (215 μmol·m ⁻² ·s ⁻¹)	Light intensity (435 μmol·m ⁻² ·s ⁻¹)	Light intensity (215 μmol·m ⁻² ·s ⁻¹)	Light intensity (435 μmol·m ⁻² ·s ⁻¹)
Star	33 ± 2 a ^z	38 ± 1 a	33 ± 6 a	41 ± 0 a
Star-Plus	36 ± 12 a	64 ± 4 b	55 ± 13 b	80 ± 7 b

^zMeans followed by the same letter are not significantly different according to Tukey’s test (*P* ≤ 0.001).

Data are measured as the percentage of white tissue in petals from a total of three flowers from three different plants.

WAS measured with a microPH meter (Sentron 501; Sentron, Federal Way, WA). The pH measurements were recorded as a mean of five replicates, each replicate representing the pooled tissue collected from a single flower.

ANTHOCYANIN ANALYSIS. Analytical high-performance liquid chromatography (HPLC) analysis was used to identify the pigments in floral tissue as previously described (Griesbach et al., 1991). The anthocyanin extracts were acid hydrolyzed at 100 °C in 3 N HCl for 1 h and the hydrolyzed products characterized by HPLC on a 7.8 × 300-mm column of 5-μm Bondapak C18 (Waters Corp., Milford, MA) using a 20-min linear gradient of 0% to 15% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid and held at 15% (v/v) for an additional 20 min. Flow rate was 1.0 mL·min⁻¹ and detection was by absorption at 540 nm. Anthocyanidins were characterized by coelution with known standards. The amount of anthocyanidin was determined by measuring the peak area using the Maxima software (Waters Corp.). The relative amount of anthocyanin was reported as the mean of the area of absorption per gram fresh weight of three replicates.

Fig. 4. (A–D) The effects of temperature and light on the expression of the *Star* flower color pattern in *Petunia xhybrida* ‘Star-Plus’. (A) Ten hours of 215 μmol·m⁻²·s⁻¹ light at 20/15 °C (day/night). (B) Ten hours of 215 μmol·m⁻²·s⁻¹ light at 30/25 °C. (C) Sixteen hours of 435 μmol·m⁻²·s⁻¹ light at 20/15 °C. (D) Sixteen hours of 435 μmol·m⁻²·s⁻¹ light at 30/25 °C.

Data were imported into SigmaStat 3.1 and ANOVA performed. In significant cases, pairwise multiple comparison between means were made using Tukey’s test for multiple comparisons.

DNA/RNA ANALYSIS. Flavonoid gene expression (WD, MYC, and MYB transcription factors; anthocyanin synthase; and chalcone synthase) was compared in white and red *Star* tissue. RNA was isolated from 100 mg fresh weight of red or white petal tissue of fully opened flowers and flower buds using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Initial reverse transcription–polymerase chain reactions (RT-PCRs) showed residual genomic DNA contamination that necessitated digestion with RNase-free DNase (Ambion Inc, Austin, TX) after RNA isolation. In addition, PCR primers were developed that spanned an intron within each of the genes. In this way, the larger PCR products amplified from genomic DNA could be readily distinguished from the smaller PCR products amplified from mRNA.

The RT-PCR reactions were performed using the Titan One Tube RT-PCR Kit (Roche Diagnostic Corp., Indianapolis, IN) and the GeneAmp 2400 PCR System instrument (Perkin–Elmer Corp., Boston, MA). Different cycle numbers were tested to determine the linear phase of amplification in conventional PCR. Under linear conditions, mRNA concentration could be quantified by agarose gel band intensity. The following temperatures and times resulted in linear amplification of all the genes: 10 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 60 s followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 60 s. Reverse transcription–polymerase chain reaction products were analyzed by agarose gel electrophoresis.

Primer sets for RT-PCR amplification of the chalcone synthase gene (*Chs*) were previously reported (Griesbach and Beck, 2005). Primers for the WD, MYC, and MYB transcription factors (*Wd_{An11}*, *Myb_{An2}*, and *Myc_{An1}*); anthocyanin synthase (*Ans*); and tubulin (*Tub*) genes were based upon sequences of genes from a number of species reported in GenBank. The *Chs* forward primer was 5′-GAACAGCCACACCTACAAAC-3′ and the reverse primer was 5′-AACCTGCTGGTACATC ATG-3′. The *Wd_{An11}* forward primer was 5′-TCAAGAATCAC AACATCTCC-3′ and the reverse primer was 5′-TAGCAAAC AAGAAGCAGC-3′. The *Myc_{An1}* forward primer was 5′-TCAAAAATCAAACCCCTTCAA-3′ and the reverse primer was 5′-CGATTCCAGCAGGAAAAGAG-3′. The *Myb_{An2}* forward primer was 5′-GATCTAGATGTCAGTTGCAGTGA-3′ and the reverse primer was 5′-TAGGATCCGTCACGATT TCAACT-3′. The *Ans* forward primer was 5′-TGGGAGGATT ATTTCTTCCA-3′ and the reverse primer was 5′-GTTGTA CTTGCCGTTGCTTA-3′. The *Tub* forward primer was 5′-TAG CGAAACCAGTGCTGGAAAG-3′ and the reverse primer was 5′-GCTTGAGGGCTCAAAAACAG-3′.

Table 2. The effect of tobacco etch virus infection on the expression of the *Star* pattern in *Petunia xhybrida* ‘Star-Plus’ under different light exposure and temperature.

Plant	White tissue [mean ± SE (%)]			
	Low light (10 h of 215 μmol·m ⁻² ·s ⁻¹)		High light (16 h of 435 μmol·m ⁻² ·s ⁻¹)	
	Low temperature (20/15 °C day/night)	High temperature (30/25 °C day/night)	Low temperature (20/15 °C day/night)	High temperature (30/25 °C day/night)
Healthy	38.68 ± 0.08 a ^z	73.60 ± 0.07 a	76.84 ± 0.10 a	92.56 ± 0.02 a
Infected	38.60 ± 0.11 a	68.95 ± 0.03 b	32.65 ± 0.01 b	93.95 ± 0.01 b

^zMeans followed by the same letter are not significantly different according to Tukey’s test (*P* ≤ 0.001). Data are reported as the percentage of white tissue in petals from a total of five flowers from five different plants.

Fig. 5. (A–D) The effects of temperature and light on the expression of the *Star* flower color pattern in *Petunia xhybrida* ‘Star-Plus’ infected with tobacco etch virus. (A) Ten hours of $215 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 20/15 °C (day/night). (B) Ten hours of $215 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 30/25 °C. (C) Sixteen hours of $435 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 20/15 °C. (D) Sixteen hours of $435 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 30/25 °C.

The RT-PCR products were cloned and sequenced to verify their identity. Reverse transcription–polymerase chain reaction products were cloned into plasmid DNA and transformed into *Escherichia coli* using the pGem-T Easy Vector System II (Promega Corp., Madison, WI). Plasmid DNA was isolated

from the bacteria using the RPM Kit (Bio 101, Carlsbad, CA). Purified plasmid DNA was cycle sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA), pUC/M13 forward and reverse sequencing primers (Promega Corp.), and a GeneAmp 2400 PCR System instrument (Perkin–Elmer Corp.). Samples were analyzed on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems).

Messenger RNA concentration was quantified by agarose gel band intensity using Scion Image (Scion Corp., Frederick, MD). Intensity data were imported into SigmaStat 3.1 and ANOVA performed. Gene expression is reported as the mean $\pm$ SE.

Results and Discussion

It was originally reported that the star pattern was greatly influenced by both light intensity and temperature (Schröder, 1934). However, we found that the star pattern in all the commercially available cultivars was quite stable. In reviewing images of *Star* mutants from the late 1800s, we noticed that many of the plants produced a few flowers with a white border instead of the typical white star pattern. There are a few modern cultivars that have been selected to express this white border phenotype: picotee. Thus, it appears that the *Star* mutation has two phenotypes (picotee or star). The picotee and star phenotypes have a similar biochemical basis (Saito et al., 2006). The difference between these two phenotypes is most likely the result of development timing. If the mutant gene is expressed early during flower development, a star pattern results, whereas a picotee pattern results if expression is late during flower development.

We crossed a *Star* mutant with a picotee phenotype (‘Celebrity Burgundy Frost’) with a *Star* mutant with a star phenotype (‘Primetime Burgundy Star’). In the F_2 population, nearly half the seedlings produced solid burgundy flowers. The remaining seedlings expressed differences in when the *Star* mutation was expressed during flower development, as described earlier. We were not able to perform a genetic analysis on the segregation data. Because the expression of the *Star* mutation depends on environment, it is likely that many of the solid-flower seedlings would have expressed the *Star* mutation under an appropriate environment. The large population size precluded screening all the seedlings under controlled environmental conditions to determine which of the solid-flower seedlings were wild type.

Three F_2 seedlings (‘Star’, ‘Star-Minus’, and ‘Star-Plus’) were clonally propagated and used to evaluate the extent of the star pattern under different environmental conditions (Fig. 1). The color of the pigmented tissue in the star pattern in all three seedlings was 7.5RP 1.8/4.9 and is designated red for convenience. The star pattern in

Fig. 6. (A–F) The change in floral symptoms of tobacco etch virus-infected *Petunia xhybrida* ‘Star-Plus’ as plants age from view A to view F. The youngest flowers (A) are 3 weeks old, whereas the oldest flowers (F) are 6 months old. (C–E) Flowers are $\approx$ 1, 2, and 4 months old respectively. Plants were grown under 16 h of $435 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 20/15 °C.

Table 3. The effect of tobacco etch virus infection on the anthocyanin content and pH of white and red tissue of *Petunia ×hybrida* ‘Star-Plus’ flowers.

Plant	Anthocyanin [mean ± SE (ng·mL ⁻¹ dry wt)]		pH (mean ± SE)	
	Red tissue	White tissue	Red tissue	White tissue
Healthy	7.1 ± 1.8 a ²	0 a	4.82 ± 0.04 a	5.10 ± 0.07 a
Infected	14.3 ± 11.9 a	7.5 ± 1.4 b	5.36 ± 0.05 b	5.58 ± 0.04 b

²Means followed by the same letter are not significantly different according to Tukey’s test ($P \leq 0.001$). Anthocyanin was reported as the combined concentration of malvidin 3-caffeoylrutinoside-5-glucoside and malvidin 3-*p*-coumaroylrutinoside-5-glucoside. Data are reported as the mean of a total of five flowers from five different plants.

Fig. 7. (A, B) High-pressure liquid chromatography profiles of anthocyanidins from healthy (A) and tobacco etch virus-infected (B) *Petunia ×hybrida* ‘Star-Plus’. The peaks at 36 and 40 min are malvidin 3-caffeoylrutinoside-5-glucoside (a) and malvidin 3-*p*-coumaroylrutinoside-5-glucoside (b) respectively.

‘Star’ (Fig. 2) and ‘Star-Minus’ (data not shown) was very stable and varied little under different environments, unlike the pattern in ‘Star-Plus’ (Fig. 3). Under 23 °C day/18 °C night temperature and high light exposure, the star pattern was accentuated in ‘Star-Plus’ (Table 1). At 16 h of 435 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light, flowers had about twice as much white tissue compared with flowers grown under 10 h of 215 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Temperature also influenced the amount of white tissue in ‘Star-Plus’ (Fig. 4, Table 2). A 10-degree difference in temperature had a significantly greater effect at 10 h of 215 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light than at 16 h of 435 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. Under low light, there was a 47% reduction in the amount of white tissue at 20/15 °C (day/night) compared with 30/25 °C, whereas under high light there was only a 17% reduction. In summary, the star pattern in ‘Star-Plus’ was enhanced with more white tissue under high temperature, long days, and high light intensity, whereas the star pattern in ‘Star’ was not influenced by either temperature or light. Our observations of ‘Star-Plus’ grown under different light intensities are similar to those of Schröder (1934) and Harder and Marheineke (1936). They reported that flowers of *P. ×hybrida* ‘Klons 90’ had less white tissue when grown under

low light intensity. There was $\approx 50\%$ less white tissue when ‘Klons 90’ was grown at 25 °C under 7000 lx than at 16,000 lx (Harder and Marheineke, 1936). Mol et al. (1983) observed the same phenomenon with *P. ×hybrida* ‘Red Star’. Interestingly, our observations of ‘Star-Plus’ grown under different temperatures are the opposite of what Schröder (1934) and Harder and Marheineke (1936) observed. They reported that flowers of ‘Klons 90’ had less white tissue when grown under high temperature. There was $\approx 90\%$ less white tissue when ‘Klons 90’ was grown under 12,000 lx at 35 °C than at 15 °C (Harder and Marheineke, 1936). Flower color is well-known to be significantly influenced by the environment (Griesbach, 1992; Maekawa et al., 1980; Shaked-Sachray et al., 2002; Weiss and Halevy, 1991). It is not unreasonable to expect that epistatic gene interactions between ‘Klons 90’ and ‘Star-Plus’ would be different. One possibility is that there is a difference in the temperature response of an anthocyanin gene promoter between the two plants. The ‘Klon 90’ promoter might require a higher temperature for optimal expression. The effects of virus infection on the star pattern in ‘Star’ and ‘Star-Plus’ were studied. Three viruses were selected: TEV, PepMoV, and PVY. In both ‘Star’ and ‘Star-Plus’, very few floral symptoms were observed after PVY infection (data not shown). Although both TEV (Figs. 5 and 6; Table 2) and PepMoV (data not shown) reduced the amount of white tissue after infection, TEV had the greatest effect. Plants of ‘Star-Plus’ had a more severe floral phenotype than plants of ‘Star’ (data not shown). Tobacco etch virus infection increased the anthocyanin concentration in white tissue and had no statistically significant affect on the concentration in red tissue (Table 3). This suggests that TEV does not affect structural gene expression in red wild-type tissue. Unlike in healthy plants, there was considerable variability in the concentration of anthocyanin between flowers from different virus-infected plants. The anthocyanin profile in both healthy and infected flowers was the same: $\approx 40\%$ malvidin 3-caffeoylrutinoside-5-glucoside and 60% malvidin 3-*p*-coumaroylrutinoside-5-glucoside (Fig. 7).

Table 4. The relative expression of flavonoid genes in healthy and virus-infected red and white tissue from *Petunia ×hybrida* ‘Star-Plus’ flowers.

Gene	Relative flavonoid gene expression (mean ± SE)							
	Healthy				Virus infected			
	Flower bud		Open flower		Early infection		Late infection	
	Red tissue	White tissue	Red tissue	White tissue	Red tissue	White tissue	Red tissue	White tissue
<i>Chs</i>	1.08 ± 0.16	0.88 ± 0.04	0.95 ± 0.04	0.74 ± 0.03	1.06 ± 0.33	1.11 ± 0.24	0.58 ± 0.30	0.77 ± 0.60
<i>Ans</i>	1.18 ± 0.12	1.14 ± 0.07	1.01 ± 0.24	1.09 ± 0.22	0.84 ± 0.10	0.96 ± 0.23	0.47 ± 0.12	0.62 ± 0.22
<i>Wd_{An11}</i>	0.62 ± 0.01	0.64 ± 0.11	0.61 ± 0.07	0.60 ± 0.01	0.31 ± 0.04	0.32 ± 0.08	0.38 ± 0.08	0.37 ± 0.04
<i>Myb_{An2}</i>	0.95 ± 0.12	0.91 ± 0.16	0.73 ± 0.05	0.60 ± 0.30	0.47 ± 0.13	0.45 ± 0.05	0.59 ± 0.02	0.43 ± 0.02
<i>Myc_{An1}</i>	0.92 ± 0.15	0.95 ± 0.16	0.89 ± 0.04	0.85 ± 0.07	0.56 ± 0.16	0.65 ± 0.06	0.67 ± 0.15	0.62 ± 0.06

Expression was measured 3 months (early) and 6 months (late) after inoculation. Expression was measured as the ratio of agarose gel band intensity of the reverse transcription–polymerase chain reaction product standardized to tubulin. Data are reported as the mean of three different RNA isolations.

Fig. 8. Reverse transcription–polymerase chain reaction products in healthy red (R) or white (W) floral tissue of *Petunia ×hybrida* ‘Star-Plus’. *Ans*, anthocyanin synthase; *Chs*, chalcone synthase; *Tub*, tubulin; *Myc*, MYC_{An1} transcription factor; *Myb*, MYB_{An2} transcription factor; *Wd*, WD_{An11} transcription factor.

These results are similar to those reported by Teycheney and Tepfer (2001). They observed that TEV infection in *P. ×hybrida* ‘Starmania’ produced the most severe floral phenotype compared with PVY or cucumber mosaic virus (CMV). In ‘Red Star’, TEV infection caused stunting that prevented flowering, whereas in *P. ×hybrida* ‘Soie Violet Blanc Etoile’ and ‘Bravo Rouge Etoile Blanc’ floral symptoms were less severe than in ‘Starmania’.

In our studies, the environment greatly influenced the degree of TEV-induced floral symptoms. Under low light or high temperature, there was no statistically significant difference in the amount of white tissue between healthy and TEV-infected ‘Star-Plus’ plants (Figs. 4 and 5; Table 2). However, under 16 h of 435 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light at 20/15 °C, there was a 57% reduction in the amount of white tissue in infected versus healthy plants (Figs. 4C and 5C). Besides decreasing the amount of white tissue, TEV infection shifted the flower color toward the blue. The difference in color was the result of an increase in pH (Table 3) (Griesbach, 2005).

The expression of *Chs*, *Ans*, *Wd_{An11}*, *Myb_{An2}*, and *Myc_{An1}* in virus-free and virus-infected white and red star tissue under different light and temperature conditions was determined. In both red and white tissue, there was no significant difference in the level of expression of *Chs*, *Ans*, *Wd_{An11}*, *Myb_{An2}*, and *Myc_{An1}* in flower buds 2 to 3 d before opening compared with flowers opened for 1 d (Table 4, Fig. 8). Because there was no difference, further studies were done with opened flowers.

The only gene displaying a statistically significant differential expression between red and white tissue was *Chs* (Table 4). The other anthocyanin structural genes had the same level of expression in two tissues. In white tissue, the *Chs* transcript concentration was $\approx 75\%$ of that found in red tissue. Based upon the literature, we expected *Chs* to be differentially expressed. In ‘Red Star’, white tissue had 1.5% of CHS enzyme activity found in red tissue (Mol et al., 1983). In ‘Carnival Red Star’, the *Chs* transcript concentration in white tissue was $\approx 1\%$ of that found in red tissue (Koseki et al., 2005). The decrease in CHS in the white tissue is the result of miRNA silencing (Koseki et al., 2005).

Because virus infection produced the greatest floral symptoms under 16 h of 435 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ light at 20/15 °C, gene expression studies were carried out under these conditions. There was a significant temporal difference in the effect TEV had on anthocyanin gene expression (Table 4; Fig. 6). Three months after inoculation, there was an $\approx 25\%$ increase in *Chs* transcript levels in white tissue and little or no difference in red tissue. These results are in agreement with the literature. When ‘Red Star’ and ‘Starmania’ plants were infected with either PVY, CMV, or TEV, the increased pigmentation of white tissue was the result of an increase in *Chs* mRNA levels (Koseki et al., 2005; Teycheney and Tepfer, 2001).

Viral infection is known to modify host gene expression by suppressing RNA silencing. Similar to the *Star* mutation, *Glycine max* L. seeds are yellow because of the presence of *Chs* miRNAs (Senda et al., 2004). When *G. max* was infected with either CMV or soybean mosaic potyvirus, there was an increase in *Chs* mRNA that restored anthocyanin seed color. The increase in mRNA was not the result of a decrease in miRNA. In fact, there was a significant increase in miRNA levels. Senda and others proposed that there is a threshold level of CHS required for pigmentation. Virus-encoded suppressor activity increases the level of *Chs* mRNA that results in reaching the CHS threshold level for pigmentation. The increase in *Chs* mRNA then acts as a template for the generation of higher levels of miRNA.

Fig. 9. (A, B) Model of anthocyanin gene expression at the transcriptional (A) and translational (B) levels. Proteins shaded in gray are virus encoded. MYC, MYB, and WD40 are transcription factors. E-box and MYB recognition element (MRE) are promoter binding sites. eIF and eIFiso are elongation factors for protein translation. PABP, messenger RNA poly-A binding protein; HC-Pro, potyvirus helper component protein; VPg, potyvirus genome-linked viral protein.

In potyviruses, the helper component (HC-Pro) suppresses silencing by inhibiting miRNA methylation (Yu et al., 2006). Unmethylated miRNAs are uridylylated at their 3'-end, resulting in a loss of function (Li et al., 2005). It is also possible that HC-Pro could suppress silencing by binding to either miRNAs or siRNAs. Two independent RNA binding domains are found within its central region (Urcuqui-Inchima et al., 2001). In tomato bushy stunt tobravirus, the p19 protein suppresses silencing by binding to siRNAs (Baulcombe and Molnar, 2004).

As TEV-infected plants became older, the star pattern in newly formed flowers varied (Fig. 6). Several weeks after infection, newly opened flowers have a few flecks of color within the white tissue. As the plants age, the floral pattern in newly opened flowers becomes red veined. Six-month-old plants produce flowers with reduced pigmentation in both the red and white sectors (Fig. 6F). This phenomenon corresponds to the decrease in structural gene transcript levels that are seen in flowers from progressively older plants (Table 4). Three months after TEV inoculation (Table 4), we found that the transcript levels of the structural genes (*Chs* and *Ans*) in 'Star-Plus' red tissue decreased less than the transcript levels of the regulatory genes (*Myc_{An1}*, *Myb_{An2}*, and *Wd_{An11}*). Six months after inoculation, the transcript levels of the structural genes in red tissue decreased to 30% to 40% of their level found after 3 months, whereas the transcript levels of the regulatory genes (*Myc_{An1}*, *Myb_{An2}*, and *Wd_{An11}*) slightly increased.

The initial decrease in regulatory gene transcription levels upon virus infection could explain why the red tissue in TEV-infected flowers was bluer than in healthy flowers (Table 3). Previously, we have shown that *Myc_{An1}* regulates the expression of two genes involved in vacuolar acidification (Griesbach, 1998). The bHLH domain in *Myc_{An1}* is required for the regulation of pH, but not for dihydroflavonol reductase (DFR) expression (Spelt et al., 2002). When mutant *Myc_{An1}* alleles

lacking the bHLH domain were overexpressed, near-normal levels of anthocyanin biosynthesis occur without vacuolar acidification or normal seed coat development (Spelt et al., 2002). A decrease in *Myc^{An1}* should result in bluer colored flowers.

Another means by which TEV infection might influence anthocyanin expression is by modifying mRNA translation through interaction of the genome-linked viral protein, *V_{PG}*, with the eukaryotic initiation factors eIF4E or eIF(iso)4E (Leonard et al., 2000; Schaad et al., 2000; Yoshii et al., 2004). In uninfected cells (description compiled from Brown- ing, 1996; Gallie and Browning, 2001; Johnston et al., 1998; Ruffel et al., 2004), eIF4E binds to another initiation factor, eIF4G, to form the eIF4F complex; then the eIF4F complex binds to the cap structure of mRNAs (m⁷GpppN-). Bound eIF4G acts as a scaffold for the binding of 1) eIF3, which binds the 40S ribosomal subunit; 2) eIF4A helicase, which unwinds the 5' untranslated region of the mRNA; and 3) the poly-A binding protein (PABP), which stabilizes the binding of eIF4F to the cap and its binding to the poly(A) tail. Poly-A binding protein, eIF4G, and eIF4E form a bridge between the 5' and 3' ends of the mRNA, allowing circularization and promoting translation. A second cap-binding complex, eIF(iso)4F, is composed of the isoforms eIF(iso)4E and eIF(iso)4G. The eIF4F and eIF(iso)4F complexes facilitate translation of distinct populations of mRNAs.

Potyvirus selectively use either eIF4F or eIF(iso)4F, depending on the host-virus combination. In TEV, *V_{PG-TEV}* binds to eIF(iso)4E, preventing it from binding to eIF(iso)4G to form the eIF(iso)4F complex, whereas in clover yellow vein virus (CIYVV), *V_{PG-CIYVV}* binds to eIF4E. In addition, the *V_{PG}* precursor peptide *V_{PG-Pro}* can bind to PABP (Leonard et al., 2004). The 2a proteinase (2A^{pro}) of mammalian rhinoviruses can cleave the eIF4F complex, drastically reducing translation of capped mRNAs (Haghighat et al., 1996). It is possible that one of the potyviral proteinases could have the same function.

The changes in pigmentation pattern after TEV-infected plants age could be due in part to *V_{PG-TEV}* interactions with the elongation factor complexes. As eIF4F and eIF(iso)4F interact with distinct mRNA populations, it is possible that eIF(iso)4F is required for regulatory gene translation and eIF4F for structural gene translation. Because TEV uses the iso forms, host genes that use the iso forms would have a reduced rate of translation as a result of TEV sequestration of eIF(iso)4e. As virus-infected plants become older, rates of virus replication typically decrease (Wang and Maule, 1995); eIF(iso)4E and PABP would then become more available for host mRNA translation, resulting in higher levels of MYC and MYB.

In the earlier stages of infection, *Chs* translation is increased, presumably as a result of HC-Pro interference with miRNAs targeting *Chs* mRNA. At the same time, *V_{PG}* titrates out eIF(iso)4E, reducing translation of *WD*, *MYC*, and *MYB* mRNAs. The color flecking of the white sector of the flowers at the earlier stages of infection is "veinally" associated, and is presumed to result from the mosaic distribution pattern of viral replication spreading from the veins. As the infection progresses, the suppression of *Chs* silencing becomes more complete, resulting in fully red flowers. However, at later stages of infection, TEV replication slows and less *V_{PG}* is formed, allowing translation of more *WD*, *MYC*, and *MYB*. Concurrently, less HC-Pro is available to suppress miRNA activity, and silencing of *Chs* is gradually restored, leading to flowers with white flecking in the red, as well as the white,

sectors of the flowers. The relative concentrations of *CHS*, *WD*, *MYC*, and *MYB*, and complex interactions between these multiple factors rather than the absolute concentration of any single factor, appear to regulate pigmentation. This could explain why it takes ≈4 months after infection for *Star* flowers to become completely solid red. Initially after TEV infection, *MYC* and *MYB* levels decrease, resulting in a reduction in the rate of structural gene transcription. As plants age and viral replication decreases, *MYC* and *MYB* levels increase, leading to an increase in structural gene transcription, even though the transcript level of the structural genes decreases.

In summary, TEV may be acting at several points in the anthocyanin biosynthetic pathway within *Star* petunia. First, TEV, through HC-Pro, may be interfering with the host miRNA pathway (Fig. 9A). As discussed earlier, miRNAs could be involved in regulating *Chs*, *Myc*, *Myb* and *WD40* mRNAs. Second, TEV, through *V_{PG}*, may be interfering with eIF(iso)4E (Fig. 9B). As discussed earlier, eIF(iso)4E could be involved in *Myc*, *Myb*, and *WD40* translation.

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