Tobacco streak virus (strain dahlia) suppresses post-transcriptional gene silencing of *flavone synthase II* in black dahlia cultivars and causes a drastic flower color change

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Main Conclusion

Tobacco streak virus suppressed post-transcriptional gene silencing and caused a flower color change in black dahlias, which supported the role of cyanidin-based anthocyanins for black flower appearance.

Author contribution

MH and SO conceived the research. MD and SO designed the research. AD conducted the research. FT contributed pigment analysis. AD and SO wrote the manuscript. All authors read and approved the manuscript.

Abstract

Black flower color of dahlia (*Dahlia variabilis)* has been attributed, in part, to the high accumulation of cyanidin-based anthocyanins that occurs when flavone synthesis is reduced because of post-transcriptional silencing (PTGS) of *flavone synthase II* (*DvFNS*). There are also purple-flowering plants that have emerged from a black cultivar 'Kokucho'. We report that the purple color is not caused by a mutation, as previously thought, but by infection with tobacco streak virus (TSV_{dahlia}), which suppresses the PTGS of *DvFNS*. When TSV_{dahlia} was eliminated from the purple-flowering 'Kokucho' by leaf-primordia-free shoot apical meristem culture, the resulting flowers were black. TSV_{dahlia}-infected purple flowers had lower numbers of siRNAs to *DvFNS* than black flowers, suggesting that TSV_{dahlia} has a silencing suppressor. The graft inoculation of other black cultivars with TSV_{dahlia} altered their flower color drastically except for 'Fidalgo Blacky', a very deep black cultivar with the highest amount of cyanidin-based anthocyanins. The flowers of all six TSV_{dahlia}-infected cultivars accumulated increased amounts of flavones and reduced amounts of cyanidin-based anthocyanins. 'Fidalgo Blacky' remained black despite the change in pigment accumulation, and the amounts of cyanidin-based anthocyanins in its TSV_{dahlia}-infected plants were still higher than those of other cultivars. We propose that black flower color in dahlia is controlled by two different mechanisms that increase the amount of cyanidin-based anthocyanins: *DvFNS* PTGS-dependent and independent mechanisms. If both mechanisms occur simultaneously, the flower color will be blacker than if only a single mechanism is active.

Key words

Cyanidin, *Dahlia variabilis*, FNS II, PTGS, Silencing suppressor

Abbreviations

ANS	Anthocyanidin synthase
AV-2	Asparagus virus 2
bHLH	Basic helix-loop-helix
CHI	Chalcone isomerase
CHS	Chalcone synthase
CMV	Cucumber mosaic virus
CP	Coat protein
CSVd	Chrysanthemum stunt viroid
DFR	Dihydroflavonol 4-reductase
DMV	Dahlia mosaic virus
F3H	Flavanone 3-hydroxylase
F3′H	Flavonoid 3′-hydroxylase
FNS II	Flavone synthase II
HPLC	High performance liquid chromatography
INSV	Impatiens necrotic spot virus
LP-free SAM	Leaf-primordia-free shoot apical meristem
MP	Movement protein
PTGS	Post-transcriptional gene silencing
PSTVd	Potato spindle tuber viroid
RT-PCR	Reverse transcription-PCR
siRNA	Small interfering RNA
TSV	Tobacco streak virus
TSWV	Tomato spotted wilt virus

Introduction

Flower color is one of the most important traits for ornamental plants. There are various flower colors (e.g., red, purple, pink, orange, yellow, blue, and white) as well as variegated and bicolor flowers. Among flower colors, black is considered to be attractive because it is rare and present in few genera. Black flower color is found in wild pansy (*Viola tricolor*) (Clausen 1930) and wild *Lisianthius nigrescens* (Markham et al. 2004). Recently, some black flower cultivars of petunia (*Petunia hybrida*), hollyhock (*Althaea rosea*), and dahlia (*Dahlia variabilis*) have begun to be produced commercially. While black flowers are increasingly popular, the mechanism producing this color is unclear.

To elucidate this mechanism, we have studied dahlia that has a wide range of flower colors and comparatively large numbers of the black cultivars. Moreover, the pigments, enzymes and genetic factors related to flower colors have been largely elucidated in this species. The pigments accumulating in dahlia petals belong to three major flavonoid compounds: anthocyanins (pelargonidin-based and cyanidin-based), flavones (apigenin-based and luteolin-based), and chalcones (isoliquiritigenin-based and butein-based) (Nordström and Swain 1953; 1956; 1958; Harborne et al. 1990). These contribute to red ~ purple, ivory, and yellow colors, respectively. Generally, anthocyanidins, the aglycone of anthocyanins are synthesized by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) (Grotewold 2006; Tanaka et al. 2008). Anthocyanins result from the addition of sugar moieties. Flavones are synthesized by flavone synthase (FNS I and FNS II) from flavanones, the products of CHI (Martens and Mithöfer 2005). Chalcones are synthesized by CHS, chalcone reductase (CHR) (Davies et al. 1998), and chalcone 3-hydroxylase (CH3H) (Schlangen et al. 2010a). In the pathway leading to anthocyanins and flavones, cyanidin and luteolin are synthesized via flavonoid 3′-hydroxylase (F3′H) activity as counterparts to pelargonidin and apigenin, respectively (Ayabe and Akashi 2006; Tanaka and Brugliera 2013). In dahlia, these enzymes have been studied (Fischer et al. 1988; Wimmer et al. 1998; Halbwirth et al. 2008; Thill et al. 2012) and the genes encoding the enzymes have been isolated (Schlangen et al. 2010b; Ohno et al. 2011a; Ohno et al. 2011b; Deguchi et al. 2013). In addition, a bHLH transcription factor, DvIVS, regulates anthocyanin levels by regulating the expression of *DvCHS1*, *DvF3H*, *DvDFR*, and *DvANS* (Ohno et al. 2011a; Ohno et al. 2013).

In a previous study (Deguchi et al. 2013), the black dahlia cultivars showed lower petal lightness (*L**) and chroma (*C**) values than the other color cultivars. This appeared to be a phenotypic indicator for the black appearance. Black dahlias indeed accumulate high amounts of total anthocyanins (Thill et al. 2012; Deguchi et al. 2013), however, total anthocyanins are lower in black cultivars than in some purple cultivars, suggesting that the black appearance cannot be explained solely by total anthocyanin levels. Black dahlias exhibit two other distinct features of pigment accumulation. One is high accumulation of cyanidin-based anthocyanins. In cultivars with similar levels of total anthocyanins, the *L** and *C** values were inversely proportional to the cyanidin-based anthocyanin levels, suggesting that the cyanidin-based anthocyanins contribute more strongly to the black appearance than pelargonidin-based ones (Deguchi et al. 2013). Therefore, we considered that the high accumulation of cyanidin-based anthocyanins was also related to the black appearance in addition to the higher accumulation of total anthocyanins. The other feature of black dahlias is low accumulation of flavones. Interestingly, a comparison between the black cultivar ‘Kokucho’ and its purple-flowering plant showed that the suppression of flavone

synthesis is caused by post-transcriptional gene silencing (PTGS) of *FNS II* (*DvFNS*), leading to the high accumulation of both total anthocyanins and cyanidin-based anthocyanins. This may be due to the abolishment of competition between anthocyanidin and flavone synthesis.

The purple-flowering 'Kokucho', which had been previously used as a putative mutant (Deguchi et al. 2013) sometimes produces mosaic-colored flowers (black parts in purple petals) (Supplementary Fig. S1), raising the question of whether it is truly a mutant. Some reports have shown flower color changes to be induced by plant virus infection. In tulip (*Tulipa gesneriana*) and petunia, the alteration of anthocyanin pigmentation patterns are attributed to infection with certain plant viruses (Dekker et al. 1993; Lesnaw and Ghabrial 2000; Teycheney and Tepfer 2001). Thus, we assumed that purple-flowering 'Kokucho' is infected by some virus. In the present study, we examined virus infection and our data indicated that the flower color change from black to purple in 'Kokucho' was induced by a tobacco streak virus (TSV) infection. The inoculation of other black cultivars with the virus provided us further insights into black flower coloring.

Materials and Methods

Plant materials

Eighteen tuberous roots of the black dahlia cultivar 'Kokucho' were grown in the experimental field at Kyoto University (Kyoto, Japan). Sets of five plants of nearly the same height, each with either normal black flowers (B1–B5) or color changed purple flowers (P1–P5), were selected for the subsequent experiments. Along with 'Kokucho', three black cultivars ('Fidalgo Blacky', 'Ms. Noir', and 'Black Cat'), two black–white bicolor cultivars ('Kazusa-shiranami' and 'Kageboushi'), and one pink cultivar ('Jyunn-ai') were also grown and the petals were collected for analysis.

Detection of viruses and viroids

The detection of viruses and viroids was performed with reverse transcription-PCR (RT-PCR). The total RNA of leaves was extracted using Sepasol- RNA I Super G (Nacalai Tesque, Kyoto, Japan) followed by purification using High-Salt Solution for Precipitation (Takara Bio Inc., Ohtsu, Japan). The viruses and viroids tested were as follows; cucumber mosaic virus (CMV), dahlia mosaic virus (DMV), impatiens necrotic spot virus (INSV), TSV, tomato spotted wilt virus (TSWV), chrysanthemum stunt viroid (CSVd), and potato spindle tuber viroid (PSTVd), which have been reported to be infectable to dahlias. The primer set used for the detection of each virus or viroid is given in Supplementary Table S1. Total RNA was reverse-transcribed with ReverTra Ace (Toyobo Co. Ltd., Osaka, Japan) using a specific reverse primer for each virus or viroid (Supplementary Table S1) or with ReverTra Ace qPCR RT Master Mix (Toyobo). The plasmids containing the full or partial sequence for coat protein (CP) of CMV, DMV, TSV or TSWV and the full sequence of CSVd were used in RT-PCR as positive controls, and water was used as a negative control.

Elimination of viruses and viroids by leaf-primordia-free shoot apical meristem culture

‘Kokucho’ purple plants (P3, P4, and P5) were used for leaf-primordia-free shoot apical meristem (LP-free SAM) culture (Hosokawa et al. 2004) to eliminate viruses and viroids. Regenerated shoots from these three plants were transplanted onto modified MS medium (Murashige and Skoog 1962), and five rooted plants were transplanted in the experimental field. The flowers of these five lines were used for the color and pigment accumulation analyses.

Sequence analysis of TSV RNA

The reverse primers for RNA2 and RNA3 of TSV were designed around the 3′ ends with reference to the existing TSV sequences in the BLAST database. The primer sets used to amplify the full-length 2b protein gene on RNA2 and the movement protein (MP) and CP genes on RNA3 were also designed (Supplementary Table S2). Total RNA extracted from the leaves of TSV-infected ‘Kokucho’ was subjected to reverse transcription using RNA2 and RNA3 full reverse primers. Then, the 2b protein, CP, and MP genes were amplified using RT-PCR. The amplified PCR products were cloned into pTAC-1 vector (BioDynamics Laboratory Inc., Tokyo, Japan) according to the manufacturer’s instructions. Eight plasmids for each gene were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3100 Genetic Analyzer (Applied Biosystems). Because the TSV sequence isolated from ‘Kokucho’ was not identical to known TSV sequences, we refer to it here as TSV_{dahlia}.

Measurements of plant growth

‘Kokucho’ line 4, which remained infected by TSV_{dahlia} even after LP-free SAM culture, and line 5, from which TSV_{dahlia} had been eliminated, were used to compare plant growth characteristics. Nine plants propagated from each line were grown in 24 cm in diameter pots in a glasshouse from July to December 2014. The height, node number, inflorescence diameter, leaf color, and tuber weight of these plants were measured.

In vitro graft inoculation of virus

In vitro grown TSV_{dahlia}-infected ‘Kokucho’ line 4 was used as a root stock and black, black–white bicolor, and pink cultivars were used as a scion. The shoots of these cultivars not infected with TSV_{dahlia} were *in vitro* grafted onto the ‘Kokucho’ line 4 root stock. About one month later, TSV_{dahlia} infection was confirmed in petals using RT-PCR and the rooted shoots of TSV_{dahlia}-infected plants were transplanted in the field. The plants without grafts (originals) and those grafted onto TSV_{dahlia}-uninfected ‘Kokucho’ line 5 (graft controls) were also grown in the same experimental field. Flowers were used for the analyses of color, pigment accumulation, and transcript abundance.

Color analysis

To evaluate flower color, the color components of the CIE $L^*a^*b^*$ coordinate system, namely lightness (L^*) and chroma [C^*, calculated as $C^* = (a^{*2} + b^{*2})^{1/2}$], were measured using a hand spectrophotometer (NR-3000, Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). Three areas of the adaxial petal surface were subjected to color measurement. In the case of bicolor cultivars, colored parts (petal bases) were measured. Scores from three expanded, completely colored petals taken from three different flowers were averaged.

Quantification of pigments

Pigments were quantified with high performance liquid chromatography (HPLC). Fresh petals (100 mg) used in the color analysis were homogenized with a mortar and a pestle under liquid nitrogen. Added 1 mL of extraction solution (5% hydrochloric acid in 50% methanol), the mixture was stored overnight at 4°C in darkness. The mixture was then centrifuged at 4°C at 15,000 rpm for 15 min, and the supernatant was collected and diluted 50 times with the same solvent. For hydrolysis, 1.2 mL of the diluted solution was boiled at 95°C for 2 h and 20 µl of the hydrolyzed solution was injected into the HPLC apparatus. The analysis was performed using HPLC system (Hitachi L-7100, L-7200, L-7420, L-7500: Hitachi Systems, Ltd., Tokyo, Japan) with a C18 column (Nihon Waters K. K., Tokyo, Japan), maintained at 40°C. The detection wavelength was 350 nm for flavones and 520 nm for anthocyanidins. Eluent preparation and HPLC analysis proceeded according to Ohno et al. (2011b). For the standard curves, 1 mg of pelargonidin chloride (Sigma-Aldrich Corporation, St. Louis, USA), cyanidin chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), apigenin (Wako), and luteolin (LKT Laboratories Inc., St. Paul, MN, USA) were dissolved in 1 mL of 5% hydrochloric acid in 50% methanol, and one-half dilution series were made. Twenty micro liters of each dilution was injected, and the relationship between peak areas and pigment concentrations was calculated. Calculations for pelargonidin and cyanidin were converted from those for each chloride. The assay was performed with three petals from three different flowers.

Real-time RT-PCR

Quantitation of *DvFNS*, *DvF3'H*, *DvIVS*, *DvCHS1*, *DvCHS2*, *DvCHI*, *DvF3H*, *DvDFR*, and *DvANS* transcripts was performed by real-time RT-PCR. Total RNA was reverse transcribed with ReverTra Ace (Toyobo) using oligo dT primer, and real-time RT-PCR was performed with SYBR Premix EX *TaqII* (Takara) using the LightCycler 480 system (Roche Diagnostics K.K., Tokyo, Japan). The real-time RT-PCR was performed as follows: 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec and 60°C for 30 sec. Single-target product amplification was checked using a melting curve. To measure the highest levels of transcript abundance, half-colored, unexpanded petals were used for analysis of *DvFNS* and *DvF3'H* and completely colored, unexpanded petals were used for other genes. Completely colored, unexpanded petals of bicolor cultivars were separated into bases and tips. Three or two biological replications were performed and *DvActin* was used as an internal standard. Primers for *DvActin* and *DvF'3H* are given in Supplementary Table S3; primers for other genes are described in Ohno et al. (2011b) and Deguchi et al. (2013).

Detection of siRNAs

The detection of small interfering RNA (siRNA) was performed according to Ohno et al. (2011b). Total RNA was extracted from TSV_{dahlia}-uninfected and TSV_{dahlia}-infected petals of black, black–white bicolor, and pink cultivars. Total RNA of the red–white bicolor cultivar ‘Yuino’ wherein the PTGS of *DvCHS* occurs (Ohno et al. 2011b), was used as a positive control for the detection of *DvCHS* siRNA. A digoxigenin-labeled RNA probe was synthesized using T7 RNA polymerase (Roche, Basel, Switzerland) by *in vitro* transcription of a 3′-digested pTAC-1 vector (BioDynamics Laboratory) carrying the exon 2 region of *DvFNS* and the full length of *DvCHS2* in the antisense orientation.

Mapping of small RNAs

Small RNAs were extracted from 200 mg of half-colored fresh petals of TSV_{dahlia}-infected 'Kokucho' (line 4) using a MirVana miRNA Isolation Kit (Applied Biosystems) and sequenced with the Illumina HiSeq System (Illumina Inc., San Diego, CA, USA) in a single-end read. Small RNAs (18–32 nt) were mapped onto the *DvFNS* genomic sequence of 'Kokucho' (AB769841) as a reference sequence, with no mismatches allowed.

Result

Virus detection and elimination from purple-flowering 'Kokucho'

Purple 'Kokucho' produces entirely purple flowers from autumn to winter, but black sectors are sometimes observed in summer (Supplementary Fig. S1). The presence of the mosaic flower color prompted us to investigate whether virus infection could explain the color change. Five of 18 'Kokucho' plants grown from tuberous roots produced color-changed purple flowers, while the other 13 plants produced normal black flowers. These five purple-flowering plants (P1–P5) and five black-flowering plants (B1–B5) of almost the same plant size were used for virus and viroid detection. RT-PCR analysis revealed that TSV_{dahlia} was present in all purple flowered plants, but not in black flowered plants. (Fig. 1). CMV and DMV were also detected in some purple and black plants but the infection did not correlate with flower color. TSWV, CMV, and CSVd were not detected in any plants. Moreover, the bands of expected lengths for INSV or PSTVd were not detected (data not shown). These findings suggest that infection with TSV_{dahlia} correlated with flower color in 'Kokucho', but CMV, DMV, INSV, TSWV, CSVd, and PTSVd were irrelevant to its flower color change.

To eliminate viruses from purple 'Kokucho', LP-free SAM cultures (Hosokawa et al. 2004) were prepared. This method is superior to ordinary apical meristem culture for eliminating viruses. Five regenerated lines (1–5) were obtained and grown in the experimental field. One of the lines (line 4) remained to produce purple flowers like its mother plant (P3). However, the other lines (1–3, 5) completely recovered the black flower color (Table 1). As a result of RT-PCR analysis, TSV_{dahlia} was detected by RT-PCR only in line 4, whereas CMV was detected in all lines (Fig. 2). These results indicate that elimination of TSV_{dahlia} succeeded in four lines (1–3, 5) but failed in line 4 and that purple flower color corresponded to TSV_{dahlia} infection. The detection of CMV in all lines may be due to the failure of elimination or re-infection during growth because two mother plants (P3 and P4) were infected and a mother plant (P5) was not infected. The elimination of TSV_{dahlia} completely correlated with the recovery of black flower color, suggesting that purple 'Kokucho' is not a mutant and that purple flower color is caused by TSV_{dahlia} infection.

The comparison of plant growth between TSV_{dahlia}-infected line 4 and TSV_{dahlia}-uninfected line 5, which were regenerated from the same mother plant (P3), showed no significant differences ($P > 0.05$) in plant height, node number, inflorescence diameter, tuberous root weight, and leaf color (Supplementary Fig. S2, Table S4). Therefore, infection with the virus did not affect aspects of plant growth, aside from flower color.

The sequence of TSV isolated from 'Kokucho'

TSV, belonging to the genus *Ilarvirus* and the family Bromoviridae, can infect a wide variety of plant species, including dahlia. The virus isolated from purple 'Kokucho' shares high homology with registered TSV, but the absence of identical sequences in the database implied that TSV_{dahlia} is a new strain of TSV. For TSV_{dahlia} isolated from 'Kokucho', the full-length sequences of genes encoding 2b protein (LC030106), MP (LC030107), and CP (LC030108) were 618, 873, and 717 bp long and encoded 205, 290, and 238 putative amino acid residues, respectively. The sequences of genes for 2b, MP, and CP share 89%, 88%, and 92% identity, respectively, with reported TSV sequences [strain WC: U75538 (Scott et al. 1998), X00435 (Cornelissen et al. 1984)]. They share 91%, 95%, and 96% identity, respectively, with those isolated from crownbeard (*Verbesina encelioides*; JX463338, JX463339) (Sharman and Thomas 2013); 89%, 92%, and 90% identity, respectively, with soybean (*Glycine max*; FJ403376, FJ403377); and 87%, 91%, and 90% identity, respectively, with *Cucurbita pepo* (KM504247, KM504248) (Padmanabhan et al. 2014). The sequence of CP also shares 98% identity with that isolated from *Xanthium occidentale* (JX463349) (Sharman and Thomas 2013) and fava bean (*Vicia faba*, AM933669).

Mapping of small RNAs on the DvFNS *genomic sequence in* TSV_{dahlia}*-infected purple 'Kokucho'*

Purple-flowering 'Kokucho' line 4, which remained infected by TSV_{dahlia} accumulated flavones, while color-recovered black-flowering line 5 accumulated low amounts of flavones (Fig. 3a). *DvFNS* transcript abundance was higher in petals of line 4 than in petals of line 5 (Fig. 3b), as shown in Deguchi et al. (2013) in which purple-flowering 'Kokucho' was described as a mutant.

In original black 'Kokucho', siRNA of *DvFNS* had been detected and abundant small RNAs were mapped onto the *DvFNS* gene, suggesting that *DvFNS* is post-transcriptionally silenced (Deguchi et al. 2013). Therefore, the obvious increase in *DvFNS* transcript abundance in purple 'Kokucho' may result from PTGS suppression by virus infection. To confirm this, we mapped small RNAs extracted from the petals of TSV_{dahlia}-infected purple 'Kokucho' (line 4) onto the *DvFNS* genomic sequence. Of the 24,846,705 reads, which were 18–32 nt long, 2,126 reads were successfully mapped (Fig. 4). The mapped small RNAs of 21 nt accounted for only 1,779 reads and were mostly mapped onto exon 2. The number of mapped reads in purple 'Kokucho' was much fewer than in black 'Kokucho', wherein 11,574 of 14,895,667 reads were mapped onto *DvFNS* genome (Deguchi et al. 2013). Moreover, the 21 nt antisense small RNA, which had the highest number with 4,192 reads in black 'Kokucho', was not mapped at all in purple 'Kokucho'. These results indicated that the PTGS of *DvFNS* was suppressed in color-changed purple 'Kokucho' by TSV_{dahlia} infection.

Changes in flower color, pigment accumulation, and transcript abundance in black and black–white bicolor cultivars with TSV_{dahlia} *inoculation*

To verify whether TSV_{dahlia} changes flower color in other cultivars, we performed graft inoculations. Four black cultivars ('Fidalgo Blacky', 'Ms. Noir', 'Black Cat', and TSV_{dahlia}-uninfected 'Kokucho'), two black–white bicolor cultivars ('Kazusa-shiranami' and 'Kageboushi'), and one pink cultivar ('Jyunn-ai') were *in vitro* grafted onto purple-flowering 'Kokucho' (line 4) because the mechanical inoculation hardly succeeded in infecting these cultivars with TSV_{dahlia}. The transmission and infection with TSV_{dahlia} was confirmed using RT-PCR (Supplementary Fig. S3). TSV_{dahlia}-infected plants of each black and bicolor cultivar produced flowers differing in color from the

originals. The altered color was purple in most cultivars, but it remained black only in ‘Fidalgo Blacky’, despite a slight bluish color change (Fig. 5). All TSV_{dahlia}-infected plants of the black and bicolor cultivars showed higher petal lightness (*L**) values than plants of the original cultivars, and they also showed higher petal chroma (*C**) values except for ‘Kageboushi’, whereas graft control showed the original color (Fig. 6, Supplementary Table S5). ‘Fidalgo Blacky’, which has the blackest appearance, with low *L** and the lowest *C** values, showed the smallest increase in *L** score as a result of the virus infection.

These TSV_{dahlia}-infected plants accumulated flavones in their color-changed petals and accumulated lower amounts of anthocyanins (anthocyanidins) than their TSV_{dahlia}-uninfected counterparts (originals and graft controls) (Fig. 7). Moreover, in these cultivars, the reducing ratio of cyanidin was higher than that of pelargonidin (Fig. 7). In contrast, the pink cultivar ‘Jyunn-ai’, which originally accumulated flavones, exhibited no changes in flower color or pigment accumulation (Fig. 5, 6, 7; Supplementary Table S5). ‘Kazusa-shiranami’ original cultivar was reported to accumulate flavone in its petals (Ohno et al. 2011b), but it actually accumulated low flavones similar to the other black cultivars in this study.

According to the results of real-time RT-PCR, *DvFNS* transcript abundance was markedly increased in TSV_{dahlia}-infected plants of black and bicolor cultivars (Fig. 8). The transcript abundance in TSV_{dahlia}-infected plants were about 7–100 times higher than those of TSV_{dahlia}-uninfected plants. In contrast, the genes involved in anthocyanin biosynthesis, *DvIVS*, *DvCHS1*, *DvCHS2*, *DvCHI*, *DvF3H*, *DvDFR*, *DvANS*, and *DvF3'H*, which are also involved in the synthesis of cyanidin, showed no marked changes in the transcript abundance upon virus infection in all black and bicolor cultivars (Supplementary Fig. S4). In these cultivars, siRNA of *DvFNS* was detected in TSV_{dahlia}-uninfected plants, while it was not detected in TSV_{dahlia}-infected plants (Fig. 9). These results suggest that the suppression of *DvFNS* PTGS by TSV_{dahlia} alters the accumulation of pigments and flower color in these black and black–white bicolor cultivars, as demonstrated in ‘Kokucho’ line 4.

Interestingly, in the cultivars ‘Kazusa-shiranami’ and ‘Kageboushi’, the white tips of the bicolor petals disappeared upon virus infection, and the flower color turned entirely purple (Fig. 5e, f, l, m). The accumulation of pigments was similar in petal tips and petal bases, both anthocyanidins and flavones were accumulated (data not shown). The white tips of bicolor petals are known to result from simultaneous PTGS of *DvCHS1* and *DvCHS2* (Ohno et al. 2011b). In color-changed petal tips of ‘Kazusa-shiranami’ and ‘Kageboushi’, *DvCHS2* transcript abundance increased compared with the same parts of TSV_{dahlia}-uninfected plants and in ‘Kageboushi’, *DvCHS1* transcript abundance also showed the same tendency (Supplementary Fig. S4). Moreover, the siRNA of *DvCHS* was detected in TSV_{dahlia}-uninfected plants but was not detected in TSV_{dahlia}-infected plants (Fig. 9). These results suggest that the PTGS of *DvCHS1* and *DvCHS2*, in addition to *DvFNS*, was suppressed by TSV_{dahlia} infection in petal tips of bicolor cultivars, causing the color change from white to purple. Furthermore, in ‘Jyunn-ai’, the siRNA of either *DvFNS* or *DvCHS* was not detected in TSV_{dahlia}-uninfected plants (Fig. 9) and no changes of transcript abundance occurred with TSV_{dahlia} infection (Fig. 8, Supplementary Fig. S4). This suggests that the PTGS of these genes does not occur in ‘Jyunn-ai’; hence, no change in flower color was observed upon infection with TSV_{dahlia}.

Discussion

Flower color change is induced by TSV_{dahlia} through the suppression of endogenous PTGS

The flower color of purple 'Kokucho' is sometimes partially recovered to black in summer (Supplementary Fig. S1), suggesting that purple 'Kokucho' is not a mutant. There have been reports of flower color changes caused by virus infection, called *color-breaking*. In tulip, this is caused by potyviruses, such as tulip-breaking virus and tulip tip-breaking virus (Dekker et al. 1993; Lesnaw and Ghabrial 2000). Bicolor daffodils (*Narcissus pseudonarcissus*) result from potexvirus narcissus mosaic virus (Hunter et al. 2011) and RedStar-type petunia cultivars result from cucumovirus CMV, potyviruses potato virus Y and tobacco etch virus (Teycheney and Tepfer 2001). Furthermore, some reports showed that high temperature enhances plant resistance to viruses by activating the silencing of virus RNA (Szittya et al. 2003; Chellappan et al. 2005; Ghoshal and Sanfaçon 2014). Therefore, we speculated that a similar phenomenon which enhanced host resistance against TSV_{dahlia} may occur in mosaic black petals of purple-flowering 'Kokucho'. TSV_{dahlia} was detected in purple-flowering plants of this cultivar, whereas it was not detected in original black-flowering plants (Fig. 1). Purple flower color was recovered to black after elimination of the virus using LP-free SAM culture, whereas 'Kokucho' line 4, which remained infected with TSV_{dahlia}, retained purple flowers (Fig. 2, Table 1). Among the viruses and viroids we examined, only TSV_{dahlia} completely corresponded to purple flower color of 'Kokucho'. Although we could not completely rule out the possibility that an unknown virus caused the color change, our results suggested the flower color change observed in 'Kokucho' was not caused because of a mutation but by TSV_{dahlia} infection. The fact that flower color was recovered from purple to black in all lines wherein TSV_{dahlia} had been eliminated (lines 1–3, 5), strongly supports our conclusion that TSV_{dahlia} caused the flower color change.

Purple 'Kokucho' accumulated more flavones but lower amount of particularly cyanidin-based anthocyanins than the original black 'Kokucho' (Fig. 3a). These differences caused the observed flower color change. The transcript abundance of *DvFNS* was higher in purple 'Kokucho' than in the black original (Fig. 3b). Similar phenomena were observed in color-changed TSV_{dahlia}-infected plants of all black and black–white bicolor cultivars (Fig. 7, 8). A previous study suggested that the low transcript abundance of *DvFNS* in most black dahlias was induced by the PTGS of *DvFNS* (Deguchi et al. 2013). Fewer small RNAs mapped onto the *DvFNS* genomic sequence in TSV_{dahlia}-infected purple 'Kokucho' than in original cultivar (Fig. 4, Deguchi et al. 2013), suggesting the suppression of *DvFNS* PTGS in TSV_{dahlia}-infected 'Kokucho'. In addition, the lower levels of *DvFNS* siRNA were detected in color-changed TSV_{dahlia}-infected plants than TSV_{dahlia}-uninfected plants (Fig. 9), strongly suggesting that the suppression of *DvFNS* PTGS in these cultivars was caused by the virus. Some existing studies report that color changes in plant tissues can result from the viral suppression of endogenous PTGS (Teycheney and Tepfer 2001; Senda et al. 2004). Thus, the suppression of *DvFNS* PTGS may alter pigment accumulation, resulting in flower color change from black to purple in most black dahlia cultivars. In black–white bicolor cultivars ('Kazusa-shiranami' and 'Kageboushi'), TSV_{dahlia} infection changed flower color not only from black to purple at the petal base but also from white to purple at the petal tip (Fig. 5e, f, l, m). The white tips of bicolor petals are due to simultaneous PTGS of *DvCHS1* and *DvCHS2* (Ohno et al. 2011b). Our analysis of transcript abundance and siRNA detection (Fig. 9, Supplementary Fig. S4) suggested that the suppression of *DvCHS* PTGS also occurred in petal tips in these TSV_{dahlia}-infected plants, inducing the accumulation of anthocyanins and resulting in flower color

change from white to purple. Furthermore, the pink cultivar 'Jyunn-ai', which lacks PTGS showed no flower color change when infected by TSV_{dahlia} (Fig. 5g, n). Therefore, these results indicate that flower color changes in dahlia cultivars may be induced through the suppression of endogenous PTGS by TSV_{dahlia} infection.

The result from this study suggests that TSV_{dahlia} possesses a silencing suppressor. Some plant viruses are known to have silencing suppressors to prevent silencing of viral genes and also to suppress endogenous PTGS in the host. While there are no current reports of a silencing suppressor in TSV, the 2b protein on RNA 2 of asparagus-virus 2 (AV-2), which belongs to the same genus (Ilarvirus), is known to act as a silencing suppressor (Shimura et al. 2013). Therefore, it is possible that the 2b protein (LC030106) acts as the silencing suppressor in TSV_{dahlia}, although its amino-acid sequence shares only 41% identity with that of AV-2. The 2b protein of CMV acts as a silencing suppressor (Brigneti et al. 1998), but it failed to suppress *DvFNS* PTGS and did not change flower color in 'Kokucho' (Fig. 1, 2). TSWV, which has a silencing suppressor NSs protein (Takeda et al. 2002), also did not change flower color in either black or bicolor dahlias. This may be because these viruses poorly invade reproductive organs in dahlia; in fact, neither virus could be detected in dahlia petals (data not shown). In contrast, TSV_{dahlia} was clearly detected in dahlia petals (Supplementary Fig. S3). The ability of TSV to invade reproductive organs is indicated by its high seed transmission ratio [2.6%–30.6% in soybean (Ghanekar and Schwenk 1974); 6.8%–48% in *Parthenium hysterophorus* (Sharman et al. 2009); and 0%–35% in strawberry (Johnson et al. 1984)] and its low elimination ratio by ordinary apical meristem culture (Naka et al. 2007) or LP-free SAM culture in dahlia (Hosokawa 2008). Owing to its ability to invade floral organs, TSV_{dahlia} may be able to suppress PTGS efficiently in dahlia petals and induce flower color change.

Detail consideration of the mechanism for black flower color

Upon TSV_{dahlia} infection, flowers of black and black–white bicolor cultivars tended to change to a purplish color, with higher L^* and C^* values (Fig. 5, 6; Supplementary Table S5). Flowers of infected plants never appeared black, except for 'Fidalgo Blacky'. TSV_{dahlia}-infected plants accumulated flavones through increased *DvFNS* transcript abundance, thereby reducing the amounts of anthocyanins, independently of the transcript abundance of the genes related to anthocyanin synthesis (Fig. 7, Supplementary Fig. S4). Increased flavone accumulation resulted from the suppression of *DvFNS* PTGS by TSV_{dahlia}, and the resultant change in anthocyanin accumulation may be explained by substrate competition between flavone synthesis and anthocyanidin synthesis, as discussed previously (Thill et al. 2012; Deguchi et al. 2013). Thus, these results demonstrate that endogenous PTGS of *DvFNS* is a general characteristic of black dahlias and essential for black flower coloring in most black cultivars.

'Fidalgo Blacky' showed little change and retained the black appearance despite flavone accumulation and anthocyanin reduction (Fig. 5, 6, Supplementary Table S5). It proved the following two points: The first is that the existence of flavone itself has little effect on black flower appearance. Flavones are nearly colorless pigments but influences the color of anthocyanins, a phenomenon called co-pigmentation (Asen et al. 1972). The flowers of TSV_{dahlia}-infected 'Fidalgo Blacky' remained black even though flavones accumulated and abaxial petals turned from reddish to bluish due to co-pigmentation (data not shown). This suggests that flavone itself and its co-pigmentation effects do not prevent black flower coloring. The second point is that black flower coloring can occur without PTGS of *DvFNS*. The fact that TSV_{dahlia}-infected 'Fidalgo Blacky' remained black suggesting that the PTGS

of *DvFNS* and subsequent low flavone accumulation and increased total and cyanidin-based anthocyanins are not absolutely necessary for black flower coloring.

The question remains of how TSV_{dahlia}-infected 'Fidalgo Blacky' is able to maintain black flower coloring. *L** and *C** scores were markedly lower for TSV_{dahlia}-infected 'Fidalgo Blacky' than for other TSV_{dahlia}-infected cultivars, despite nearly identical amounts of total anthocyanins (Fig 6, 7; Supplementary Table S5). The only substantial difference among these TSV_{dahlia}-infected plants was in the ratio between cyanidin-based and pelargonidin-based anthocyanins, and 'Fidalgo Blacky' had the highest levels of cyanidin-based anthocyanins. We previously proposed that cyanidin-based anthocyanins contribute much more to lowering *L** (described as the "darkness unit") than equivalent levels of pelargonidin-based anthocyanins (Deguchi et al. 2013). In *Matthiola incana*, 'Vintage Burgundy' accumulates only cyanidin-based anthocyanins and has lower petal *L** scores than 'Vintage Red' which accumulates nearly equal levels of pelargonidin-based anthocyanins (Tatsuzawa et al. 2012), supporting the higher contribution to lowering *L** of cyanidin-based anthocyanins. Cyanidin-based anthocyanins may also contribute more to lowering *C**. Therefore, it may be that TSV_{dahlia}-infected 'Fidalgo Blacky' retained sufficient levels of cyanidin-based anthocyanins to maintain the black flower appearance. These findings suggest that the high accumulation of cyanidin-based anthocyanins is the proximate causal factor in black flower appearance in dahlias.

High accumulation of cyanidin-based anthocyanins can be induced via two mechanisms: a *DvFNS* PTGS-dependent mechanism and a *DvFNS* PTGS-independent mechanism. In the latter one, high ability of cyanidin synthesis may induce the high accumulation of cyanidin-based anthocyanins independently of *DvFNS* PTGS, as indicated by TSV_{dahlia}-infected 'Fidalgo Blacky' in this study. Because the black color expressed by this mechanism is immune to be changed by TSV_{dahlia} infection, it may be stable and useful for breeding. We must note that both mechanisms may be premised on the high potential for anthocyanin synthesis conferred by the Type 1 *DvIVS* promoter (Ohno et al. 2013), which was present in all black and black–white bicolor cultivars used in this study (data not shown). The flower appearance of most black dahlias we examined was induced only by the former mechanism, but that of 'Fidalgo Blacky' was induced by both the mechanisms and its appearance was the most black because of having the highest accumulation of cyanidin-based anthocyanins. Thus, the combination of these two mechanisms, involving *DvFNS* PTGS and high ability of cyanidin synthesis, can produce blacker flower appearance than with a single mechanism.

Acknowledgements

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Supplementary data

Table S1. Primer sets for virus and viroid detection

Figure legends

Fig. 1

Virus and viroid detection in purple-flowering and original black-flowering ‘Kokucho’.

The cDNA from the leaves of five purple-flowering plants (P1–P5) and five black-flowering plants (B1–B5) was used for PCR. Primers used to detect each virus or viroid are listed in Supplementary Table S1. Plasmids containing the sequence of each virus or viroid were used as positive controls (PC) and water was used as a negative control (NC). TSV_{dahlia}, tobacco streak virus in dahlia; CMV, cucumber mosaic virus; DMV, dahlia mosaic virus; TSWV, tomato spotted wilt virus; CSVd, chrysanthemum stunt viroid.

Fig. 2

Virus and viroid detection from lines regenerated from leaf-primordia-free shoot apical meristem (LP-free SAM) cultures of purple-flowering ‘Kokucho’.

Primers used to detect each virus and viroid are listed in Supplementary Table S1. Leaf cDNA from each line was used. Line 1 was regenerated from purple ‘Kokucho’ P5, line 2 and line 3 were regenerated from P4, line 4 and line 5 were regenerated from P3. Plasmids containing the sequence of each virus and viroid were used as positive controls (PC). TSV_{dahlia}, tobacco streak virus in dahlia; CMV, cucumber mosaic virus; DMV, dahlia mosaic virus; TSWV, tomato spotted wilt virus; CSVd, chrysanthemum stunt viroid.

Fig. 3

Flavonoid aglycone contents (a) and relative transcript abundance of *DvFNS* (b) in petals of ‘Kokucho’ line 4 (purple) and line 5 (black), derived from leaf-primordia-free shoot apical meristem cultures.

All data represent averages of two biological replications. Completely colored, expanded petals were analyzed. The transcript abundance of *DvFNS* in line 4 is relative to that in line 5. *DvActin* was used as the internal standard.

Fig. 4

Mapping of small RNAs of TSV_{dahlia}-infected purple ‘Kokucho’ onto the *DvFNS* genomic sequence.

Small RNAs (18–32 nt long) with no mismatches to ‘Kokucho’ *DvFNS* genomic DNA (AB769841) were mapped onto either the sense strand (above the X-axis) or the antisense strand (below the X-axis). The numbers of matched reads are calculated per million total reads. The total number of matched reads after calculation is about 86 in TSV_{dahlia}-infected ‘Kokucho’ and 777 in TSV_{dahlia}-uninfected ‘Kokucho’ (Deguchi et al. 2013). The specific 21 nt antisense small RNA mapped in greatest number to position 2,307–2,327 of the *DvFNS* genomic sequence in TSV_{dahlia}-uninfected one (Deguchi et al. 2013) was not mapped at all in TSV_{dahlia}-infected ‘Kokucho’.

Fig. 5

Flowers of TSV_{dahlia}-uninfected original cultivars (a–g) and TSV_{dahlia}-infected plants (h–n).

a and h, ‘Kokucho’; b and i, ‘Fidalgo Blacky’; c and j, ‘Ms. Noir’; d and k, ‘Black Cat’; e and l, ‘Kazusa-shiranami’; f and m, ‘Kageboushi’; g and n, ‘Jyunn-ai’.

Fig. 6

Distributions of *L** (lightness) and *C** (chroma) values of TSV_{dahlia}-uninfected (original and graft control) plants and TSV_{dahlia}-infected plants. (a) 'Kokucho'; (b) 'Fidalgo Blacky'; (c) 'Ms. Noir'; (d) 'Black Cat'; (e) 'Kazusa-shiranami'; (f) 'Kageboushi'; (g) 'Jyunn-ai'.

Each data point represents the average of 27 total values: 3 locations × 3 petals × 3 independent flowers. Bars represent SE. The raw data are shown in Supplementary Table S5.

Fig. 7

Flavonoid aglycone contents of petals of TSV_{dahlia}-uninfected (original and graft control) plants and TSV_{dahlia}-infected plants.

All data represent the average ± SE of each flavonoid (three flowers; the values for 'Ms. Noir' graft control and 'Jyunn-ai' original each represent two flowers). Completely colored, expanded petals were used. Although values are presented as masses (mg), the same trends apply to molar masses because the molecular weights of pelargonidin, cyanidin, apigenin, and luteolin are nearly equal, 271, 287, 270, and 286 g mol^{-1}, respectively.

Fig. 8

Relative transcript abundance of *DvFNS* in the petals of TSV_{dahlia}-uninfected (original and graft control) plants and TSV_{dahlia}-infected plants.

RNA was extracted from half-colored, unexpanded petals. All data points represent averages ± SE of three biological replications (the values for 'Kazusa-shiranami' original, TSVdahlia-infected 1, 2 ,'Jyunn-ai' original and grafted control represent two flowers). *DvActin* was used as the internal standard. Calculations were conducted using a standard curve prepared from a diluted series of 'Fidalgo Blacky' TSV_{dahlia}-infected 2 cDNA.

Fig. 9

RNA gel blot hybridization for *DvFNS* siRNA and *DvCHS* siRNA detection.

U, TSV_{dahlia}-uninfected plant; I, TSV_{dahlia}-infected plant. Ribosomal RNA on the electrophoresis gel is shown in the lower photograph.

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Tables and Figures

Table 1. Petal color described with lightness (*L**) and chroma (*C**).

	Black 'Kokucho' (Original)	Purple 'Kokucho'	Individuals regenerated by leaf primordia-free shoot apical meristem (LP-free SAM) culture from purple 'Kokucho'				
			1	2	3	4	5
*L**(Lightness)	14.8 ± 0.3	18.3 ± 6.1	15.3	15.0	15.0	18.4	14.8
*C**(Chroma)	88.2 ± 0.8	117.3 ± 1.2	89.7	87.6	93.5	121.2	83.7
Flower color	Black	Purple	Black	Black	Black	Purple	Black

Data of black and purple 'Kokucho' represent the average value ± SE of five plants each (B1-B5, P1-P5). Data of LP-free SAM cultured lines represent the average of two biological replications of each line (1-5).

Fig. 1

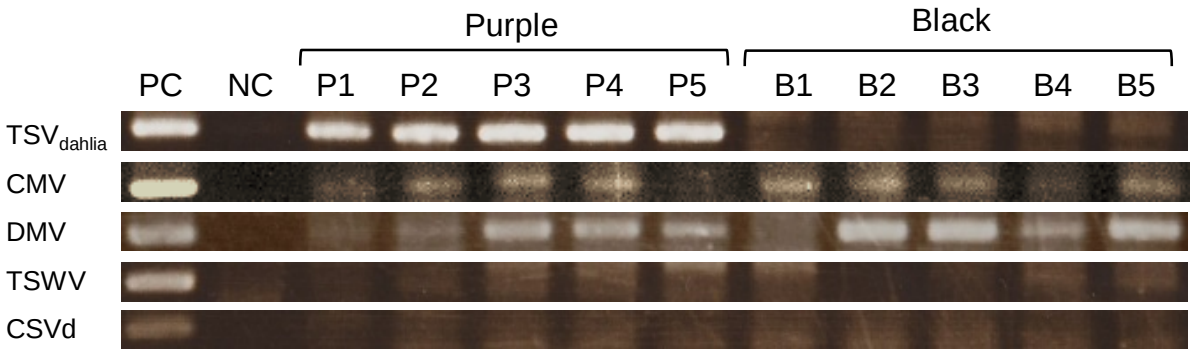

Purple
Black
PC
NC
P1
P2
P3
P4
P5
B1
B2
B3
B4
B5
TSV dahlia
CMV
DMV
TSWV
CSVd

Fig. 2

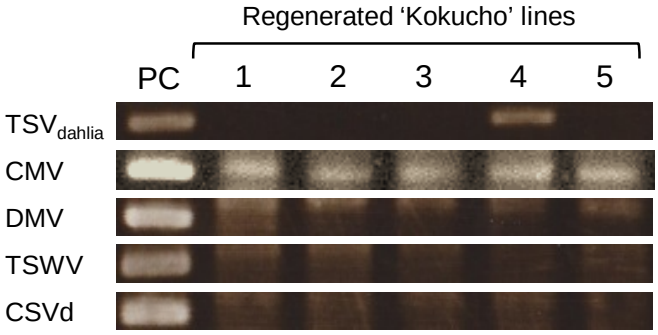

Regenerated 'Kokucho' lines
PC
1
2
3
4
5
TSV dahlia
CMV
DMV
TSWV
CSVd

Fig. 3

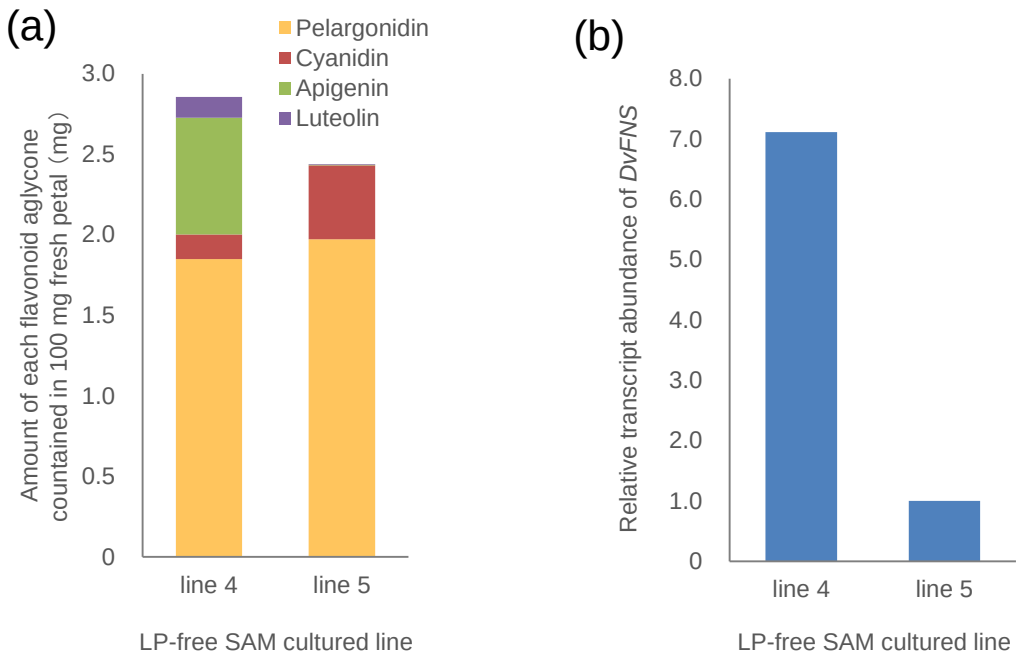
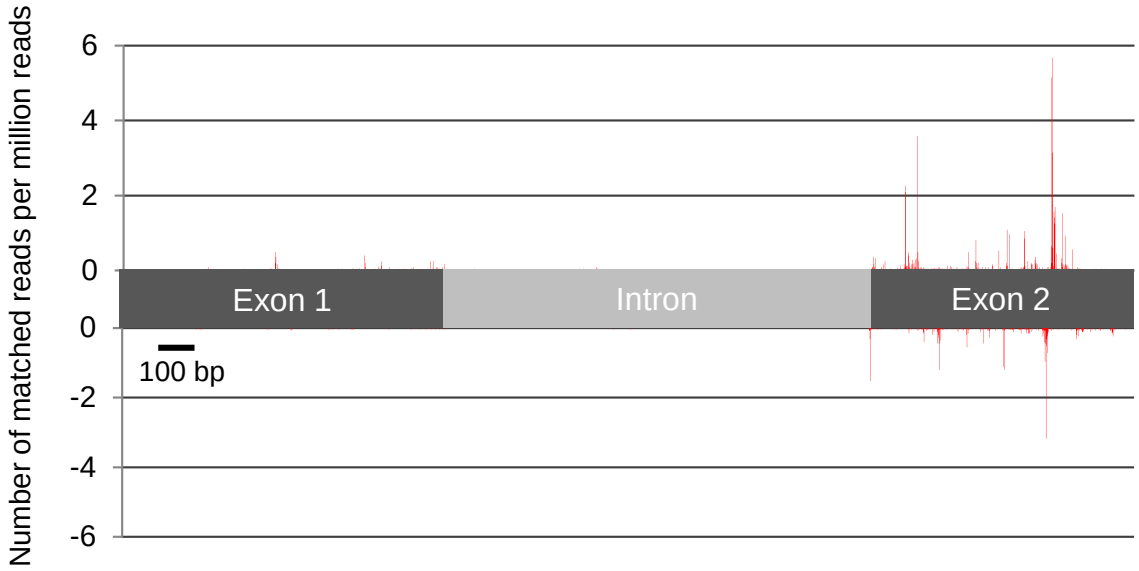

(a)
Pelargonidin
Cyanidin
Apigenin
Luteolin
Amount of each flavonoid aglycone countained in 100 mg fresh petal (mg)
3.0
2.5
2.0
1.5
1.0
0.5
0
line 4
line 5
LP-free SAM cultured line
(b)
Relative transcript abundance of DvFNS
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
line 4
line 5
LP-free SAM cultured line

Fig. 4


Number of matched reads per million reads
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2
0
0
-2
-4
-6
Exon 1
Intron
Exon 2
100 bp

Fig. 5

Fig. 6

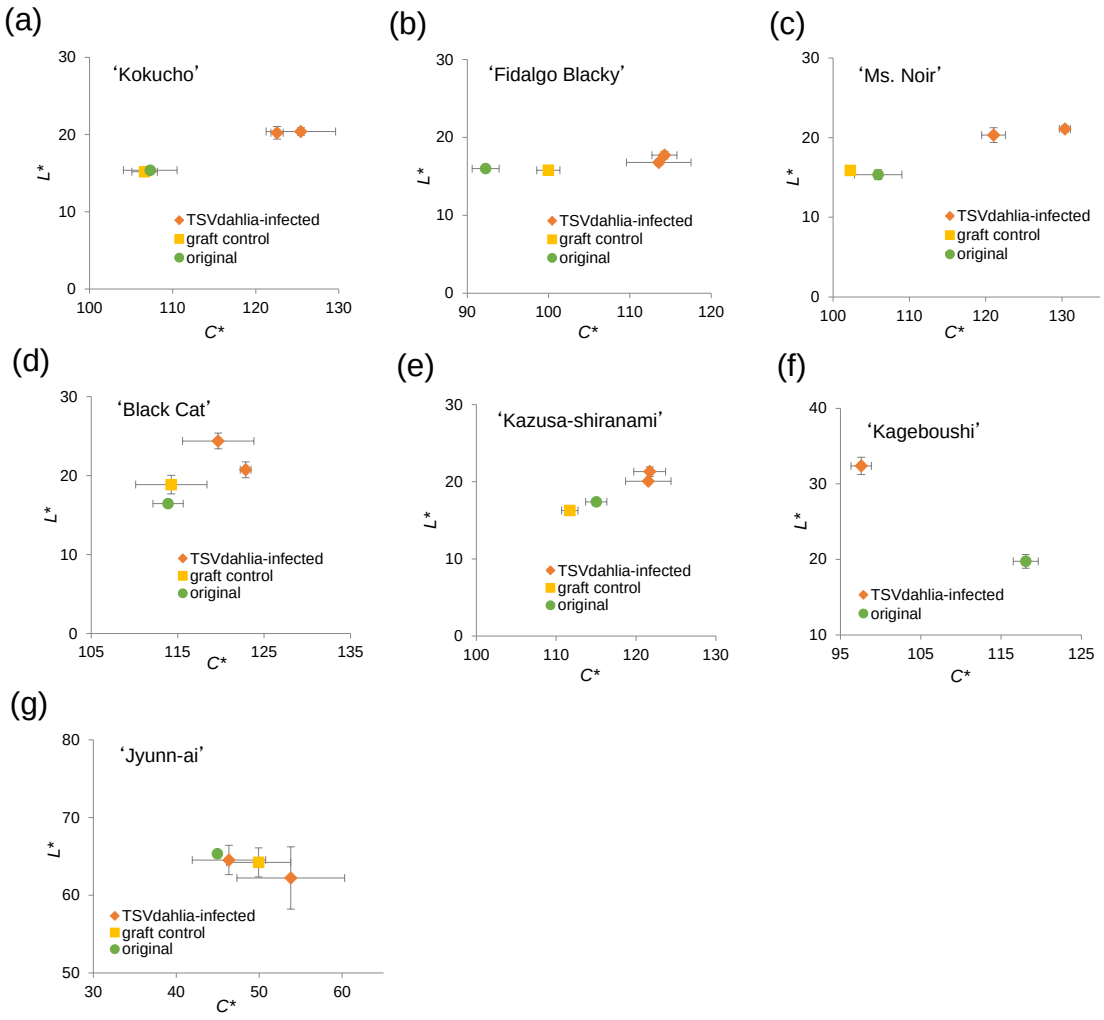

(a)
'Kokucho'
L*
C*
TSVdahlia-infected
graft control
original
(b)
'Fidalgo Blacky'
(c)
'Ms. Noir'
(d)
'Black Cat'
(e)
'Kazusa-shiranami'
(f)
'Kageboushi'
(g)
'Jyunn-ai'

Fig. 7

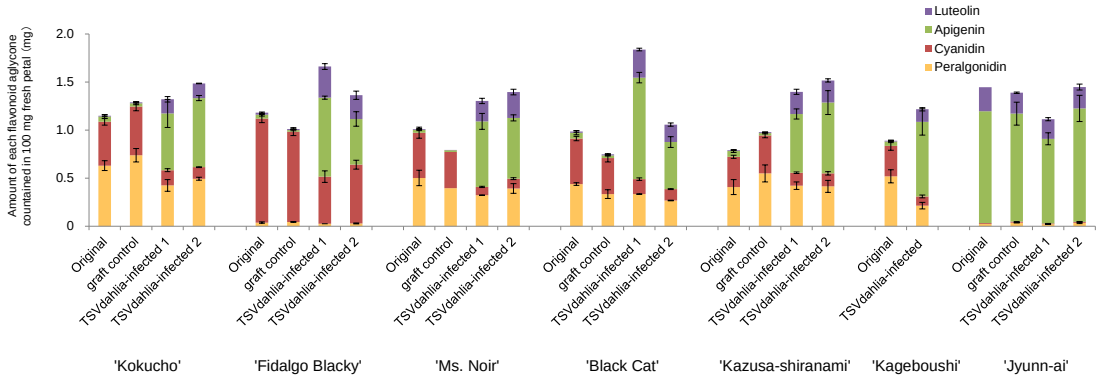

Luteolin
Apigenin
Cyanidin
Peralgonidin
Amount of each flavonoid aglycone countained in 100 mg fresh petal (mg)
2.0
1.5
1.0
0.5
0
Original
graft control
TSVdahlia-infected 1
TSVdahlia-infected 2
'Kokucho'
'Fidalgo Blacky'
'Ms. Noir'
'Black Cat'
'Kazusa-shiranami'
'Kageboushi'
'Jyunn-ai'

Fig. 8

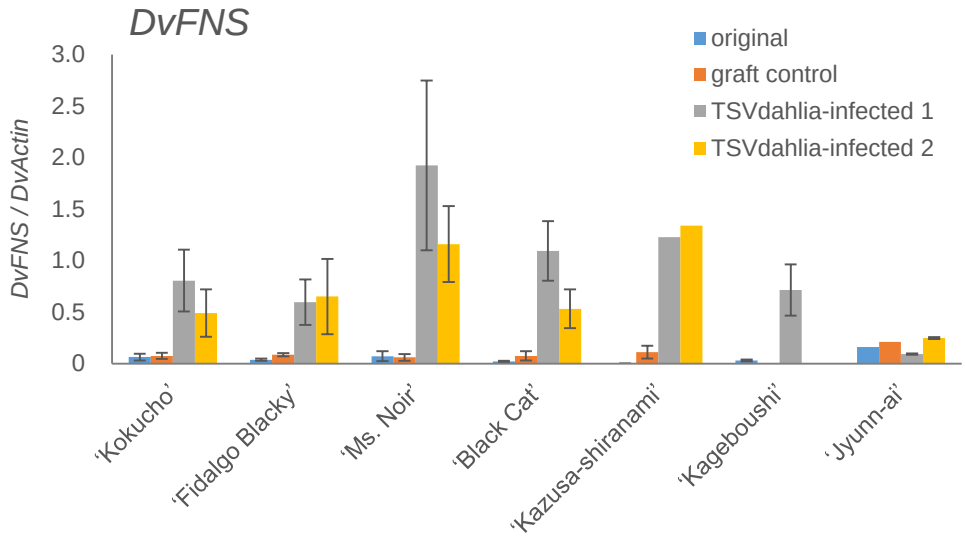
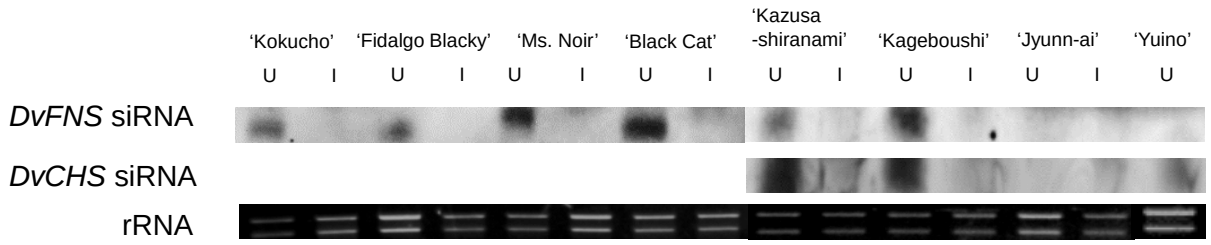

DvFNS
DvFNS / DvActin
3.0
2.5
2.0
1.5
1.0
0.5
0
original
graft control
TSVdahlia-infected 1
TSVdahlia-infected 2
'Kokucho'
'Fidalgo Blacky'
'Ms. Noir'
'Black Cat'
'Kazusa-shiranami'
'Kageboushi'
'Jyunn-ai'

Fig. 9


'Kokucho'
'Fidalgo Blacky'
'Ms. Noir'
'Black Cat'
'Kazusa -shiranami'
'Kageboushi'
'Jyunn-ai'
'Yuino'
U I U I U I U I U I U I U I U
DvFNS siRNA
DvCHS siRNA
rRNA

Supplementary Tables and Figures

Tobacco streak virus (strain dahlia) suppresses post-transcriptional gene silencing of *flavone synthase II* in black dahlia cultivars and causes a drastic flower color change

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Supplementary Fig. S1

Purple ‘Kokucho’ flowers with partially black petals occurring in summer.

Arrows indicate black mosaic regions in purple petals.

Supplementary Fig S2.

Comparison of growth parameters between TSV_{dahlia}-infected ‘Kokucho’ (line 4) and TSV_{dahlia}-uninfected ‘Kokucho’ (line 5). (**a**) plant height at four time points; (**b**) node number per plant at three time points; (**c**) diameter of the first-opened inflorescence; (**d**) weight of tuberous roots per plant measured on December 29, 2014. The rooted cuttings of both lines were transplanted to 24 cm pots on July 11, 2014. Nine plants from each line were grown in a greenhouse. All data represent the average ± SE. No significant differences ($P > 0.05$) were observed for any parameter, according to *t*-test.

Supplementary Fig. S3

Detection of TSV_{dahlia} in *in vitro* grafted plants.

cDNA from half-colored, unexpanded petals from each plant was used. O, original cultivars without grafting; GC, graft controls (plants grafted onto TSV_{dahlia}-uninfected ‘Kokucho’ line 5); TSV1 and TSV2, plants grafted onto TSV_{dahlia}-infected ‘Kokucho’ line 4 independently. Plasmid containing the full sequence of TSV CP was used as a positive control (PC) and water served as a negative control (NC). Primers used are listed in Table S1.

Supplementary Fig. S4

Relative transcript abundance of the genes involved in anthocyanidin synthesis in petals of TSV_{dahlia}-uninfected (original and graft control) plants and TSV_{dahlia}-infected plants. (**a**) *DvIVS*, (**b**) *DvCHS1*, (**c**) *DvCHS2*, (**d**) *DvCHI*, (**e**) *DvF3H*, (**f**) *DvDFR*, (**g**) *DvANS*, and (**h**) *DvF3'H*

RNA was extracted from half-colored, unexpanded petals for *DvF3'H* and from completely colored, unexpanded petals for the other genes. Petal bases of ‘Kazusa-shiranami’ and ‘Kageboushi’ corresponded to the colored parts of TSV_{dahlia}-uninfected petals and the tips corresponded to the uncolored parts. All data points represent the average of two or three biological replications, excluding some abnormal scores. Standard error bars are applied to the data with three biological replications. *DvActin* was used as the internal standard. Calculation was conducted using a standard curve prepared from a serial dilution of ‘Kokucho’ TSV_{dahlia}-infected 2 cDNA and ‘Fidalgo Blacky’ TSV_{dahlia}-infected 2 cDNA.

Supplementary Table S1. Primer sets for virus and viroid detection

Virus / Viroid	sense (5'-3')	antisense (5'-3')
TSV_{dahlia}	CCCATAATACCGTGAACACT	CCTGTTACTCCATCAACCAT
CMV	TATTACCCTAAAGCCACCAA	GTTAGCTTGGACTCCAGATG
DMV	AAAAAGAGGCTACCATACCC	ACTTCCTGCTAGGACACTCA
TSWV	GTCAGGGGACAATAACTG	CTGCTTCTCACTGTTTCC
INSV	AAATCAATAGTAGCATTAAA	CTTCCTCAAGAATAGGCAAT
CSVd	CAACTGAAGCTTCAACGCCTT	AGGATTACTCCTGTCTCGCA
PSTVd	TAAACTCGTGGTTCCTGTGG	GCCCCGAAGCAAAGAAAGAT

Supplementary Table S2. Primers for sequence analysis of TSV_{dahlia}

Name	Orientation	Sequense (5'-3')
TSV-RNA2 Full-R2	antisense	GCATCTCCATTTGGAGGC
TSV-RNA3-Full-R2	antisense	GCATCTCCTATAAAGGAGGCATCAGTAG
TSV-2b-F1	sense	GTTTGTCGAAGAACCGC
TSV-2b-R1	antisense	AAAGCGGCATCTCTC
TSV-CP-F1	sense	GCGAAGGCGTCGTTGAGGTT
TSV-CP-R1	antisense	GATTTCGGGAATCCCCTCGAC
TSV-MP-F	sense	ATGGCGTTAGTACCAACGATG
TSV-MP-R	antisense	TCAGGCTGAAAGCAGGTTCC

Supplementary Table S3. Primers designed for real-time PCR

Gene	Orientation	Sequense (5'-3')
DvActin	antisense	GCTGACAGGATGAGCAAG
	sense	TCCACATCTGTTGGAAGG
DvF3'H	antisense	CATAACTGCCTTACTATTGTAC
	sense	GTAGTTATACGCAATATGCTC

Supplementary Table S4. Leaf color described with CIE *L*a*b*C** coordinates.

	*L**	*a**	*b**	*C**
line 4 (TSV_{dahlia}-infected)	43.32 ± 0.46	6.11 ± 0.28	13.69 ± 0.51	15.03 ± 0.45
line 5 (TSV_{dahlia}-uninfected)	44.49 ± 0.48	5.34 ± 0.46	15.48 ± 0.71	16.50 ± 0.51

n = 9. All values represent the average ± SE of three point in the uppermost leaf at 1st flower opening.

Supplementary Table S5. Petal color described with CIE *L*a*b*C** coordinates

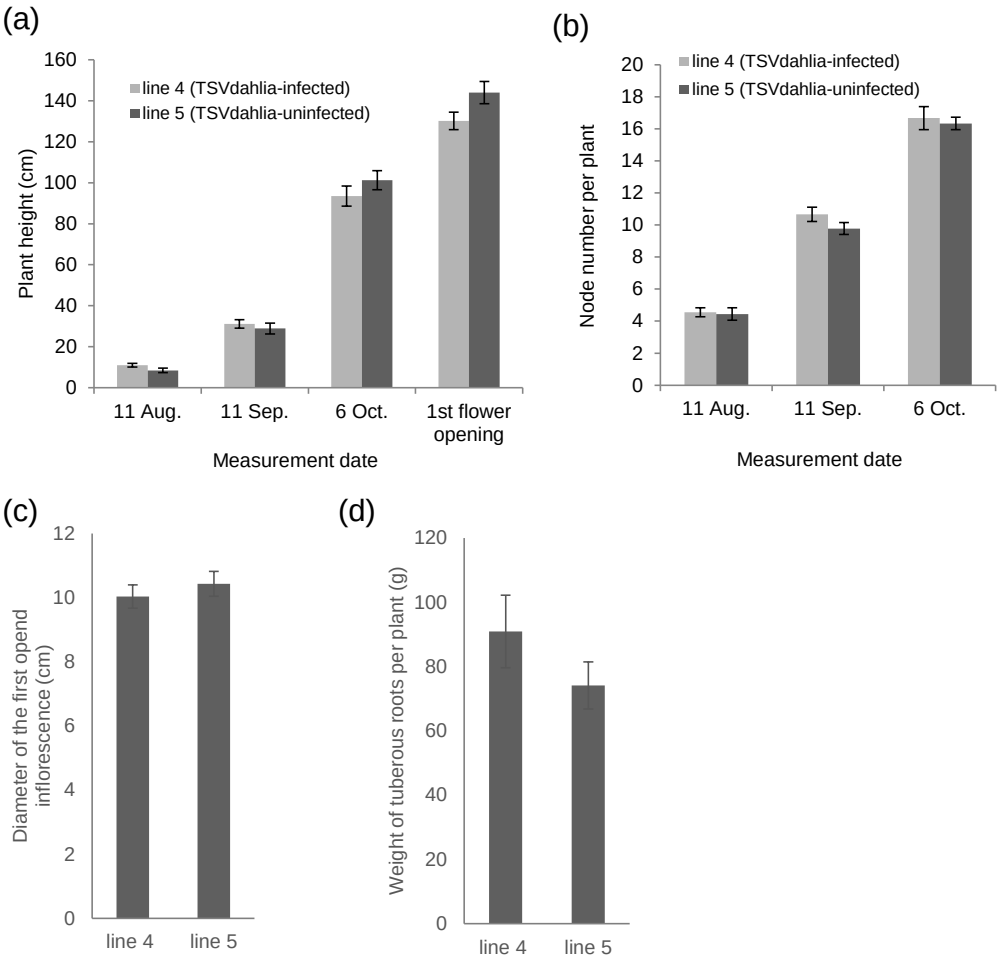
Cutivar	*L**				*C**			
	original	graft control	TSV_{dahlia}-infected 1	TSV_{dahlia}-infected 2	original	graft control	TSV_{dahlia}-infected 1	TSV_{dahlia}-infected 2
'Kokucho'	15.38 ± 0.38	15.18 ± 0.17	20.22 ± 0.82	20.38 ± 0.48	107.32 ± 3.23	106.63 ± 1.53	122.57 ± 0.73	125.44 ± 4.18
'Fidalgo Blacky'	15.99 ± 0.13	15.79 ± 0.45	16.79 ± 0.37	17.72 ± 0.47	92.26 ± 1.65	99.98 ± 1.42	113.55 ± 3.97	114.26 ± 1.54
'Ms. Noir'	15.35 ± 0.60	15.88 ± 0.67	20.32 ± 0.92	21.10 ± 0.38	105.90 ± 3.10	102.26 ± 3.24	121.03 ± 1.56	130.38 ± 0.72
'Black Cat'	16.45 ± 0.44	18.86 ± 1.17	20.74 ± 0.49	24.38 ± 1.51	113.89 ± 1.75	114.26 ± 1.55	122.86 ± 0.62	119.69 ± 4.13
'Kazusa-shiranami'	17.40 ± 0.04	16.27 ± 0.16	21.31 ± 0.61	20.07 ± 0.23	115.01 ± 1.34	111.70 ± 1.04	121.72 ± 1.99	121.54 ± 2.85
'Kageboushi'	19.73 ± 0.91	—	32.38 ± 1.13	—	118.07 ± 1.56	—	97.59 ± 1.27	—
'Jyunn-ai'	65.34 ± 1.34	64.22 ± 1.86	64.53 ± 1.89	62.22 ± 4.01	44.96 ± 1.55	49.93 ± 3.86	46.36 ± 4.42	53.82 ± 6.50

Cutivar	*a**				*b**			
	original	graft control	TSV_{dahlia}-infected 1	TSV_{dahlia}-infected 2	original	graft control	TSV_{dahlia}-infected 1	TSV_{dahlia}-infected 2
'Kokucho'	107.13 ± 3.20	106.49 ± 1.53	122.17 ± 0.73	124.84 ± 4.16	6.35 ± 0.53	5.39 ± 0.08	9.23 ± 2.03	12.06 ± 1.26
'Fidalgo Blacky'	92.17 ± 1.64	99.87 ± 1.40	113.52 ± 3.98	114.21 ± 1.57	3.95 ± 0.40	4.61 ± 0.35	2.86 ± 0.25	2.43 ± 1.28
'Ms. Noir'	105.77 ± 3.09	102.08 ± 3.26	120.53 ± 1.55	129.73 ± 0.71	5.28 ± 0.43	6.11 ± 0.19	11.02 ± 0.39	12.85 ± 1.08
'Black Cat'	113.64 ± 1.70	113.78 ± 1.58	122.38 ± 0.67	118.92 ± 4.35	7.55 ± 0.71	10.36 ± 0.89	10.81 ± 0.68	12.88 ± 2.12
'Kazusa-shiranami'	114.69 ± 1.29	111.43 ± 1.01	121.08 ± 2.04	121.16 ± 2.83	8.52 ± 0.80	7.79 ± 0.39	11.94 ± 1.92	9.34 ± 1.15
'Kageboushi'	117.49 ± 1.49	—	94.94 ± 1.05	—	11.38 ± 1.59	—	22.54 ± 1.06	—
'Jyunn-ai'	43.00 ± 1.82	48.35 ± 3.88	44.50 ± 4.56	52.31 ± 6.53	-13.01 ± 0.66	-12.41 ± 0.70	-12.83 ± 0.15	-12.53 ± 0.78

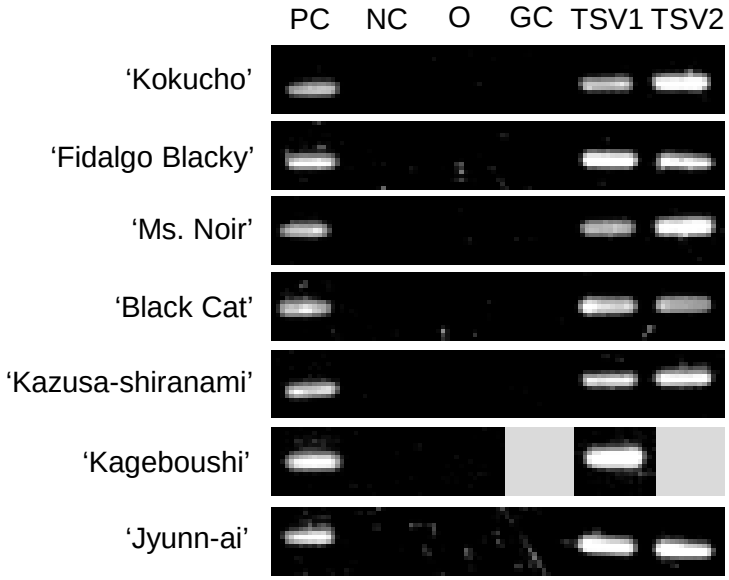
All data represent the average±SE of three independent flowers. $C^* = (a^{*2}+b^{*2})^{1/2}$

Supplementary Fig. S1

Supplementary Fig. S2



Supplementary Fig. S3



Supplementary Fig. S4