Virus-specific spatial differences in the interference with silencing of the chs-A gene in non-transgenic petunia

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Potyviruses, such as potato virus Y and tobacco etch virus, as well as cucumber mosaic cucumovirus, interfere with post-transcriptional gene silencing (PTGS). When RedStar-type Petunia hybrid cultivars, whose flowers have alternating white and pigmented sectors, were infected with these viruses, each virus induced a different pattern of restoration of floral anthocyanin pigmentation. Local reversion to coloured phenotypes in the white sectors, which occurred through interference with PTGS of the chalcone synthase A (chs-A) gene, was correlated with locally increased levels of chs-A mRNA and virus concentration. Our results show that virus infection can interfere with PTGS of a native plant gene, and that this can have profound effects on symptom expression.

Gene silencing, whether transcriptional or post-transcriptional, is a general regulatory mechanism that is thought to counteract the invasion of eukaryotic genomes by viruses and transposable elements (for review, see Fire, 1999; Kooter et al., 1999). Specific to plants is the observation that phenomena related to post-transcriptional gene silencing (PTGS) are involved in certain forms of natural resistance to infection by RNA viruses (e.g. Ratcliff et al., 1999). Moreover, in certain transgenic plants expressing viral sequences, the homology-based resistance observed also clearly relies on PTGS mechanisms (for review see Kooter et al., 1999; Waterhouse et al., 1999). The recent observation that certain plant viruses have the ability to inhibit PTGS (e.g. Voinnet et al., 1999; Llave et al., 2000) is consistent with PTGS playing a long-standing role in the coevolution of plant-virus interactions. Several viral PTGS inhibitors have been identified, of which some, like the 2b protein of cucumber mosaic cucumovirus (CMV), prevent PTGS initiation, whereas others, like the potyviral helper-component-protease (HC-Pro), have the ability to suppress PTGS in already silenced tissues (Voinnet et al., 1999). One of the most extensively studied models for characterizing the roles and mechanisms of PTGS is based on the expression of a chalcone synthase gene (chs-A) in Petunia hybrida petals (for review see Jorgensen, 1995). This gene encodes a key enzyme in the anthocyanin biosynthesis pathway, which leads to the synthesis of red, blue and purple pigments. When a 35S:chs-A transgene was expressed in a purple-flowered petunia cultivar, instead of the expected darker purple flowers, either white flowers or variegated flowers with purple sectors alternating with white sectors were observed (Napoli et al., 1990; van der Krol et al., 1990). This has been shown to result from spatially regulated PTGS of both the endogenous chs-A gene and the 35S:chs-A transgene (van Blokland et al., 1994). It was later shown that the variegated flowers with white and pigmented sectors that occur in certain petunia cultivars that were created by inter-specific crosses, such as RedStar, are also due to PTGS of the chs-A gene (Metzlafl et al., 1997). This situation is surprising in the light of evidence for a systemic signal that transmits PTGS induction within a plant (Palaquii et al., 1997), although spatially uneven PTGS has been reported in transgenic tobacco (Höfgen et al., 1994; Boerjan et al., 1994; Palaquii et al., 1996) or Nicotiana sylvestris (Kunz et al., 1996).

Although sectoring of chs-A PTGS in petunia flowers is at this time unexplained, it could result either from the absence of diffusion of the PTGS-inducing signal into the purple sectors, or from the inability of the cells in purple sectors to recognize or respond to this signal. Petunia is susceptible to numerous plant viruses, including several that have been shown to interfere with PTGS by at least two different mechanisms (Voinnet et al., 1999). This has made it possible for us to investigate the spatial regulation of chs-A PTGS by infection with different viruses in non-transgenic RedStar-type petunia cultivars.

Four RedStar-type petunia cultivars (RedStar, Soie Violet Blanc Etoilé, Bravo Rouge Etoilé Blanc and Starmania) were mechanically inoculated at the three-to-four leaf stage with either CMV-R, a subgroup II strain that induces mild mosaic symptoms on tobacco (Carrière et al., 1998), a necrotic strain of potato potyvirus Y (PYY-nysa) originally isolated from potato in Poland (Chranowska, 1991) or a strain of tobacco etch potyvirus (TEV-CAA10) that induces severe mosaic symptoms on tobacco (Legnani et al., 1996). All experiments were performed on cuttings from a single plant to insure that all individuals were genetically identical. Four plants were either
mock- or virus-inoculated in each experiment, which was carried out at least three times. On the first three petunia cultivars, TEV infection caused severe stunting with, in addition, generalized necrosis in the case of RedStar, which prevented plants from reaching the flowering stage. When infected with the other viruses, the leaves displayed only light chlorotic symptoms during the first weeks following inoculation, after which essentially no leaf symptoms were observed (data not shown). In all cases where flowers could develop on the virus-infected plants, exactly the same patterns of reversion of chs-A PTGS were observed on the flowers of the different cultivars with each virus (data not shown). Similar observations were made on plants inoculated at an early flowering stage. Since petunia cv. Starmania was the only one in which the effects of all three viruses could be evaluated, it was used in further experiments.

Four weeks after inoculation with PVY, the first emerging flowers displayed distinct ca. 1 mm diameter purple spots in the white sectors, while the dimensions of the white sectors were not otherwise affected (Fig. 1B). As plants infected with PVY grew older, the number and size of purple spots increased but nearly always remained located adjacent to the main or secondary veins. Infection by CMV did not induce spotting, but instead a narrowing of the white sectors that became more

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**Fig. 1.** Virus-induced phenotypes on petunia flowers. Next to a flower from a mock-inoculated plant (A) are shown typical symptoms induced by PVY-nysa (B), CMV-R (C) and TEV-CAA10 (D) on *Petunia hybrida* cv. Starmania flowers 24 weeks post-inoculation.
pronounced later in infection, with the remaining white parts turning a light purple (Fig. 1C). As a result, by 6 weeks post-inoculation, many flowers on CMV-infected plants were entirely purple. Infection by TEV caused a rapid invasion of the white sectors by purple veins and spots evenly located throughout these sectors, with no apparent association with vascular tissue (Fig. 1D). By 4 weeks post-inoculation, white sectors were reduced in both size and number, and were strongly invaded by purple veins and spots, with the remaining white parts of the sectors turning a light purple. After 6 weeks, flower phenotypes remained stable throughout the life of the plants.

Northern blot analysis was performed in order to further characterize virus-induced phenotypes (Fig. 2). As expected, the level of chs-A mRNA was high in purple sectors of variegated flowers from mock-inoculated petunia, and no chs-A mRNA could be detected either in the white flower sectors or in the leaves of these plants, even after a longer exposure of autoradiograms. These results are consistent with previously reported data from RT–PCR experiments (Metzlaff et al., 1997). The level of chs-A mRNA in the white sectors of infected flowers showed an increase that corresponds to the observed level of purple pigmentation within these sectors, whereas hybridization of the filter with ribosomal RNA probe showed that approximately equal amounts of total RNA were transferred onto the filter. Given that the absence of chs-A mRNA in the white sectors of flowers from mock-inoculated plants results from PTGS of the chs-A gene (Metzlaff et al., 1997), it is likely that local restoration of the purple colouration within the white sectors results from inhibition of PTGS by PVY, CMV or TEV infection, since these three viruses have the ability to interfere with PTGS (Voinnet et al., 1999). It is of interest that infection with PVY increased the amount of chs-A mRNA to a detectable level in systemic leaves (Fig. 2). This suggests that the absence of chs-A mRNA in leaves of uninfected plants may also be due to PTGS, since it has been shown by Metzlaff et al. (1997) that although full-length chs-A mRNA was undetectable in RedStar petunia leaves, even when using such highly sensitive detection methods as RT–PCR followed by Southern blot hybridization, shorter RNAs believed to be mRNA degradation products were detected. It should be noted, however, that infection with neither CMV nor TEV increased chs-A mRNA levels to a detectable level in systemically infected leaves.

Slot-blot analysis of chs-A and viral RNA was performed in order to determine whether virus accumulation was spatially correlated with restoration of purple colour. As expected from the results obtained with Northern blots, chs-A mRNA levels were specifically increased in the purple spots observed within the white sectors (pw) of the flowers from PVY-, CMV- or TEV-infected plants (Fig. 3A). In fact, chs-A mRNA levels were actually higher in these parts than in white parts (w) of the white sectors of inoculated plants, or even the purple sectors of virus-infected or mock-inoculated plants (p). The chs-A mRNA levels were also higher in the white parts of the white sectors in flowers from infected plants than in flowers from mock-inoculated plants (Fig. 3A), which is as expected since these remaining white parts turned a light purple. Expression of chs-A could be detected in leaves of either mock- or virus-inoculated plants. This can be attributed to a 10-fold increase in the load of nucleic acids relative to the amounts used for Northern blot analysis. As shown in Fig. 3(B), accumulation of viral RNA was higher in the purple spots within the white sectors (pw) than in any other part of the flower, whether purple (p) or white (w), and was also higher than in the leaves (l). However, for TEV-infected plants, accumulation of viral RNA was only slightly higher in purple than in white parts. This result shows that increased concentrations of PVY, TEV or CMV correlate with the restoration of the purple colouration within white sectors.

Our results show that PVY, TEV and CMV interfere in a pattern-specific manner with PTGS of the chs-A gene in
petunia flowers, leading to striking differences in symptom expression. The observed spatial correlation between blockage of chs-A PTGS and virus accumulation suggests that infected cells are unable to transmit to neighbouring cells the ability to overcome PTGS of the chs-A gene. Although it has been proposed that certain viral suppressors of PTGS are targeted against the systemic signal of silencing (Voinnet et al., 1999), our observation of correlation between virus concentration and blockage of PTGS suggests that, at least in petunia flowers, virus-induced interference with PTGS is not mediated by a diffusible PTGS-blocking signal. The observed differences in pigment pattern would thus be the reflection of differences in the ability of each of the viruses to effectively invade groups of petal cells, in which they block chs-A PTGS.

Non-uniform distribution of virus in infected plants is a widely observed phenomenon, such as in the alternating green and chlorotic patches typical of leaf mosaics. In many cases the green islands are essentially virus-free, and in some cases it has been shown that they are resistant to superinfection by the same or closely related viruses (e.g. Loebenstein et al., 1977). In the light of current evidence that certain forms of natural virus resistance are based on PTGS-like mechanisms, it is tempting to propose that the green islands of a mosaic are due to this type of resistance. The specific mosaic patterns induced by different viruses may in fact reflect complex spatial interactions between induction of and interference with PTGS-related phenomena, such as we observed in petunia flowers.

Virus interference with PTGS raises the possibility that virus infection could lead to loss of agronomically desirable traits obtained by PTGS in transgenic plants, including not only virus resistance (Mäki-Valkama et al., 2000), but also modified seed-oil composition (Kinney, 1997) or flower colour (Napoli et al., 1990; van der Krol et al., 1990). The results presented here underline the complexity of the interaction between factors inducing and blocking PTGS, so it will be interesting to further investigate the effects of different viruses that interfere with PTGS on other natural and transgenic PTGS systems.

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References


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