

SHORT COMMUNICATION

**HOMOLOGY-DEPENDENT VIRUS RESISTANCE AGAINST
CYMBIDIUM RINGSPOT VIRUS IS INHIBITED BY POST-TRANSCRIPTIONAL
GENE SILENCING SUPPRESSOR VIRUSES**

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Plants expressing viral sequences may show homology-dependent virus resistance (HDR), which is characterized by specific degradation of transgenic RNA and viral RNAs homologous to the transgene. This resistance mechanism is a form of post-transcriptional gene silencing (PTGS), which can be suppressed by virus-encoded proteins. We analysed the effect of *Potato virus Y*, *Cucumber mosaic virus* and *Potato virus X* infections on HDR to *Cymbidium ringspot virus* (CymRSV) shown by *Nicotiana benthamiana* transgenic line 92KA1 expressing the full-length replicase gene of CymRSV. We observed breaking of resistance in transgenic plants doubly infected with PTGS suppressor viruses and CymRSV. The need of considering the effect of PTGS suppressor viruses on resistance or PTGS-dependent phenotype in transgenic crops is discussed.

Key words: Post-transcriptional gene silencing, transgenic resistance, PVY, PVX, CMV CymRSV.

Post-transcriptional gene silencing (PTGS) in plants involves specific RNA degradation of invasive RNAs (for review see Meins, 2000; Waterhouse *et al.*, 2001). Phenomena related to PTGS are 'quelling' observed in fungi (Cogoni and Macino, 1997) and 'RNA interference' observed in nematodes, insects and mammals (Bosher and Labouesse, 2000). In PTGS-related systems no change in transgene transcription rate is observed, whereas a specific degradation mechanism is targeted against transgenic RNA and all RNAs containing sequences homologous or complementary to the transgene. Several lines of evidence suggest links between PTGS and natural plant defence mechanisms against invading RNAs such as those deriving from plant viruses and transposons (reviewed by Waterhouse *et al.*, 2001; Voinnet, 2001).

Transgenic plants expressing viral sequences may show either homology-dependent virus resistance (HDR) against viruses with genomes containing genes highly homologous to the transgene (English *et al.*, 1996), or, if susceptible, may show disease 'recovery', where upper leaves that develop after systemic infection are symptomless and contain a low concentration of virus (Lindbo *et al.*, 1993). HDR and recovery are phenomena related to PTGS because both transgenic RNA and viral RNAs are degraded. Moreover, it has been recently demonstrated that PTGS can be inhibited by several virus-encoded proteins that are also suppressors of natural anti-viral defence in the host (Anandalakshimi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Voinnet *et al.*, 1999; Carrington *et al.*, 2001).

The PTGS suppressor activity of potyviruses and *Cucumber mosaic virus* (CMV) has been extensively studied (Anandalakshimi *et al.*, 1998; Béclin *et al.*, 1998; Brigneti *et al.*, 1998; Lucy *et al.*, 2000). Indeed, it is known that the HC-Pro and the 2b proteins encoded by *Potato virus Y* (PVY) and CMV, respectively, break PTGS by interfering with different steps of the specific RNA degradation pathway. Recently, it has also been shown that *Potato virus X* (PVX) is able to block systemic spread of PTGS signal (Voinnet *et al.*, 2000). These findings open the question of whether PTGS-suppressor viruses may also interfere with HDR. Since HDR confers resistance to plant viruses, and is being used to protect crops (see Martelli, 2001 and references therein), this question has practical relevance.

PVY, CMV and PVX were used to test the interference of virus infections with HDR in two previously characterized *Nicotiana benthamiana* transgenic lines expressing the full-length replicase gene of *Cymbidium ringspot virus* (CymRSV) (Rubino *et al.*, 1993). Line 92KA1 shows high levels of HDR against CymRSV, to which is immune, whereas line 92KB1 is fully susceptible. Transgenic plants of T₁ progeny of both lines were selected by germinating seeds on agar medium containing kanamycin (100 mg ml⁻¹). The presence of the transgene in the kanamycin resistant plants was confirmed by PCR using primers homologous and complementary to sequences in *Cauliflower mosaic virus* (CaMV) tran-

scriptional control regions bordering the cloning site of the replicase gene. Genomic DNA extraction, PCR and analysis of products were done as described (Rubino *et al.*, 1993). PCR confirmed the presence of the transgene in all kanamycin-resistant plants (data not shown).

Transgenic lines, when at the stage of three fully expanded leaves (about 20 days post-germination), were used for virus inoculation. Two expanded leaves of transgenic 92KA1 plants (immune) were mechanically inoculated with PVY (ordinary strain), PVX or CMV (FN strain), respectively, or were mock inoculated. As controls, 92KB1 (susceptible) and wild type plants were inoculated with CMV and PVY. One week post-inoculation typical systemic symptoms appeared on both transgenic lines and wild type plants and virus infection was confirmed by dot-blot analysis of total RNA extracted as described by Dalmay *et al.* (1993), using ³²P-labelled probes prepared by nick-translation (Sambrook *et al.*, 1989) of cDNA clones of the coat protein genes of PVY, PVX or CMV.

Two systemically infected leaves of each plant were then super-inoculated with infectious full-length CymRSV RNA transcripts as reported (Rubino *et al.*, 1993). Eight to ten days after super-inoculation, the typical systemic symptoms induced by CymRSV infections became evident. Total RNA from the new emerging systemic leaf of all super-inoculated plants was extracted from 200 mg tissue samples as described (Dalmay *et al.*, 1993), separated in formaldehyde-permeated agarose gel, blotted to nylon membrane and hybridized with ³²P-labelled probes prepared by nick-translation of cDNA clones representing the 3' terminal 1000 nucleotides of CymRSV RNA using standard protocols (Sambrook *et al.*, 1989).

As shown in Table 1, resistance of 92KA1 plants to CymRSV was broken to varying extents by PVY, CMV and PVX. By contrast, CymRSV resistance was maintained in all mock inoculated 92KA1 plants. As expected, plants of line 92KB1 were totally susceptible to CymRSV when the infectious RNA transcript was super-inoculated in previously mock-inoculated plants. However, only six and seven out of ten plants of the same line were susceptible to CymRSV, if previously infected with PVY or CMV, respectively (Table 1). The lower CymRSV RNA infectivity observed in 92KB1 plants previously inoculated with PTGS suppressor viruses was unexpected, but it could be a consequence of a lower virus susceptibility of stressed plants. Indeed, in parallel experiments it was observed that the susceptibility of wild-type plants to CymRSV RNA infection also decreased when these had been previously inoculated with PVY and CMV, as compared with mock inoculated plants that were totally infected by CymRSV (Table 1).

Table 1. Results of superinfection with CymRSV of CymRSV-immune (92KA1), CymRSV-susceptible (92KB1) or wild type *N. benthamiana* plants which had been mock inoculated or rinoculated with PVY, CMV and PVX. The last column shows the number of infected/inoculated plants.

Line	Mock	PVY	CMV	PVX	CymRSV	CymRSV infected plants
92KA1	+				+	0/20
92KA1		+			+	9/30
92KA1			+		+	5/10
92KA1	+			+	+	8/14
92KB1	+				+	10/10
92KB1		+			+	6/10
92KB1			+		+	7/10
Wt	+				+	10/10
Wt		+			+	9/12
Wt			+		+	6/9

The present results show that PVY, CMV and PVX interfere with CymRSV resistance expressed by *N. benthamiana* plants of the 92KA1 line. As expected, 92KA1 plants previously inoculated with PVY were not silenced and became susceptible to infection by CymRSV. However, for unknown reasons not all of the plants were infected. A comparable number of plants were also infected when they were previously inoculated with PVX or CMV. It was shown that CMV blocks initiation of PTGS (Brigneti *et al.*, 1998) and that PVX blocks spreading of PTGS systemic signal (Voinnet *et al.*, 2000). In both cases, suppression occurs only if PTGS has not yet initiated.

Although 92KA1 plants were not analysed for the spatial pattern of HDR in leaves of different developmental stage, it is conceivable, as reported for other systems (Jorgensen, 1995; Palauqui *et al.*, 1996; Kunz *et al.*, 1996, 2001), that in these plants the leaves above those inoculated with CMV or PVX had not yet developed PTGS before they are infected systemically. Further experiments are needed to confirm that PTGS, thus the efficiency of HDR in transgenic 92KA1 plants, depends on the developmental stage of the host. However, this was not in the scope of this work, which wanted to investigate if the immunity conferred to *N. benthamiana* by transformation with the full replicase of CymRSV could be broken by superinfection with any suppressor virus. Suppression of HDR induced by CMV and PVY was also observed in other systems by Mitter *et al.* (2001) and Savenkov *et al.* (2001). Interference of PVX with HDR has not been previously reported.

Our data suggest that for protecting crops by HDR it may be useful to defend them concomitantly from attacks by pathogenic viruses and by viruses possessing efficient PTGS suppressor genes. Transgenic plants expressing HDR to a specific virus of major economic importance are still susceptible to other viruses of minor economic importance. If any of these viruses encodes a PTGS suppressor protein, its spread in resistant transgenic crops could have serious consequences, as resistance to the virus of major economic importance would be broken. In principle, this problem could be overcome by engineering plants with chimeric transgenes containing sequences derived from viruses of economic importance for the crop and from potential HDR suppressors. This approach is technically possible. Recent findings show that homology-dependent multi-virus resistance can be induced in transgenic plants by a single chimeric transgene derived from distinct viruses (Jan *et al.*, 2000).

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Received 25 September 2001

Accepted 8 February 2002