Posttranscriptional Gene Silencing Does Not Play a Significant Role in *Potato virus X* Coat Protein-Mediated Resistance

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ABSTRACT

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The expression of a gene that encodes coat protein (CP) of *Potato virus X* (PVX) in transgenic tobacco plants confers a high level of CP-mediated

resistance (CP-MR) against PVX infection. To determine if posttranscriptional gene silencing (PTGS) plays a role in resistance, transgenic plants expressing PVX CP were challenged against PVX under conditions in which PTGS was suppressed by low temperatures or using viruses carrying PTGS suppressors. The data demonstrate that PTGS does not play a significant role in PVX CP-MR.

Using the concept of pathogen-derived resistance (20), many attempts have been made to generate virus resistance in transgenic plants. The first report of successful pathogen-derived resistance resulted from the expression of a gene encoding the coat protein (CP) of *Tobacco mosaic virus* (TMV); plants that produce the CP exhibited delay in virus infection and disease (1). The phenomenon was referred to as CP-mediated resistance (CP-MR) (6). CP-MR has been used to confer resistance to TMV, *Alfalfa mosaic virus* (15,26,27), *Cucumber mosaic virus* (CMV) (9), *Potato virus X* (PVX) (12,24), *Tobacco streak virus* (28), and other viruses. CP-MR cannot be explained by a single mechanism that applies to all examples. In the case of TMV CP-MR, CP interferes with the disassembly of virus particles; thus, CP-MR is overcome when plants are inoculated with TMV RNA (18). In addition, CP regulates production of the TMV movement protein (3,8). In contrast, transgenic plants expressing PVX CP are resistant to infection by both PVX and PVX RNA, showing that disruption of PVX disassembly is not a significant component of CP-MR (12). It was proposed that PVX CP either interacts with the viral origin of assembly and restricts replication or interferes with translation of the replicase gene (12,24).

Posttranscriptional gene silencing (PTGS) is part of the defense system of plants and other organisms (29,31). Virus infection, including PVX infection, can induce gene silencing (10,19). Nevertheless, Spillane et al. (24) discounted the involvement of PTGS on PVX CP-MR based on experiments (conducted in transgenic CP(+) protoplasts) that showed that CP-MR is effective against a PVX mutant that lacks the CP gene. However, since the PVX mutant used in these studies and the CP transgene shared 133 contiguous nucleotide residues (in the 3' noncoding region of

the PVX genome), it raised the possibility that PTGS was involved. At the time of the previous work, it was considered unlikely that short regions of nucleotide homology could confer strong resistance through PTGS. However, during the past 10 years it has become known that RNAs containing less than 100 nucleotide residues can confer strong resistance to virus infection (31).

In response to PTGS, viruses have evolved to carry genes that suppress PTGS (31). The first described suppressor of PTGS is the helper component proteinase (HC-Pro) from potyviruses (2). PTGS can also be suppressed by low temperatures (11,13,14, 16,25).

To determine if PTGS plays a role in PVX CP-MR, transgenic plants expressing PVX CP were challenged with PVX under conditions in which PTGS was suppressed by low temperatures (25) and by infection with *Potato virus Y* (PVY), which encodes HC-Pro (30).

MATERIALS AND METHODS

Plant material and virus inoculation. Transgenic plant line 6665 is derived from *Nicotiana tabacum* Samsun and accumulates PVX CP (12). Transgenic and nontransgenic plants were grown under standard greenhouse conditions or maintained in growth chambers at 24°C, 16-h light/8-h dark cycle. Plants infected at 15°C were grown at 24°C, and 10 days before the infection were placed in chambers at 15°C and maintained in comparable light/dark conditions.

Leaves of *N. benthamiana* infected with a mutant of PVX that produces yellow fluorescent protein (YFP) during infection, (PVX-YFP), (4) or PVY⁰ were ground with phosphate buffered saline (PBS), pH 7.2, and extracts were mechanically inoculated to 6-week-old *N. tabacum* plants. Infection by particle bombardment was carried out as described by Bazzini et al. (5). Plants infected with PVX-YFP were monitored with a stereoscopic Olympus ZX9 microscope (Olympus, Tokyo) equipped with an epifluorescent unit.

Quantification and detection of viral proteins. Total proteins were extracted from leaf tissues using PBS-Tween 20 (0.05%), and proteins were quantified using the Quick Start Bradford Protein Assay Kit (Bio-Rad, CA).

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Fig. 1. Effects of *Potato virus X* (PVX) on nontransgenic and transgenic tobacco plants. **A**, Nontransgenic plant 3 weeks after inoculation (a.i.) with buffer solution. **B**, Nontransgenic plant 3 weeks a.i. with PVY. **C**, Transgenic tobacco plant that produced PVX coat protein (CP^{PVX}) 3 weeks a.i. with PVX. **D**, Nontransgenic plant infected with PVX 3 weeks a.i. **E**, Nontransgenic and **F**, CP^{PVX} transgenic plants preinfected with *Potato virus Y* (PVY) and inoculated with PVX 10 days later. **G**, Enzyme-linked immunosorbent assay detection of PVY CP in soluble proteins extracted from transgenic and nontransgenic (NT) plants infected with PVY, PVX, or both viruses (first with PVY and 10 days later with PVX). **H**, Immune dot blot of total proteins from infected nontransgenic (NT) and CP^{PVX} transgenic plants following inoculation with PVY, PVX, or both viruses. Reactions were developed following exposure to antiserum raised against the PVX CP. **I**, Transgenic tobacco plant in which the magnesium chelatase gene is silenced (albino phenotype); noninfected plant (left panel); 3 weeks a.i. with PVY (right panel).

Accumulation of PVY and PVX was analyzed with an enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instruction (Bioreba AG, Switzerland).

For immune dot blot experiments, total proteins were blotted into a nitrocellulose membrane by applying a partial vacuum. Immune detection was performed using a rabbit polyclonal antiserum to the PVX CP, followed by reaction with alkaline phosphatase-conjugated goat anti-rabbit antibody; reactions were visualized by NBT-BCIP staining.

Quantification of transgenic PVX CP messenger RNA. Total messenger RNA (mRNA) was purified using Trizol (Invitrogen, CA) according to the manufacturer's instructions. Approximately 10 µg of RNA was subjected to electrophoresis as described by Bazzini et al. (5). Sequences from the untranslated region of the mRNA derived from the E9 3' transcription stop signal were labeled with ³²P and used as hybridization probe (12).

Fig. 2. Effects of infection with *Potato virus X* (PVX)-yellow fluorescent protein (YFP). **A**, Nontransgenic (NT) and transgenic plants producing PVX coat protein (CP^{PVX}) 12 days a.i. with a PVX construct expressing free YFP (PVX-YFP). Following inoculation, plants were held at 24 or 15°C and leaves were photographed under UV illumination. **B**, Leaves of nontransgenic and CP^{PVX} plants infected with PVX at 24 and 15°C, and photographed under white light.

RESULTS AND DISCUSSION

Reducing PTGS through virus-mediated suppression of silencing. It is known that plants doubly infected with PVX and PVY develop a synergistic disease that is distinguished initially by severe vein-clearing and subsequent necrosis of the first systemic leaf, accompanied by 3- to 10-fold increase in PVX accumulation compared with plants infected with only PVX (17). HC-Pro produced during PVY replication plays a key function in this synergistic reaction (22) by blocking the host PTGS that limits PVX infection (2). A previous report demonstrated that PVY can suppress CP-mediated PTGS resistance (21). To investigate the role of PTGS on PVX CP-MR, we preinfected transgenic plants with PVY to suppress PTGS prior to inoculation with PVX.

Transgenic tobacco plants that produce PVX CP and exhibit CP-MR, and nontransgenic plants were inoculated with PVY alone, PVX alone, or first with PVY and 10 days later with PVX. Transgenic and nontransgenic plants infected by PVY displayed typical mild yellow mosaic patterns and dark green vein-banding by 3 weeks after inoculation (Fig. 1B). In agreement with previous reports (12,24), transgenic plants that contain PVX CP were highly resistant to PVX (Fig. 1H) and did not show disease symptoms (Fig. 1C). Nontransgenic plants showed characteristic PVX symptoms (Fig. 1D) and accumulated high levels of PVX CP at 3 weeks postinfection (Fig. 1H). Nontransgenic plants that

Fig. 3. Effects of *Potato virus X* (PVX) infection on accumulation of transgenic PVX coat protein (CP) messenger RNA. **A**, Northern blot of nontransgenic (NT) and transgenic PVX CP plants inoculated or not with PVX. The 3' nontranslated sequence of the transgenic construct was used as a probe. **B**, Enzyme-linked immunosorbent assay detecting PVX CP in soluble protein extracted from transgenic and NT plants 10 days postinoculation with PVX.

were first infected with PVY followed by PVX showed classic severe mosaic disease symptoms, evidence of PVX-PVY synergism (Fig. 1E), and accumulated higher levels of PVX CP compared with singly infected plants (Fig. 1H) (17). However, transgenic plants infected with both viruses did not show severe disease symptoms (Fig. 1F) and did not accumulate detectable PVX CP (Fig. 1H). Furthermore, these transgenic plants presented symptoms of PVY comparable to those of plants infected by a single virus (transgenic and nontransgenic) (Fig. 1F similar to Fig. 1B) and accumulated similar amounts of PVY CP (Fig. 1G).

To verify PTGS suppression by PVY infection, transgenic tobacco plants in which the magnesium chelatase gene is silenced were infected with PVY. Three weeks after infection, the albino phenotype of the transgenic line (provided by C. Taylor, Donald Danforth Plant Science Center) was partially restored to green pigmentation, confirming that the PVY isolate used in these experiments is capable of suppressing PTGS (Fig. 1I).

The results of these studies indicate that PTGS does not play an important role in PVX CP-MR in this plant line.

Suppressing PTGS by low temperature. Szittyta et al. (25) demonstrated that low temperature inhibits PTGS and showed that gene silencing phenotypes can be lost under low temperatures. For example, PTGS-mediated resistance to *Cymbidium ringspot virus* is highly effective at 24°C, but plants are susceptible to infection at 15°C. Similar results were shown in transgenic plants silenced for infection by CMV (13). These studies demonstrated that at low temperatures the levels of virus-derived or transgene-derived small RNAs (sRNA) are dramatically reduced compared with higher temperatures (25); other authors demonstrated that in the particular case of silencing mediated by antisense, RNA is not always sensitive to low temperature (23). PTGS suppression by low temperature has been reported in *Arabidopsis thaliana*, *N. benthamiana*, *N. tabacum*, and *Solanum tuberosa* as well as in insect and mammalian cells (11,13,14,16,25).

We tested the resistance of transgenic plants that produce PVX CP at 15 and 24°C. We reasoned that if PTGS plays an important role in PVX CP-MR, these plants should be susceptible to infection by PVX at low temperatures. In this experiment, plants were held for 10 days at 15 or 24°C before they were inoculated with PVX-YFP (4). In this study, plants were inoculated by particle bombardment with cDNA of the virus under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (4). Infection was monitored using a dissecting fluorescence microscope. As shown in Figure 2A, plants that produce PVX CP are resistant to PVX-YFP at 15 or 24°C, i.e., they produce very few, small sites of infection following inoculation. In contrast, infection of non-

transgenic plants results in production of a high number of sites of infection (shown as fluorescent areas) at both temperatures. The data show that PVX CP-MR is not temperature dependent and support the conclusion that resistance is not dependent on PTGS.

Figure 2B shows leaves of inoculated plants photographed under white light. As expected, transgenic plants did not show symptoms of PVX infection at either temperature. Nontransgenic plants infected at 15°C presented stronger PVX disease symptoms than plants held at 24°C (Fig. 2B). We take this to indicate that virus infection induced gene silencing in these plants at 24°C but not at 15°C (25).

PVX infection does not reduce amount of transgenic mRNA. To determine if the transgene is silenced following infection, the amount of CP mRNA derived from the transgene was analyzed by northern blot using sequences from the 3' end as a probe. As shown in Figure 3, the level of PVX CP mRNA in the inoculated leaf was not changed by 10 days postinoculation with PVX, suggesting that CP-MR does not involve PTGS. It is possible, however, that PTGS effects could have been masked by low sensitivity of this assay. Nevertheless, this experiment indicates that virus infection does not reduce the accumulation of transgene mRNA.

Although the mechanism(s) that confer PVX CP-MR are unclear, this study demonstrates that PTGS is not significantly involved in CP-MR. PVX CP can elicit expression of Rx genes in some potato cultivars (7), and it is formally possible that transgenic expression of PVX CP in tobacco induces an undefined component of a host response that is not detected in nontransgenic plants. To date, there is no experimental support for this hypothesis. Based on the data presented here, we conclude that PTGS does not play a significant role in CP-MR to PVX. Furthermore, these studies indicate that CP-MR will be a useful tool to confer resistance to crops under environmental conditions in which PTGS may be suppressed.

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