Tobacco mosaic virus (TMV) and potato virus X (PVX) coat proteins confer heterologous interference to PVX and TMV infection, respectively

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Replication of Potato virus X (PVX) was reduced in transgenic protoplasts that accumulated wild-type coat protein (CPWT) of Tobacco mosaic virus (TMV) or a mutant CP, CP T42W, that produced highly ordered states of aggregation, including pseudovirions. This reaction is referred to as heterologous CP-mediated resistance. However, protoplasts expressing a CP mutant that abolished aggregation and did not produce pseudovirions, CP T28W, did not reduce PVX replication. Similarly, in transgenic tobacco plants producing TMV CP WT or CP T42W, there was a delay in local cell-to-cell spread of PVX infection that was not observed in CP T28W plants or in non-transgenic plants. The results suggest that the quaternary structure of the TMV CP regulates the mechanism(s) of heterologous CP-mediated resistance. Similarly, transgenic protoplasts that produced PVX CP conferred transient protection against infection by TMV RNA. Transgenic plants that accumulated PVX CP reduced the cell-to-cell spread of infection and resulted in a delay in systemic infection following inoculation with TMV or TMV RNA. Heterologous CP-mediated resistance was characterized by a brief delay in systemic infection, whilst homologous CP-mediated resistance conferred reduced or no systemic infection.

INTRODUCTION

In 1986, it was shown that transgenic plants that accumulate the coat protein (CP) of Tobacco mosaic virus (TMV) are resistant to TMV infection (Abel et al., 1986), a reaction referred to as CP-mediated resistance (CP-MR) (Beachy, 1999). Subsequently, CP-MR against Alfalfa mosaic virus (AMV) (Loesch-Fries et al., 1987; van Dun et al., 1987, 1988), Cucumber mosaic virus (CMV) (Cuozzo et al., 1988), Potato virus X (PVX) (Hemenway et al., 1988; Spillane et al., 1997), Tobacco streak virus (van Dun et al., 1988) and other viruses was reported. In each example, plants were resistant to the virus from which the CP gene was taken and to closely related strains. The available data suggest that several CP-MR mechanisms exist. For example, plants that contain the CP of TMV are resistant to TMV, but not to TMV RNA; subsequent studies support the hypothesis that the CP interferes with disassembly of the challenge virus (Register & Beachy, 1988; Bendahmane & Beachy, 1999). Plants that contain PVX CP are resistant to PVX, as well as to PVX RNA. Hemenway et al. (1988) and Spillane et al. (1997) proposed that PVX CP interacts with the origin of assembly and restricts replication, or interferes with translation of the replicase gene. In other cases, resistance due to expression of CP sequences in transgenic plants is mediated by the RNA transcript rather than by the CP per se (Bendahmane & Beachy, 1999).

TMV and PVX contain positive-sense RNA molecules of a similar size. PVX is a flexuous rod-shaped particle, whilst TMV is a rigid rod. PVX RNA contains five open reading frames (ORFs) (see Fig. 2a) (Batten et al., 2003) that encode the replicase, triple-gene block (TGB) proteins and CP. TGBp1 induces plasmodesmata gating (Howard et al., 2004). TGBp2 and TGBp3 are associated with the endoplasmic reticulum network and are required for virus movement (Krishnamurthy et al., 2003; Mitra et al., 2003). TMV encodes replicase proteins, a 30 kDa movement protein (MP) and a CP; recent studies have shown that the CP can regulate the production of MP and may thereby regulate cell-to-cell spread of infection (Bendahmane et al., 2002; Asurmendi et al., 2004; Kawakami et al., 2004).

In CP-MR against tobamoviruses, resistance is effective against virus strains that have significant similarity to the transgenic CP (Nejidat & Beachy, 1990). Nevertheless, Anderson et al. (1989) demonstrated that transgenic tobacco plants that contained wild-type TMV CP (CPWT) exhibited a low level of resistance to, or interfered with, systemic spread and disease development when infected with
heterologous viruses, including AMV, CMV, PVX or Potato virus Y (PVY) (Anderson et al., 1989). The nature of 'heterologous resistance' was not determined in these studies.

Bendahmane et al. (1997) reported a strong correlation between assembly mutants of the TMV CP and resistance to TMV. Mutant CP^T42W, in which residue 42 (threonine, T) was mutated to tryptophan (W), exhibited increased protein aggregation compared with CP^WT and produced virus-like particles (VLPs), but not infectious virophils. Plants expressing CP^T42W showed higher levels of CP-MR than transgenic plants containing CP^WT following challenge infection by TMV and TMV RNA or infection by grafting. In contrast, mutant CP^T28W, which does not form VLPs, did not confer protection against TMV in transgenic plants (Bendahmane et al., 1997). In a subsequent study, Bendahmane et al. (2002) showed that CP^T42W reduced the production of MP and effectively reduced cell-to-cell spread of infection.

Asurmendi et al. (2004) proposed a regulatory role for the CP in establishing virus replication complexes. In that study, it was shown that mutant CPs could affect the structure of virus replication complexes, which apparently changed the efficiency of virus replication and resulted in restriction of cell-to-cell movement and spread of the infection.

In this study, we examined the role of the CPs of TMV and PVX on infection of the heterologous virus in transgenic protoplasts and whole plants. Our data indicate that the CP reduces the rate of heterologous virus replication and production of viral proteins, resulting in a delay in cell-to-cell spread of infection. However, the effect was temporary and did not confer strong resistance to disease.

**METHODS**

**Plant lines and BY-2 cell line.** The following transgenic lines were used: Nicotiana tabacum cv. Xanthi-nn that accumulates TMV CP^WT (line 3646; Abel et al., 1986), CP^T42W (line nn-T42W; Bendahmane et al., 1997), CP^T28W (line nn-T28W; Bendahmane et al., 1997) and cp42w-mp*, a homozygous plant line resulting from a genetic cross between line CP^T42W and line 277. Plant line 277 produces the TMV MP (Deom et al., 1990). The transgenic line 6665 is N. tabacum cv. Samsun-nn, which accumulates the CP of PVX (Hemenway et al., 1988). All transgenic and non-transgenic plants were grown under standard greenhouse conditions or maintained in growth chambers at 24–26 °C, with a 14 h light/10 h dark cycle. Transgenic Bright Yellow-2 tobacco (BY-2) cell lines that produced CP^WT, CP^T42W or CP^T28W (Bendahmane et al., 2002) and non-transgenic BY-2 cells were grown at 28 °C in the dark.

**In vitro transcription and inoculation.** Cloned DNAs from TMV or PVX infectious viral RNAs were linearized with KpnI or SpeI, respectively, and transcribed in vitro with a MEGAscript Transcript kit (Ambion). Reactions were supplemented with m^3G(5)'ppp(5)'G cap analogue (Ambion). Transcripts were inoculated onto 6-week-old plants as described previously (Bendahmane et al., 1997). Tobacco protoplasts were prepared from BY-2 suspension cell cultures as described by Watanabe et al. (1982) or, in the case of plant line 6665, from leaf tissues as described by Otsuki et al. (1972). Protoplasts were inoculated with RNA transcripts via electroporation (Watanabe et al., 1987). A PVX–YFP clone, encoding the PVX genome under the control of the 35S promoter with the yellow fluorescent protein (YFP) gene cloned under the control of the duplicated subgenomic CP promoter (see Fig. 2b), was blasted into the plant leaf.

Particle bombardment was carried out with a PDS-1000/He System gene gun (Bio-Rad). Approximately 3 µg plasmid DNA or RNA was coated onto gold particles and resuspended in 95% ethanol by sonication. Coated particles were deposited onto plastic filter holder screens (Bio-Rad) and accelerated on to plant leaves with a 450 ms pulse of helium (450 p.s.i.). Plants infected with virus that produced fluorescent proteins during replication were monitored with a stereoscopic Olympus ZX9 microscope equipped with an epifluorescent unit.

**Quantification of viral proteins.** Total proteins extracted from protoplasts and leaf tissues by using PBS (pH 7-2) were quantified with a Quick Start Bradford Protein Assay kit (Bio-Rad). Protein extracts were used to coat ELISA plates and antiserum against TMV CP was used as the primary antibody. Goat anti-rabbit antibody conjugated to horseradish peroxidase was used as the secondary antibody and positive reactions were detected by using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). To detect PVX CP, sheep anti-PVX antibody was coated onto the ELISA plate, after which tissue extracts were incubated in the plate for 2 h. Rabbit anti-PVX CP secondary antibody and conjugated goat anti-rabbit antibody were used as described above.

For Western immunoblot assays, total protein extracts were loaded onto 12% polyacrylamide gels containing SDS and, following electrophoresis, proteins were electroblotted onto nitrocellulose membranes. Immunodetection was performed by using a rabbit polyclonal anti-serum to the TMV CP, followed by alkaline phosphatase-conjugated goat anti-rabbit antibody, and visualized by NBT/BCIP staining. Ponceau S red solution was used to confirm that similar amounts of protein from each sample were loaded on the gel.

**Accumulation of viral RNAs.** Total RNA was purified from 5 x 10^5 BY-2 cells by using an RNeasy Plant Mini kit (Qiagen). Approximately 5 µg RNA was subjected to electrophoresis in 1-6% agarose containing 6% formaldehyde. Following electrophoresis, RNA was blotted onto Hybond-XL membrane (Amersham Biosciences). A DNA fragment encoding the TGB and CP sequences (nt 5427–5796) of the PVX genome was labelled with ^32P and used as a probe. RNAs were quantified by using a Typhoon Imager (Amer sham Biosciences) and normalized based on the amount of rRNA on the gel.

**RESULTS**

**Transgenic TMV CPs reduce the replication of PVX**

Previous studies have shown that the spread of infection by PVX, PVY, CMV and AMV is reduced in transgenic plants that produce TMV CP (Anderson et al., 1989). To explore the mechanism responsible for this effect, we initiated studies in non-transgenic and transgenic BY-2 protoplasts that accumulated TMV CP^WT, CP^T42W and CP^T28W. Western immunoblot assays confirmed that, as reported previously, the transgenic plant lines accumulated similar amounts of transgenic CP. Similarly, the transgenic BY-2 cell lines accumulated similar amounts of transgenic CP (Fig. 1). Protoplasts were inoculated with PVX RNA (see
The accumulation of PVX CP was measured by Western immunoblot assays following inoculation of protoplasts from transgenic BY-CP\textsuperscript{T42W} cells and non-transgenic BY-2 cells. As shown in Fig. 3(a), accumulation of PVX CP was detected at 11 h p.i. in non-transgenic protoplasts and continued to accumulate up to more than 24 h p.i. In contrast, accumulation of PVX CP in BY-CP\textsuperscript{T42W} protoplasts was first detected at 16 h p.i. and increased up to 36 h p.i.

To confirm these results in quantitative experiments, transgenic protoplasts that contained CP\textsuperscript{WT}, CP\textsuperscript{T42W} or CP\textsuperscript{T28W} were inoculated with PVX RNA and samples were collected at different time points up to 36 h p.i. and subjected to ELISA. As shown in Fig. 3(b), the accumulation of PVX CP was delayed and was lower in transgenic BY-2 cells that produced CP\textsuperscript{WT} or CP\textsuperscript{T42W} compared with protoplasts that accumulated CP\textsuperscript{T28W} or non-transgenic protoplasts.

To determine the effect of TMV CP on PVX replication, Northern blot assays were performed using RNAs from infected protoplasts collected at 0, 10, 15 and 24 h after inoculation (Fig. 4a). PVX RNAs were detected by using a
32P-labelled probe (see probe in Fig. 2) and quantified using a Typhoon Imager. The accumulation of genomic and subgenomic PVX RNAs was lower in transgenic BY-CPT42W and BY-CPWT protoplasts than in transgenic BY-CPT28W and non-transgenic protoplasts (Fig. 4b–d). Therefore, the reduction in CP accumulation was assumed to be a consequence of reduced virus replication.

These results indicated a strong positive correlation between the capacity of TMV CP to produce VLPs and a reduction in PVX replication (compare replication in protoplasts that contain CPWT, CPT42W with protoplasts containing CPT28W), indicating that the quaternary structure of TMV CP may be involved in heterologous CP-MR.

Transgenic TMV CPs delay cell-to-cell movement of PVX infection

Based on the finding that TMV CP reduced PVX replication, we predicted that the CP would reduce the local spread of PVX in transgenic plants. Transgenic tobacco plant lines that accumulated equivalent amounts of CPWT, CPT42W or CPT28W (Fig. 1; Bendahmane et al., 1997) were inoculated with a variant of PVX that produced YFP (PVY–YFP) during replication (see Fig. 2b for construct) (Chapman et al., 1992). Plants were either inoculated with purified PVX or bombarded with an infectious cDNA clone of PVX–YFP to simulate RNA inoculation. At 4 days p.i., the diameters of PVX infection sites were measured with a dissecting fluorescence microscope under blue light and compared with each other. No difference was detected between the two methods of inoculation, indicating that resistance was not a consequence of interference with virus disassembly per se (Fig. 5). In plants that contained CPWT and CPT42W, the size of the infection sites was significantly smaller and the intensity of fluorescence was lower than in non-transgenic plants (Fig. 5), suggesting that CPWT and CPT42W interfered with virus replication and with cell-to-cell movement of the virus. However, CPT28W did not reduce cell-to-cell spread of infection or the intensity of fluorescence (Fig. 5).

As an additional control in this experiment, we used a plant line named cp42w-mp* (S. Asurmendi, unpublished data). This line resulted from a genetic cross between line CPT42W (Bendahmane et al., 1997) and transgenic tobacco plant line 277; the latter accumulates TMV MP (Deom et al., 1990). The presence of both transgenes was confirmed in cp42w-mp* by DNA analysis; however, these plants did not accumulate MP or CP mRNA or protein due to post-transcriptional gene silencing (unpublished data). In plant line cp42w-mp*, the size of the PVX infection sites was comparable to the size of infection sites in non-transgenic plants (Fig. 5). These data confirmed the conclusion that restriction of PVX infection was the result of accumulation of TMV CP in plant lines that accumulated CPWT and CPT42W and was not due to other non-specific effects of the transgenic plant lines.

Although plant lines that contained CPWT and CPT42W caused a delay in cell-to-cell spread of PVX, all of the plants became infected equally (as determined by accumulation of PVX) by 15 days p.i. (data not shown). As previously
reported, transgenic plants that produced PVX CP exhibited homologous CP-MR to PVX (Hemenway et al., 1988) and resulted in low numbers of sites of infection (data not shown). Infection sites that developed remained small in diameter throughout the study (Fig. 5) and virus did not accumulate in the upper leaves during the period of the experiment (data not shown).

PVX CP restricts infection by TMV in protoplasts and in plants

To determine whether there was a reciprocal effect with PVX CP, protoplasts were isolated from leaves of non-transgenic plants and transgenic tobacco plants that produced PVX CP. Protoplasts were inoculated with TMV strain U1 RNA (see Fig. 2c for construct) and the amount of TMV CP was monitored throughout infection by using ELISA. We observed a significant reduction in the amount of TMV CP in transgenic protoplasts compared with non-transgenic protoplasts up to 36 h p.i. (Fig. 6).

To investigate the effect of PVX CP on TMV cell-to-cell spread in transgenic tobacco plants that accumulated PVX CP, transgenic and non-transgenic plants were inoculated by particle bombardment with RNA transcripts of an infectious clone encoding TMV-CP–GFP (see Fig. 2d for construct). This construct produced TMV replicase, MP and a CP–GFP fusion protein. Infection sites were measured at 5 days p.i. (Fig. 7). Transgenic plants infected with TMV-CP–GFP produced smaller infection sites than non-transgenic plants, indicating that PVX CP delayed the cell-to-cell spread of TMV infection. These results were in agreement with the protoplast studies, indicating that PVX CP reduced accumulation of TMV (Fig. 6), presumably as a consequence of reduced virus replication.

To determine whether PVX CP delayed the systemic spread of TMV infection, transgenic plants expressing PVX CP and non-transgenic plants were inoculated with TMV or TMV RNA (using transcripts from cloned cDNA). The accumulation of TMV CP in upper uninoculated leaves was quantified by ELISA. At 15 days p.i., transgenic plants that had been

Fig. 5. Size of PVX infection sites on leaves of transgenic and non-transgenic tobacco plants. The diameter (mm) of PVX infection sites was measured under blue light at 4 days p.i. with DNA (open bars) or virions (filled bars). TMV CP WT, transgenic plants expressing TMV CP<sup>WT</sup>; CPT42W, plant line expressing mutant CP<sup>T42W</sup>; CPT28W, plant line expressing mutant CP<sup>T28W</sup>; cp42w-mp<sup>a</sup>, cross between the CP<sup>T42W</sup> plant and a transgenic tobacco plant expressing the TMV MP (note that both transgenes were silenced spontaneously); CP PVX, plant line expressing PVX CP. A statistically significant difference in mean lesion size compared with non-transgenic line (t, 0.05 confidence level with SD) is indicated by (a).

Fig. 6. Accumulation of TMV CP in the presence and absence of PVX CP. Protoplasts from non-transgenic (■) and transgenic tobacco leaves expressing PVX CP (○) were inoculated with TMV RNA. ELISAs were conducted on protoplasts collected at various times after infection. Values represent the mean ± SD of three independent assays.

Fig. 7. Size of TMV infection sites at 5 days p.i., observed by fluorescence microscopy. Infectious transcripts of a TMV construct that produced the fusion protein CP–GFP were inoculated into non-transgenic and transgenic tobacco plants that produced PVX CP. Results are shown as mean diameter ± SD.
inoculated with either TMV or TMV RNA had accumulated approximately half of the level of virus compared with non-transgenic plants. However, by 20 days p.i., all plants had accumulated equivalent levels of TMV (Fig. 8) and exhibited equivalent disease symptoms. There were no differences between plants that were inoculated with TMV RNA or virions, indicating that the mechanism of resistance in these plants is different from that in transgenic plants that exhibit CP-MR against TMV (Abel et al., 1986).

**DISCUSSION**

It has been reported previously that transgenic tobacco plants that accumulate TMV CP delay infection by PVX and a small number of other viruses, a phenomenon referred to as heterologous CP-MR (Anderson et al., 1989). In the present study, we demonstrated that TMV CP reduced PVX replication and cell-to-cell spread of infection; the latter effect was presumably a consequence of reduced virus replication. Moreover, no differences in heterologous CP-MR were found when plants were challenged with PVX RNA or PVX, indicating that resistance was not a consequence of interference with virus disassembly per se (Fig. 5). This is in contrast to homologous CP-MR against TMV (Register & Beachy, 1988). Similarly, transgenic plants that contained PVX CP reduced virus accumulation and cell-to-cell spread following infection with TMV or TMV RNA. The results suggest that the mechanism of heterologous CP-MR conferred by these CPs is general. Culver (1996) reported that pre-infecting plants with PVX caused a brief delay in the appearance of necrotic local lesions after infection with TMV; it is possible that the results of Culver’s study and those reported herein are the result of similar mechanisms.

Based on the data presented here, we suggest that interference by TMV CP with replication and cell-to-cell spread of PVX is affected by the quaternary structure of the TMV CP. CPWT and CP T42W, which produce ordered aggregates of CP, including VLPs with sedimentation coefficients of >4S (Bendahmane et al., 1997), interfere with PVX infection in transgenic BY-2 cells and transgenic plants. In contrast, CP T28W, which does not form VLPs and produces aggregates of CP of <4S (similar to A protein; Bendahmane et al., 1997; Klug, 1999), does not interfere with PVX infection in either transgenic BY-2 cells or transgenic plants.

It is important to point out that whilst CPWT and CP T42W produce aggregates of CP and VLPs, the VLPs are not identical to each other; nevertheless, each protein confers comparable levels of heterologous CP-MR. In contrast, CP T28W confers higher levels of homologous CP-MR than CPWT (Bendahmane et al., 1997, 2002; Asurmendi et al., 2004). Heterologous CP-MP is a relatively weak reaction compared with homologous CP-MR. CP T28W did not confer heterologous CP-MR (Figs 3, 4 and 5) or homologous CP-MR (Bendahmane et al., 1997).

The plant line cp42w-mp*, which contained the CP T42W gene but did not accumulate CP due to gene silencing, did not exhibit heterologous CP-MR. Results of experiments with this plant line, in combination with the results described above, support the conclusion that the negative effects on virus infection and replication result from accumulating CPs and not as a result of the site of T-DNA insertion or other effects.

The strong positive correlation between structure and function of the CP of TMV in BY-2 cells and plants led to the hypothesis that the formation of quaternary structures of TMV CP is important for heterologous CP-MP against PVX, as well as homologous CP-MR against TMV (Bendahmane et al., 1997; Lu et al., 1998). This may indicate that certain aspects of resistance are shared between homologous and heterologous CP-MR. However, this remains to be confirmed by other studies.

We propose three possible models that may act together or independently to restrict PVX replication. In the first model, TMV CP interacts with PVX to sequester viral RNA using a primary or secondary origin of assembly and restricts availability of viral RNA for replication. Hemenway et al. (1988) and Spillane et al. (1997) suggested that this mechanism might account for homologous CP-MR against PVX. It is well known that TMV CP can encapsidate heterologous RNAs (Fritsch et al., 1973; Sleat et al., 1988).

The second model proposes that transgenic CP interferes with a putative ‘internal receptor’ for virus infection and replication that is used by both TMV and PVX and thereby restricts infection or replication. It is known that replication of TMV and PVX is performed on cellular membranes (Reichel & Beachy, 1998; Más & Beachy, 1999; Carette et al.,...
2000; Asurmenidi et al., 2004). We suggest that plant viruses may use one or more common ‘receptor-like mechanisms’ to establish sites of replication. If such a ‘receptor’ is already occupied by the transgenic CP, the virus may not establish sites of replication or sites may be less productive than in the absence of CP. As heterologous CP-MR is not as effective as homologous CP-MR, this may indicate that the putative receptor has a degree of specificity or that the receptor can be in competition during virus replication. This may explain the lack of complete resistance in heterologous CP-MR. It may also indicate that there are several steps or stages in homologous CP-MR that are not achieved in heterologous CP-MR.

The third model proposes that heterologous CP-MR is the result of a response triggered by interactions between the transgenic CP and components of a host response to the CP. In this model, larger aggregates of CP, but not monomeric or smaller aggregates of CP, activate gene expression or existing (basal/innate) defence mechanisms to confer heterologous CP-MR. Although the trigger of a putative response mechanism may be virus-specific, the response itself apparently is not specific. We reported previously that transgenic plants that contain TMV CP and accumulate VLPs do not exhibit elevated levels of common pathogenesis-related proteins (Carr et al., 1989).

Our studies demonstrate that expression of the CP of TMV and PVX interferes with infection of the heterologous virus, including replication, cell-to-cell movement and spread. A more complete understanding of this phenomenon may lead to applications in biotechnology that result in the design of proteins conferring broad resistance to infection by multiple plant viruses.

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