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Tobacco Mosaic Virus Replicase-Mediated Cross-Protection: Contributions of RNA and Protein-Derived Mechanisms

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Specific sequences of the tobacco mosaic virus (TMV) RNA-dependent RNA-polymerase (RdRp) gene were investigated for their ability to confer cross-protection. Nine overlapping segments ranging from 713 to 1070 nucleotides in length and covering the methyltransferase, helicase, and polymerase (POL) domains of the TMV RdRp open reading frame were systemically expressed in *Nicotiana benthamiana* using a potato X virus (PVX) vector [Chapman, S., Kavanagh, T., and Baulcombe, D. C. (1992). *Plant J.,* 1, 549–557]. PVX-infected plants were subsequently challenge inoculated with 10 μg of wild-type TMV and monitored for TMV accumulation. Mock inoculated plants and plants preinfected with the unmodified PVX vector rapidly accumulated high levels of challenge virus. In contrast, plants preinfected with PVX vectors expressing segments of the TMV RdRp open reading frame displayed either high or low levels of protection. High protection levels were observed for PVX constructs expressing segments of the TMV POL domain, whereas low protection levels were observed for PVX constructs expressing segments covering the methyltransferase and helicase domains. Frameshift mutations that blocked protein expression from RdRp segments disrupted only the high levels of protection derived from POL segments and not the low levels derived from the other segments. However, all RdRp segments conferred similarly high levels of protection against a TMV construct with restricted local movement. Thus both RNA and protein sequences in conjunction with the speed of the infecting challenge virus can affect the protection derived from the TMV RdRp gene.

INTRODUCTION

Cross-protection, which is defined as the ability of one virus to inhibit or prevent infection by a second virus, was first observed by McKinney (1929). Since this description, numerous studies have sought to provide understanding of the mechanisms responsible for this phenomenon as well as to develop its use for field applications (Sherwood, 1987). During the past two decades, transgenic and viral vector technologies have permitted the exploration of this phenomenon at the molecular level. From these studies, several different viral and host processes have been implicated in conferring protection. In general, cross-protection derived from specific viral sequences have been attributed to RNA- or protein-based mechanisms. However, the collective contributions of these different mechanisms to the observed protection have not been fully explored.

Several lines of evidence indicate that RNA-based protection is derived from a nucleotide sequence-specific host defense mechanism, termed posttranscriptional gene silencing (PTGS), that targets viral RNAs for destruction (Dougherty and Parks, 1995; Baulcombe, 1999). For example, Hamilton and Baulcombe (1999) identified PTGS-associated RNA molecules that were complementary to viral RNAs and accumulate only in the presence of viral replication. In addition, Ratcliff *et al.* (1999) used viral vectors to demonstrate that PTGS can be activated to induce cross-protection by a diverse group of RNA viruses. Furthermore, viral proteins, such as the potyvirus HC-pro, act as suppressors of PTGS in transgenic plants (Anandalakshmi *et al.,* 1998; Beclin *et al.,* 1998; Brigneti *et al.,* 1998; Kasschau and Carrington, 1998). Thus viruses have evolved specific countermeasures to suppress plant-derived PTGS. Taken together, these findings demonstrate that PTGS is a common plant defense response active against diverse viruses and likely to play an important role in cross-protection.

Cross-protection derived from specific viral proteins has also been demonstrated. One well-studied system involves the ability of the tobacco mosaic virus (TMV) coat protein to mediate cross-protection. Evidence for the role of coat protein in conferring protection comes from several sources (Bendahmane and Beachy, 1998). In particular, the ability of unencapsidated viral RNA to overcome the protection led Sherwood and Fulton (1982) to speculate that cross-protection between TMV strains was the result of the protecting virus blocking the disassembly of the challenge virus. Subsequent TMV studies have confirmed that coat protein-derived protection is dependent on the ability of coat protein to properly as-

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sociate with and block the disassembly of the challenge virus (Bendahmane *et al.,* 1997 and Lu *et al.,* 1998). Other forms of protein-derived protection that utilize defective viral gene products have also been described. For instance, the transgenic expression of a defective TMV 30-kDa cell-to-cell movement protein has been shown to interfere in the movement of a broad spectrum of viruses (Cooper *et al.,* 1995). Thus it is clear from these examples that the preexpression of viral proteins also plays an important role in cross-protection.

The TMV RNA-dependent RNA polymerase (RdRp) gene encodes a 126-kDa protein that is terminated by a single amber stop codon. Readthrough of this termination codon results in the production of a larger 183-kDa protein (Pelham, 1978). In addition, a subgenomic mRNA derived from the readthrough portion of the 183-kDa protein and encoding a putative 54-kDa protein has also been observed (Sulzinski *et al.,* 1985). However, this protein has not been detected *in vivo* (Sulzinski *et al.,* 1985; Golemboski *et al.,* 1990). Homology comparisons reveal the presence of methyltransferase (MT) and helicase (HEL) domains within the 126-kDa protein and a polymerase (POL) domain within the readthrough, 54-kDa portion of the 183-kDa protein (Koonin, 1991; Koonin and Dolja, 1993; Buck, 1996).

The ability of a viral RdRp gene to mediate protection was first described by Golemboski *et al.* (1990) in plants transformed with the 54-kDa POL domain of the TMV 183-kDa protein. Subsequent studies have produced conflicting reports regarding the mechanism(s) behind this resistance. For example, transgenic plants that display silencing typically accumulate little transgene transcript. However, resistant 54-kDa plants accumulated significant levels of the transgene transcript. In addition, Carr *et al.* (1992) demonstrated that a nontranslatable 54-kDa open reading frame (ORF) failed to confer protection in a transient protoplast assay. These lines of evidence suggest a protein-mediated mechanism is involved in conferring resistance. However, Marano and Baulcombe (1998) demonstrated that 54-kDa transgenic plants were resistant against a potato virus X (PVX) vector designed to express TMV 54-kDa gene sequences as small as 383 nucleotides. This finding is consistent with an RNA-based PTGS mechanism. Related studies using the 54-kDa ORF of another tobamovirus, pepper mild mottle virus, also indicated that resistance in this system was derived from an RNA-based PTGS mechanism (Tenllado *et al.,* 1996). In addition, other portions of the TMV RdRp gene have been found to confer protection when expressed as transgenes. Most notably, Donson *et al.* (1993) produced transgenic plants containing a 183-kDa RdRp gene disrupted by the insertion of a bacterial transposon. Plants containing this gene displayed resistance against several different tobamoviruses in a non-homology-dependent fashion. This lack of specificity suggests the conferred resistance was not derived from a PTGS mechanism. Collectively, these findings suggest that multiple mechanisms may contribute to the resistance conferred by TMV RdRp domains.

To further dissect the role of the TMV RdRp in cross-protection, a PVX vector (Chapman *et al.,* 1992) was used to individually express nine overlapping segments comprising the entire TMV 183-kDa RdRp ORF. These plants were subsequently challenged with TMV and monitored for virus accumulation. Results demonstrated that all nine RdRp segments conferred low levels of protection that were independent of protein expression. However, three segments covering the POL domain conferred significantly higher levels of protection that were dependent on protein expression. In contrast, no distinctions in the levels of protection conferred by either RNA or protein were observed when plants were challenge inoculated with a TMV virus deficient in local movement. Thus the effectiveness of the protection conferred by either RdRp RNA or protein sequences is greatly influenced by the speed of challenge virus infection.

RESULTS

Construction and expression of TMV RdRp segments

For this study, nine overlapping RdRp segments ranging from 713 to 1070 nucleotides in length and covering the entire 183-kDa TMV ORF were individually cloned into the viral vector PVX2C2S (Fig. 1). Each segment was engineered to contain its own translational start and stop codons to permit protein expression from the PVX2C2S vector. PVX2C2S constructs containing the different TMV RdRp sequences produced mild mosaic symptoms in *Nicotiana benthamiana* that were similar in appearance to those produced by the unmodified PVX vector. Maintenance of each TMV RdRp segment within the PVX vector was confirmed by RT–PCR analysis of viral RNA isolated from systemically infected tissue. PCR analysis using TMV segment-specific primers indicated that each RdRp segment was maintained within the PVX vector (Fig. 2A). In addition, PCR analysis using PVX-specific primers that flank the insert also confirmed the maintenance of the TMV RdRp segments (data not shown). However, on occasion several faint smaller bands were also observed, indicating that a small proportion of the PVX population had deleted all or part of the TMV RdRp insert. To prevent the buildup of PVX populations that lack TMV RdRp sequences, only transcript RNA derived from the cDNA clones was used as inoculum in this study.

Hexa-histidine tags were engineered at the amino-terminus of each TMV RdRp segment to allow protein expression from PVX vector constructs to be monitored. Western immunoblot analysis demonstrated that segments 1–5, 7, and 8 accumulated detectable levels of protein in systemically infected leaf tissue (Fig. 2B). In contrast, this method failed to detect protein from seg-

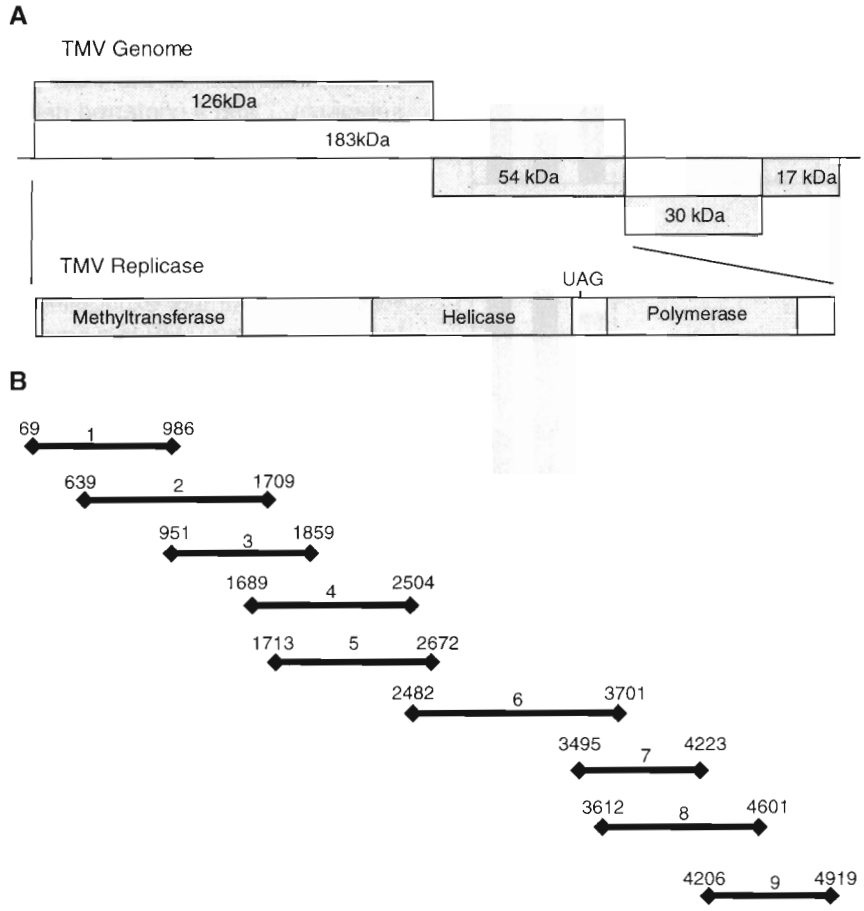

FIG. 1. Schematic representation of the TMV genome including RdRp domains (A) and the locations of the nine expressed TMV RdRp segments (B). TMV nucleotide number is from Goelet *et al.* (1982).

ments 6 and 9. Western immunoblot results were also variable with respect to the band intensity and substrate incubation time needed to observe the tagged segments. Variations in protein accumulation likely represent differences in the translation and/or stability of each RdRp segment.

Protection conferred by individual TMV RdRp segments

TMV challenge inoculations of *N. benthamiana* plants either mock inoculated or inoculated with the unmodified PVX vector or a vector construct containing 850 nucleotides of the *Escherichia coli Cpd*B gene resulted in the rapid accumulation of TMV in inoculated leaves (Fig. 3). These plants also displayed severe necrosis at the local and systemic level that prevented sampling beyond 4 days postinoculation (d.p.i.). In contrast, all nine PVX constructs expressing segments of the TMV RdRp ORF resulted in significantly lower accumulations of challenge TMV at 2 and 4 d.p.i. However, at 6 d.p.i., PVX vector constructs containing TMV RdRp segments 1–6, covering the 126-kDa MT and HEL domains, showed significant increases in the accumulation of TMV. At

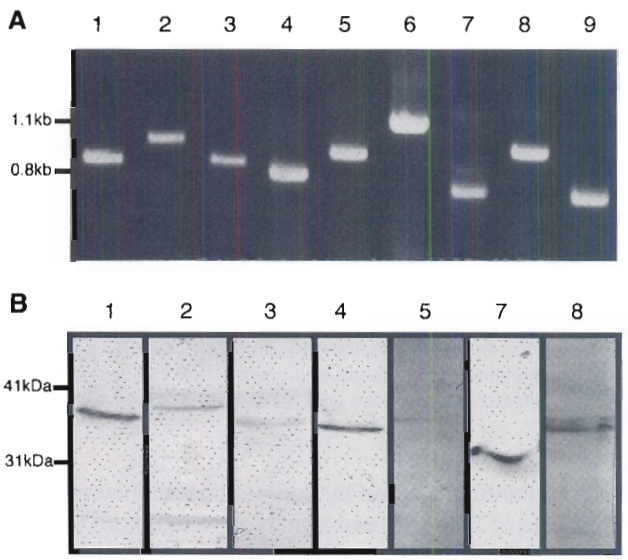

FIG. 2. PVX2C2S maintenance and expression of TMV RdRp segments. (A) RT–PCR amplification of TMV RdRp segments from total RNA purified from *N. benthamiana* leaves systemically infected with PVX2C2S constructs containing RdRp segments 1–9. (B) Western immunoblot detection of hexa-histidine tagged TMV RdRp proteins derived from segments 1–5, 7, and 8. Approximately 30 μg of total protein was loaded in each lane.

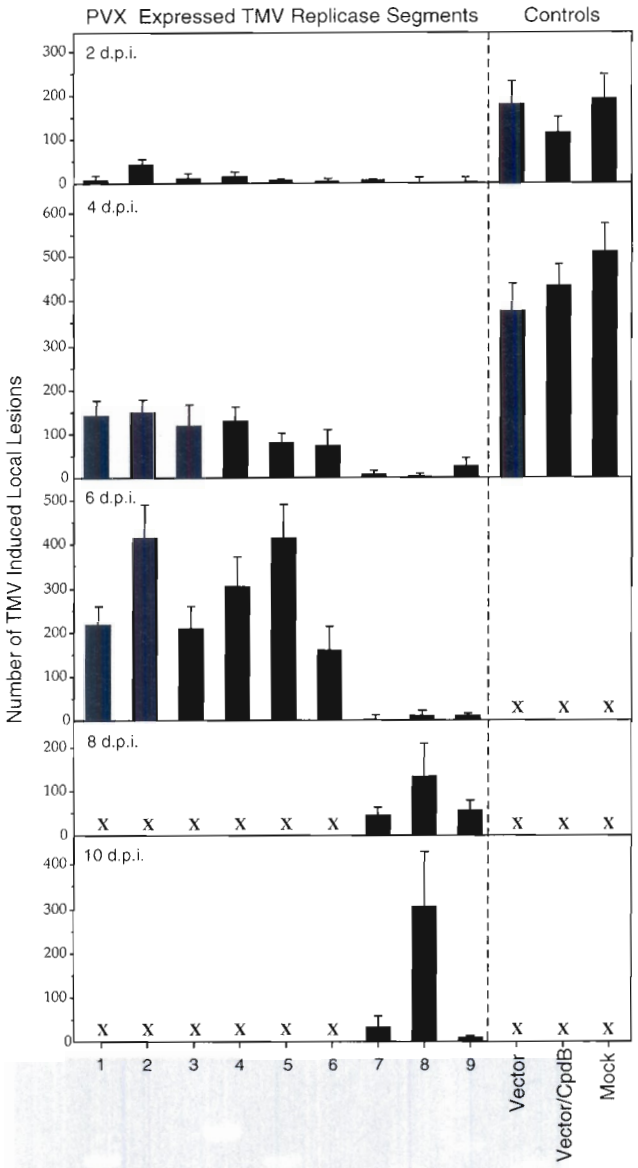

FIG. 3. The accumulation of challenge TMV over time in the inoculated leaves of plants preinfected with PVX2C2S-TMV RdRp constructs. Samples were collected at 2, 4, 6, 8, and 10 d.p.i. with 10 μg of purified TMV. TMV accumulations were determined by local lesion assay on *N. tabacum* cv Xanthi nc. Bars represent the average ± SE of TMV lesion numbers obtained from 6–12 individual plants preinfected with PVX2C2S constructs and challenge inoculated with TMV. X indicates TMV accumulations too numerous to count and/or TMV-induced death of challenge-inoculated leaves with no further samples taken.

8 d.p.i., plants protected by segments 1–6 showed accumulations of TMV that were too high to measure via local lesion assay. Although not as severe as in control plants, increased TMV accumulations in these plants was accompanied by the appearance of TMV-induced chlorosis and necrosis in both inoculated and noninoculated tissues.

Three PVX-expressed TMV RdRp segments, 7–9, conferred significantly greater delays in the accumulation of challenge TMV (Fig. 3). These three segments cover the 54-kDa readthrough portion of the TMV RdRp ORF and contain elements of the POL domain. Of these three segments, 7 and 9 conferred delays in the accumulation of challenge TMV that lasted the entire 10-day sampling period, whereas the delay conferred by segment 8 lasted only 8 d.p.i. In addition, by 10 d.p.i., sporadic areas of TMV- induced chlorosis and necrosis were observed in the noninoculated tissue of plants preinfected with PVX2C2S constructs expressing segments 7–9, indicating that systemic TMV movement had occurred.

Effect of frameshift mutations on TMV RdRp conferred protection

Frameshift mutations were created in segments 1, 3, 4, and 6–9 to determine whether the observed protection was associated with the expression of the corresponding protein. These mutations were designed to shift the normal reading frame of each RdRp segment at a specific amino acid downstream of the engineered translational start codon. Each frameshift leads to a new stop codon, resulting in a severely truncated protein. The locations of these frameshifts relative to the start codon of each segment are amino acid position 11 for segments 1, 6, 8 and 9; position 13 for segment 3; position 58 for segment 7; and position 62 for segment 4.

Cross-protection results obtained from frameshift segments revealed no significant alteration in the level of protection conferred by segments 1, 3, 4, and 6 (Fig. 4). Thus the expression of protein from these RdRp segments is not required to provide low levels of protection against challenge TMV. In contrast, frameshift mutations significantly affected the high levels of protection conferred by segments 7–9 (Fig. 4). This finding indicates that protein expression from these segments is a key factor in their ability to confer high levels of protection. However, frameshift segments 7–9 did confer low levels

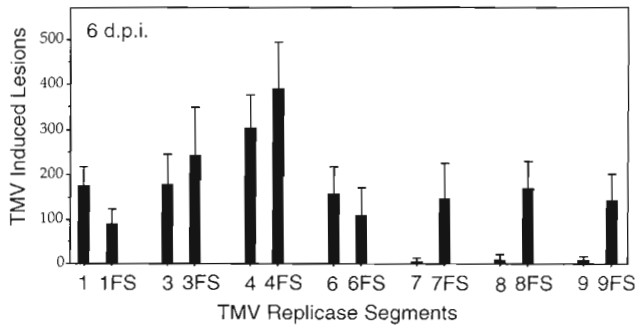

FIG. 4. Accumulation of challenge TMV in the inoculated leaves of plants preinfected with PVX2C2S-TMV RdRp and RdRp-frameshift (FS) constructs. Samples were collected at 6 d.p.i., and TMV accumulations were determined as described previously. Bars represent the average ± SE of TMV lesion numbers obtained from four to six individual plants preinfected with PVX2C2S constructs.

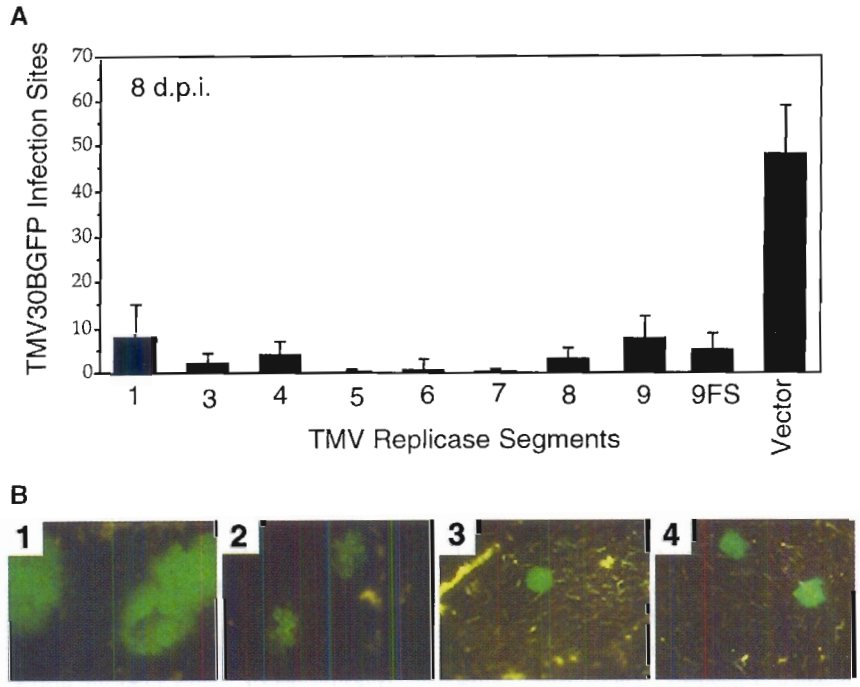

FIG. 5. TMV30BGFP challenge inoculations. (A) Number of fluorescent TMV30BGFP infection sites observed at 8 d.p.i. on *N. benthamiana* leaves preinfected with PVX2C2S constructs containing TMV RdRp segments 1, 3–9, and 9frameshift (FS). Bars represent the average ± SE of infection sites observed on 5–12 challenge-inoculated leaves. (B) Representative TMV30BGFP infection sites on leaves systemically infected with either 1, the unmodified PVX2C2S vector, or 2, 3, and 4, PVX2C2S constructs containing TMV RdRp segments 6, 9, and 9-FS, respectively. Photographs were taken at 8 d.p.i. with TMV30BGFP at 5× magnification.

of protection similar to the protection levels conferred by segments 1–6.

Specificity of the conferred protection

The specificity of the protection conferred by TMV RdRp segments 1, 3, and 5–9 was investigated using the tobamovirus tobacco mild green mosaic virus (TMGMV-U2) as challenge inoculum. TMGMV-U2 shares approximately 76% overall homology with TMV. Results demonstrated that all PVX-expressed TMV RdRp segments allowed the rapid accumulation of TMGMV-U2 within 4 days postchallenge inoculation (d.p.i.) (data not shown). The rapid accumulation of TMGMV-U2 was also accompanied by severe necrosis in both inoculated and noninoculated tissues. Thus none of the tested TMV RdRp segments were capable of conferring protection against the related tobamovirus TMGMV-U2.

Challenge inoculations with TMV-GFP

To determine whether the protection conferred by PVX expressed TMV RdRp segments was the result of reduced numbers of infection sites and/or reductions in virus spread, *N. benthamiana* leaves systemically infected with PVX constructs expressing TMV RdRp segments 1, 2, 4–9, and 9-frameshift were challenge inoculated with 10 μg of TMV30BGFP. This TMV construct expresses the green fluorescent protein (GFP) from *Aequorea victoria* downstream of the CP subgenomic promoter (Shivprasad *et al.*, 1999). Challenge-inoculated leaves were examined by fluorescence microscopy to determine the number and size of TMV30BGFP infection sites. Leaves preinfected with PVX constructs expressing any of the TMV RdRp segments showed significantly fewer TMV30BGFP infection sites compared with control leaves infected with the unmodified PVX vector (Fig. 5A). In addition, the few infection sites observed were smaller in appearance and more restricted in their expansion than infection sites found on control leaves (Fig. 5B). Furthermore, GFP fluorescence was not observed in systemic nonchallenged tissue of plants preinfected with PVX2C2S TMV RdRp constructs, indicating that TMV30BGFP did not move systemically. These findings indicate that all TMV RdRp segments confer equally high levels of protection against TMV30BGFP.

DISCUSSION

Cross-protection can be mediated by both viral RNA, as is the case for PTGS, or viral proteins, as is the case for coat protein-mediated protection (Ratcliff *et al.*, 1999; Bendahmane *et al.*, 1997; and Lu *et al.*, 1998). Thus depending on the system, different and sometimes multiple mechanisms may contribute to the protection phenomenon. In this study, the viral vector PVX2C2S was used to determine the ability of different TMV RdRp

domains to confer cross-protection. By using this strategy, specific features of the TMV RdRp, such as individual domains or the ability to express protein, can be assessed and compared for their role in providing protection. The identification of RdRp features that confer protection should provide insight into the mechanisms responsible for this phenomenon.

Cross-protection assays using the PVX2C2S system revealed two distinct levels of protection. TMV segments covering the 126-kDa ORF including MT and HEL domains all conferred a similar 2-day delay in the accumulation of challenge TMV, whereas segments 7–9, covering elements of the 54-kDa POL domain, conferred significantly higher levels of protection. However, in all cases the protection was incomplete and challenge TMV could eventually be recovered from both inoculated and noninoculated tissues. The ability of the challenge virus to establish initial infection sites may reflect the inability of the PVX2C2S vector to uniformly infect all leaf cells (Chapman *et al.,* 1992; Baulcombe *et al.,* 1995). Cells not infected by the PVX2C2S RdRp constructs presumably remain susceptible to TMV, providing replication sites that ultimately lead to the breakdown of protection. In addition, both low and high levels of protection were not effective against the related tobamovirus TMGMV-U2. The specificity of this protection is consistent with results previously obtained from the transgenic expression of the TMV 54-kDa ORF (Golemboski *et al.,* 1990) and suggests an underlying homology-dependent mechanism.

Frameshift mutations engineered into specific RdRp segments demonstrated that protein translation was required to maintain the high levels of protection derived from the POL domain but not the lower protection levels derived from the other RdRp segments. Similarly,(Carr *et al.* (1992) demonstrated that a nontranslatable TMV 54-kDa ORF did not confer protection in a transient expression system. This suggests that translation of the 54-kDa POL domain plays an important role in conferring protection. In contrast, frameshift mutations did not affect the low level of protection conferred by RdRp segments covering the 126-kDa ORF, indicating that this level of resistance was RNA mediated. Furthermore, frameshift constructs 7–9, covering the 54-kDa ORF, conferred a 2-day delay in TMV accumulation that was similar to the delay conferred by RdRp segments covering the 126-kDa ORF (Fig. 4). Thus the RNA sequences of the POL domain are also capable of conferring low protection levels.

The TMV POL domain was divided into three overlapping segments, all of which conferred significant levels of protection, with the highest levels being obtained from segments 7 and 9 (Fig. 3). However, no correlation was observed between the level of protein accumulation and the level of protection. In particular, protein derived from segments 7 and 8 were readily detected by Western immunoblotting, whereas repeated attempts to detect the accumulation of segment 9 failed. However, frameshift studies indicate that translation of the segment 9 protein is required to obtain the high level of protection. Thus the hexa-histidine tag on segment 9 may be unstable or the protein itself may be sequestered within the host in some fashion that prevents its detection. Our failure to detect segment 9 is reflected in the inability of previous studies to detect the TMV 54-kDa RdRp protein in either virus-infected or transgenic plants (Sulzinski *et al.,* 1985; Golemboski *et al.,* 1990).

The simplest explanation for these data are that the polypeptides derived from TMV 54-kDa POL segments function in a dominant-negative fashion to disrupt challenge virus infection. Segment 7 covers the N-terminal unique region of the POL as well as elements of the fingers domain. Segment 9 covers the carboxyl half of the POL, including the palm, thumb, and the carboxyl end of the fingers domains. Segment 8 overlaps significantly with segments 7 and 9 and contains the fingers and palm domains. Structure and function comparisons with similar elements in other polymerases indicate that several of these features are directly involved in RNA template binding (Buck, 1996; O'Reilly and Kao, 1998). Interestingly, Nguyen *et al.* (1996) demonstrated that viral RNA trafficking within transgenic 54-kDa plants was inhibited. Thus nonproductive interactions between truncated POL proteins and the challenge virus RNA could explain the mechanism of protection as well as account for its specificity.

An alternative explanation for these data are that translation of the POL segments enhances the ability of the RNA sequences to confer protection. Marano and Baulcombe (1998) determined that the resistance observed in 54-kDa transgenic plants was active against a specific antisense region of the TMV POL domain and occurred in a fashion that suggested a gene silencing-like mechanism. In addition, Lewandowski and Dawson (1998) observed that TMV POL sequences inhibit the replication of truncated TMV RNAs by the wild-type virus. Thus POL domain sequences appear to play a critical role in controlling TMV replication. Precisely how these sequences modulate virus replication and whether this process contributes to the observed protection via a protein- and/or RNA-based mechanism remain to be determined.

TMV30BGFP inoculations demonstrated that the RdRp-derived protection significantly blocked challenge virus infection and spread (Fig. 5). The inability of TMV30BGFP to establish an infection or to travel systemically in *N. benthamiana* plants displaying virus-induced PTGS has been previously observed (Ratcliff *et al.,* 1999). Interestingly, no significant difference was observed in the appearance or number of TMV30BGFP infection sites on plants protected by RdRp segments that conferred either high or low levels of protection against wild-type TMV (Fig. 5). The ability of all TMV RdRp segments to confer high protection levels against TMV30BGFP but

FIG. 6. Half-leaf assays showing the local movement of wild-type TMV in comparison with TMV30BGFP on the local lesion host *N. tabacum* cv. Xanthi nc. Photograph was taken 4 d.p.i. Arrows denote individual lesions.

not against wild-type TMV suggests that the RNA mechanism is considerably more effective against TMV30BGFP than against the wild-type virus. We speculate that the variation in protection levels conferred against TMV30BGFP and wild-type TMV reflects differences in the speed at which these viruses spread in the inoculated leaf. Local lesion comparisons between TMV30BGFP and wild-type TMV demonstrate that TMV30BGFP is restricted in its ability to spread in a cell-to-cell manner (Fig. 6). This is consistent with previous studies that show insertion of gene sequences within the 3′ end of TMV dramatically reduce the accumulation of the 30-kDa cell-to-cell movement protein (Culver *et al.*, 1993). The reduced spread of TMV30BGFP likely renders it more susceptible to the protection mediated by the RdRp RNA. In contrast, the wild-type virus apparently moves/replicates with sufficient speed to allow it to rapidly overcome the RNA-mediated mechanism. Whether the ability of wild-type TMV to overcome this protection is due solely to its speed of movement or is linked to the production of a suppressor of the RNA-mediated protection remains to be investigated.

In summary, the protection conferred by segments of the TMV RdRp gene expressed from within a heterologous viral vector can be attributed to at least two different mechanisms. The first involves an RNA-mediated mechanism that is apparently activated by the presence of any RdRp gene sequence. In fact, the ability of a nontranslatable TMV coat protein ORF to confer a similar 2-day delay in the accumulation of challenge TMV when expressed from the PVX2C2S vector suggests that any TMV sequence can function to activate this form of protection (Culver, 1996). Furthermore, the nucleotide-specific nature of this protection suggests the presence of a PTGS-like mechanism. In addition, the levels of protection conferred by the RNA-based mechanism varied depending on the movement speed of the challenge virus. Rapid infection speeds have been proposed as a possible counterdefense strategy used by viruses to overcome PTGS (Ratcliff *et al.*, 1999). The second mechanism of protection required protein expression from segments of the POL domain and conferred substantially greater delays in the accumulation of challenge TMV in comparison with the RNA-based mechanism. However, the protein-mediated protection occurs in conjunction with the RNA-mediated protection. It is feasible that the protein-mediated mechanism functions only to slow wild-type virus replication to a level that allows the RNA-derived mechanism to be more effective. This type of combined effect could also explain the homology-dependent nature of the observed protection. However, additional studies will be needed to determine whether these two mechanisms actually cocontribute to produce an increased level of protection.

MATERIALS AND METHODS

Virus constructs and expression analysis

pPVX2C2S contains the cloning sites *Eco*RV and *Sal*I engineered downstream of the PVX coat protein subgenomic promoter, allowing the transcription and translation of any inserted ORF (Chapman *et al.*, 1992). Linker modification at the *Eco*RV site was done to incorporate a novel start codon followed by 6 histidine codons. This modification results in the addition of a hexa-histidine tag at the amino-terminus of any ORF cloned into the pPVX2C2S vector.

Nine overlapping segments of the TMV 183-kDa RdRp ORF were individually amplified using the polymerase chain reaction (PCR) (Fig. 1; Culver, 1996). The 5′ PCR primers encoded *Eco*RV restrictions sites and were designed to maintain the reading frames of each TMV RdRp segment. The 3′ PCR primers included a translational stop codon and an *Sal*I restriction site. The presence of each TMV RdRp sequence within the PVX vector was confirmed by DNA sequencing. To disrupt protein expression, frameshift mutations were also introduced into TMV RdRp segments 1, 3, 4, and 6–9. These mutations were created either by PCR mutagenesis (Higuchi *et al.*, 1988) or through the direct incorporation of frameshift mutations into the 5′ PCR primers used to amplify the segments. Each frameshift mutation would result in the severe truncation of the protein segment.

Infectious RNA transcripts were generated from each

PVX vector construct and used to inoculate leaves of *N. benthamiana*. At 10–14 d.p.i., systemically infected *N. benthamiana* tissue displaying mild mosaic symptoms was harvested, and total protein or RNA was extracted (Laemmli, 1970; Qiagen Inc., Valencia, CA). The extracted proteins were resolved by SDS–PAGE and electroblotted onto nitrocellulose paper. Monoclonal antibody specific to hexa-histidine tracts (Sigma Chemical Co., St. Louis, MO) was used to visualize RdRp segments. Predicted protein products derived from TMV RdRp segments should range between 26 and 45 kDa in size. Extracted RNA was subjected to RT–PCR analysis using primers specific to each TMV RdRp segment.

Cross-protection assays

Infectious transcripts from PVX TMV–RdRp constructs were used to inoculate *N. benthamiana* plants at the three- to five-leaf stage. Additional plants either mock inoculated or inoculated with the unmodified PVX vector or a vector construct containing 850 nucleotides of the *Cpd*B phosphodiesterase gene from *E. coli* were used as controls. All plants were maintained in environmental growth chambers at 25°C under a 12-h photoperiod. The first two leaves of each plant to fully show systemic PVX symptoms were dusted with Carborundum. Each leaf (two per plant) was challenge inoculated with 5 μg of purified TMV (Gooding and Hiebert, 1967) in 50 μl of 10 mM sodium phosphate buffer, pH 7.4. A 1-cm-diameter leaf punch was taken from each of the two challenge inoculated leaves at 2, 4, 6, 8, and 10 d.p.i. To determine the accumulation of challenge TMV, leaf punches were macerated in 200 μl of 10 mM phosphate buffer, pH 7.4, and 50 μl was used to inoculate a Carborundum-dusted leaf of *Nicotiana tabacum* cv. Xanthi nc, a local lesion host for TMV but not for PVX. Each macerated sample was similarly assayed two or three times, and the average lesion number was used to represent the accumulation of TMV in that sample. Similar cross-protection assays were performed using purified TMGMV-U2 as challenge inoculum. In addition, PVX2C2S constructs expressing regions of the TMV RdRp involved in the elicitation of the *N* gene hypersensitive response (Erickson *et al.*, 1999) did not induce lesion formation when inoculated by themselves onto leaves of *N. tabacum* cv. Xanthi nc. Thus PVX2C2S RdRp constructs did not affect the local lesion assays.

pTMV30BGFP contains the GFP ORF inserted downstream of the TMV coat protein subgenomic promoter (Shivprasad *et al.*, 1999). Purified TMV30BGFP virus (10 μg) was used to challenge inoculate *N. benthamiana* leaves systemically infected with PVX2C2S constructs expressing TMV RdRp segments. At 8 d.p.i., TMV30BGFP infections sites were visualized and counted by fluorescence microscopy.

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