Transgene translatability increases effectiveness of replicase-mediated resistance to Cucumber mosaic virus

William M. Wintermantel and Milton Zaitlin

Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA

Transgenic tobacco plants expressing an altered form of the 2a replicase gene from the Fny strain of Cucumber mosaic virus (CMV) exhibit suppressed virus replication and restricted virus movement when inoculated mechanically or by aphid vectors. Additional transformants have been generated which contain replicase gene constructs designed to determine the role(s) of transgene mRNA and/or protein in resistance. Resistance to systemic disease caused by CMV, as well as delayed infection, was observed in several lines of transgenic plants which were capable of expressing either full-length or truncated replicase proteins. In contrast, among plants which contained nontranslatable transgene constructs, only one of 61 lines examined exhibited delays or resistance. Once infected, plants never recovered, regardless of transgene translatability. Transgenic plants exhibiting a range of resistance levels were examined for transgene copy number, mRNA and protein levels. Although ribonuclease protection assays demonstrated that transgene mRNA levels were very low, resistant lines had consistently more steady-state transgene mRNA than susceptible lines. Furthermore, chlorotic or necrotic local lesions developed on the inoculated leaves of transgenic lines containing translatable transgenes, but not on inoculated leaves of lines containing nontranslatable transgenes. These results demonstrate that translatability of the transgene and possibly expression of the transgene protein itself facilitates replicase-mediated resistance to CMV in tobacco.

Introduction

The mechanisms responsible for replicase-mediated resistance to various plant viruses have been studied extensively for a number of years (reviewed by Palukaitis & Zaitlin, 1997). There is evidence that different mechanisms may be responsible for engendering replicase-mediated resistance to different virus species. Studies on replicate-mediated resistance to Tobacco mosaic virus (TMV) and Pea early browning virus concluded that translatability of the transgene, encoding the 54 kDa ORF of each virus, was necessary for resistance (Carr et al., 1992; MacFarlane & Davies, 1992). Similarly, translation of the 2a replicase protein of Alfalfa mosaic virus was required for replicase-mediated resistance to this virus (Brederode et al., 1995). In contrast, transgenic tobacco plants containing potyvirus coat protein sequences (Lindbo et al., 1993; Smith et al., 1994), and replicase sequences of Potato virus X (Mueller et al., 1995) engender resistance through silencing of transgene mRNA and homologous viral RNA.

In some cases, transgenic plants exhibit more than one resistance phenotype, which can complicate analysis of mechanisms. For example, Pang et al. (1994) found that coat protein-mediated resistance against tospoviruses was RNA-mediated when directed against closely related isolates, but protein-mediated when directed against more distantly related tospoviruses. Tenllado et al. (1995) found that transgenic plants containing the 54 kDa gene of Pepper mild mottle virus exhibited two different resistance responses, one complete and the other delayed. Further studies have suggested protein involvement in this system as well (Tenllado et al., 1996). In addition, although TMV replicase-mediated resistance was shown to be protein-mediated in protoplasts (Carr et al., 1992), research by Marano & Baulcombe (1998) found that transgenic tobacco plants containing a gene encoding the TMV 54 kDa protein exhibited transgene silencing. The silencing was shown to contribute to virus resistance.
Replicate-mediated resistance to *Cucumber mosaic virus* (CMV) is complex, and encompasses at least two separate mechanisms which contribute to the resistance, one reducing virus replication (Carr et al., 1994), the other restricting long-distance virus movement (Wintermantel et al., 1997). A second type of movement restriction may exist at the cell-to-cell level (Nguyen et al., 1996; Canto & Palukaitis, 1999). CMV RNA2 is the target of both components of the resistance; however, the block on virus movement involves interactions in the central region of the RNA, while replication inhibition involves sequences throughout its entire length (Hellwald & Palukaitis, 1995). Although the viral target of the resistance has been identified, it has not been determined whether the transgene mRNA, derived from CMV RNA2, or its protein product is the effector of replicate-mediated resistance. We address this question, beginning with a series of transgenic plants containing translatable and nontranslatable transgenes, designed to determine whether transgene translatability or protein expression is required for resistance. This work is coupled with an analysis of the relationship between resistance and transgene copy number, steady-state transgene mRNA levels and symptomatology. Together, the results indicate a complex resistance which is difficult, although not impossible, to achieve without a translatable transgene, and probably protein expression. While transgene translatability has sometimes been identified as a facilitator of homology-dependent gene silencing (Cassidy & Nelson, 1995; Tanzer et al., 1997), some aspects of the resistance described here clearly do not fit the typical gene-silencing model for transgene-mediated resistance.

**Methods**

**Plants and virus inoculations.** All transgenic and nontransformed plants used were *Nicotiana tabacum* cv. Turkish Samsun NN, and were grown under greenhouse conditions. Transgenic plants were either self-fertilized progeny of lines 1-2, 1-8 or 2-7, described in Anderson et al. (1992), or were developed during the course of the studies described herein. The Fny strain of CMV (Roossinck & Palukaitis, 1990) was used for all virus inoculations. Plants were mechanically inoculated either by rubbing Carborundum-dusted leaves with sap diluted in 50 mM sodium phosphate buffer, pH 7.2, or by rubbing leaves with freshly purified Fny-CMV virions (at concentrations of 5-50 µg/ml, as indicated in the text) suspended in the same buffer containing Celite. Tobacco plants grown from seed were inoculated when plants had approximately four leaves, and were no more than 13 cm high. Plants derived from tissue culture were inoculated on small and mid-sized leaves when plants were no more than 10 cm high.

**Plant transformation constructs and transformations.** All constructs used for plant transformation (Fig. 1A) were developed from pCMV N/B-23 (Anderson et al., 1992). First, the CMV RNA2 transgene sequence in pCMV N/B-23 was excised by digestion with BamHI, which flanks the transgene, and subsequently cloned into the BamHI site of pBluescriptII SK+ (Stratagene), to yield pBW40. The Spdi site in pBW40 was removed by digestion with Spdi, followed by treatment with T4 DNA polymerase (Sambrook et al., 1989). Restriction enzyme analysis and DNA sequencing were used to verify removal of the start codon. This new construct, pBW42, was digested with BamHI, and the restriction fragment containing the CMV RNA 2 sequence was inserted into the binary plant transformation vector pROK2 (Sleat et al., 1988) to form pCMV2-PP (Fig. 1A). Restoration of the deletion in pCMV N/B-23 was accomplished by replacing a Spfi fragment from pBW42, containing a deletion between nucleotides 1850–1951, with a Spfi fragment from pFny209, a full-length cDNA clone of Fny-CMV RNA2 (Fig. 1A). Restriction analysis and DNA sequencing were used to verify complete restoration of the missing sequence. The new construct was digested with BsiHI, and the fragment containing the CMV RNA 2 sequence was inserted into the plant transformation vector pROK2 to form pCMV2-FLP. To generate a nontranslatable transgene, pBW42 was digested with SalI, and the overhang was filled in using the Klenow fragment of DNA polymerase I (Sambrook et al., 1989), creating a new *SalI* site, which shifted translation out of frame, resulting in early termination (Fig. 1A, B). This new construct was cleaved with BsiHI, and the fragment containing the CMV RNA 2 sequence was inserted into the plant transformation vector pROK2 to form pCMV2-NP (Fig. 1A). Restriction analysis and DNA sequencing were used to verify the sequence changes and the orientation of the transgene in pCMV2-NP, pCMV2-PP and pCMV2-FLP. Translation of all constructs was tested in a wheat germ cell-free *in vitro* translation system (Roberts & Patterson, 1973) prior to insertion of the transgene cassette into the plant transformation vector. All translatable constructs produced translation products of the expected size. It was not possible to clearly identify the small 3 kDa polypeptide of pCMV2-NP; however, no significant translation products were detected with this construct (data not shown).

Transfer of the constructs for plant transformation from *E. coli* to *Agrobacterium tumefaciens* was by triparental mating, and *Agrobacterium*-mediated plant transformation and regeneration were performed according to the methods of Rodgers et al. (1986). Primary transformants (R0) were tested for resistance by inoculating young plants with 5 µg/ml freshly purified virions of Fny-CMV. Screening was based on the appearance of systemic mosaic symptoms. Primary transformants which did not develop symptoms of infection, or which showed delays in the rate of development of systemic symptoms, were allowed to self-pollinate, and seed was collected. A few randomly selected primary transformants which were not resistant were also saved for seed. Sets of R1 progeny were subsequently inoculated and tested for resistance with 5 and 50 µg/ml freshly purified Fny-CMV virions. R1 progeny exhibiting resistance at 50 µg/ml, and several susceptible R1 progeny containing each of the different constructs, were allowed to self-pollinate and seed was collected. R2 progeny were subsequently tested for resistance by inoculation with 50 µg/ml freshly purified Fny-CMV virions.

**Tobacco genomic DNA isolation and analysis.** Genomic DNA was isolated from several small (1-5 cm long) tobacco leaves according to the procedure of Fulton et al. (1995) with the following modifications: leaves were pulverized in liquid nitrogen prior to grinding, and extracted twice to eliminate proteinaceous contaminants. DNA was resuspended in sterile, nuclease-free water and either used immediately or stored frozen at −20 °C. Ten µg of tobacco genomic DNA was digested with either EcoRI or BamHI, and electrophoresed at 40 V overnight in 1% agarose-1 x TBE. Southern (alkali) blotting and hybridization of tobacco genomic DNA were performed on Amersham Hybond-N+ nylon membrane at 65 °C, according to the manufacturer’s recommendations. Blots were hybridized to a denatured PCR-amplified, gel-purified DNA fragment corresponding to RNA2 of Fny-CMV, labelled with [32P]dATP, with detection by autoradiography.

**Total plant RNA isolation and analysis.** Total plant RNA was prepared by grinding 15 to 20 small (3-4 cm long) tobacco leaves in
liquid nitrogen, followed by suspension, vortexting and extraction three times in a mixture of 5 ml RNA extraction buffer (50 mM Tris–HCl, pH 8.0; 10 mM EDTA, 2% SDS) and 5 ml phenol–chloroform, followed by ethanol precipitation of the aqueous phase. Total RNA concentration and quality were determined by spectrophotometry and gel electrophoresis. Ribonuclease protection assays were performed using the RPAII kit (Ambion) and $^{32}P$UTP-labelled antisense transcript probes made from a cDNA clone containing a 417 nucleotide XbaI–XhoI fragment from Fny-CMV RNA2 (Fig. 1A).

Results

Transformation with translatable and nontranslatable transgene sequences

Three types of constructs (Fig. 1A) were designed for use in plant transformation experiments to determine whether the mRNA and/or protein product of the transgene is responsible for replicase-mediated resistance to CMV. All constructs (Fig. 1A) were developed from pCMV N/B-23 (Anderson et al., 1992). This construct contained the cloned DNA sequence of a full-length RNA2 from the Fny strain of CMV, with a 94 nucleotide internal deletion. The 94 nucleotide internal deletion (nucleotides 1857–1950) removed sequences encoding a Gly-Asp-Asp (GDD) polymerase motif, known to be conserved in the replicases of positive-strand RNA viruses (Argos et al., 1984; Bruenn, 1991), and simultaneously shifted the protein to an alternate reading frame, resulting in early termination of translation. The original transgene also contained an in-frame AUG 5′ of the viral AUG in a good translational context, creating the potential for early translation initiation, and the addition of 29 amino acids to the N terminus of the truncated CMV 2a protein (Anderson et al., 1992). The resulting product, a 75 kDa protein, is considerably smaller than the 97 kDa full-length CMV 2a protein. The upstream start codon was part of an SphI site used during construction of pCMV N/B-23. To resolve any question as to the role of the additional protein sequence in resistance, the new translational stop codon (UGA), which shifted translation out of frame, and the original 97 kDa full-length CMV 2a protein, was developed. These two constructs were identical, except that the SphI restriction site containing the upstream AUG was
removed in pCMV2-PP, resulting in translation of a 71 kDa protein from the authentic 2a start codon, rather than the 75 kDa protein encoded by pCMV N/B-23. The C terminus of the 2a protein is not translated in pCMV2-PP or pCMV N/B-23, due to a frameshift resulting from the internal deletion (Fig. 1). Preliminary plant transformation studies demonstrated that the additional N-terminal amino acids did not contribute to the initial resistance phenotype, and that both pCMV N/B-23 and pCMV2-PP were comparable for generating resistant plants (data not shown). As a result, the remaining two new constructs also lacked the additional AUG. The second construct, pCMV2-FLP (Fig. 1 A), was designed to determine if resistance could be obtained from plants transformed with the full-length Fny-CMV RNA2 sequence, encoding a full-length, 97 kDa 2a protein. The third construct, pCMV2-NP, was generated from pCMV2-PP to determine if resistance could be obtained from plants transformed with a nontranslatable CMV RNA2 sequence. A 4 nucleotide insertion near the 2a start codon of pCMV2-NP shifts translation to an alternate reading frame encoding a small 3 kDa polypeptide, but in all other respects pCMV2-NP is identical to pCMV2-PP.

None of the plants transformed with vector sequence alone (23 lines) showed delays or any level of resistance as primary transformants or progeny (data not shown). Only one of 61 lines transformed with the nontranslatable frameshift construct, pCMV2-NP (hereafter referred to as NP lines), produced resistance (line NP7), and one other NP line exhibited a slight delay in the appearance of symptoms. All other NP lines became infected at the same rate as nontransformed control plants (Table 1, Fig. 2). Because line NP7 produced a partial resistance phenotype, unlike all other lines containing nontranslatable transgenes, the transgene from this line was amplified by PCR and sequenced to determine if the reading frame had been restored. Results verified that the transgene in line NP7 remained nontranslatable (data not shown). When R2 progeny of resistant line NP7 were tested for resistance, approximately 50% of the plants tested exhibited delayed infection or resistance (data not shown). In contrast, transformation with the two constructs which allowed for translation of considerable portions of the transgene (pCMV2-PP and pCMV2-FLP) resulted in many transgenic lines exhibiting delayed infection compared with controls (Table 1, Fig. 2), as well as more lines showing substantial levels of resistance (Table 1). This implied that transgene translatability or the CMV 2a protein encoded by the transgene may have a role in replicated-mediated resistance to CMV.

Symptomatology also suggested a role for the transgene protein in resistance. When small transgenic and nontransformed tobacco plants (four-leaf stage) were inoculated with concentrated virus, initial symptoms on inoculated leaves differed substantially among plants containing each of the three types of transgene constructs (Fig. 3). Plants containing a transgene from any of the protein-producing constructs (pCMV N/B-23, pCMV2-PP and pCMV2-FLP) exhibited chlorotic and/or necrotic lesions on the inoculated leaves in side-by-side tests (Fig. 3). In lines containing pCMV2-PP or pCMV N/B-23 (PP and NB23 lines, respectively) chlorotic lesions appeared first, with necrosis beginning at the periphery of the chlorotic lesion, and with time the entire lesion became necrotic (Fig. 3C). Inoculated leaves of lines containing pCMV2-FLP (FLP lines) often exhibited much more extensive necrosis than PP or NB23 lines, with necrosis occurring in a ringspot pattern (Fig. 3D). Chlorotic areas on inoculated leaves of FLP lines were only observed with lower concentrations of inoculum. In contrast, all NP lines exhibited a light green mottle on inoculated leaves, identical to the symptoms observed on nontransformed control plants (Fig. 3A, B). No

Table 1. Response of transgenic tobacco lines to inoculation with Fny-CMV

<table>
<thead>
<tr>
<th>Construct used in transformation</th>
<th>Percent resistant lines</th>
<th>Lines with delayed symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV2-NP</td>
<td>1/61 (1.6%)</td>
<td>1/61 (1.6%)</td>
</tr>
<tr>
<td>pCMV2-PP</td>
<td>4/61 (6.6%)</td>
<td>7/61 (11.5%)</td>
</tr>
<tr>
<td>pCMV2-FLP</td>
<td>8/67 (11.9%)</td>
<td>11/67 (16.4%)</td>
</tr>
</tbody>
</table>

Plants were inoculated with 50 µg/ml freshly purified CMV virions and scored at 3 weeks post-inoculation. Data represent a summary of three experiments.

![Fig. 2. Cumulative rate of systemic symptom development on upper leaves of nontransformed tobacco plants (NT), and resistant or partially resistant R2 transgenic tobacco plants encoding either a nontranslatable protein (NP), a partial protein missing the C terminus (PP) or a full-length protein (FLP). Plants from several different lines per each transgene construct were inoculated with sap from newly infected squash, showing strong CMV symptoms. Total plants tested per construct are as follows: NT, 32 plants; NP, 146 plants; PP, 236 plants, FLP, 141 plants. Data for line NP7 were included in the data presented here for plants containing the nontranslatable transgene protein (NP).](image-url)
distinct lesions or necrosis occurred on the inoculated leaves of NP lines, including line NP7, the line in which resistance was observed. When systemic symptoms developed on NP plants, they matched those that occurred on nontransformed control plants. The presence of lesions on the inoculated leaves of transgenic plants containing translatable versions of the CMV RNA2 transgene was associated with either resistance to systemic disease or with delayed systemic-infection.

Is the transgene protein alone responsible for replicase-mediated resistance?

To determine if there was any correlation between transgene copy number and resistance, Southern blots were performed on DNA isolated from R1 plants of each of five well-characterized transgenic tobacco lines exhibiting a range of resistance phenotypes. Although all resistant lines contained more than one copy of the transgene, there was no correlation between the number of copies and the potential degree of resistance (data not shown).

The results of the plant transformation experiments suggested that transgene protein or mRNA translatability contributes to CMV replicase-mediated resistance. The occurrence of limited resistance in one nontranslatable line, however, implies that the transgene mRNA can also engender resistance, even if nontranslatable, suggesting that gene silencing or another RNA-based mechanism could sometimes be involved in CMV replicase-mediated resistance. In order to characterize the relationship between transgene mRNA and resistance, Northern blots were performed on total mRNA extracts from the same well characterized, R1 transgenic-resistant tobacco lines examined by Southern analysis (NB23 lines). Although the blots were capable of detecting as little as 10 pg of control RNA, transgene mRNA levels were too low for comparison (data not shown). Consequently, ribonuclease protection assays were used to examine steady-state transgene message levels in these plants, and their relationship with resistance. Ribonuclease protection assays were performed with 100 µg of total plant mRNA from each of the transgenic lines. Total RNA was hybridized to antisense CMV RNA2 transcript probe (Fig. 4A). Lines 1-2 and 1-8, which have the strongest resistance based on testing at very high inoculum doses (Anderson et al., 1992) and which also exhibit suppression of virus replication (Carr et al., 1994), also contained the highest steady-state levels of transgene mRNA. In contrast, line 1-1, which was chosen to represent a transgenic line with an extremely low level of resistance, contained the lowest levels of transgene mRNA among the lines examined. Lines 2-3 and 2-7, showing intermediate levels of resistance, contained low to intermediate levels of transgene mRNA compared with the other lines studied (Fig. 4A). When sibling plants of lines representing each of the three levels of resistance were examined for steady-state transgene mRNA by ribonuclease protection assay, identical patterns were observed among siblings (Fig. 4B). These results indicated a direct correlation between higher relative amounts of steady-state transgene message and resistance. Ribonuclease protection assays were also done on R1 plants of several FLP Lines. These plants also exhibited a range of resistance levels between lines (Fig. 4C). In these experiments, the level of resistance was based on the percentage of plants infected in resistance tests at inoculum concentrations of either 5 or 50 µg/ml (data not shown). As with the partial protein constructs, those lines exhibiting the highest levels of resistance contained the most steady-state transgene mRNA, while those exhibiting progressively lower levels of resistance contained lower steady-state levels of transgene mRNA, although none of the mRNA levels were very high with any of the constructs in any plants, resistant or
Ribonuclease protection assays performed on R1 transgenic plants containing the nontranslatable transgene construct, pCMV2-NP, also contained low levels of steady-state transgene mRNA (Fig. 4D). Only one of 61 lines containing the nontranslatable transgene exhibited resistance (line NP7), and this line was included in these experiments. Other than line NP7, all nontranslatable lines tested were completely susceptible to CMV. A range of different steady-state transgene mRNA levels was observed, and resistant line NP7 contained an intermediate level of steady-state transgene mRNA, compared with other NP lines as well as when compared with the translatable transgene constructs (Fig. 4D; see line 1-8, used as internal standard). Additional plants of line NP7 exhibited levels of steady-state transgene mRNA comparable to levels in the sample present in Fig. 4(D) (data not shown). Susceptible line NP11, however, exhibited a steady-state transgene mRNA level comparable to highly resistant control line 1-8 (Fig. 4D). In contrast to transgenic plants containing translatable transgenes, these results demonstrate there is no correlation between higher steady-state transgene mRNA levels and resistance in plants containing nontranslatable transgenes.

Transgenic plants containing fully or partially translatable transgenes were far more effective in generating resistance or delayed symptom development than plants containing nontranslatable transgenes. To determine if stronger resistance was directly correlated with higher transgene protein levels in lines containing translatable transgenes, Western blots were performed on total protein extracts from transgenic plants exhibiting different levels of resistance. Although it was possible to detect the transgene protein by Western blotting (Carr et al., 1994), levels were very low, and it was not possible during the course of our studies to detect the transgene protein consistently or to compare protein levels between lines differing in resistance. In fact, the only lines in which the protein could be detected at all were those exhibiting the highest levels of resistance (data not shown). Gal-On et al. (1998) confirmed the low level of the 75 kDa transgene protein in tomato plants transformed with pCMV N/B-23.

Discussion

Replicase-mediated resistance to CMV is complex and impedes the ability of the CMV to infect plants by interfering with both virus replication and movement (Carr et al., 1994; Nguyen et al., 1996; Wintermantel et al., 1997; Canto & Palukaitis, 1999). Studies involving pseudorecombinant and chimeric forms of CMV composed of sequences from strains which could overcome resistance, and those which could not, demonstrated that the transgene affected the viral RNA (Hellwald & Palukaitis, 1995). These studies did not, however, determine whether the transgene mRNA or protein was susceptible (Fig. 4C). These results indicated the same mechanism was responsible for resistance with both constructs.

Fig. 4. Ribonuclease protection assays of total RNA from R1 transgenic and nontransformed tobacco comparing steady-state transgene mRNA levels with resistance. The level of resistance for each line is listed at the bottom of each lane: 0 = susceptible, 1 = low resistance, 5 = high resistance, based on the results of resistance testing described in the text. NT refers to nontransformed tobacco. Highly resistant transgenic line 1-8, containing pCMV N/B-23, was used as an internal control in (C) and (D). Hybridization is to a [32P]UTP-labelled, 417 nucleotide, antisense RNA transcript of Fny-CMV RNA2. (A) Steady-state transgene mRNA levels in the tobacco lines containing the pCMV N/B-23 (partial protein) transgene and exhibiting different levels of resistance; (B) steady-state transgene mRNA levels in sibling lines exhibiting different levels of resistance; (C) steady-state transgene mRNA levels in the tobacco lines containing the pCMV2-FLP (full-length protein) construct, and exhibiting different levels of resistance; (D) steady-state transgene mRNA levels in the tobacco lines containing the pCMV2-NP (nontranslatable transgene) construct.
Translatability of the transgene is important for replicase-mediated resistance to CMV

The analysis of transgenic plants containing translatable and nontranslatable transgenes, and examination of steady-state mRNA levels, suggest that, like some examples of RNA-mediated resistance, translatability of the transgene plays an important role in replicase-mediated resistance to CMV. Resistance can be obtained with either a full-length, fully translatable CMV RNA2 construct, or a CMV RNA2 construct with a small internal deletion, but which translates only the N-terminal approximately two-thirds of the protein (Anderson et al., 1992; Fig. 1, Fig. 2, Table 1). In contrast, as shown here, a nearly identical construct that only differs by a small, 4 nucleotide insertion which shifts translation out of frame shortly after initiation, was unable to produce resistance in tests of 60 of 61 independent transgenic lines (Table 1).

Local lesions formed on transgenic plants capable of producing full-length or nearly full-length transgene proteins, but not on those transgenic plants containing the non-translatable transgene construct. These lesions are a visible representation of the extent of virus movement in the inoculated leaf (Wintermantel et al., 1997). CMV is unable to enter the vascular system within these lesions, and is confined to localized areas on the inoculated leaf. Some plants that exhibit lesions on the inoculated leaf do eventually become infected, although infection is delayed compared to infection of either nontransformed controls or lines containing the non-translatable construct (Fig. 2). In highly resistant lines, however, further cell-to-cell movement beyond the lesion and entry into the vascular system were precluded (Wintermantel et al., 1997). The localized symptoms may be indicative of a role for the transgene protein in restricting virus cell-to-cell and/or long-distance movement through as yet unidentified cellular processes.

Line NP7, the one nontranslatable line exhibiting some level of resistance differed from other resistant transgenic lines in that it did not produce the local response on inoculated leaves that occurs when resistant lines expressing translatable transgene constructs are inoculated with high concentrations of CMV virions. The inability of CMV to produce lesions on NP7 inoculated leaves suggests that this line is fundamentally different from resistant lines containing translatable constructs. A possible explanation is that NP7 may not exhibit the restricted cell-to-cell or long-distance movement of CMV that is observed in resistant lines containing a translatable transgene (Nguyen et al., 1996; Wintermantel et al., 1997). Alternatively, NP7 may simply represent a transformation anomaly in which transgene insertion has somehow interfered with normal processes necessary for virus infection.

Do our results mean RNA is not involved in replicase-mediated resistance to CMV?

This study demonstrates a role for transgene translatability in CMV replicase-mediated resistance, and suggests a role for the transgene protein in resistance as well. Others have also identified transgene translatability as contributing to RNA-mediated resistance (Cassidy & Nelson, 1995; Tanzer et al., 1997). Transgenic plants containing a nontranslatable coat protein transgene of Peanut stripe virus, for example, exhibited either resistance or delayed symptom development following inoculation, and like the plants in our study, failed to show recovery once infected. Plants containing translatable coat protein constructs produced either complete resistance, or delayed infection followed by recovery (Cassidy & Nelson, 1995). Unlike the Cassidy & Nelson study, however, none of our nontranslatable lines exhibited a complete resistance phenotype, and recovery was not observed for any line containing translatable or nontranslatable CMV RNA 2 constructs. Furthermore, nearly all nontranslatable lines in our study became infected at the same rate as nontransformed controls.

The partial resistance to CMV exhibited by line NP7, the lone resistant line containing the nontranslatable construct, however, indicates that in some cases RNA may also facilitate resistance in the absence of protein. In addition, steady-state transgene mRNA levels were fairly low in all plants tested. This alone could be suggestive of transgene silencing (reviewed by Baulcombe, 1996), which is characterized by low steady-state levels of transgene mRNA correlated with high levels of resistance. Such inverse correlations have been found in transgenic resistant plants expressing translatable and nontranslatable coat protein genes (Lindbo et al., 1993; Smith et al., 1994), replicate genes (Mueller et al., 1995) and other foreign genes (Flavell, 1994). If replicase-mediated resistance to CMV were the result of a gene silencing effect, one might expect to find lower levels of steady-state transgene mRNA in highly resistant plants than in susceptible transgenic controls. Interestingly, our CMV resistant transgenic plants contain higher relative levels of transgene mRNA than more susceptible lines (Fig. 4).

We do not rule out the possibility that translatability enhances RNA effects in this resistance, but there are a number of points suggesting that the protein itself contributes to CMV-replicase mediated resistance. (1) Only one of 60 transgenic NP lines producing a nontranslatable transgene mRNA with only minor sequence alterations produced resistance, and systemic symptoms appeared in susceptible NP
lines at the same rate as in nontransformed plants (Fig. 2). In contrast, effective resistance, as well as many delayed infections, were obtained with plants containing any of the translatable constructs. (2) The lack of symptom production on inoculated leaves (Fig. 3) of NP plants suggests the inability to produce a transgene protein changes the nature of the interaction between the virus and the transgenic host. (3) Lines containing translatable constructs with stronger resistance contain higher steady-state transgene mRNA levels than lines exhibiting less resistance. Although this does not mean RNA-mediated resistance is not operating in these plants, the pattern is not what would typically be expected for this type of resistance. (4) There is never recovery from infection as often occurs in RNA-mediated resistance.

Arguably, the most interesting aspect of CMV replicase-mediated resistance is the existence of at least two separate elements contributing to the resistance: one resulting in a reduced level of virus replication, the other restricting virus movement (Carr et al., 1994; Hellwald & Palukaitis, 1995; Nguyen et al., 1996; Wintermantel et al., 1997; Canto & Palukaitis, 1999). We do not contend that RNA-mediated resistance does not occur in CMV replicase-mediated resistance, rather that this resistance is likely the result of a complex mechanism in which both transgene mRNA and protein can contribute separately, or in a cumulative manner, to engender resistance to CMV.

The authors would like to thank Nanditta Banerjee for performing several of the ribonuclease protection assays and for assistance with various aspects of this project, and Stewart M. Gray and Karen-Beth Schollhof for critical review of this manuscript.

References


Transgene translation contributes to CMV R-MR


Received 22 September 1999; Accepted 24 November 1999