Infection and coaccumulation of tobacco mosaic virus proteins alter microRNA levels, correlating with symptom and plant development

A. A. Bazzini14, H. E. Hopp1, R. N. Beachy15, and S. Asurmendi14

1Instituto de Biotecnología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria Castelar, Las Cabanas y Los Reseros, B1712WAA Buenos Aires, Argentina; and 2Donald Danforth Plant Science Center, 975 North Warson Road, St. Louis, MO 63132

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Infections by plant virus generally cause disease symptoms by interfering with cellular processes. Here we demonstrated that infection of Nicotiana tabacum (N. t.) by plant viruses representative of the Tobamoviridae, Potyviridae, and Potexviridae families altered accumulation of certain microRNAs (miRNAs). A correlation was observed between symptom severity and alteration in levels of miRNAs 156, 160, 164, 166, 169, and 171 that is independent of viral posttranscriptional gene silencing suppressor activity. Hybrid transgenic plants that produced tobacco mosaic virus (TMV) movement protein (MP) plus coat protein (CP)T42W (a variant of CP) exhibited disease-like phenotypes, including abnormal plant development. Grafting studies with a plant line in which both transgenes are silenced confirmed that the disease-like phenotypes are due to the coexpression of CP and MP. In hybrid MPxCP T42W plants and TMV-infected plants, miRNAs 156, 164, 165, and 167 accumulated to higher levels compared with nontransgenic and noninfected tissues. Bimolecular fluorescence complementation assays revealed that MP interacts with CP T42W in vivo and leads to the hypothesis that complexes formed between MP and CP caused increases in miRNAs that result in disease symptoms. This work presents evidence that virus infection and viral proteins influence miRNA balance without affecting posttranscriptional gene silencing and contributes to the hypothesis that viruses exploit miRNA pathways during pathogenesis.

miRNAs are highly conserved among plant families, from mosses to angiosperms (9).

Expression of suppressors of PTGS can produce developmental defects, presumably by altering miRNAs pathways (10–13). These results suggested that interference with miRNA-directed processes might be a general feature of pathogenicity.

To determine whether viruses that encode strong PTGS suppressors, but not those that do not encode suppressors, interfere with miRNAs, five different (+) sense single-stranded RNA viruses from three different virus families were analyzed: Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV) from the tobamovirus family, Tobacco etch virus (TEV) and Potato virus Y (PVY) from the potyvirus family, and Potato virus X (PVX) from the potexvirus family. TEV and PVY have strong PTGS suppressor activity (14) whereas TMV and PVX have weak PTGS suppressor activity (15, 16).

TMV encodes two replicate proteins, a 30-kDa movement protein (MP) (17) and a 17.5-kDa coat protein (CP) (18). Transgenic expression of TMV CP confers CP-mediated resistance to infection by TMV (19), whereas expression of MP modifies plastomesmata size-exclusion limit and enhances cell-to-cell movement of the virus (17, 20). Transgenic expression of mutant CP T42W, in which residue 42 (threonine, T) was mutated to tryptophan (W), exhibited increased protein aggregation compared with WT CP and conferred higher CP-mediated resistance than WT CP (21–24). Neither TMV MP nor CP is apparently involved in suppression of PTGS; Ding et al. (25) reported that TMV replicate delays transgene silencing in Nicotiana benthamiana. In these studies, TMV infection weakly suppressed PTGS of GFP mRNA in tissues that are in or close to leaf veins (15).

To determine the effects of simultaneous expression of MP and CP on TMV infection, we crossed transgenic tobacco lines that produce TMV CP T42W (21) and MP (26). Surprisingly, two independent lines that accumulate TMV MP and CP T42W exhibited severe abnormal phenotypes. We analyzed the impacts of expression of MP and CP T42W on accumulation of specific miRNAs and found that expression of both proteins interfered with miRNA accumulation in N. tabacum. However, neither MP nor CP suppressed PTGS. These data support the hypothesis that viral proteins that do not suppress PTGS are capable of suppressing miRNAs.

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The authors declare no conflict of interest.

Abbreviations: sRNA, small RNA; CP, coat protein; TMV, tobacco mosaic virus; MP, movement protein; PTGS, posttranscriptional gene silencing; miRNA, microRNA; ToMV, tomato mosaic virus; TEV, tobacco etch virus; PVX, potato virus X; PVY, potato virus Y; BiFC, bimolecular fluorescence complementation; YFP, yellow fluorescent protein.

To whom correspondence may be addressed. E-mail: rnbeachy@danforthcenter.org or sasurendi@cinia.inta.gov.ar.

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altering miRNA pathways, an effect that may impact disease symptoms in infected plants.

Results

Virus Infections Cause Different Types of Symptoms and Alter Accumulation of miRNAs. Groups of 20 N. tabacum [cv Samsun nn (Sx)] plants were mechanically inoculated with TMV, ToMV, PVX, TEV, or PVX in two independent experiments. All plants were placed in the same greenhouse for the duration of each experiment, and the percentage of plants exhibiting disease symptoms and severity of symptoms was recorded [supporting information (SI) Fig. 5]. We also recorded the number of infected plants with flowers and the height of the plants 30 days after inoculation.

Under the conditions used in this study, TMV and ToMV developed disease symptoms in a shorter period than TEV, PVY, and PVX. TMV and ToMV infection caused delays in flowering, and plants were taller than noninfected plants. Infection by TEV and PVX produced symptoms in an intermediate time period compared with TMV and PVY; plants infected with PVY showed symptoms later in time than did infection with TMV. Symptoms caused by PVX infection were relatively severe; TEV infection produced chlorosis in leaves with mild leaf distortion, and PVY cause mild mottling of infected leaves (SI Fig. 5).

Based on these data, a disease severity rating was created (Table 1). In sum, in this study, TMV and ToMV were the most aggressive, PVX and TEV were less aggressive, and PVY was the least aggressive.

To determine whether infection by TMV, ToMV, PVX, PVY, or TEV altered miRNA accumulation, groups of 12 N. tabacum plants were separately infected with each virus. At 10 days after infection, sRNAs were isolated from six to eight leaves per group, and accumulation of a selection of miRNAs was analyzed by Northern blots by using sequences from Arabidopsis thaliana that are correlated with plant development. Fig. 1 shows the relative accumulation of miRNAs in two independent biological replicates. Hybridization with miRNA was measured by using a radioactivity-scanning device and normalized based on the amount of rRNA quantified by using ethidium bromide staining of gels. The amount of miRNA species in noninfected plants was arbitrarily set at 1.0, and other data were computed relative to these plants. We observed that infection by TMV and ToMV caused highly significant increases in the levels of most of the 10 miRNAs tested. TEV and PVX caused moderate changes in the miRNAs tested, whereas infection with PVY caused the fewest changes in miRNAs (Fig. 1). miRNAs 156, 160, 164, 166, 169, and 171 were most severely affected: miR171* was the only complementary strand of all miRNAs that was detected, although complementary strands of all miRNAs were tested (data not shown). Failure to detect a sequence is not conclusive evidence of lack of presence and can be explained by low levels of miRNA* and/or problems with detection.

Suppression of PTGS by Viral Infections. To analyze PTGS suppressor activity by these viruses, 3-week-old transgenic N. benthamiana plants that constitutively express a gene encoding GFP [gfp; line 16c (14)] were used. When plants of this line were infiltrated with Agrobacterium tumefaciens carrying an inverted repeat of the gfp construct (27), the bright green fluorescence normally produced in these plants suppressed by 25–30 days, as expected, as a consequence of gene silencing (14). Silenced plants were then separately inoculated with the five viruses. When systemic symptoms were observed, the plants were analyzed under UV illumination. As expected, most of the plants infected with PVY and TEV recovered green fluorescence, indicating that infection with either virus suppressed PTGS (Table 1) (15). In contrast, the majority of plants inoculated with TMV, ToMV, and PVX did not show a GFP signal, indicating these viruses did not have a strong suppressor of PTGS (Table 1). However, small areas of GFP fluorescence, mainly around leaf veins, were observed in a low number of ToMV and TMV infected plants (Table 1). The level and tissue specificity of PTGS suppression activity of the viruses reported here are in agreement with previous studies that used similar assays (Table 1) (15).

Molecular Characterization of Transgenic Plants Expressing MP and/or CP. To study the effect of transgenic expression of TMV MP and CP on CP-mediated resistance, and miRNAs, we crossed a transgenic line that produces the TMV MP (plant line 277; refs. 19 and 28) with a transgenic line that produces a mutant of TMV CP, CP142W (23); both plant lines were developed in N. tabacum cv Xanthi and have been extensively characterized (17, 22, 24, 26). F1 progeny of the

![Fig. 1. miRNAs accumulation is altered by viral infections. (Left) Northern blot analysis to detect the accumulation of various miRNAs and miRNA* after infection with selected viruses. Ethidium-bromide-stained rRNA shown below each blot was used to normalize data. (Right) Average and standard error of miRNA level of two independent biological replicates. The data were derived based on hybridization of RNAs derived from noninfected plants, established as 1.0.](image-url)
crosses were normal in appearance and were selfed to obtain double-homozygous lines; three F3 lines were selected for further study.

The presence of both transgenes in F1 plants was confirmed by genomic PCR (SI Fig. 6), and accumulation of MP and CP mRNAs and proteins was established via Northern and Western blot assays (SI Fig. 6). Two of the three F3 homozygous lines accumulated similar levels of CP and MP RNA and protein (lines nos. 21 and 22). In the third line (no. 18), neither MP nor CP RNA nor protein was detected, suggesting that both genes were silenced. This hypothesis was supported by an analysis that detected the accumulation of sRNA that includes CP gene sequences (SI Fig. 6). The silenced line, named mpxcpT42W*, and line 22, referred to as MPxCPT42W, were selected for further studies.

**Coexpression of TMV MP and CP T42W Alters Plant Development.** MPxCPT42W lines 22 and 21 exhibited severe morphological changes and poor fertility. F3, F4, and F5 progeny exhibited mild mosaic patterns on leaves, reduced number of plants that produce flowers, and reduced number of flowers per flowering plant (summarized in Table 2 and Fig. 2). Other phenotypes include reduced seed and reduced number of flowers per flowering plant (summarized in Table 2). Western blot assays of scion tissues of grafted plants were performed to monitor the accumulation of MP and CP; most samples did not accumulate detectable levels of MP and CP. However, MP and CP were detected in one particular plant, showing that the PTGS was not established in this scion. As expected, this plant showed phenotypes similar to MPxCPT42W. The reciprocal graft (i.e., MPxCPT42W, used as rootstock) did not show unusual phenotypes (Table 2).

**Table 2. Quantitative description of altered development of MPxCPT42W plants**

<table>
<thead>
<tr>
<th>Plants</th>
<th>No. of germinated seeds/planted seeds (%)</th>
<th>No. of abnormal/normal plants (%)</th>
<th>Width/length leaves (SD)</th>
<th>Epidermal cell surface, mm² (SD)</th>
<th>Height, cm (SD)</th>
<th>Plants with flowers, %</th>
<th>Plants with buds, %</th>
<th>No. of flowers per plant</th>
<th>Tripartite stigma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sx</td>
<td>272/424 (64)</td>
<td>0/272 (0)</td>
<td>0.56 (0.02)</td>
<td>1,419 (216)</td>
<td>90.17</td>
<td>100</td>
<td>100</td>
<td>17.33</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>MP</td>
<td>262/436 (60)</td>
<td>0/262 (0)</td>
<td>0.51 (0.2)</td>
<td>1,360 (175)</td>
<td>63.53</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>CPT42W</td>
<td>247/375 (65)</td>
<td>0/247 (0)</td>
<td>0.53 (0.02)</td>
<td>1,236 (196)</td>
<td>86.36</td>
<td>100</td>
<td>100</td>
<td>—</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>mpmpCT42W</td>
<td>253/400 (63)</td>
<td>0/253 (0)</td>
<td>0.54 (0.03)</td>
<td>1,305 (202)</td>
<td>81.28</td>
<td>100</td>
<td>100</td>
<td>15.50</td>
<td>0/35 (0)</td>
</tr>
<tr>
<td>MpxCP42W/mpmpCT42W</td>
<td>87/387 (22)§</td>
<td>19/87 (22)§</td>
<td>0.58 (0.05)§</td>
<td>2,517 (299)§</td>
<td>43.93</td>
<td>22.2§</td>
<td>44.4§</td>
<td>5.57§</td>
<td>16/39 (41)§</td>
</tr>
<tr>
<td>MpxCP42W/mpmpCT42W</td>
<td>—</td>
<td>—</td>
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<tr>
<td>MpxCP42W — — — — 83.47 91.6 100 12.27 0/106 (0)</td>
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<td>100</td>
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</tr>
<tr>
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<td></td>
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Number and percentage of germinated seeds and number of seedlings that exhibited abnormal phenotypes were measured in three independent experiments. Average ratio of width/length from >10 leaves. Epidermal cells observed with a scanning electron microscope: at least 25 cells measured using Image J to calculate average area. Height and percentage of plants presenting flowers and buds as well as number of flowers per plant and number of tripartite stigmas were averaged from >10 plants measured at 3 months (2 months after grafting); lower two lines indicate grafted plants, in which information above the slash (/) represents the scion, and that below the line indicates rootstock of the grafted plants.

1Measured 11 days after planting.

2Measured 2 months after grafting.

*Student’s t test, 95% confidence interval of this difference.

As controls in this study, we developed grafted plants comprising MPxCPT42W as scion with rootstocks of transgenic plants that produce CPT42W, MP or nontransgenic plants. None of these grafted plants restored the normal phenotype to the scion (data not shown). We concluded from these studies that coexpression of MP + CP is responsible for the abnormal development observed on MPxCPT42W lines.

The phenotypes exhibited in line MPxCPT42W are similar to...
those exhibited by a group of transgenic *A. thaliana* plants in which either miRNAs or targets of miRNA were altered (10, 13, 30–32). As described in Fig. 2, flowers of line MPxCP*T42W* exhibit a loss of symmetry (Fig. 2C), altered number and shape of reproductive organs (Fig. 2B, J, and N), and stigmas were frequently tripartite (Fig. 2F and Table 1). Other changes were also observed (Fig. 2) and may be the cause of low fertility in this plant line (compare Fig. 2 J–I).

A high percentage of seedlings of the F₃ progeny of MPxCP*T42W* (see Table 2) produce abnormal cotyledons (cup-shaped or partially fused cotyledons) (Fig. 2 G and H) and asymmetrically shaped leaves (Fig. 2L) compared with nontransgenic plants (Fig. 2K). Similar phenotypes were described in *A. thaliana* with increased miR164 levels (31, 32).

Leaves of MPxCP*T42W* have an unusual round shape: the length/width ratio of the leaves was 0.68 and is statistically different from WT plants (Table 2; Fig. 2D). In addition, MPxCP*T42W* leaves appear more waxy with a rough/hard texture (Fig. 2D) and epidermal cells are larger than nontransgenic leaves (Table 2; compare Fig. 2Q and P with Q). Similar changes were observed in transgenic *A. thaliana* plants with changes in miRNAs (30, 31). Recently, it was reported that miR160 regulates genes that alter epidermal cell shape in *A. thaliana* (33). These observations led us to consider whether or not the activity of this and/or other miRNAs are affected in plant line MPxCP*T42W* (33).

**Accumulation of miRNAs Is Altered in MPxCP*T42W* Plants.** We investigated the accumulation of selected 21-nt miRNAs that are involved in plant development in *A. thaliana* by using miRNA probes designed from *A. thaliana* sequences. Fig. 3 shows the results of Northern blot hybridization that detects specific miRNAs. In each experiment, we also included samples of leaf tissues 15 days after inoculation with TMV, as well as tissues from parent plant lines, and line mpxCF*T42W*. The radioactive signal was normalized to the amount of ribosomal RNA in each sample. Numbers presented above each subfigure represent the average relative accumulation of each miRNA from two biological replicates, compared with noninfected, nontransgenic plant tissues (set at 1.0). Standard errors are given in parentheses.

miRNAs 156, 164, 165, and 167 accumulated to higher levels in MPxCP*T42W* and TMV-infected plants compared with nontransgenic and noninfected tissue (Fig. 3A). On the other hand, transgenic plants MP, CPT42W, and mpxCF*T42W* and nontransgenic plants accumulated similar amounts of these miRNAs (Fig. 3A). Therefore, there is a strong correlation between increased miRNA accumulation and the aberrant phenotype exhibited in plant line MPxCP*T42W* and in TMV-infected plants. Accumulation of miR 156 was also elevated in the MP plant line (line 277) compared with nontransgenic plants, although not to the level of the MPxCP*T42W* line (Fig. 3A). Although accumulation of miR160 was also somewhat increased in line 277, as was miRNA 156, 164, 165, and 167, the differences were not considered significant (Fig. 3B). Accumulation of miRNA 171 and its complement (miR171*) was altered only in TMV-infected plants, in agreement with Fig. 1 (see Fig. 3B).

**MP Interacts with CPT42W in Vivo.** Because MPxCP*T42W* plants, but not either of the parent lines, exhibit abnormal phenotypes and changes in miRNAs, we investigated the possibility that TMV MP interacts in vivo with CP*T42W*. For this study, we used bimolecular fluorescence complementation (BiFC). BiFC is based on the formation of a fluorescent complex when two fragments of yellow fluorescent protein (YFP) are brought together by interaction between proteins fused to the fragments (34, 35). Sequences encoding YFP a.a. 1–155 and a.a. 156–239 (YFPN and YFPC, respectively) were fused to sequences encoding the MP or CP*T42W* to produce MP–YFPN, MP–YFPC, CP–YFPN, and CP–YFPC. Constructs encoding YFPN and YFPC (E–YFPN and E–YFPC) and constructs encoding MP–YFPC, MP–YFPN, CP–YFPC, and CP–YFPN were developed (SI Fig. 7).

Leaves of *N. benthamiana* were infiltrated with a suspension of *A. tumefaciens* harboring the BiFC constructs, and sites were examined via fluorescence microscopy. Controls that induced coexpression of YFPN and YFPC or constructs encoding only MP or CP did not produce fluorescence (Fig. 4). In contrast, YFP fluorescence was detected after infiltration because coexpression of MP–YFPC and CP–YFPN, and CP–YFPC, respectively, were fused to sequences encoding the MP or CP*T42W* producing MP–YFPN, MP–YFPC, and CP–YFPC. Constructs encoding YFPN and YFPC (E–YFPN and E–YFPC) and constructs encoding MP–YFPC, MP–YFPN, CP–YFPC, and CP–YFPN were developed (SI Fig. 7).

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Because plants that produce both MP and CP exhibit abnormal development, we suggest that complexes comprising MP +
Table 3. MP and CPT42W do not suppress local and systemic PTGS

<table>
<thead>
<tr>
<th>Constructs</th>
<th>GFP signal†</th>
<th>Constructs</th>
<th>Systemic silencing (%)†</th>
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<tbody>
<tr>
<td>GFP</td>
<td>Yes</td>
<td>GFP</td>
<td>68/76 (89.5)</td>
</tr>
<tr>
<td>GFP + dsGFP</td>
<td>No</td>
<td>dsGFP</td>
<td>0/32 (0)</td>
</tr>
<tr>
<td>GFP + dsGFP + HC-Pro</td>
<td>Yes</td>
<td>dsGFP + p25</td>
<td>3/42 (7.1)</td>
</tr>
<tr>
<td>GFP + dsGFP + MP</td>
<td>No</td>
<td>dsGFP + MP</td>
<td>55/62 (88.7)</td>
</tr>
<tr>
<td>GFP + dsGFP + CPT42W</td>
<td>No</td>
<td>dsGFP + CPT42W</td>
<td>51/57 (89.5)</td>
</tr>
<tr>
<td>GFP + dsGFP + MP + CPT42W</td>
<td>No</td>
<td>dsGFP + MP + CPT42W</td>
<td>56/63 (88.9)</td>
</tr>
</tbody>
</table>

†Local silencing. Results of fluorescent signal in N. benthamiana leaves infiltrated with A. tumefaciens carrying different constructs.

TMV MP and CP Do Not Suppress PTGS. Viruses proteins that function as suppressors of PTGS can alter plant development and miRNA accumulation or/and activities (10–12). Therefore, we conducted experiments to determine whether TMV MP, CPT42W, or MPxCPT42W suppress PTGS to elicit the phenotypes in plant line MPxCPT42W.

Local Silencing. Leaves of nontransgenic N. benthamiana were infiltrated with A. tumefaciens carrying a GFP gene construct (35S-GFP), resulting in transient GFP expression (SI Fig. 8). Coinfiltration with Agrobacterium strains that carry genes encoding 35S-GFP and an inverted repeat of GFP sequences (35S-dsGFP) does not result in green florescence, because PTGS is triggered by the 35-dsGFP gene (SI Fig. 8) (40). Similarly, transient expression of the PTGS suppressor HC-Pro (35S-HC-Pro) simultaneously with 35GFP + 35-dsGFP inhibits silencing of the 35S-GFP gene, resulting in fluorescence comparable to that induced by 35S-GFP (SI Fig. 8).

Leaves of N. benthamiana plants were coagroinfiltrated with genes encoding 35S-GFP, 35S-dsGFP, 35S-MP, 35S-MP produces TMV MP, 35S-GFP + 35S-dsGFP, 35S-CPT42W (35S-CPT42W produces TMV CPT42W), or 35S-GFP + 35S-dsGFP + 35S-MP + 35S-CPT42W (SI Fig. 8). In none of these experiments did we observe GFP fluorescence. These data, summarized in Table 3, suggest that transient expression of MP or CPT42W did not suppress PTGS in this system and led us to propose that these proteins do not suppress PTGS in transgenic lines of N. tabacum used in this study.

Systemic Silencing. We conducted experiments to determine whether TMV MP and/or CPT42W prevent spread of the gene silencing signal as does p25 of PVX (16). Three week old transgenic N. benthamiana plants expressing GFP (14) were agroinfiltrated with 35S-dsGFP or coinfiltrated with 35S-dsGFP + 35S-HC-Pro, 35S-p25, 35S-MP, 35S-CPT42W, or 35S-MP + 35S-CPT42W. Systemic silencing of GFP was obtained when plants were inoculated with 35S-dsGFP alone, and when 35S-dsGFP was coinfiltrated with 35S-MP, 35S-CPT42W, or 35S-MP + 35S-CPT42W (Table 3). As expected, coinfiltrating genes encoding HC-Pro or p25 prevented the spread of gene silencing (Table 3) (16). In contrast, expression of genes encoding MP, CPT42W, or both did not prevent systemic silencing of GFP in transgenic N. benthamiana.

Discussion

Recent studies in plants and animals suggest that viruses can suppress gene expression and use endogenous RNA-silencing pathways to regulate host gene expression, presumably to benefit virus replication (3, 4, 41, 42). However, the underlying mechanisms that control these activities remain unclear. Epstein–Barr virus and other DNA viruses (reviewed in ref. 41) encode miRNAs that directly down- or up-regulate host and/or viral mRNAs.

Several studies have demonstrated that viral suppressors of RNA silencing can interfere with miRNA-mediated regulation of host genes (10–13). These studies revealed that viral proteins interfere with miRNA pathways, although it is unclear whether it is part of the virus replication strategy or a side effect due to interference with the miRNA pathways by the action of suppressors of both PTGS and the miRNA pathway.

In this work, we showed that viruses with weak or no PTGS suppression activity (TMV and ToMV) altered accumulation of miRNAs (Fig. 1 and Table 1). On the other hand, viruses that strongly inhibit gene silencing (e.g., TEV and PVY) did not modify miRNA accumulation to similar levels. (These data do not necessarily mean that miRNA activity per se is not affected.) Hence, this work provides evidence that PTGS suppression activity is not essential for virus induced changes in amounts of miRNAs, and that viruses may exploit or use the miRNA pathway independent of suppression of PTGS.

Our studies revealed that viruses that produced the most severe symptoms on tobacco under the conditions tested (TMV and ToMV; Table 1) altered miRNA accumulation to a greater extent than viruses that produced mild symptoms (i.e., TEV and PVY; Fig. 1). This result suggests that certain disease symptoms depend on miRNA levels.

We also report that transgenic plants that accumulate TMV MP + CPT42W (MPxCPT42W) exhibit abnormal development (Fig. 2 and Table 2), including phenotypes similar to those exhibited in mutants of A. thaliana that in which miRNA pathways are altered. Our data support the hypothesis that coexpression of MP and CP in the hybrid lines MPxCPT42W are directly or indirectly responsible for the abnormal development observed on these lines (see Fig. 2 and Table 2). Furthermore, BiFC experiments support the conclusion that MP and CP interact in vivo. In other studies, immunoprecipitation by using anti-MP or -CP antibodies and extracts from infected cells recovered the second protein (S. Kawakami and R.N.B., unpublished data). In addition, MP and CP are colocalized in infected cells (23). We propose that complexes made up of MP + CP possess functions not found in either protein alone, and that these functions could induce abnormal plant development by altering miRNA accumulation.

Some of the developmental phenotypes exhibited by hybrid lines MPxCPT42W resemble those observed in transgenic A. thaliana and N. benthamiana that overexpress pre-miRNAs (15, 30, 33). For example, overexpression of miR164 altered embryonic, vegetative, and floral development, including cotedyledons that appear similar to those in line MPxCPT42W (Fig. 2 F–H, and L) (31, 32). miR165 plays a role in leaf radial symmetry and meristem formation (Fig. 2 C, O, and K) (13). These characteristics are similar to those observed in line MPxCPT42W. Likewise, accumulation of miRNAs 156, 164, 165, and 167 are altered in line MPxCPT42W and may be responsible for abnormal development in this hybrid.

Although our studies did not show a clear change in the amount of miR160, we cannot rule out a putative effect on miRNA160 activity. Epidermal cell shape is modified in MPxCPT42W (similar to Fig. 2 O–Q), a phenotype that may be controlled by miR160 (33).

Differences among the types of disease symptoms caused by TMV infection and those in MPxCPT42W plant lines may reflect temporal and/or spatial differences between localization of viral proteins in transgenic plants compared with virus infection (13). The transgenes studied here are under the control of a promoter known to be expressed in the meristem. In contrast, virus infections rarely invade the meristem.
A number of viruses have reported that viruses and viral proteins can modify miRNA pathways and thereby alter plant development (11–13). For example, Dunoyer et al. (11) reported that accumulation of several miRNAs were not altered by Peanut clump virus p15, although the accumulation of transcripts of target gene was affected (11). To date, the viral proteins known to alter the miRNA pathway also suppress PTGS. In contrast, we did not detect PTGS suppressor activity of TMV MP or CP (Fig. 4). Our data support the hypothesis that CP and MP act together to alter miRNA pathways.

Altering accumulation of miRNA likely affects miRNA targets, some of which could be part of host–pathogen interactions (3). We report (SI Table 4 and SI Text) that line MPxCP T42W was somewhat more resistant to TMV infection than MP and CP T42W plants under several different conditions.

In conclusion, coexpression of MP and CP T42W alters the accumulation of multiple miRNAs in transgenic plants, an effect that causes abnormal development similar to that in plants with altered accumulation of miRNAs. Similarly, infection by TMV alters accumulation of these miRNAs. Furthermore, these effects are apparently independent of the PTGS system. Studies with five different viruses showed (i) that PTGS suppressor activity is not essential for changes in abundance of miRNAs, and (ii) that changes in miRNA accumulation are a common feature of infection, an effect that may cause disease symptoms. Further studies are necessary to define the nature of the interactions between infection, miRNAs, and symptoms, by using approaches other than those used here. It is expected that these studies will lead to a more complete understanding of mechanisms that control disease symptoms in plants.

**Methods**

**Viral Infections.** For virus infection, a single leaf per plant was inoculated with semiclarified virus in 20 mM NaHPO4 (pH 7). All virus inocula were used at similar concentrations.

**miRNA Analysis.** Total RNA was isolated from leaves by using TRIzol reagent (Invitrogen, Carlsbad, CA), repeating the chloroform extraction three times. Twenty micrograms of RNA was resolved in 17% polyacrylamide gels containing 7 M urea; after electrophoresis, RNA was blotted to GeneScreen Plus membrane (PerkinElmer Life Science, Waltham, MA). Probes homologous to Arabidopsis miRNAs were end-labeled. The intensity of each band, based on rRNA loaded in each well, was quantified by using a Typhoon Trio (Amersham Biosciences, Piscataway, NJ). RNA loaded was quantified by using Typhoon software. The value for the miRNA species in nontransgenic/noninfected plants was set at 1.0 and other data calculated relative to this value. The data shown in Figs. 1 and 3 are the average of two independent biological replicates.

**BiFC Experiments.** YFP fragments encoding amino acids 1–155 (N fragment) and 155–239 (C fragment) were amplified by PCR using primers that add five glycine residues to a BamHI restriction site and cloned into pMON999 (pMON-YFP N and pMON-YFP C). TMV MP and CP T42W were amplified by PCR to delete the stop codon and include a BamHI restriction site. PCR products were cloned into pMON999 vectors with the N or C YFP fragments and subsequently cloned into the pART binary vector, resulting in MP-YFP C, MP-YFP N, CP-YFP C, CP-YFP N, E-YFP C, and E-YFP N. All constructs were transferred to A. tumefaciens strain GV3101 and infiltrated in 4-week-old N. benthamiana plants. The YFP signal was photographed at 2-4 days after infection.

**PTGS Studies.** Local and systemic PTGS experiments were performed as described in Bazzini et al. (43). TMV MP and CP T42W were amplified from pTMV-T42W (21), first cloned into pGEM-T Easy Vector Systems (Promega, Madison, WI), then subcloned via EcoRI into the pMON999 vector and finally into pART27 via NotI. The resulting constructs were called 35S-MP and 35S-CP T42W, respectively.

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