Differential Effects of Mild and Severe *Cucumber mosaic virus* Strains in the Perturbation of MicroRNA-Regulated Gene Expression in Tomato Map to the 3' Sequence of RNA 2

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Viral infections interfere with the microRNA (miRNA)-mediated regulation of gene expression, determining developmental defects. In tomato leaves, the accumulation levels of six miRNA species and their target transcripts corresponding to transcription factors with roles in plant development and leaf morphogenesis and two genes involved in the short RNA processing, *DCL1* and *AGO1*, were significantly enhanced upon infection with the severe strain *Cucumber mosaic virus* (CMV)-Fny, while that of *AGO4* was reduced. In plants harboring the infection of the mild strain CMV-LS, the effects on miRNA pathway were reduced, although *AGO1*, *DCL1*, and *NAC1* also were shown to overaccumulate during infections exhibiting a mild phenotype. The use of the recombinant strain CMV-Fny(LS2b), in which the 3'-terminal region of CMV-Fny RNA 2, including the 2b coding sequence, was replaced with the corresponding region of CMV-LS RNA 2, provided evidence that the exchanged region was implicated in the perturbation of miRNA metabolism. In tomato plants infected with CMV-Fny supporting the ameliorative satellite (sat)RNA variant Tfn-satRNA, the symptomless phenotype correlated, with the exception of *NAC1* upregulation, with the absence of effects on mitochondrial RNA and miRNA expression. Some of the aspects of miRNA pathway perturbation described were peculiar to CMV–tomato interactions and involved in the etiology of the disease phenotype elicited in this host.

Plants possess different RNA-silencing pathways based on small RNAs, and this diversity is exploited for the regulation of a number of physiological functions, including regulation of gene expression and control of viruses, invasive elements, and repeated sequences (Chapman and Carrington 2007). MicroRNA (miRNA) metabolism is an RNA-silencing pathway that controls endogenous gene expression (Jones-Rhoades et al. 2006). It starts with the processing of approximately 1-kb miRNA precursors in the nucleus (pri-miRNAs), which produce imperfectly paired double-stranded (ds)RNAs of approximately 70 nucleotides (nt) (pre-miRNAs) that serve as sub-

strate for Dicer-like proteins (DCL1 in *Arabidopsis thaliana*) for the production of mature 21- to 22-nt miRNAs. Sense miRNAs are then incorporated in the RNA-induced silencing complex (RISC), a ribonucleoprotein complex including Argonaute proteins (AGO1 in *A. thaliana*), and target for degradation complementary endogenous mitochondrial (m)RNAs in a sequence-specific manner, while minus-sense miRNAs, or miRNA*, are very unstable and quickly degraded in the cytoplasm (Jones-Rhoades et al. 2006).

Small interfering RNAs (siRNAs) are chemically similar to miRNAs but show a size range between 21 and 24 nt (Hamilton et al. 2002; Llave et al. 2002). SiRNAs are processed from precursors containing dsRNA structure, such as transcripts containing inverted repeats or intermediates formed during RNA virus replication, and, therefore, are part of the RNA-silencing pathway operating as a defense of hosts against invading or noxious nucleic acids (Voinnet 2005). There is substantial overlapping between miRNA- and siRNA-dependent RNA-silencing pathways: for instance, virus-derived dsRNA are reduced to small 21- to 24-nt dsRNAs by DCL proteins such as DCL2 and DCL4 in *A. thaliana* (Aliyari and Ding 2009), and then siRNAs complementary to viral targets are recruited by AGOs (AGO1 and AGO7 in *A. thaliana*) (Qu et al. 2008) and incorporated into the RISC for sequence-specific viral RNA cleavage.

Most plant viruses are able to counteract the siRNA-mediated antiviral immune system by expressing proteins that block or interfere to some extent with RNA silencing (Palukaitis and MacFarlane 2006; Voinnet 2005). Because there are so many common features shared between the siRNA and miRNA pathways, an outcome of the viral RNA silencing-suppression activity is the alteration of miRNA metabolism that some authors interpreted as a side effect of the antiviral silencing suppression (Dunoyer et al. 2004; Qu and Morris 2005), although functions as a type of adaptive response or a mechanism beneficial to viral infection cannot be ruled out. A number of modifications in the normal miRNA-dependent gene expression regulation have been observed by both expressing viral silencing suppressors in transgenic plants and inoculating plants with viruses. These modifications have been proposed to include direct interaction between viral silencing suppressors and AGO1; functional inactivation of RISC complexes; and overaccumulation of stabilized and inactive miRNA/miRNA* duplexes, null or reduced miRNA-guided cleavage, and consequent upregulation of host target mRNAs (Chapman et al. 2004; Zhang et al. 2006; Bazzini et al. 2007; Lewsey et al. 2007; Shiboleth et al. 2007; Tagami et al. 2007).

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Because *DCL1* and *AGO1* are themselves post-transcriptionally regulated by miRNAs (miR162 and miR168, respectively) through a negative feedback mechanism controlling their expression, miRNA pathway perturbation also may lead to the significant increase of their accumulation levels (Zhang et al. 2006). However, miRNA target genes in plants are preferentially transcription factors (TF) which, in fact, account for over 65% the predicted targets in *A. thaliana* (Rhoades et al. 2002). Most of the targeted TF are known to regulate plant tissue and organ development, suggesting that miRNAs play a central role in the coordination of a wide range of cell division and cell fate decisions throughout the plant (Rhoades et al. 2002). Organogenesis from meristems and leaf morphogenesis, for instance, depends on the participation of several miRNAs that work as a synchronized network determining spatial and temporal regulation of gene expression. MiR164, miR165/166, and miR319, with their respective targets belonging to the NAC, HD-ZIP, and TCP families of TF, are among the essential regulators of meristem initiation and maintenance, axillary meristem differentiation, and leaf morphology, together with another numerous family of TF, the *auxin response factor* (*ARF*) genes, that are targeted by both miR167 and another family of small RNA regulators known as trans-acting siRNAs (Chuck et al. 2009). Modifications of the complex equilibrium of miRNA-dependent TF expression, such as the virus-induced disruption of the miRNA pathway, are then expected to manifest with clear phenotypes involving defects of plant development.

Different viral silencing-suppressor proteins have been associated with phenomena of miRNA pathway alteration including, among others, the P1/HCP of different *Potyvirus* spp., *Potato virus X* p25, *Tomato bushy stunt virus* p19, *Turnip crinkle virus* coat protein, and the 2b protein of *Cucumber mosaic virus* (CMV). Interestingly, transgenic expression in *Arabidopsis* of the 2b protein from mild CMV strains did not induce disruption of the miRNA pathway (Chapman et al. 2004; Zhang et al. 2006; Lewsey et al. 2007), whereas expression of the 2b protein from a severe strain, in addition to inducing a symptom-like phenotype in transgenic plants, also showed altered accumulation levels of miRNAs and miRNA-regulated genes (Zhang et al. 2006; Lewsey et al. 2007).

According to molecular and serological features, CMV strains are currently classified into three subgroups indicated as IA,

IB, and II (Palukaitis and Garcia-Arenal 2003). The CMV genome is constituted by three linear, plus-sense, single-stranded RNAs (RNAs 1, 2, and 3) and the subgenomic RNAs 4 and 4A, which contain five genes encoding proteins designated 1a, 2a, 2b, 3a, and 3b (Palukaitis and Garcia-Arenal 2003). The CMV 2b is a multifunctional and well-characterized protein of approximately 12 kDa, encoded by a sequence located at the 3' end of RNA 2 that overlaps, on a different reading frame, the 3' end of the 2a gene. It is expressed from RNA 4A, a subgenomic RNA derived from RNA 2 (Ding et al. 1994). The 2b proteins from CMV and another member of the *Cucumovirus* genus, *Tomato aspermy virus*, were shown to function as suppressors of post-transcriptional gene silencing (Beclin et al. 1998; Brigneti et al. 1998; Li et al. 1999). The 2b serves as a determinant for viral long-distance transport (Ding et al. 1995; Ji and Ding 2001; Soards et al. 2002; Wang et al. 2004), as well as for local cell-to-cell movement (Soards et al. 2002; Shi et al. 2003). Moreover, 2b represents the major virus pathogenicity determinant, as demonstrated by replacing the 2b gene of a mild CMV strain with that of a more severe one or by deleting the 2b gene sequence obtaining a symptomless infection (Ding et al. 1996; Shi et al. 2002; Soards et al. 2002). Thus, the difference in virulence observed between the severe subgroup IA strain CMV-Fny and the mild subgroup II strain CMV-LS on different hosts (Zhang et al. 1994; Wang et al. 2002) also could be determined, in addition to other viral factors, by differences in the respective 2b protein sequences.

In the present work, we used a system based on a chimeric CMV inoculum to investigate the role of 2b and adjacent RNA 2 sequences in perturbing miRNA pathways in tomato in the course of CMV infections. We found that, unlike what previously was observed in 2b-transgenic *Arabidopsis* (Lewsey et al. 2007), both CMV-Fny and CMV-LS provoked significant changes in the miRNA-regulated expression of several genes. We also demonstrated that TF having a key role in leaf morphogenesis, and corresponding miRNAs that post-transcriptionally regulate their turnover, overaccumulated specifically during CMV-Fny infections but not during either mild CMV infections or when CMV-Fny was inoculated together with a mild variant of satellite (sat)RNA co-determining symptomless infections on tomato.

RESULTS

Infectivity, symptoms, and RNA accumulation levels of severe, mild, and recombinant CMV strains.

We constructed a chimeric infectious clone in the background of CMV-Fny RNA 2, in which its 3' terminus was replaced with a homologous fragment derived from CMV-LS RNA 2. Nucleotide sequence analysis showed that the resulting pFny209(2b) clone contained a recombinant cDNA joining fragments 1 to 2,296 from CMV-Fny RNA 2 and 2,297 to 3,041 from CMV-LS RNA 2. It putatively coded for a chimeric 2a protein consisting of the 736 N-terminal amino acids from CMV-Fny and the 106 C-terminal amino acids from CMV-LS, in addition to the 2b protein entirely derived from CMV-LS (Fig. 1A). The recombinant strain CMV-Fny(2b) was reconstituted in tomato plants inoculated with infectious transcripts of CMV-Fny RNAs 1 and 3 plus the chimeric RNA 2 derived from the transcription of pFny209(2b).

CMV-Fny(2b), its parental strains CMV-Fny and CMV-LS, and the combination of CMV-Fny supporting the ameliorative variant of the satRNA from CMV-Tfn (Tfn-satRNA) were inoculated onto tomato plants to compare symptoms development and viral RNA accumulation. As the first step, tomato plants infected with the different CMV inocula were

Fig. 1. Wild-type and recombinant *Cucumber mosaic virus* (CMV) strains show different pathogenicity on tomato plants. **A**, Schematic representation of CMV RNA2 vectors used in this work. cDNAs corresponding to CMV-Fny and CMV-LS RNA2s are cloned in pFny209 and pLS209, respectively. The vector pFny209(2b) was obtained by substituting a 741-bp region of pLS209, that includes the C-terminus of the 2a protein, the complete 2b protein, the 2a/2b overlapping fragment, and the 3' untranslated region, to the corresponding region of CMV-Fny. **B**, Effects of decreasing severity on leaf morphology in tomato cv. UC82 are displayed upon infection with CMV-Fny, CMV-LS, and CMV-Fny(2b). Scale bar represents 1 cm.

monitored for disease symptoms between 12 and 14 days postinoculation (dpi). CMV-Fny severely altered leaf morphogenesis, inducing the typical reduction of leaflet blades (Fig. 1B, shoestring leaf) and of whole-plant growth. CMV-LS infection induced a moderate leaf blade reduction not associated with decrease of plant size (Fig. 1B). CMV-Fny(FL2b) infected tomato plants systemically, eliciting very mild symptoms consisting of moderate bubbling of leaflet blades (Fig. 1B), with no other evident alteration of whole-plant morphology. The genetic stability of the recombinant CMV-Fny(FL2b) RNA 2 was assessed at 30 dpi by reverse-transcription polymerase chain reaction (RT-PCR) amplification of a 900-bp DNA fragment corresponding to the 3'-terminal region of RNA 2 using a forward primer designed on a region of CMV-Fny approximately 150 nt upstream from the recombination breakpoint. Three of these DNA fragments deriving from three individual plants were directly sequenced, showing no sequence variations from the original cDNA clone (not shown). The infection of CMV-Fny/Tfn-satRNA, as expected, showed a symptomless phenotype on tomato (not shown).

As the second step, the RNA accumulation profiles of the four inocula in infected tomato plants were examined. Samples were collected from the systemically infected leaves at 9 dpi. The levels of accumulation of CMV RNAs 2 and 3 in these leaf tissues were determined by quantitative real-time RT-PCR (qRT-PCR), using β -tubulin as the reference gene.

Accumulation levels of CMV-Fny RNAs 2 and 3 were two and four times higher than the corresponding RNAs of CMV-LS (Fig. 2), while abundance of CMV-Fny(FL2b) RNAs 2 was reduced by 5- and 10-fold, respectively, compared with the corresponding RNA of CMV-LS and CMV-Fny. The accumulation profiles of CMV-Fny RNAs observed in plants harboring CMV-Fny/Tfn-satRNA were very similar to those of CMV-Fny(FL2b), in the case of RNA 2, and both CMV-Fny(FL2b) and CMV-LS, in the case of RNA 3. The CMV-Fny/Tfn-satRNA quantification result was consistent with previous observations which demonstrated a positive correlation between latent infection and viral RNA downregulation (Cillo et al. 2007).

These results suggest that viral RNA accumulation, which is also an indirect indicator for the accumulation of viral proteins in infected tissues, positively correlates with the severity of induced symptoms and is in agreement with the notion that the 3' sequence of CMV RNA 2 cannot be replaced by a homologous region from a different strain without affecting both the accumulation of specific viral RNAs and disease phenotype. The mild phenotype induced by the chimeric strain CMV-Fny(FL2b) correlated with the mildness of the viral strain from which the RNA 2 3' terminus had been cloned, which codes for the 2b protein, a viral pathogenicity determinant.

Accumulation levels of miRNA-regulated genes are differentially altered by different inocula in CMV-infected tomato plants.

We tested whether the accumulation of selected miRNA-regulated and non-miRNA-regulated transcripts in tomato could be altered by severe as well as mild CMV strains, and whether the sequence substitution between a severe and mild CMV strain in a region including the 2b open reading frame (ORF), as in CMV-Fny(FL2b), might be sufficient to interchange the strain-specific ability to interfere with miRNA pathway.

The comparison of the expression levels of the gene set from infected and mock-inoculated plants was made by qRT-PCR using RNA preparations extracted from leaves at 9 dpi (i.e., before symptom appearance). The abundance of *DCL1* and *AGO1* transcripts was significantly increased by all the CMV inocula compared with mock-inoculated plants but with

evident strain specific differences. For *DCL1* mRNA, there was a clear increase in plants infected by CMV-LS (relative quantity [RQ] > 4) compared with plants infected by either CMV-Fny or CMV-Fny(FL2b) (RQ between 2.5 and 3) (Fig. 3A). Conversely, the level of accumulation of *AGO1* transcripts was found to be higher in plants infected by CMV-Fny (RQ = 4.9) than in those infected by either CMV-LS or CMV-Fny(FL2b) (RQ between 3.4 and 4) (Fig. 3A).

Two of five TF transcripts, *ARF8* and *TCP3*, did not show overaccumulation upon virus infection regardless of the inoculated strain. The two inocula containing the CMV-LS RNA 2 3' sequence induced a clear increase in the transcript levels of *NAC1* (RQ > 7) whereas, in samples infected with CMV-Fny, the abundance of *NAC1* was only double that of mock-inoculated plants. *HD-ZIP* and *TCP4* were increased in plants infected with CMV-Fny to levels of approximately RQ = 2, while CMV-LS caused only a limited increase of *HD-ZIP* accumulation and CMV-Fny(FL2b) did not result in higher accumulation of the two transcripts (Fig 3A).

We included in the analysis the quantification of the Argonaute-like gene *AGO4* and another TF, *PHAN*, which have not been associated thus far with miRNA-guided expression regulation. Although there was no statistical difference between *AGO4* expression in mock- or virus-inoculated plants, mean abundance of its transcripts in CMV-LS was almost double that in CMV-Fny infected samples, a difference determined by weak upregulation in the former and downregulation in the latter case. The *PHAN* mRNA accumulation levels in plants infected by CMV-Fny were four times higher than in mock-inoculated plants, while the other inocula did not cause any alteration of *PHAN* transcripts (Fig. 3B).

Fig. 2. Accumulation levels of *Cucurbit mosaic virus* (CMV) RNAs 2 and 3 in tomato leaves 9 days postinoculation (dpi) with four CMV inocula, determined by quantitative real-time reverse-transcription polymerase chain reaction analysis. According to the comparative method (relative quantity [RQ] = $2^{-\Delta\Delta C_t}$), cycle threshold (C_t) values of the target viral RNA were first normalized to the expression levels of β -tubulin mRNA (reference gene), and then made relative to the amount of the target viral RNA in an individual CMV-LS-infected (L) sample, representing the calibrator. For the calibrator, $-\Delta\Delta C_t = 0$ and RQ = 1 and, for the remaining samples, the value of RQ indicates the fold change of viral RNA accumulation relative to the calibrator. Columns represent mean values from three biological replicates and vertical bars indicate standard errors. M, mock-inoculated plants; F, CMV-Fny; L, CMV-LS; FL2b, CMV-Fny(FL2b); FTFN, CMV-Fny/Tfn-satRNA.

Altogether, these results indicate that, in tomato leaf tissues, CMV strains are able to interfere differentially with the accumulation of transcripts of the selected genes and that differences in the 3'-terminal region of RNA 2 may account for different mRNA accumulation. For example, whereas some genes were upregulated only (*TCP4* and *PHAN*) or to higher amounts (*AGO1* and *HD-ZIP*) in the presence of CMV-Fny, there was a marked overregulation for *NAC1* in plants infected by the two strains having the RNA 2 3' region derived from CMV-LS.

Accumulation levels of miRNA and miRNA* are differentially altered in CMV-infected tomato plants.

The interference of viral infection with miRNA pathways in tomato, predicted by the observed stable accumulation of some mRNAs that are target of miRNA-guided cleavage, was further investigated by qRT-PCR quantification of the involved miRNAs and miRNA*s. Tomato leaves infected by CMV-Fny showed a significant level of accumulation of *DCL1* (Fig. 3) and a parallel stabilization of miR162 and, in particular, of miR162* (Fig. 4), while the infection of CMV-LS and CMV-Fny(LS2b) did not induce the accumulation of these miRNA species despite the observed increase of *DCL1* transcript (Fig. 3). MiR168 and miR168* showed accumulation levels with the same strain-specific variation trend of mRNA target *AGO1* (Fig. 3), although the differences in miR168 values were not statistically significant, probably because of the high variability observed among biological replicates (Fig. 4).

MiR164 and miR164* accumulation levels reflected those of *NAC1*, with the only difference for CMV-LS that stabilized the accumulation of miR164* so that it was more abundant than in tomato leaves infected by CMV-Fny(LS2b) (Fig. 4).

Also miR165, miR319, and their star strands showed accumulation levels varying with the same tendency of their target genes (*HD-ZIP* and *TCP4*, respectively), with higher values due to CMV-Fny infection and no or very limited variations induced by the other CMV strains compared with mock-inoculated plants (Fig. 4). Finally, miR167, which targets *ARF8* for degradation, did not show any variation of accumulation levels, thus confirming that CMV infections do not alter the expression levels of this gene (Fig. 4).

These results indicated that, with some exceptions as in the notable case of *DCL1*/miR162, there is a substantial correlation between the accumulation levels of target mRNAs and the corresponding miRNA species, consistent with the proposed mechanism by which infection of mild and severe CMV strains would differentially perturb miRNA-guided regulation of gene expression.

Minor modifications of miRNA accumulation and gene expression regulation in symptomless CMV/satRNA-infected plants.

Having shown that CMV infections induced modifications of miRNA pathways for the regulation of different classes of genes, we addressed the question of whether asymptomatic infections deriving from the inoculation of CMV-Fny in the presence of the ameliorative Tfn-satRNA might be associated with some modifications on miRNA-guided regulation of the genes selected for this study.

Results of these experiments are shown in Figure 5. In agreement with what was shown previously, all analyzed transcripts, except for *ARF8* and *TCP3*, were upregulated upon infection with CMV-Fny, reaching RQ levels comparable with what was shown in Figure 3. CMV-Fny/Tfn-satRNA infec-

Fig. 3. Expression levels of nine genes modulated by viral infection in tomato plants 9 days postinoculation (dpi) with *Cucurbit mosaic virus* (CMV). Relative quantities (RQs) of **A**, seven microRNA (miRNA)-regulated transcripts: *Dicer-like 1* (*DCL1*), *Argonaute 1* (*AGO1*), *ARF8*, *NAC1*, *HD-ZIP*, *TCP4*, and *TCP3*; and **B**, two non-miRNA-regulated transcripts: *Argonaute 4* (*AGO4*) and *AS1/PHANTASTICA* (*PHAN*) determined by quantitative real-time reverse-transcription polymerase chain reaction analysis. Total RNA samples were extracted at 9 dpi with three different CMV strains or from mock-inoculated plants as the negative controls, as indicated by the gray-scale color legend. According to the comparative method ($RQ = 2^{-\Delta\Delta Ct}$), cycle threshold (Ct) values of the target transcripts were first normalized to the expression levels of β -tubulin mRNA (reference gene) and then made relative to the amount of the target mRNA in an individual noninfected (mock-inoculated) sample, representing the calibrator. For the calibrator, $-\Delta\Delta Ct = 0$ and $RQ = 1$ and, for the remaining samples, the value of RQ indicates the fold change of gene expression relative to the calibrator. Columns represent mean RQ values from three biological replicates and vertical bars indicate standard errors. Statistical significance of expression differences of individual genes upon infection by different CMV strains was analyzed with one-way analysis of variance. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$ (Tukey post-hoc test).

tions, on the contrary, did not cause stabilization of miRNA-regulated transcripts, with the important exception of the *NAC1* mRNA, for which accumulation levels were approximately sevenfold higher than mock-inoculated plants and double than CMV-Fny-infected plants (Fig. 5A). CMV-Fny, in the absence and, more evidently, in the presence of Tfn-satRNA, reduced the accumulation of *AGO4* transcripts (Fig. 5B). On the other hand, *PHAN* mRNA abundance increased in CMV-Fny-infected plants but did not change expression levels upon CMV-Fny/Tfn-satRNA infection (Fig. 5B).

Also in this case, mRNA accumulation correlated with CMV-Fny-induced stabilization of the respective miRNA and miRNA* (Fig. 6). However, compared with the results shown in Figure 4, in this second set of qRT-PCR experiments, miR164* levels, unlike miR164, were not different from those recorded in healthy plants. With CMV-Fny/Tfn-satRNA, the abundance of the selected miRNA was substantially similar to that of healthy controls, with the exception of miR168* and miR165*, whose accumulation levels were increased (Fig. 6).

These data, which confirmed the positive correlation between accumulation levels of miRNAs and their target transcripts in virus-infected tomato plants, support the original hypothesis of minimal perturbation of gene expression in symptomless CMV/satRNA infections.

Interference with host gene expression and miRNA accumulation is not active in CMV-inoculated tomato leaves.

All the data presented above refer to analyses on RNA extracted from systemically infected leaf tissues at 9 dpi. We also monitored the abundance of the selected library of mRNAs and miRNAs in cotyledons 2 dpi with CMV-LS and CMV-Fny. The results of these experiments, reported in Table 1, showed that, at an early stage of infection, neither CMV-Fny nor

CMV-LS induced significant expression variations in any of the six transcripts analyzed by qRT-PCR. The accumulation levels at 2 dpi were also tested for two miRNAs, miR168 and miR164, and, in both cases, the abundance in healthy plants did not differ from that measured in CMV-infected tomato plants. These results indicate that disruption of miRNA pathways is not an early effect of CMV infection on tomato and that the deriving accumulation of mRNAs and miRNAs is evident in systemically infected but not in directly inoculated leaf tissues.

DISCUSSION

In this study, we have shown that, in tomato, some pathogenic properties of two different CMV strains are affected by the 3'-terminal portion of RNA 2, a region including the 2b ORF, and correlate to some extent with the alteration of miRNA metabolism.

In *A. thaliana*, CMV-Fny symptoms are particularly severe compared with those induced by CMV-LS, and the effect has been attributed to the differential ability of the 2b protein of mild (CMV-LS) and severe (CMV-Fny) strains to interfere with miRNA metabolism (Lewsey et al. 2007). We have shown that, in tomato, three TF with a known role in leaf morphogenesis, *TCP4*, *HD-ZIP*, and *PHAN*, were upregulated specifically by CMV-Fny infections. The *lanceolate* mutation in tomato revealed the role of the related gene, a homolog of the *A. thaliana* *TCP4* TF, in the proper development and shaping of compound leaves (Ori et al. 2007). Both *HD-ZIP*, coding for a homeodomain-leucine zipper DNA-binding protein homologous to the *A. thaliana* TF *PHAVOLUTA* and *PHABULOSA*, and *PHAN*, the ortholog of *A. thaliana* MYB *ASYMMETRIC LEAVES1* (*AS1*), are important for establishing the adaxial cell fate during leaf dorso-ventral patterning (Chitwood et al. 2007; Willmann and

Fig. 4. Expression levels of selected microRNA (miR) and microRNA* (miR*) modulated by viral infection in tomato plants 9 days postinoculation (dpi) with *Cucurbit mosaic virus* (CMV). Relative quantities (RQs) were determined by quantitative real-time reverse-transcription polymerase chain reaction analysis. Total RNA samples were extracted at 9 dpi with three different CMV strains, or from mock-inoculated plants as the negative controls, as indicated by the gray-scale color legend. According to the comparative method ($RQ = 2^{-\Delta\Delta Ct}$), cycle threshold (Ct) values of the target microRNA were first normalized to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mitochondrial (m)RNA (reference gene), and then made relative to the amount of the target microRNA in an individual noninfected (mock-inoculated) sample, representing the calibrator. According to the comparative method ($RQ = 2^{-\Delta\Delta Ct}$), cycle threshold (Ct) values of the target transcripts were first normalized to the expression levels of GAPDH mRNA (reference gene) and then made relative to the amount of the target miRNA in an individual noninfected (mock-inoculated) sample, representing the calibrator. For the calibrator, $-\Delta\Delta Ct = 0$ and $RQ = 1$ and, for the remaining samples, the value of RQ indicates the fold change of miRNA expression relative to the calibrator. Columns represent mean RQ values from three biological replicates and vertical bars indicate standard errors. Statistical significance of expression differences of individual genes upon infection by different CMV strains was analyzed with one-way analysis of variance. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$ (Tukey post-hoc test).

Poethig 2007). Strict spatial and temporal transcriptional control of *TCP4* and *HD-ZIP* by miR319 and miR165/166, respectively, are indispensable for correct leaf development (Garcia 2008), which explains why mutations of *AGO1*, generating dysfunctional miRNA processing and enhanced accumulation of these and other TF transcripts in the absence of miRNA-guided mRNA cleavage, show defects in lateral organ polarity and leaf and flower morphology (Kidner and Martienssen 2004; Vaucheret et al. 2004). In this scenario, it is not surprising that the alteration of miRNA-regulated expression of *AGO1*, *TCP4*, and *HD-ZIP* by CMV-Fny in infected tomato plants led to a strong phenotype displaying leaf-altered morphology. A causal correlation between virus-induced perturbation of miRNA pathways and disease symptoms has already been suggested by several authors (Kasschau et al. 2003; Zhang et al. 2006; Bazzini et al. 2007; Shibolet et al. 2007). Our work shows that, in tomato, genes such as *PHAN*, whose expression is not, according to present knowledge, directly regulated by miRNA, are also abnormally expressed and are likely to play an important part in generating the observed disease phenotype. We can hypothesize that primary events due to blocked miRNA activity and abnormal TF accumulation may lead to a secondary cascade of events involving other genes important in leaf morphogenesis. Nevertheless, a second hypothesis could implicate a direct participation of *PHAN* in the host response to CMV infection. In fact, the activation or repression of *ASI* or *PHAN* genes in response to viroidal,

fungal, and bacterial pathogens has been described in different plants (Martin et al. 2007; Nurmberg et al. 2007). The disease phenotype of CMV-LS on tomato was milder than that induced by CMV-Fny, a trait also reported on different hosts such as tobacco and cucurbits (Zhang et al. 1994; Wang et al. 2002), and correlated with the observation that CMV-LS 2b protein did not interfere with miRNA-regulated gene expression (Lewsey et al. 2007). The results of our work also support this hypothesis in that TF required for leaf morphogenesis (*HD-ZIP*, *TCP* genes, and related miRNA/miRNA* as well as *PHAN*) showed unaltered or slightly altered expression upon CMV-LS inoculation. However, in the light of our results, that show that the expression of genes other than the mentioned TF is indeed markedly altered by CMV-LS infection (discussed below), it would be more appropriate to conclude that the two CMV strains induce different symptoms in tomato because of their ability to differently interfere with the miRNA-guided expression of some genes. CMV-Fny(LS2b) was a useful tool for addressing the question on whether, in CMV infections, the 2b protein was the viral factor eliciting modifications of miRNA-regulated gene expression, as supported by studies on transgenic plants expressing the 2b protein (Zhang et al. 2006; Lewsey et al. 2007). This hypothesis seems to be supported by the coincidence of gene and miRNA expression data in samples inoculated with CMV-LS and CMV-Fny(LS2b) that exhibited a similar phenotype and significantly differed from CMV-Fny despite the fact that, out of

Fig. 5. Expression levels of nine genes modulated by viral infection in tomato plants 9 days postinoculation (dpi) with *Cucumber mosaic virus* (CMV). Relative quantities (RQs) of **A**, seven microRNA (miRNA)-regulated transcripts: *Dicer-like 1* (*DCL1*), *Argonaute 1* (*AGO1*), *ARF8*, *NAC1*, *HD-ZIP*, *TCP4*, and *TCP3*; and **B**, two non-miRNA-regulated transcripts: *Argonaute 4* (*AGO4*) and *ASI/PHANTASTICA* (*PHAN*) determined by quantitative real-time reverse-transcription polymerase chain reaction analysis. Total RNA samples were extracted at 9 dpi with CMV-Fny (Fny, white columns) or CMV-Fny/TfnsatRNA (Fny/TfnsatR, light-gray columns), or from mock-inoculated plants (dark-gray columns) as the negative controls. According to the comparative method ($RQ = 2^{-\Delta\Delta Ct}$), cycle threshold (Ct) values of the target transcripts were first normalized to the expression levels of β -tubulin mRNA (reference gene) and then made relative to the amount of the target mRNA in an individual noninfected (mock-inoculated) sample, representing the calibrator. For the calibrator, $-\Delta\Delta Ct = 0$ and $RQ = 1$ and, for the remaining samples, the value of RQ indicates the fold change of gene expression relative to the calibrator. Columns represent mean RQ values from three biological replicates and vertical bars indicate standard errors. Statistical significance of expression differences of individual genes upon infection by different CMV strains was analyzed with one-way analysis of variance. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$ (Tukey post-hoc test).

the 3' fragment of RNA 2, the recombinant strain CMV-Fny (LS2b) shared the rest of the genome with CMV-Fny. However, the chimeric CMV-Fny(LS2b) RNA 2 does not permit isolation of the 2b functions from those of the RNA 2 3' untranslated region and the 2a/2b overlapping region, whose possible role in specific pathogenic processes has been recently discussed (Du et al. 2008).

The ability of CMV-Fny to induce severe symptoms in tomato plants was dramatically affected by the coinoculation of Tfn-satRNA, which gave rise to symptomless infections. Our work demonstrates unaltered expression of the selected miRNAs and their target genes, associated with a substantial downregulation of CMV RNA 2 upon Tfn-satRNA coinfection, to levels that were much lower than those detected in infections with CMV-Fny and other symptomatic CMV/satRNA combinations (Fig. 2) (Cillo et al. 2007). Thus, we propose that low levels of RNA 2 in the presence of Tfn-satRNA would probably imply the translation of reduced amounts of 2b and, consequently, the reduced ability to interfere with *AGO1* activity and miRNA-mediated gene expression. Therefore, this work provides the first direct evidence that the differential alteration of miRNA pathway for the regulation of gene expression could contribute to elucidating the mechanisms underlying symptom suppression co-determined by certain satRNAs in CMV-infected plants.

In previous studies, the analysis of CMV effects on the miRNA pathway was mainly limited to the expression of 2b proteins belonging to different strains, which led to the conclusion that subgroup I (e.g., CMV-Fny) 2b but not subgroup II (e.g., CMV-LS, CMV-Q) 2b proteins were able to block *AGO1* activity and impair proper miRNA-guided mRNA cleavage in *A. thaliana* (Chapman et al. 2004; Zhang et al. 2006; Lewsey et al. 2007). Our study, on the other hand, explored the effects on miRNA pathways of authentic CMV infections on tomato, and produced some original results on the alteration of *DCL1*, *AGO1*, *AGO4*, and *NAC1* expression levels that could be due

to changes in both experimental strategy and host plant system. According to Zhang and associates (2006), CMV-Fny 2b, unlike CMV-Q 2b, is able to stabilize to high levels *DCL1* and miR162/miR162* accumulation. In CMV-infected tomato plants, we showed that (i) *DCL1* mRNA levels did not always correlate with miR162 amounts, both of which accumulated to higher levels than healthy plants only in the case of CMV-Fny infection; and (ii) *DCL1* modulation was significantly different between CMV-LS and CMV-Fny(LS2b), with the former inducing higher transcript levels than the latter despite the fact that the abundance of the respective miRNAs was not different from that of healthy plants. Thus, the two strains sharing the 3'

Table 1. Expression levels of microRNA (miRNA)-regulated transcripts and relative miRNAs at 2 days postinoculation in *Cucumber mosaic virus* (CMV)-Fny- and CMV-LS-infected tomato plants, determined by quantitative real-time reverse-transcription polymerase chain reaction and expressed as relative quantity (RQ)^a

Gene/miRNA	Mock	Fny	LS
<i>DCL1</i>	1.00 ± 0.10	0.78 ± 0.09	0.72 ± 0.09
miR162	nd	nd	nd
<i>AGO1</i>	0.92 ± 0.05	0.80 ± 0.04	0.72 ± 0.08
miR168	1.14 ± 0.16	1.18 ± 0.26	1.28 ± 0.09
<i>NAC1</i>	1.07 ± 0.15	1.08 ± 0.04	1.02 ± 0.19
miR164	1.13 ± 0.06	1.16 ± 0.14	1.18 ± 0.06
<i>HD-ZIP</i>	1.17 ± 0.08	1.25 ± 0.05	0.93 ± 0.09
miR165	nd	nd	nd
<i>TCP4</i>	1.33 ± 0.16	1.19 ± 0.12	1.02 ± 0.13
miR319	nd	nd	nd
<i>PHAN</i>	1.03 ± 0.02	0.99 ± 0.05	0.95 ± 0.08

^a Values were first normalized to the expression levels of β -tubulin (mRNA quantification) or glyceraldehyde 3-phosphate dehydrogenase (miRNA quantification) and then made relative to the amount of the target mRNA or miRNA in noninfected (mock-inoculated) plants. Data represent RQ mean values from three biological replicates $\pm$ standard errors; nd = not determined.

Fig. 6. Expression levels of selected microRNA (miR) and microRNA* (miR*) modulated by viral infection in tomato plants 9 days postinoculation (dpi) with *Cucumber mosaic virus* (CMV). Relative quantities (RQs) were determined by quantitative real-time reverse-transcription polymerase chain reaction analysis. Total RNA samples were extracted at 9 dpi with CMV-Fny or CMV-Fny/Tfn-satRNA, or from mock-inoculated plants as the negative controls. Cycle threshold (Ct) values of the target microRNA (miRNA) were first normalized to the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mitochondrial (m)RNA (reference gene), and then made relative to the amount of the target miRNA in an individual noninfected (mock-inoculated) sample. According to the comparative method ($RQ = 2^{-\Delta\Delta Ct}$), cycle threshold (Ct) values of the target transcripts were first normalized to the expression levels of GAPDH mRNA (reference gene) and then made relative to the amount of the target miRNA in an individual noninfected (mock-inoculated) sample, representing the calibrator. For the calibrator, $-\Delta\Delta Ct = 0$ and $RQ = 1$ and, for the remaining samples, the value of RQ indicates the fold change of miRNA expression relative to the calibrator. Columns represent mean RQ values from three biological replicates and vertical bars indicate standard errors. Statistical significance of expression differences of individual genes upon infection by different CMV strains was analyzed with one-way analysis of variance. Differences were assumed to be statistically significant, and indicated with different letters, for $P < 0.05$ (Tukey post-hoc test).

terminus of the CMV-LS RNA 2 and its 2b might alter *DCL1* accumulation levels via additional, 2b- or miRNA-independent regulation mechanisms that could be promoted by CMV-LS and, to a lesser extent, also by CMV-Fny(LS2b) (Fig. 3). We showed that CMV-Fny infections were able to induce overaccumulation of *AGO1* and miR168/miR168* in infected tomato plants, similarly to what was evidenced in *Arabidopsis* plants infected with the same CMV strain or constitutively expressing CMV-Fny 2b (Zhang et al. 2006). In our experiments, CMV-LS and CMV-Fny(LS2b) also elicited the accumulation of *AGO1* and miR168/miR168*, denoting a remarkable difference with CMV-Q 2b-transgenic *A. thaliana* plants (Zhang et al. 2006). This result could be an indication of a higher responsiveness of *AGO1* miRNA-regulated expression to actual viral infections compared with transgenic expression of individual viral proteins, as also was shown on different virus–host models (Shibboleth et al. 2007).

We also looked at the accumulation levels of *AGO4*, another member of the *Argonaute* gene family that is not believed to be controlled by the miRNA pathway. This was the only gene among the selected set whose abundance was weakly down-regulated by CMV-Fny infection to accumulation levels that were significantly lower than those recorded in the course of CMV-LS infections. Therefore, our results are in agreement with evidence suggesting that some CMV 2b silencing suppression functions, mediated by either transcriptional suppression of *AGO4* (Ye et al. 2009) or by a direct 2b-*AGO4* interaction at the protein level (Tomas Canto, *personal communication*), may counteract plant RNA-based defense mechanisms by interfering with *AGO4*-dependent RNA-directed DNA methylation activity and systemic RNA silencing (Jones et al. 2006).

A peculiar pattern of expression emerged by analyzing *NAC1* transcripts in infected tomato plants. Numerous members of the NAC family of TF has been shown to be responsive to both biotic and abiotic stresses (Olsen et al. 2005), and specific NAC genes of solanaceous species are transcriptionally activated following infections by viral or fungal pathogens (Collinge and Boller 2001; Oh et al. 2005; Selth et al. 2005). However, the originality of our findings resides in the miRNA-mediated upregulation of *NAC1* transcripts in infections characterized by a mild or symptomless disease phenotype (i.e., with CMV mild strains or in the presence of a benign satRNA). Because CMV-LS 2b expression in transgenic *Arabidopsis* plant did not affect *NAC1* and miR164 accumulation levels in either leaf or root tissues (Lewsey et al. 2007), it is tempting to speculate that the CMV-LS 2b protein could trigger an altered *NAC1* miRNA-guided regulation in tomato but not in *Arabidopsis*. On the other hand, because *NAC1* overaccumulation also occurred in plants infected by CMV-Fny/Tfn-satRNA and, in this case, the mechanism of upregulation appears to be non-miRNA mediated, it seems possible that other viral factors can affect levels of *NAC1* accumulation through different mechanisms, as has been demonstrated in tomato plants infected with *Tomato leaf curl virus* (Selth et al. 2005). It would be very interesting to further investigate whether *NAC1*-enhanced expression may play a direct role in plant defense against CMV infections in tomato.

The alteration of miRNA pathways, as described above, was observed at 9 dpi in systemically infected leaf tissues but not at 2 dpi on directly inoculated leaves. It is possible that, at earlier time points, the disturbing interaction between viral infection and the miRNA-dependent gene regulation activity is not yet detectable in terms of altered mRNA and miRNA accumulation levels. Nevertheless, a microarray analysis showed that a massive alteration of gene expression profiles is clearly present at 2 dpi, with over 1,000 differentially expressed genes already detectable in CMV-Fny-infected tomato plants (F. Cillo, A.

Polverari, and D. Gallitelli, *unpublished data*). The mechanisms by which the 2b protein has been shown to suppress RNA silencing, targeting initiation of the process at the growing points of the plants (Brigneti et al. 1998), might explain the absence of effects on miRNA pathways in developed, inoculated tissues.

In conclusion, this study provides evidence, as also shown by a very recent article (Feng et al. 2009), of the existence of virus-induced and miRNA-mediated pathogenic processes in infected tomato plants. Moreover, we show that fundamental similarities but also remarkable differences arise from comparing interaction mechanisms of viral pathogens with model plants, such as *A. thaliana*, versus important crop plants like tomato. Further work is needed to clarify the role of these host-specific but also strain-specific differences in the onset of susceptible or tolerant response of the host to viral infection, and to understand whether the new information may be exploited to introduce new strategies for crop protection from viruses.

MATERIALS AND METHODS

Recombinant plasmid construct.

A recombinant CMV RNA 2 clone has been obtained, in which the original 3'-terminal sequence of CMV-Fny, including the whole 2b protein coding sequence, has been replaced by the corresponding region of CMV-LS.

Plasmid pFny209 (Rizzo and Palukaitis 1990), carrying the cDNA for CMV-Fny RNA 2, was mutagenized in order to introduce a *SacII* restriction site. Changes applied to pFny209 were T2297G and A2298G, using the PCR-based protocol of the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.), and the mutagenized clone was named pFny209_*SacII*. The thermal cycles for PCR were as follows: initial denaturation at 95°C for 30 s; 12 cycles at 95°C for 30 s, 55°C for 1 min, and 68°C for 6 min. At the same time, a DNA fragment equivalent to the 3'-terminal region (nucleotides 2,294 to 3,038) of CMV-LS RNA 2 was amplified from pLS-CMV2, the vector carrying the corresponding cDNA (Zhang et al. 1994), using AccuPrime *Taq* DNA Polymerase High Fidelity (Invitrogen, Paisley, U.K.). The primers for this amplification were designed in order to introduce *SacII* and *PstI* restriction sites at the 5' and 3' ends of the fragment, respectively. The PCR product was first cloned into a pGEM-T vector (Promega Corp., Madison, WI, U.S.A.), then introduced by T4 DNA ligase (Promega Corp.) in pFny209_*SacII* previously digested with *SacII* and *PstI* restriction endonucleases. All fragments obtained during the cloning procedure and the final vector pFny209(LS2b) were confirmed by sequencing before use.

Plants and viruses.

Seedlings of tomato (*Solanum lycopersicum* cv. UC82) were grown in glasshouse with controlled temperature at 22°C (±2°C) with a 16-h photoperiod.

The two strains CMV-Fny (Owen and Palukaitis 1988) and CMV-LS (Wahyuni et al. 1992), received from P. Palukaitis (SCRI, Dundee, U.K.), were maintained on UC82 tomato and purified from systemically infected leaves, collected 12 days after inoculation, according to Lot and associates (1972).

The recombinant strain CMV-Fny(LS2b) was obtained by *in vitro* transcription of the following three CMV genomic RNAs: CMV-Fny RNA 1 and RNA 3, transcribed from the vectors pFny109 and pFny309, respectively (Rizzo and Palukaitis 1990), and the chimeric RNA 2, transcribed from the vector pFny209(LS2b), described in the previous paragraph. The mechanical coinoculation of the three transcribed RNAs resulted in systemic infection of tomato plants, from which the

strain was purified as indicated above. The inoculum combining CMV-Fny plus the CMV-Tfn-satRNA was obtained by coinoculation of the corresponding in vitro transcripts, according to published methods (Cillo et al. 2004).

Inoculation of test plants was carried out mechanically at the cotyledon stage with sap from systemically infected tomato leaf tissues at 12 to 15 dpi homogenized in 20 volumes of 100 mM phosphate buffer, pH 7.2.

RNA extraction and qRT-PCR analysis.

Total RNA was extracted either from CMV-inoculated or mock-inoculated cotyledon leaf tissues of tomato plants 2 dpi, or from systemically infected second true leaves 9 dpi, using TRIzol reagent (Invitrogen). RNA preparations were subjected to on-column DNase digestion (RNase-free DNase set and RNeasy mini kit; Qiagen, Hilden, Germany), denatured at 95°C for 3 min, and reverse-transcribed in the presence of random hexam-

Table 2. List of genes and microRNAs (miRNAs) and primer sequences for quantitative real-time reverse-transcription polymerase chain reaction analysis

Gene	Gene name	Protein function	Biological process	Accession	Primer sequence ^a	Related miRNA	Primer name ^b	Primer sequence ^c
<i>DCL1</i>	<i>DICER-LIKE 1</i>	Ribonuclease III	RNA silencing	BI209312	for actgtatcatgatgtgtgcacag rev gagttccaatagaagagctg ctg	miR162	sly-miR162 sly-miR162*	for cgccgctcgataaac ctctgcat for cgccgctcgaggca gcggtaccat
<i>AGO1</i>	<i>ARGONAUTE 1</i>	Ribonuclease H	RNA silencing	BP901659	for ggaattgctgatttcctccg tcg rev ctgatatttggttctaaa gatgcac	miR168	ath-miR168a deg-miR168*	for cgccgctcgcttggt gcaggtcgggaa for cgctccgcttgca ycaastga
<i>ARF8</i>	<i>ARF8</i>	Transcription factor (<i>AUXIN RESPONSE FACTOR</i> family)	Hypocotyl elongation, flower and root development	EF667342	for tagcgacagcatgcacatt gga rev tccggaacgcatccaac aga	miR167	sly-miR167 sly-miR167*	for cgccgctgaagctg ccagcatga for cgcgagatcatgtg gcagcctta
<i>NAC1</i>	<i>NAC1</i>	Transcription factor (NAM/ATAF/CUC domain-encoding family)	Lateral root development	BG791220	for caccaggatttagattccat cca rev tctctccccaacacatgc	miR164	ath-miR164a deg-miR164*	for cgccgctggagaag cagggcacgtgca for cgtgcacgtgctch hcttctcca
<i>HD-ZIP</i>	<i>HD-ZIP protein</i>	Transcription factor (class III HOMEODOMAIN-LEUCINE ZIPPER family)	Leaf morphogenesis	X91212	for ctgattaggagagctctctattct ctc rev gacacttgcttctgtaatcg gtc	miR165/166	ath-miR165a deg-miR165*	for cgccgctggacca ggcttcac for ccggggamtgtgtc tggmtcga
<i>TCP3</i>	<i>TCP3</i>	Transcription factor (TB1/CYC/PCF family)	Leaf morphogenesis	EF091574	for gcctccatggaaaccaac taccact rev gagctagcgctattgttctgcc cta	miR319	sly-miR319 sly-miR319*	for ccgccccttgagact gaaggagctcc for ccagctgccgcgag cttcttagtcca
<i>TCP4</i>	<i>TCP4/LANCEOLATE</i>	Transcription factor (TB1/CYC/PCF family)	Leaf morphogenesis	EF091571	for agcaagtgcagggtcaagc rev ctggtggcgaattgaacaag	miR319	sly-miR319 sly-miR319*	for ccgccccttgagact gaaggagctcc for ccagctgccgcgag cttcttagtcca
<i>AGO4</i>	<i>ARGONAUTE 4</i>	Ribonuclease H	RNA-directed DNA methylation	CD002775	for tgtgctccgataagttatg cca rev tggagctagcaacgtttgaa for aagcgtcgagggaagaacag			
<i>PHAN</i>	<i>AS1/PHANTASTICA</i>	MYB transcription factor [AS1/RS2/PHAN (ARP) family]	Leaf morphogenesis	AF148934	rev ccgccattaggttcagcaag for cctgacagcttctgcatgt			
<i>TUB</i>	<i>β-TUBULIN</i>	(Reference gene for miRNAs)		DQ205342	rev catcttcagcccagttgggtg for cgccgcgaaacaatcaaac tttac			
<i>GAPDH</i>	<i>GLYCERALDEHYDE 3 PHOSPHATE DEHYDROGENASE</i>	(Reference gene for miRNAs)		U97257			Poly(T) adapter Poly(T) adapter reverse ^d	gcgagcacagaattaata cgactcactatagg(t)1 2vn gcgagcacagaattaata cgact

^a For, forward primer; rev, reverse primer.

^b All miRNA and miRNA* sequences derived from Sanger Institute; sly-, sequence from *Solanum lycopersicum* miRNA; ath-, sequence from *Arabidopsis thaliana* miRNA (when corresponding sly-miRNA not available on database); deg-, degenerated primer sequence complementary to corresponding miRNA* from *Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera*, and *Populus trichocarpa* (when corresponding sly-miRNA* not available on database).

^c Degenerated bases are indicated by the following one-letter code: C + T = Y; C + G = S; A + C = M; A + T + C = H; A + C + G = V; A + C + G + T = N.

^d Common reverse primers for all miRNAs, miRNA*s, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference sequence.

ers (High-capacity reverse-transcription kit; Applied Biosystems, Foster City, CA, U.S.A.) in 20 µl of total reaction volume containing 1 µg of total RNA and incubated at 42°C for 2 h and at 85°C for 1 min according to the manufacturer's instructions.

On the same cDNA material, CMV RNA and host mRNA quantifications were performed via qRT-PCR. Primer pairs, used for qRT-PCR of viral RNAs 2 and 3, were designed to anneal to identical sequences between CMV-Fny and CMV-LS, and were Fny_LS_RNA2 for (5'-tgagttgcttggtgtatgacac-3', forward), Fny_LS_RNA2 rev (5'-catcaccttagcttccatgttgaa-3', reverse), Fny_LS_RNA3 for (5'-gtagacatctgtgacgcgatgccg-3', forward), and Fny_LS_RNA3 rev (5'-tcgcggagaagcatcatgagaa-3', reverse). Primers pairs used for tomato genes of interest were designed with the support of the Integrated DNA Technologies's PrimerQuest software and are listed in Table 2. Tomato β-tubulin, shown in preliminary analyses implemented in the software GeNorm (Vandesompele et al. 2002) to be stably expressed in healthy and virus-infected plants, was chosen as the reference endogenous gene for both viral RNA and mRNA relative quantification.

RT-PCR reaction volumes were set up to 10 µl that contained Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl of a 1:10 dilution of the cDNA reaction, and 300 nM each of the corresponding forward and reverse primers. Three different biological replicates, represented by total RNA extracted from two individually inoculated plants and converted into cDNA, were used for statistical analysis of the quantification, and each cDNA sample was amplified in duplicate on a single 48-well optical plate using a StepOne Real-Time PCR System (Applied Biosystems). The cycling profile consisted of 95°C for 10 min followed by 40 cycles of 15 s at 95°C and 70 s at 60°C, as recommended by the manufacturer. Immediately after the final PCR cycle, a melting curve analysis was done to determine specificity of the reaction. Quantities of RNA accumulation levels were calculated as RQ values using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta C_t}$) method (Livak and Schmittgen 2001). Before quantitative analyses, validation experiments were carried out to confirm equal amplification efficiencies between reference and target genes and the applicability of the comparative method. Assessing the relative amplification efficiencies was achieved by running standard curves for each amplicon (i.e., five serial log₁₀ dilutions of starting cDNA were amplified and Ct values of target and reference genes were measured in triplicate and plotted against the log of the input cDNA amount). The efficiencies were considered comparable when falling within a range of 100 ± 10%, corresponding to a curve slope of -3.3 ± 0.33 (Livak 1997), and primers pairs that did not allow PCR performances matching these criteria were discarded and substituted.

For qRT-PCR of miRNAs, the strategy of Shi and Chiang (2005) was adopted, with some modifications. The same total RNA preparations used for cDNA synthesis were polyadenylated as follows: 2 µg of RNA was added to 25 µl of a reaction mixture containing 2.5 mM MnCl₂, 80 µM ATP, 1× reaction buffer, and 0.5 µl (1 U) of *Escherichia coli* Poly(A) Polymerase from Ambion's Poly(A) Tailing Kit (Applied Biosystems). The reaction was incubated at 37°C for 15 min in order to add a short adenine tail to non-poly(A), low molecular weight RNA molecules. Then, 4 µl (320 ng) of this Poly (A) RNA was primed with 7.2 µM Poly(T) adapter (Table 2) and reverse transcribed in 10 µl with the High-capacity reverse-transcription kit (Applied Biosystems) using the following temperature conditions: 10 min at 25°C, 50 min at 42°C, and 1 min at 85°C. For PCR amplification of miRNAs, a common reverse primer was employed named Poly(T) adapter reverse (Table 2), homologous to 23 nt in the Poly(T) adapter sequence, while forward primers sequences were homologous to the positive- or negative-sense

(star strand) miRNA, as obtained from the specific database available online from the Sanger Institute website. Forward primers included a 6-nt additional sequence at the 5' terminus for stabilizing the amplicon, as described in Table 2. Forward primers for miRNA with no tomato sequences yet available on the database were designed either on the corresponding *A. thaliana* sequence or were degenerated by inserting multiple nucleotides at single positions in order to match the corresponding miRNA sequences from *A. thaliana*, *Oryza sativa*, *Vitis vinifera*, and *Populus trichocarpa* (Table 2). Tomato glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose stable expression in healthy versus infected plants was assessed by GeNorm, was used as the reference gene for miRNA qRT-PCR: an amplicon of the same size of RT-PCR-amplified miRNAs was generated by using a forward primer homologous to the 3'-terminal sequence of the GAPDH mRNA (Table 2). Amplification of miRNA targets was performed from 1 µl of a 1:10 dilution of the cDNA reaction, in a volume of 10 µl containing 200 nM primers and reagents from the Fast SYBR Green Master Mix (Applied Biosystems), and the cycling profile consisted of 95°C for 30 s followed by 40 cycles of 3 s at 95°C and 15 s at 60°C, as recommended by the manufacturer. Also, for miRNA qRT-PCR reactions, before quantitative analysis with the comparative method, equal amplification efficiencies were calculated by comparing standard curves (discussed above), and melting curve analyses were performed to monitor primer-dimer formation and to confirm individual gene-specific products.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Integrated DNA Technologies's PrimerQuest software:
eu.idtdna.com/Scitools/Applications/Primerquest
 Wellcome Trust Sanger Institute miRBase::Sequences database:
microrna.sanger.ac.uk/sequences