The 2b Silencing Suppressor of a Mild Strain of *Cucumber mosaic virus* Alone Is Sufficient for Synergistic Interaction with *Tobacco mosaic virus* and Induction of Severe Leaf Malformation in 2b-Transgenic Tobacco Plants

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Tobacco plants infected simultaneously by *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) are known to produce a specific synergistic disease in which the emerging leaves are filiformic. Similar developmental malformations are also caused to a lesser extent by the severe strains (e.g., Fny) of CMV alone, but mild strains (e.g., Kin) cause them only in mixed infection with TMV. We show here that transgenic tobacco plants expressing 2b protein of CMV-Kin produce filiformic symptoms when infected with TMV, indicating that only 2b protein is needed from CMV-Kin for this synergistic relationship. On the other hand, transgenic plants that express either the wild-type TMV genome or a modified TMV genome with its coat protein deleted or movement protein (MP) inactivated also develop filiformic or at least distinctly narrow leaves, while plants expressing the MP alone do not develop any malformations when infected with CMV-Kin. These results show that either TMV helicase/replicase protein or active TMV replication are required for this synergistic effect. The effect appears to be related to an efficient depletion of silencing machinery, caused jointly by both viral silencing suppressors, i.e., CMV 2b protein and the TMV 126-kDa replicase subunit.

In plants, multiple regulatory and defensive reactions are mediated by RNA silencing (Brodersen and Voinnet 2006; Voinnet 2009). Common features for all these reactions are that they are triggered by double-stranded (ds) RNA, initiated by processing of the dsRNA to 21-, 22-, or 24-nucleotide-long fragments by Dicer-like endoribonucleases and employment of these small RNAs for sequence-specific targeting of the silencing machinery to homologous RNA sequences. RNA silencing pathways are essential for numerous developmental functions mediated either by micro-RNAs (miRNAs) or by various *trans*- and *cis*-acting siRNAs, either on the transcriptional or posttranscriptional level (Flynt and Lai 2008). They are also effectively induced by various viral RNAs, including both the genomic RNAs of RNA viruses and transcripts of single-stranded and dsDNA viral genomes (Chellappan et al. 2004; Ho et al. 2006; Moissiard and Voinnet 2006; Molnar et al. 2005; Yoo et al. 2004), and lead to elimination or strong sup-

pression of the viral RNA sequences. RNA silencing thus constitutes an inducible, sequence-specific protection against viral invaders (Ding and Voinnet 2007; Hamilton and Baulcombe 1999; Li and Ding 2006; Voinnet 2005; Wadsworth and Dunoyer 2009).

To combat this major line of plant defense, viral pathogens have developed various silencing-suppressor proteins (viral suppressors of RNA silencing [VSRS]). It now appears plausible that most, if not all, plant viruses code for gene products to counteract their hosts' silencing activity and that these viral products are essential for the infectivity of viruses in their hosts (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998; Wadsworth and Dunoyer 2009). Many VSRS are known to disturb the silencing machinery of the host and produce varied developmental defects and malformed phenotypes when expressed in transgenic plants (Chapman et al. 2004; Dunoyer et al. 2004; Lewsey et al. 2007; Mallory et al. 2002; Shibolet et al. 2007; Zhang et al. 2006). For example, symptoms caused by *Tobacco mosaic virus* (TMV) (genus *Tobamovirus*, family *Virgaviridae*) and *Cucumber mosaic virus* (CMV) (genus *Cucumovirus*, family *Bromoviridae*) are at least partly due to interference of the VSRS with the host's silencing machinery (Bazzini et al. 2007; Cillo et al. 2009; Ye et al. 2009).

In some cases, mixed infections of two or more viruses cause synergism, i.e., symptoms that are more severe than what would be the additive effect of the two viruses. In the synergistic interaction, the titers, movement, or both, may be enhanced for one or both viruses, but it is also possible that only the symptoms (host response) are enhanced (Zhang et al. 2001). In many cases, these interactions are related to the function of the VSRS proteins. For instance, *Potato virus Y* (PVY) (genus *Potyvirus*, family *Potyviridae*) is known to significantly enhance the replication and symptoms of *Potato virus X* (genus *Potexvirus*, family *Flexiviridae*) in mixed infections (Vance 1991). This effect, as well as enhancement of other viruses, can be reproduced also in transgenic plants expressing only the P1-HCPro silencing suppressor polypeptide of PVY, indicating that it is due to the dampening of the host defense by this VSRS (Pruss et al. 1997). The class I RNase III protein, a viral silencing suppressor, encoded by *Sweet potato chlorotic stunt virus* (SPCSV) (genus *Crinivirus*, family *Closteroviridae*), and its ability to enhance accumulation of other viruses by eliminating the antiviral defense in sweet potato plants

(Cuellar et al. 2009) represents the other example in which the severe synergistic effect between two viruses is due to a single viral protein.

CMV causes synergistic infections with a number of other viruses (Wang et al. 2004). A special case of viral synergism is known to occur in tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*) plants infected both with TMV and CMV, in which the mixed infection causes so-called filiformic leaves, i.e., a developmental defect leading to a complete lack of the leaf blades (Garces-Orejuela and Pound 1957; Matthews 1991). The aim of this study was to investigate which proteins of CMV-Kin and TMV are responsible for the synergistic effects of these viruses.

RESULTS

Filiformic leaf malformation

in 2b-transgenic plants infected with TMV.

Transgenic tobacco lines expressing the 2b VSR protein of CMV (strain Kin) (Siddiqui et al. 2008) were inoculated with TMV strain U1. The plants became rigorously infected and, 2 weeks postinoculation (wpi), displayed systemic mosaic symptoms typical of wild-type (wt) TMV infection, with virus titers similar to the inoculated wt plants. However, after 4 to 5 wpi, the newly forming leaves in the 2b-transgenic plants were of the typical filiformic type (Fig. 1A and B). When examined under a stereomicroscope, the filiformic leaves were observed to mostly consist of the leaf mid rib, with occasional minor

initials of the leaf blade (Fig. 1A-d and -e), and contained malformed leaf hairs (Fig. 1A-e). The new leaves continued to develop the filiformic phenotype for about 2 weeks (Fig. 1B-a), after which the newly emerging leaves developed normally (Fig. 1B-b). Just before flowering, the plants reproduced the filiformic leaf type (Fig. 1B-c). Similar malformations were observed in wt tobacco plants coinoculated with TMV and CMV-Kin, but they were never observed in wt tobacco plants infected solely by TMV or CMV-Kin. In three repeated experiments, when the CMV-Kin was either coinoculated with TMV to wt plants or was solely inoculated to 2b-transgenic plants, its accumulation in these plants was strongly enhanced at 4 to 5 wpi (Fig. 1C) as compared with the wt control plants, indicating that the expression of 2b did not induce effective resistance against the homologous viral sequence, but instead, it improved the replication of the virus, maybe by providing additional silencing suppression.

The transgenic plants expressing the 2b of CMV-Kin could be effectively infected with the severe Fny strain of CMV, causing severe filiformisms of leaves (Fig. 1D-a). The wt plants infected with CMV-Fny also developed filiformic leaves, but the symptoms were mild (Fig. 1D-b).

Filiformic leaf malformation

in 2b-transgenic plants is correlated with TMV titers.

To investigate the causes of the filiformic leaf formation, different virus-host interaction parameters were analyzed using plants of two independent 2b-transgenic tobacco lines in all

Fig. 1. Symptoms induced by *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) infections in wild-type (wt) tobacco (*Nicotiana tabacum*) and 2b-transgenic tobacco plants. **A**, Tobacco leaves (wt) displaying TMV symptoms (a), or CMV-Kin symptoms (b), as compared with a healthy leaf (c). TMV-infected leaves of a 2b-transgenic plant showing filiformic symptoms are displayed in (d) and as seen under a microscope to view the malformed leaf hairs (e), as compared with those of a healthy 2b-transgenic plant (f). **B**, A 2b-transgenic plant infected with TMV showing the initial development of filiformic leaves at 5 weeks postinoculation (wpi) (a), recovery from the initial filiformism at 7 wpi (b), and reappearance of filiformic leaves at 12 wpi (c). Thin arrows point to filiformic leaves, whereas the leaves developing normally following recovery are pointed out with thick arrows. **C**, Accumulation of the CMV coat protein antigen in wt tobacco plants and the 2b-transgenic plants infected with CMV-Kin and the wt tobacco plants coinoculated with CMV-Kin and TMV, as detected by double-antibody sandwich-enzyme-linked immunosorbent assay. **D**, Development of filiformic leaves (pointed by arrows) caused by the severe strain CMV-Fny in 2b-transgenic tobacco plants (a) and wt plants (b).

experiments. First, both of these lines were tested by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for expression of the 2b mRNA, which was detected in both lines (Fig. 2A). The expression levels changed in both lines over time, as tested 2 to 6 wpi with TMV. Initially (2 to 3 wpi), the transgene mRNA expression was strongly enhanced in both lines and, then, declined but remained significantly higher than in the non-TMV-infected 2b-transgenic plants (Fig. 2A). Results of both transgenic lines were similar albeit the transgene expression exhibited somewhat different kinetics.

Subsequently, we analyzed the role of TMV accumulation, if any, in the severe leaf malformation of the 2b-transgenic plants mentioned above. The results indicated that TMV accumulation was strongly enhanced in the 2b-transgenic plants at 3 wpi (Fig. 2B), but a week later, TMV titers were significantly reduced in all plants, indicating strong induction of de-

Fig. 2. Relative changes in the 2b transgene mRNA expression levels and accumulation of *Tobacco mosaic virus* (TMV) RNA in 2b-transgenic tobacco plants, as determined by reverse transcription quantitative polymerase chain reaction. **A**, Mean 2b mRNA levels ($n = 6$) in healthy (H) plants and in two independent 2b-transgenic tobacco lines infected with TMV, at 2, 3, 4, 5, and 6 weeks postinoculation (wpi). **B**, Mean TMV RNA levels determined in the same 2b-transgenic lines as above at 2, 3, 4, 5, and 6 wpi. The columns indicate $\log_{10}$ transformation fold differences with 2b mRNA in healthy plants (A) and TMV RNA measured at 2 wpi (B). Standard error is indicated by thin error bars on the top. The experiment was repeated five times with similar results.

Fig. 3. Immuno-localization of *Tobacco mosaic virus* (TMV) coat protein (CP) in the shoot tips of TMV-infected wild-type (wt) and 2b-transgenic tobacco plants. **A**, In wt plants, the purple signals for TMV CP were observed in the fourth and fifth leaf primordia (4 and 5, indicated by arrows) but not in the younger leaf primordia (1 to 3) at 4 weeks postinoculation (wpi). **B**, In the 2b-transgenic plants, virus signals were observed in the third and fourth leaf primordia and also in the apical dome (indicated by arrow) at 4 wpi. Note initiation of leaf malformation in leaf primordium 3. **C**, An inset of the view observed in B at 40× magnification is shown at a higher magnification (100×). **D**, Virus signals in the leaf primordia of the 2b-transgenic plant at 5 wpi (40× magnification).

fense against TMV. At 5 wpi (i.e., the time of appearance of the filiformic leaves) TMV titers were enhanced again. The fluctuations of TMV titers indicated that the level of antiviral defense against TMV varied in the course of infection. Strong reduction of the 2b transgene mRNA accumulation (Fig. 2A) coincided with the decrease of the TMV titers at 4 wpi (during initiation of the leaf malformations), suggesting that the host defense against TMV and transgene expression was strongly activated at this stage.

In the TMV-infected 2b-expressing plants, the filiformic leaves were observed only in the upper parts of mature plants. To test this interaction at different growth stages, the transgenic tobacco line expressing CMV 2b and a transgenic line expressing infectious wt genome of TMV (Siddiqui et al. 2007) were crossed. Previously, we have reported that these transgenic TMV-expressing tobacco plants germinate and grow up as virus free and resistant to exogenous TMV infections, but then, a productive virus infection breaks out about 7 weeks after germination (Siddiqui et al. 2007). The causal mechanism of this early resistance is not known, but it appears not to be mediated by RNA silencing (Siddiqui et al. 2007). The F1 seedlings obtained by crossing T3 homozygous progeny of the (self-pollinated) transgenic lines appeared phenotypically similar to the original TMV-transgenic plant. The F1 progeny remained initially virus free and broke up to a productive TMV infection at the same stage as the parental TMV-transgenic plants. About 2 weeks later, they started to produce filiformic leaves (not shown). Again, 2 weeks later, these plants recovered from the malformed phenotype, retaining only the strong TMV symptoms.

Viral entry into the shoot apices.

Lateral organ differentiation and determination of the leaf polarity take place in the apical meristem and in the leaf primordial. These processes are regulated by transcription factors. Many of these processes are regulated by specific miRNA-mediated silencing reactions, with severe developmental defects being caused by malfunctions in the silencing machinery (Emery et al. 2003; Iwakawa et al. 2007; Liu et al. 2009; Mallory et al. 2004; Ochando et al. 2006; Prigge et al. 2005; Tang et al. 2003; Zhong and Ye 2004). Thus, it was anticipated that the observed filiformic leaf phenotype could be a consequence of VSRS-mediated interference in the meristems. Clearly, the transgenically expressed 2b protein of CMV-Kin was not sufficient to cause this by itself (the noninoculated 2b-transgenic plants exhibited no filiformism), but it was possible that expression of the 2b protein in the vasculature and meristematic cells could allow TMV to enter the meristems, which is normally prevented by silencing-mediated surveillance (Martín-Hernández and Baulcombe 2008). In repeated experiments immuno-localization of TMV in the shoot apices using TMV coat protein (CP)-specific antibodies revealed low

amounts of TMV in the vascular bundles of the fourth and fifth leaf primordia of some of the systemically infected wt plants at the time when the filiformic leaves were just appearing (4 wpi). In the 2b-transgenic plants, TMV was detected also in the first and second leaf primordia and in some apical meristems in low amounts at this timepoint (Fig. 3). However, at 5 wpi, TMV was detected almost in all (five out of six) of the analyzed sections prepared from leaf primordia of the 2b-transgenic plants (Fig. 3), whereas the virus was detected only in very low amounts in the vascular bundles of the wt plants.

Role of different TMV proteins in the synergistic interaction with CMV.

To map for one or more genomic regions or gene products of TMV playing a role in the synergistic interaction with CMV, we used transgenic tobacco plants expressing the infectious wt TMV genome or modified TMV genomes either lacking the entire CP gene (*TMV-ΔCP*) (Dawson et al. 1988; Turpen et al. 1993) or containing an inactivated MP gene (*TMV-ΔMP*) due to deletion of nucleotides 5,050 to 5,430 (Siddiqui et al. 2007; unpublished results) (Fig. 4A). Furthermore, a transgenic tobacco line expressing the 30 kDa MP alone (Fig. 4C) (provided by W. O. Dawson, Citrus Research and Education Center, Lake Alfred, FL, U.S.A.) was included in the experiments. The transgenic lines that express either the wt TMV or *TMV-ΔCP* construct developed a detectable TMV infection at 7 to 8 weeks postgermination. The plants expressing wt TMV genome exhibited a typical TMV infection with mosaic disease, whereas the *TMV-ΔCP*-expressing plants developed only very mild symptoms and contain very low titers of viral RNA, as reported previously (Siddiqui et al. 2007). The *TMV-ΔMP* transgenic line expressed the TMV replicase-specific mRNA (Fig. 4B), but no viral CP or replicase proteins were detected in these plants by Western blotting (data not shown), indicating that the virus accumulation was very low and undetectable. These plants did not show any typical viral symptoms. However, they expressed a unique malformed flower phenotype (Fig. 4D), which suggests that the TMV 126-kDa replicase domain and possibly the 183-kDa replicase protein are expressed in the plants, although at a level that is too low to be detected.

All aforementioned types of transgenic plants were inoculated with CMV-Kin at the age of 5 weeks. The wt TMV transgenic plants started to show typical TMV symptoms at 2 to 3 wpi, and soon thereafter, all plants also produced filiformic leaves (Fig. 4E-a). Most (80%) of the *TMV-ΔCP* expressing plants also produced one or two filiformic leaves per plant but only 4 to 5 wpi (Fig. 4E-b), after which the new leaves developed normally. The *TMV-ΔMP* transgenic plants did not develop any typical filiformic leaves, except for some narrow leaves just before flowering (Fig. 4E-c). The wt plants, or plants expressing only the TMV-MP, did not show any altered leaf morphology following infection with CMV-Kin. Hence,

Fig. 4. Tobacco plants transformed for expression of the whole genome of *Tobacco mosaic virus* (TMV) or parts thereof. **A**, Schematic presentation of the TMV wild-type genome and its derivatives used for plant transformation. 35S = *Cauliflower mosaic virus* 35S promoter. The coat protein (CP)-deleted genome and movement protein (MP)-inactivated genome were generated by deleting nucleotides 5,712 to 6,191 and 5,050 to 5,430, respectively. Numbers indicate nucleotide positions in the genome. **B**, Detection of expression of the *TMV-ΔMP* RNA in the transgenic tobacco plants by reverse transcription polymerase chain reaction (RT-PCR) analysis of DNase-treated total RNA. An amplification product of the expected size (143 bp) from the replicase gene was detected in all three tested plants. Lanes 1 and 5, wild-type tobacco plant; lanes 2, 3, 4, 6, 7, and 8, *TMV-ΔMP* transgenic plants. Lanes 1 to 4 show results obtained by PCR using cDNAs synthesized on DNase-treated RNA, and lanes 5 to 8 show results obtained from the same DNase-treated total RNA samples without reverse-transcription (controls). **C**, Western blot analysis of MP expression in the TMV MP-transgenic plants. Besides the MP (30 kDa), the polyclonal antibodies raised to TMV MP also detected an unknown host protein (approximately 28 kDa) in both lanes. Lane 1, wt and lane 2, TMV-MP transgenic plant. **D**, Malformed flowers developing in *TMV-ΔMP* transgenic plants (a and b, at different magnifications), as compared with a flower of a nontransgenic tobacco plant (c). **E**, Development of filiformic leaves in the transgenic tobacco plants following infection with *Cucumber mosaic virus* (CMV)-Kin. The TMV-transgenic plants (a) developed many filiformic leaves (arrows), whereas the *TMV-ΔCP* transgenic plants (b) developed fewer but still characteristically filiformic leaves (arrow). In contrast, the *TMV-ΔMP* transgenic plants (c) infected with CMV-Kin did not develop perfectly filiformic leaves but leaves that were unusually narrow (arrow).

A

TMV transgenic plants

B **C** **D** **E**

these results indicate that the TMV MP alone cannot mediate the synergistic interaction with CMV and that CP is not required for it. The data suggest that (adequate levels of) the TMV helicase-replicase protein, which is a VSRS protein (Csorba et al. 2007), or active replication of TMV in the leaf primordia is required for the synergistic reaction with CMV-Kin.

DISCUSSION

Mixed infections with CMV and TMV in tobacco or tomato plants produce a specific synergistic disease characterized by development of filiformic leaves or “shoestring symptoms” (Garces-Orejuela and Pound 1957; Matthews 1991). Severe strains (Fny or WAI) of CMV alone can produce similar severe leaf malformations in tobacco and tomato plants, but mild CMV strains such as LS, Q (Cillo et al. 2009; Shi et al. 2002; Zhang et al. 1994; Ziebell et al. 2007) or Kin used in this study do not cause such symptoms unless plants are coinfecting with TMV. Our results show that the *2b* gene derived from CMV-Kin and expressed in transgenic tobacco plants is sufficient to induce a synergistic response with TMV, which is manifested by development of filiformic leaves. So far, only two other viral silencing-suppressor proteins, the P1-HCPro protein of PVY (Pruss et al. 1997) and class 1 RNase III protein of SPCSV (Cuellar et al. 2009) have been reported that alone can cause synergism with a heterologous virus and induce a similar phenotypic outcome as observed in coinfection of the respective two viruses.

The synergism developing as a consequence of TMV infection of the *2b*-transgenic plants was dynamic. It appeared at a certain time after infection, prevailed for a few weeks, then disappeared and reappeared once again during the vegetative growth phase of tobacco, followed by plant maturation and flowering. Fluctuation of the symptoms suggests that the effect depends on the quantitative balance between one or more disturbing viral molecules and one or more targets. The main factor appeared to be fluctuation of TMV titers, which was strongly reduced at 4 wpi and was then again stimulated at 5 wpi, coinciding with the time when the first signs of leaf malformations appeared. Similar fluctuation of the titers of TMV-based gene expression vectors have been observed in *Nicotiana benthamiana* plants in experiments in which the TMV vector carries a host sequence (*ChlH* gene) (Hiriart et al. 2003). In this case, the dynamic fluctuation of TMV infection is assumed to be associated with a strong silencing reaction against the vector virus and suppression of the silencing-based antiviral defense by the VSRS of TMV (the 126-kDa replicase subunit protein) (Hiriart et al. 2003). Results of the present study suggest that development of filiformic leaves was initiated at the time that TMV titers peaked, i.e., antiviral defense was at the lowest level, as these malformations started to become visible 1 to 2 weeks later. Consequently, it seems that the leaf malformations are attributable to interference with the silencing-based control of the host-gene regulation and, perhaps, to the depletion of the host's silencing machinery by the complementary effects of the viral silencing suppressors and the replicating virus. This hypothesis is supported by development of severe leaf malformations in transgenic, non-virus infected *Arabidopsis* plants that express the *2b* gene of the severe Fny strain but not in plants expressing the *2b* gene of the mild LS or Q strains of CMV (Lewsey et al. 2007; Zhang et al. 2006).

To determine which TMV-encoded factors are needed in the synergistic process with *2b* of CMV-Kin, we established the CMV-TMV combination in a reversed order by inoculating CMV-Kin to transgenic tobacco plants that express either the wt, the CP-deficient or MP-inactivated genome of TMV, or the TMV-MP alone (Siddiqui et al. 2007; *unpublished results*). In-

fection of CMV-Kin in the transgenic plants expressing the TMV-MP produced only mild symptoms typical of CMV-Kin, indicating that the MP was not able to cause the synergistic interaction. On the other hand, CMV-Kin infection in the TMV-ΔCP transgenic plants resulted in the filiformic leaf phenotype, indicating that CP was not needed for the synergism. Thus, it appeared that the helicase/replicase or the TMV replication itself would be the TMV-specific factor required for the synergism. The 126-kDa replicase subunit (the helicase) is the VSRS of TMV (Csorba et al. 2007; Ding et al. 2004), which would be consistent with its involvement in the viral synergism. It has been shown that the dosage of TMV helicase protein affects the strength of RNA-silencing suppression (Harries et al. 2008). Thus the low level of the helicase protein expressed in the TMV-ΔCP plants may relate to the low level and late appearance of the synergistic phenotype upon CMV-Kin infection. This may also explain why only mildly malformed (narrow) leaves were produced in the CMV-inoculated TMV-ΔMP plants, which expressed detectable amounts of viral RNA but undetectable levels of the TMV helicase protein.

Usually the shoot apical meristem and leaf primordia remain free of systemic virus infection (Foster et al. 2002; Hull 2002; Wang et al. 2008, 2009) due to the RNA silencing surveillance mechanism (Martin-Hernandez and Baulcombe 2008), but CMV is able to invade these tissues, at least transiently, due to the *2b*-mediated silencing suppression (Mochizuki and Ohki 2004; Sunpapao et al. 2009). The CMV *2b* protein is also known to prevent systemic spread of silencing at the perception stage of the signal (Guo and Ding 2002; Wadsworth and Dunoyer 2009). Results of the present study are consistent with the potential of *2b* of CMV-Kin to compromise systemic silencing, as indicated by the spread of TMV into the apical tissues and its enhanced accumulation in the leaf primordia in which leaf-blade differentiation takes place. Many transcription-factor families are essential for the shoot apical meristem maintenance and differentiation and leaf development and morphology, and a number of them are regulated by various miRNAs or *trans*-acting siRNAs (Chuck et al. 2009; Liu et al. 2009). Disturbances in these regulatory pathways lead to defects in leaf development and morphology (Alvarez et al. 2006; Borghi et al. 2010). TMV encodes a strong silencing suppressor, the 126-kDa helicase protein, which functions via binding small RNAs, inhibiting their HYL1-mediated methylation (Csorba et al. 2007; Ding et al. 2004; Kurihara et al. 2007). This VSRS of TMV can severely compromise both the siRNA- and miRNA-mediated silencing pathways (Csorba et al. 2007). However, mere accumulation of TMV (helicase/replicase in these tissues is not adequate for induction of the filiformic leaves, as the transgenic plants expressing the wt TMV under the constitutive 35S promoter do not develop these symptoms (Siddiqui et al. 2007). Thus, specific synergistic interactions between the TMV- and *2b*-mediated silencing suppression are required for such strong phenotypes.

Other features relevant to silencing suppression activity of the *2b* protein are, at least, binding of small RNAs, nuclear localization, self-interaction, inhibition of AGO1 and RISC function, and reduction of the accumulation, and possibly function, of AGO4 (Cillo et al. 2009; González et al. 2010; Goto et al. 2007; Lewsey et al. 2007; Ye et al. 2009; Zhang et al. 2006). These features have been characterized particularly for the *2b* of the severe Fny strain, and it is not known whether the *2b* encoded by mild strains of CMV should possess the same functions. However, it is clear that the *2b* proteins function as symptom determinants and determine the severe and mild symptom types of different CMV strains. To investigate the functional differences of the *2b* proteins of the Fny and LS strains, Cillo and associates (2009) analyzed,

from infected tomato plants, the transcript levels of selected essential effector molecules or targets of the silencing pathways or both. They found that some of them (DCL1, AGO4, ARF8, NAC1, HD-ZIP, PHAN), although not all (AGO1, TCP3), were differentially affected in tomatoes infected by these two strains (Cillo et al. 2009). Any of such differential effects may essentially contribute to the different reactions of the two 2b forms. However, many of the changes may be secondary effects, and it is difficult to pinpoint the exact primary functional difference between the two types of the 2b proteins. According to Diaz-Pendon and associates (2007), 2b is not required for symptoms but is required for successful infection, and as suggested by Sunpapao and associates (2009), 2b may enhance symptoms by viral distribution kinetics in developing tissues. Our results suggest that 2b has a similar influence also on TMV.

MATERIALS AND METHODS

Plant material, viruses and inoculations.

The transgenic *N. tabacum* cv. Xanthi plants expressing 2b protein from CMV-Kin, infectious transcripts of wt TMV, or the truncated TMV genomes TMV-ΔCP have been described previously (Siddiqui et al. 2007, 2008; Turpen et al. 1993). The TMV-ΔMP transgenic plants have been produced, in parallel with the wt TMV and TMV-ΔCP transgenic plants, by deleting nucleotides 5,050 to 5,430 from the transformation construct of the wt TMV (Fig. 4A).

The CMV strains Kin and Fny were provided by D. Baulcombe, (University of Cambridge, U.K.) and P. Palukaitis (Scottish Crop Research Institute, Dundee, U.K.), respectively. TMV inoculum was obtained by transcribing the infectious wt TMV-U1 clone (gift from W. O. Dawson) in vitro and using the transcripts to inoculate plants mechanically (Hiriart et al. 2003). Mechanical inoculations with CMV and TMV were done by grinding infected leaf tissue in 10 mM phosphate buffer (1:10, wt/vol), pH 7.0, and two full-grown leaves of 5-week-old transgenic plants and of wt tobacco plants dusted with Carborundum were inoculated by rubbing the sap on to the leaves. Inoculation experiments were repeated in three to four times, with four to six replica plants in each experiment. All plants were maintained in a growth chamber under controlled conditions under an 18-h photoperiod (light intensity of 100 $\mu\text{E s}^{-1} \text{ m}^{-2}$). Day and night temperatures were 22 and 18°C, respectively. The close-up examination of leaf samples was done using a Leica stereomicroscope, mounted with the Olympus DP 50 camera (Leica Microsystems AG, Solms, Germany).

Transgenic TMV- and 2b-expressing *N. tabacum* plants were cross-pollinated by dusting spores from 2b plants onto stigma of TMV-transgenic plants before the flower buds were open. The F1 plants were maintained in the conditions mentioned above.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

DAS-ELISA was used for detection of CMV, according to the manufacturer's instructions (Bioreba, Reinach, Switzerland) with minor modifications. Commercially available polyclonal antibodies and alkaline phosphatase conjugated antibodies to CMV were obtained from Bioreba (Reinach, Switzerland) and were diluted at 1:1,000 for use. *p*-Nitrophenyl phosphate was used as substrate to develop the reaction and absorbance at 405 nm were measured with an ELISA plate reader (Benchmark, Bio-Rad, Hercules, CA, U.S.A.) when 100 ng of purified TMV or CMV virions used as internal standards had reached the value of 2.00.

Immunolocalization of TMV.

Shoot meristems were excised from TMV-infected 2b transgenic *N. tabacum* plants 4 and 5 wpi. The excised samples were immediately fixed and sections (7 μm) were prepared as described by Wang and associates (2008). Following initial incubation in phosphate-buffered saline (PBS) containing 4% bovine serum albumin for 30 min at room temperature, the sections were incubated with alkaline phosphatase-conjugated polyclonal antibodies specific to TMV (dilution 1:50) overnight at 4°C. After three consecutive washings with PBS, sections were stained with freshly prepared fuchsin substrate solution. The sections were observed with a Leitz, Laborlux S light microscope (Leica Microsystems AG) under 40 $\times$ and 100 $\times$ magnifications.

RT-PCR.

Expression of replicase gene in the TMV-ΔMP transgenic plants was tested by RT-PCR. Total RNA was extracted from the leaves using Trizol (Invitrogen, Carlsbad, CA, U.S.A.), according to manufacturer's instructions. The first-strand cDNA was synthesized using 1 μg of DNase-treated total RNA and random hexamers as described by Vuorinen and associates (2010). PCR was done using the cDNAs and, also, the DNase-treated RNA that was not reverse-transcribed (control), with primers (forward: 5'-gtgacatcgatgattggag-3', reverse: 5'-ccgtccacaagaacaacctt-3') designed to amplify the 2,424 to 2,567 nt (143 bp) region of the TMV replicase gene.

Protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting.

To detect expression of TMV MP in transgenic plants, MP was extracted according to Lehto and associates (2003). Samples were separated by SDS-PAGE electrophoreses, followed by immunoblotting with anti-TMV-MP antibodies, and chemiluminescence detection was done using SuperSignal West Fento maximum sensitivity substrate (Thermo Scientific, Sugarland, TX, U.S.A.) (Fig. 4C), as described by Lehto and associates (2003).

RT-qPCR.

Samples were collected at 2, 3, 4, 5, and 6 wpi. Total RNA was extracted and cDNA synthesis of DNase-treated RNAs carried out as described above. Actin gene was used as an internal reference. Primers were designed with PrimerExpress v2.0 (Applied Biosystems, Foster City, CA, U.S.A.). The 2b transgene expression was detected with primers 5'-cgaagacgaa ggtctcagc-3' (forward) and 5'-catctggaaaagtctcgctatagat-3' (reverse), resulting in a product of 92 bp. TMV was detected with primers 5'-agtgaaaatcaggaacc-3' (forward) and 5'-tcgaagagc tccgattataaga-3' (reverse) annealing to the CP gene and amplifying the genomic region corresponding to nucleotides 5,998 to 6,148 (150 bp). For detection of actin gene expression, primers 5'-cgtctgtgataatgggactggaa-3' (forward) and 5'-cgtggg cgaccaacaatact-3' (reverse) were designed according to the actin gene of tobacco (GenBank accession number X63603.1). The $\log_{10}$ -transformed fold values, calculated through RT-qPCR *E* values, were used for statistical analysis as described by Vuorinen and associates (2010).

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