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# Satellite RNA-mediated Reduction of Cucumber Mosaic Virus Genomic RNAs Accumulation in *Nicotiana tabacum*

Qiansheng LIAO[1,2], Liping ZHU[2], Zhiyou DU[2], Rong ZENG[2,3], Junli FENG[2], and Jishuang CHEN[1,2]*

[1] *College of Life Sciences, Zhejiang University, Hangzhou 310029, China;*
[2] *Institute of Bioengineering, Zhejiang Sci-Tech University, Hangzhou 310018, China;*
[3] *College of Biological Safety Technology, Hunan Agricultural University, Changsha 410128, China*

**Abstract** Satellite RNAs (satRNAs) are molecular parasites that interfere with the pathogenesis of the helper viruses. In this study, the relative accumulation of cucumber mosaic virus (CMV)-Fny genomic RNAs with or without satRNAs were quantitatively analyzed by real-time RT-PCR. The results showed that satRs apparently attenuated the symptoms of CMV-Fny on *Nicotiana tabacum* by depressing the accumulation of CMV-Fny genomic RNAs, tested as open reading frames. The accumulation of CMV-Fny *1a*, *2a*, *2b*, *3a*, and *CP* genes was much higher than that of CMV-Fny with satRs added (CMV-Fsat), at different inoculation times. CMV-FnyΔ2b, in which the complete *2b* gene and 41 amino acids at the C-terminal of the *2a* gene were deleted, caused only a slight mosaic effect on *N. tabacum* seedlings, similar to that of CMV-Fsat, but the addition of satRs to CMV-FnyΔ2b showed further decrease in the accumulation of CMV-FnyΔ2b genomic RNAs. Our results indicated that the attenuation of CMV, by adding satRs or deleting the *2b* gene, was due to the low accumulation of CMV genomic RNAs, and that satRNA-mediated reduction of CMV genomic RNAs accumulation in *N. tabacum* was possibly related to the *2b* gene.

**Key words** cucumber mosaic virus; satellite RNA; deletion of *2b* gene; relative accumulation

Cucumber mosaic virus (CMV) is a plant pathogen that is prevalent all over the world, and has an extremely large host range of over 1000 plant species [1–3]. CMV has a tripartite, single-stranded, positive-sense RNA genome. RNA1- and RNA2-encoded proteins (1a and 2a respectively) are involved in virus replication, and RNA4A, as a subgenomic RNA from RNA2, has been found to correlate to viral long distance movement and to suppression of gene silencing [4–7]. RNA3 encodes the 3a protein and the viral coat protein (CP), which is expressed from subgenomic RNA4, and the *3a* gene is necessary for cell-to-cell movement [2,8,9]. In addition to the viral genomic RNAs, some CMV strains contain satellite RNAs (satRNAs) as additional RNAs associated with the virus genome. To date, over 100 CMV satRNA variants have been found to be associated with over 65 CMV isolates [2]. SatRNAs are completely dependent on their helper viruses for replication, encapsidation and dispersion. They do not contain an open reading frame (ORF), but tend to be highly structured. Despite their small size and poor protein product, CMV satRNAs might have dramatic effects on symptom expression induced by the helper virus, ranging from amelioration to severe exacerbation [2,10,11,13]. Specific nucleotide residues or structures of satRNAs determine the capability of modulating symptoms caused by helper viruses [14–18]. In most cases, the presence of satRNA attenuates the symptoms induced by CMV infection, and the presence of CMV-satRNA usually reduces the titer of helper virus, more so in *Solanaceous* than in cucurbit host plants [2,11,13,19,20]. Some reports suggest that the effect of satRNA on the helper viruses could be related to the competitiveness of replication between the helper virus and satRNA [11,21]. The suppres-

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*Corresponding author: Tel/Fax, 86-571-86843196; E-mail, chenjs@zstu.edu.cn

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sive effect of satRNA on the accumulation of the CMV genomic RNAs and encoded proteins can be considered for its effect on accumulation of RNA1 and 2, relating to 1a and 2a proteins, or the effect on accumulation of RNA3 and 4, relating to and 3a and CP [20]. A limited report was documented for quantitative determination for the effect of satRNA on the genomic RNAs and genes of its helper virus, especially on the *2b* gene, which is linked to virus distribution among plant organs and to symptom expression. Developing a sensitive method to quantitatively determine the low level of CMV genes in host plants when the *2b* gene is deleted, and the construction of a CMV *2b* deletion mutant, will help to answer these questions. Based on our previous work to determine absolute copies of CMV genomic RNAs, we searched for the effect of satRNA on the accumulation of CMV genomic RNAs and also the influence of deleting the *2b* gene on viral RNA accumulation and symptom expression.

## Materials and Methods

### Infectious clones of CMV and satRNA and *in vitro* transcription

The infectious cDNA clones (pF109, pF209 and pF309) of CMV-Fny were provided by Dr. Peter PALUKAITIS (Scottish Crop Research Institute, Dundee, UK) [21], and the full-length cDNA clone, psatRs, was obtained from a 368 nt satRNA (GenBank accession number: AF451896), namely satRNA-Rs (satRs), from a CMV strain isolated from *Raphanus sativus*. Full-length RNAs were transcribed from pF109, pF209, pF309 and psatRs with T7 RNA polymerase (Promega, Madison, USA). Plasmid pF109, pF209 and pF309 were linearized with restrictive enzymes before RNA transcription. Transcription reaction essentially followed the protocol described by Beckler [22], and quantified by agarose gel electrophoresis before inoculation.

### Construction of deletion mutant for CMV-Fny RNA2

The deletion of the *2b* gene was carried out by overlapping polymerase chain reaction (PCR). Fragments I and II were amplified with two PCR primer pairs FnyΔ2bF1/FnyΔ2bR1 and FnyΔ2bF2/FnyΔ2bR2, respectively, as listed in **Table 1**. Two amplified fragments were used together as templates for a further amplification with the primer pair FnyΔ2bF1/FnyΔ2bR2. **Fig. 1** shows the whole process of F209Δ2b construction. The resultant fragment

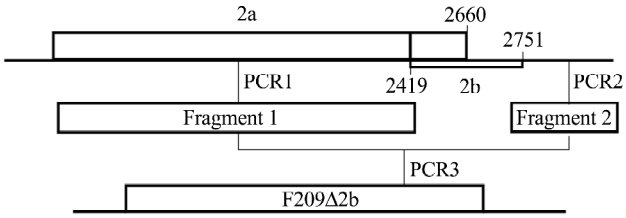


**Fig. 1 Diagram of F209Δ2b construction strategy**
PCR, polymerase chain reaction.

**Table 1 Primers used in this study**

| Gene | Primer pairs | |
|---|---|---|
| FnyΔ2b 1 | FnyΔ2bF1[a] | 5′-AATC*GGATCC*TAATACGACTCACTATAGTTTATTTACAAGAGCGTACGG-3′ |
| | FnyΔ2bR1 | 5′-AGATGCGGAAGGGGAGGTTTCAAATTCTTTCGCTGTTTGTTGGA-3′ |
| FnyΔ2b 2 | FnyΔ2bF2 | 5′-TGAAACCTCCCCTTCCGCATCT-3′ |
| | FnyΔ2bR2[b] | 5′-AATT*CTGCAG*TGGTCTCCTTTTGGAGGCC-3′ |
| satRNA | Sat F | 5′-GTTTTGTTTGWTGGAG-3′ |
| | Sat R | 5′-GGGTCCTGTAGAGGA-3′ |
| 18s rRNA | 18s rRNAF1564 | 5′-TTCCTAGTAAGCGCGAGTCATCAGC-3′ |
| | 18s rRNAR1630 | 5′-GCGACGGGCGGTGTGT-3′ |
| CP gene | CP F | 5′-GTGGGTGACGGTCCGTAA-3′ |
| | CP R | 5′-AGATGTGGGAATGCGTTGG-3′ |
| satRs | satRsF F81 | 5′-GTAGCTGCATGGTGGTGGGAC-3′ |
| | satRsRR210 | 5′-GCGGGGGCTCAAATGAAATG- 3′ |

[a] C*GGATCC*, *Bam*HI; TAATACGACTCACTATA, T7 promotor; and [b] *CTGCAG*, *Pst*I.

was digested with *Bam*HI and *Pst*I and cloned into pF209 plasmid previously digested with the same enzymes. Colonies were subcultured and plasmids were isolated by standard procedures [23]. The clone pF209Δ2b was fully sequenced to confirm the completely deletion of the *2b* gene together with 41 amino acids from the C-terminus of 2a protein.

### Plant seedlings and viral inoculation

Tobacco (*Nicotiana tabacum* cv. *Tabacum*, Huangmiaoyu) was used as the host plant. Seedlings were inoculated at the three- to four-leaf stages. Twenty microliters of DEPC $H_2O$, containing 100 mg/ml full RNA transcripts, each of CMV-Fny RNA1, 2 and 3, with or without 25 mg/ml transcripts of satRs, was used as the inocula. The inocula of CMV-FnyΔ2b and CMV-FnyΔ2b plus satRs (CMV-FnyΔ2bsat) was prepared using the same protocol as that of CMV-Fny, except that F209Δ2b was used for transcription of RNA2. Twelve sets of four tobacco seedlings were inoculated mechanically with CMV-Fny, CMV-Fsat, CMV-FnyΔ2b and CMV-FnyΔ2bsat, for detecting the accumulation of CMV genomic RNAs and satRs in host plants. Reverse transcription (RT)-PCR detection and double-stranded RNA (dsRNA) analysis were adopted to confirm whether CMV-Fny could serve as the helper virus of satRs [24]. The inoculated leaves were collected at 2 days post-inoculation (dpi), and the same amount of plant tissue was used to prepare total RNAs. The inoculated leaves with the first leaves above the inoculated leaves were sampled at 3 dpi, and the inoculated leaves with 2–4 leaves above them were sampled at 10 dpi. At 21 dpi, all the systemic leaves, except the first leaves above the inoculated leaves, were sampled to prepare total RNAs. Tobacco seedlings were grown under greenhouse conditions prior to inoculation. Inoculated plants were maintained in a growth chamber with a temperature range of 22–28 ºC and a 16 h day length. Infection of pseudorecombinant viral strains was confirmed by RT-PCR and dsRNA analysis as in our previous work [24].

### Quantification of the accumulation of viral RNAs in tobacco tissue

The relative amount of CMV genomic RNAs and satRs in systemically infected tobacco seedlings was determined by a real-time RT-PCR protocol used in our previous report [25]. Total RNA was extracted from leaf tissues of CMV inoculated plants or mock inoculated plants using Trizol reagents (Invitrogen, California, USA). RNA integrity was electrophoretically verified by staining with ethidium bromide and judged by $A_{260}/A_{280}$ absorption. Total RNA (approximately 1 μg) was reverse transcribed with 100 U of Superscript II Plus RNase H-Reverse trancriptase (Invitrogen, California, USA), using 100 μM random 6 mer primers according to the manufacturer's instructions. All the real-time PCR primer pairs were used to assay the relative accumulation of each ORF for CMV and satRs according to our previous work [25], and the real-time PCR reaction was carried out with the ABI PRISM7000 real-time PCR machine (California, USA). Relative quantitation was carried out according to the comparative $C_T$ method ($2^{-\Delta C_T}$), with 18S ribosomal RNA extracted from the same host tissue used as the internal control.

## Results

### Symptom expression on tobacco inoculated with CMV-Fsat

A mixture of RNA *in vitro* transcripts from pF109, pF209 and pF309 was mechanically inoculated onto tobacco seedlings, to build CMV-Fny, and a mixture of CMV-Fny RNAs plus satRs was inoculated to build CMV-Fsat**.** As shown in **Fig. 2**, at 3 dpi, RT-PCR detection results showed that CMV-Fny supported the replication of satRs in tobacco seedlings, and high accumulation of satRs dsRNA in host plants suggested that CMV-Fny was com-

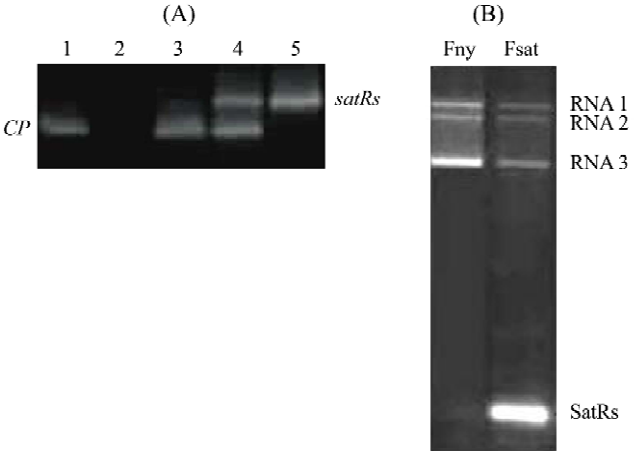


**Fig. 2 Reverse transcription–polymerase chain reaction and double-stranded RNA detection of cucumber mosaic virus (CMV)-Fny and CMV-Fsat in infected seedlings of *Nicotiana tabacum***

(A) Reverse transcription–polymerase chain reaction results amplified with the specific primer pairs for the CMV-Fny partial *CP* gene and *satRs*. 1, pF309 plasmid; 2, mock inoculation; 3, CMV-Fny inoculation; 4, CMV-Fsat inoculation; 5, psatRs plasmid. (B) Double-stranded RNAs extracted from plant tissues systemically infected with CMV-Fny or CMV-Fat at 14 d post-infection.

petent to serve as the helper virus of satRs. At 7 dpi, mosaic symptoms appeared on the newly growing leaves of seedlings inoculated with CMV-Fny but not on seedlings inoculated with CMV-Fsat. At 21 dpi, symptoms on the seedlings inoculated with CMV-Fny developed into severe mosaic, systemic deformation and were stunted, whereas the plants inoculated with CMV-Fsat expressed only slight mosaic symptoms but without obvious deformation (**Fig. 3**). This suggested that satRs postponed the emergence of symptoms induced by CMV-Fny and also attenuated symptom expression on tobacco seedlings.

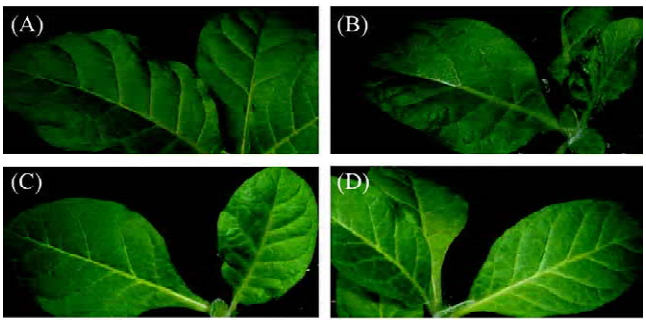


**Fig. 3 Symptoms on seedlings of *Nicotiana tabacum* infected with cucumber mosaic virus (CMV)-Fny and CMV-FnyΔ2b with or without satRs at 21 d post-infection**

(A) Mock. (B) CMV-Fny. (C) CMV-Fsat. (D) CMV-FnyΔ2b.

## Effect of satRs on the accumulation of CMV-Fny genomic RNAs

The relative accumulation of ORFs for CMV-Fny *1a*, *2a*, *2b*, *3a* and *CP* in systemic leaves was examined at 3 dpi, 10 dpi and 21 dpi by real-time RT-PCR. As shown in **Fig. 4**, the results indicated that the accumulation of CMV-Fny genomic RNAs was clearly higher than those of CMV-Fsat. At 3 dpi, satRs began to exert effects on CMV in host plants, resulting in lower accumulation of CMV-Fsat genomic RNAs. The degree of the accumulation of CMV-Fny genomic RNAs affected by satRs is shown in **Table 2**. The accumulation level of CMV-Fny ORFs was approximately 5–10 folds higher than those of CMV-Fsat. The degree of depression of each ORF by satRs was significantly different, at a tendency of *2a>2b>3a>1a>CP*. At 10 dpi, the accumulation level of CMV ORFs was still depressed by satRs, but the degrees were reduced with the exception of *2b*, in which the degree of depression was higher than at 3 dpi. The depressing degree by satRs was *2b>2a>1a>3a>CP*. At 21 dpi, the suppression effect of satRs on the accumulation of CMV ORFs had clearly

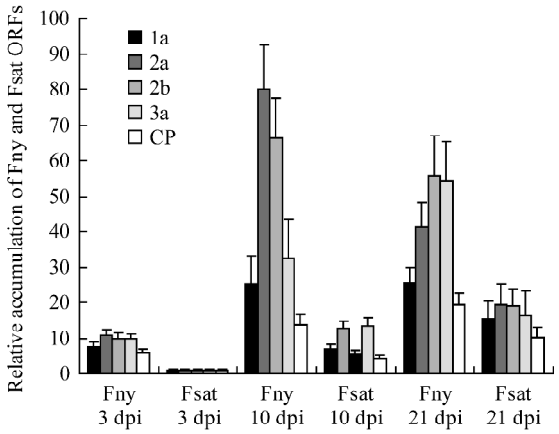


**Fig. 4 Relative accumulation of cucumber mosaic virus (CMV)-Fny open reading frames (ORFs) and CMV-Fsat ORFs in systemic leaves of *Nicotiana tabacum* at different inoculation times**

Total RNA was extracted from the tissues of eight individual plants inoculated with CMV-Fny with or without satRs. The relative accumulation of each gene was analyzed by realtime reverse transcription-polymerase chain reaction amplified with specific pairs, and was repeated three times, with 18S rRNA used as the endogenous control. Relative quantization of data was carried out according to the comparative $C_T$ method ($2^{-\Delta C_T}$), for example, the relative accumulation of CMV-Fny 1a $=2^{-(\text{aver 1a } C_T - \text{aver 18S } C_T)}$. Data for 1a, 2a, 2b, 3a and CP of CMV-Fsat at 3 dpi was normalized as 1, and the relative accumulation of the other samples was calculated based on them. The standard deviation was obtained in three repeats. The data between different ORFs could not be compared directly as each gene had different reverse transcription and polymerase chain reaction efficiency. dpi, days post-inoculation.

**Table 2 The degree of the accumulation of CMV-Fny genomic RNAs affected by satRs at different inoculation time**

| | 3 dpi | 10 dpi | 21 dpi |
|---|---|---|---|
| 1a | 7.6* | 3.6 | 1.6 |
| 2a | 10.8 | 6.28 | 2.1 |
| 2b | 9.8 | 11.8 | 2.9 |
| 3a | 9.7 | 2.4 | 3.3 |
| CP | 5.8 | 3.1 | 1.9 |

* The value was calculated as the relative accumulation of CMV-Fny 1a gene versus that of CMV-Fsat, and the data of the relative accumulation of CMV-Fny and CMV-Fsat was obtained from **Fig. 4**.

declined. The accumulation of *1a*, *2a*, *2b*, *3a* and *CP* for CMV-Fny was 1.6-, 2.1-, 2.9-, 3.3- and 1.9-fold higher than those of CMV-Fsat, respectively. In the newly growing leaves, there was especially low accumulation of satRs and CMV genomic RNAs, and the accumulation of CMV-Fny *1a*, *2a*, *2b*, *3a* and *CP* was 11.6-, 24.3-, 37.6-, 3.9- and 7.0-fold higher, respectively, than those of CMV-Fsat. Thus, satRs depressed the accumulation of CMV RNAs,

and the effect of satRs on the accumulation of the *2b* gene was relatively significant. The attenuation of symptoms in CMV-Fsat infected plants was due to low accumulation of CMV genomic RNAs.

### Symptom expression in the host plants inoculated with CMV-FnyΔ2b

Mixtures of RNA transcripts from pF109, pF209Δ2b and pF309 (to build CMV-FnyΔ2b) and RNA transcripts for CMV-FnyΔ2b plus satRs (to build CMV-FnyΔ2bsat) were mechanically inoculated in the tobacco seedlings. At 10 dpi, neither host plant expressed obvious symptoms, but RT-PCR detection of the *CP* gene and satRs in systemic leaves suggested that CMV-FnyΔ2b could systemically infect tobacco, and CMV-Fny with the *2b* (with partial *2a*) gene deleted, still supported the replication and accumulation of satRs in host plants. At the same time, RNA2 of CMV-FnyΔ2b, extracted from systemic leaf tissue, was amplified with RT-PCR and sequenced. The result showed that the RNA2Δ2b was not repaired. At 21 dpi, both host plants inoculated with CMV-FnyΔ2b and also CMV-FnyΔ2bsat appeared slightly mosaic (**Fig. 3**). But no further attenuation of the CMV-FnyΔ2b symptoms was observed when satRs was added.

### Accumulation of CMV-FnyΔ2b genomic RNAs and effect of satRs

The accumulation of CMV-FnyΔ2b genomic RNAs and satRs in leaves inoculated with different viral strains was examined at 3 dpi and 10 dpi. As shown in **Table 3**, at 3 dpi, the relative accumulation level of CMV-FnyΔ2b ORFs in the inoculated leaves was only 0.5%–5% of CMV-Fny. At 10 dpi, the accumulation levels of CMV-FnyΔ2b genomic RNAs were still much lower than those of CMV-Fny. The results suggested that attenuation of symptom expression on host plants by *2b* gene deletion was related to low replication and/or accumulation of CMV genomic RNAs. At 3 dpi, the accumulation of CMV-FnyΔ2bsat genomic RNAs in the inoculated leaves was at a similar level to that of CMV-FnyΔ2b, indicating that no suppression effect on the replication and/or accumulation of CMV-FnyΔ2b ORFs was caused by adding satRs. At 10 dpi, even though the accumulation of CMV-FnyΔ2b ORFs increased, the presence of satRs showed no obvious decrease in the accumulation of CMV-FnyΔ2b ORFs in the inoculated leaves. The results suggested satRs exerted no obvious effect on the replication and/or accumulation of CMV genomic RNAs when the *2b* gene was deleted. At 10 dpi, the accumulation levels of CMV-FnyΔ2bsat genomic RNAs in systemic leaves were found to be nearly equal to those of CMV-FnyΔ2b (**Fig. 5**). Thus, satRs showed no effect on the replication and/or accumulation of CMV genomic RNAs when the *2b* gene was deleted.

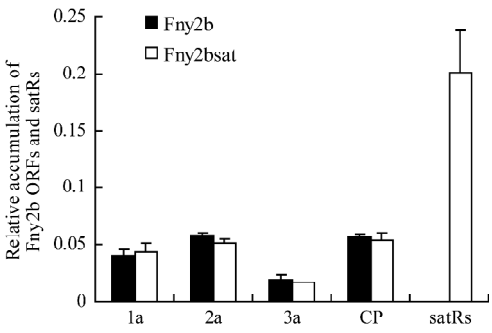


**Fig. 5 Relative accumulation of cucumber mosaic virus (CMV)-FnyΔ2b open reading frames (ORFs) with or without satRs in systemic leaves of *Nicotiana tabacum* at 10 dpi**

**Table 3 The relative accumulation of CMV-Fny and CMV-FnyΔ2b ORFs with or without satRs in the inoculated leaves at 3 and 10 dpi**

| | CMV-Fny | | CMV-FnyΔ2b | | CMV-FnyΔ2bsat | |
|---|---|---|---|---|---|---|
| | 3 dpi | 10 dpi | 3 dpi | 10 dpi | 3 dpi | 10 dpi |
| *1a* | 8.10±0.29 | 7.89±0.12 | 0.039±0.008 | 1.11±0.10 | 0.032±0.008 | 1.03±0.05 |
| *2a* | 3.78±0.10 | 1.98±0.03 | 0.063±0.002 | 1.42±0.45 | 0.055±0.015 | 1.15±0.14 |
| *2b* | 57.08±2.57 | 30.91±0.87 | 0 | 0 | 0 | 0 |
| *3a* | 0.28±0.03 | 0.23±0.007 | 0.012±0.001 | 0.14±0.02 | 0.011±0.003 | 0.10±0.03 |
| *CP* | 31.50±0.76 | 16.16±0.77 | 0. 18±0.07 | 2.33±0.11 | 0. 16±0.003 | 2.32±0.33 |

Total RNA was extracted from the tissues of 8 host plants infected by CMV-Fny or CMV-FnyΔ2b with or without satRs. The relative accumulation of each CMV-Fny gene was analyzed by ral-time T-PCR amplified with specific pairs, and the analysis of the relative accumulation of each gene was repeated three times.18s rRNA was used as an endogenous control and relative quantization with data was performed according to the comparative $C_T$ method ($2^{-\Delta C_T}$), for example the relative accumulation of CMV-Fny 1a $=2^{-(\text{aver 1a } C_T - \text{aver 18S } C_T)}$. The standard deviation was obtained in three repeats.

### Accumulation of CMV-Fny genomic RNAs in inoculated leaves and effect of satRs

To determine whether the low accumulation of CMV-Fsat genomic RNAs in systemic leaves was due to low accumulation in the inoculated leaves, the accumulation of CMV-Fny and CMV-Fsat ORFs in the inoculated leaves was tested at early stages. At 2 dpi, the relative accumulation levels of CMV-Fny *1a*, *2a*, *2b*, *3a* and *CP* were found to be 2.2-, 2.1-, 4.6-, 1.8- and 3.5-fold those of CMV-Fsat, respectively (**Table 4**). At 3 dpi, the relative accumulation levels of CMV-Fny *1a*, *2a*, *2b*, *3a* and *CP* were found to be 2.3-, 3.4-, 3.8-, 2.2- and 2.2-fold those of CMV-Fsat, respectively. These results suggested that satRs also mediated a reduction in genomic RNAs accumulation in the inoculated leaves. But, in the inoculated leaves, the replication and/or accumulation of CMV-Fny genomic RNAs was less obviously influenced by satRs, compared to that in systemic leaves.

**Table 4 The relative accumulation of CMV-Fny ORFs and CMV-Fsat ORFs in the inoculated leaves at the early stage of infection**

| | 2 dpi | | 3 dpi | |
|---|---|---|---|---|
| | CMV-Fny | CMV-Fsat | CMV-Fny | CMV-Fsat |
| 1a | 1.69±0.12 | 0.76±0.08 | 8.10±0.29 | 3.58±0.28 |
| 2a | 0.53±0.03 | 0.25±0.06 | 3.78±0.10 | 1.08±0.11 |
| 2b | 4.26±0.30 | 0.93±0.10 | 57.08±2.57 | 14.88±0.88 |
| 3a | 0.07±0.01 | 0.04±0.007 | 0.28±0.03 | 0.13±0.01 |
| CP | 3.64±0.12 | 1.03±0.26 | 31.50±0.76 | 14.76±0.88 |

## Discussion

It has been widely reported that the presence of satRNA modifies the pathogenesis of CMV. Depending on the strains of the helper virus, satRNA and species of hosts, the effect of the satRNA on virus-induced symptoms can be none, enhanced or attenuated [2,10,11,13]. In our study, satRs attenuated the symptoms induced by CMV-Fny, and depressed CMV-Fny genomic RNAs accumulation in tobacco (**Fig. 3**, **Fig. 4**, **Table 2** and **Table 4**). The results were consistent with a previous report [20]. Soards *et al*. found that CMV-Fny lacking the *2b* gene induced a symptomless systemic infection in tobacco [26]. In our study, when the *2b* gene of CMV-Fny (CMV-FnyΔ2b) was deleted with part of the *2a* gene lacking, CMV-FnyΔ2b still could infect tobacco plants, and the inoculated host plants appeared to have slight mosaic symptoms (**Fig. 3**). The differences in the symptomology might have resulted from using different tobacco varieties, as the modified RNA2 had a similar structure in both studies. *N. tabacum* cv. *Tabacum* was used in our work, whereas *N. tabacum* cv. *Xanthi-nc* was used in the study by Soards *et al*. Many reports indicate that the inactivity of the CMV *2b* gene by directed mutation of a single nucleotide acid or by gene deletion did not lead to a decrease in the accumulation of CMV genomic RNAs in protoplast, and that neither the C-terminal 41 amino acids of the 2a protein nor the 2b protein of CMV was required for replication [26–28]. Our work quantitatively determined the different ORFs of CMV in the inoculated and systemic leaf tissues, and the results showed indubitable replication of CMV in both leaf tissues when the *2b* gene was deleted. The detection also reflected the support of satRNA replication by CMVΔ2b, even though the accumulation levels of both CMV genomic RNAs and satRNA were low.

The addition of satRs to CMV-FnyΔ2b did not show further decrease in the accumulation of CMV-FnyΔ2b genomic RNAs (**Table 3** and **Fig. 4**). At 3 dpi, the accumulation of CMV-Fny ORFs was over 5-fold that of CMV-Fsat in the systemic leaves (**Table 2**); in the inoculated leaves, the accumulation of CMV-Fny ORFs was also higher than those for CMV-Fat (**Table 4**). The effect of satRs on CMV-Fny genomic RNAs replication and/or accumulation in the inoculated leaves was less obvious than in systemic leaves. At 21 dpi, the accumulation of CMV-Fny genomic RNAs in systemic leaves declined, but that of CMV-Fsat was still increasing (**Table 2**). This suggested satRs jammed the systemic infection of CMV-Fny in tobacco.

CMV-FnyΔ2b caused only slight mosaic symptoms in host plants, similar to that of CMV-Fsat, and the accumulation of CMV-FnyΔ2b genomic RNAs was much lower than that of CMV-Fny. Data in **Table 3** and **Fig. 4** shows that the efficiency of systemic infection of CMV-Fny declined greatly when the *2b* gene was deleted. So the deletion of the *2b* gene had a similar effect on CMV-Fny genomic RNAs replication and/or accumulation as adding satRs. The *2b* gene of different cucumoviruses has been shown to have a major effect on long distance movement of CMV as well as on the degree of virulence and the host range [26–30]. These effects are believed to relate to the ability of the 2b protein to inhibit various defense reactions, such as suppressing the RNA silencing mechanism and interfering with the salicylic acid-mediated defense response [5–7, 29–31]. But, to date, no report has focused on the ability of CMV to support satRNA when the *2b*

gene is deleted. Regarding the low accumulation of genomic RNAs in CMV-FnyΔ2b, the relative accumulation of CMV genomic RNAs was not further reduced by satRNA.

Taken together, through the quantitative determination of CMF ORFs in our work, the accumulation and/or replication of CMV genomic RNAs was greatly reduced by the addition of satRs or the deletion of the *2b* gene, but the CMV accumulation was not significantly altered when the *2b* gene was deleted.

## Acknowledgements

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Edited by
**Minghua XU**