Addition of nucleotides similar to deleted CAA repeats in the 5′ non-coding region of tomato mosaic virus RNA following propagation

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Previously we made a series of deletion mutants in the 5′ non-coding region of tomato mosaic tobamovirus (ToMV) RNA and checked their ability to replicate in tobacco protoplasts. Long deletions in this region caused the virus to lose the ability to replicate. Several mutants with deletions of about 10 nucleotides (short deletion mutants; SDM) retained the ability to replicate. In this study, we inoculated SDMs onto systemic host plants and observed their symptoms. One mutant (19/32) caused severe mosaic symptoms on some tobacco plants (Nicotiana tabacum cv. Samsun) but no symptoms on others. Virus accumulation in 19/32-inoculated plants paralleled the severity of symptoms. Four CAA repeat sequences were deleted in 19/32. Progeny 19/32 from plants showing severe systemic mosaic symptoms had acquired additional nucleotides in this region. We conclude that the CAA repeat sequence is related to the fitness of the virus population to replicate in whole plants rather than to translation of ToMV replicase genes.

Tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) are the best characterized tobamoviruses with respect to replication and propagation at the molecular level (Dawson & Lehto, 1990; Meshi et al., 1992). The tobamovirus genome consists of a positive-sense ssRNA of about 6400 nucleotides. The replication cycle includes synthesis of minus-strand RNA (Ishikawa et al., 1991) as well as plus-strand (virus) RNA. The 5′ and 3′ termini of the genomic RNA and/or minus-strand RNA have been shown to be involved in recognition by the replicase for initiation of accurate replication (Meshi et al., 1992; Takamatsu et al., 1990, 1991; van Belkum et al., 1985). Tobamovirus genomic RNAs carry approximately 70 and 200 nucleotide non-coding regions at their 5′ and 3′ termini, respectively. The 3′ non-coding region has been shown to fold into highly-ordered tRNA-like and upstream pseudoknot structures (Takamatsu et al., 1990; van Belkum et al., 1985). In contrast, it is unlikely that the 5′ non-coding region contains significant secondary structure (Takamatsu et al., 1991).

The 5′ non-coding region was first characterized by Richards et al. (1978) and Jonard et al. (1978). It is involved in regulation of the expression of downstream 126 kDa and 183 kDa replicase proteins and efficient uncoating of virions (Gallie et al., 1987; Wilson, 1984; Mundry et al., 1991). We previously analysed the replication of mutant ToMVs with nucleotide deletions in the 5′ non-coding region (Takamatsu et al., 1991). Deletions of nucleotides 9–47 or 25–71, for example, abolished the capacity for autonomous replication in protoplasts. Deletions of about 10 nucleotides (short deletion mutants; SDM) located between nucleotides 10–71 did not diminish the ability of ToMV RNA to replicate in single cells, while deletion of nucleotides 2–8 caused the loss of virus replication (Takamatsu et al., 1991). All SDM RNAs directed the synthesis of the 126 kDa protein at similar efficiencies in vitro in a rabbit reticulocyte lysate translation system, indicating that something other than translational efficiency was responsible for their failure to replicate (Takamatsu et al., 1991).

We inoculated these SDMs onto Nicotiana tabacum cv. Samsun plants and observed the symptoms caused. Mutants used in this work were 1/9, 8/18, 19/32, 47/62 and 8/31 (Takamatsu et al., 1991; Fig. 1). Mutation 19/32 from plants showing severe systemic mosaic symptoms had acquired additional nucleotides in this region. We conclude that the CAA repeat sequence is related to the fitness of the virus population to replicate in whole plants rather than to translation of ToMV replicase genes.

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Fig. 1. ToMV 5' non-coding region mutants used in this study. Construction of these mutants was described previously (Takamatsu et al., 1991), except for the 25/19 mutant (see text).

Fig. 2. (a) Symptoms of Samsun tobacco plants inoculated with ToMV mutant 19/32. Five plants are shown, giving rise to mutants 19/32-1 to 19/32-5. (b) Accumulation of CP. Proteins extracted from each plant were subjected to SDS-PAGE, Western blotting and immunodetection with anti-CP antiserum.

When Samsun tobacco plants were challenged with mutant 19/32, no consistent results were obtained. Disease severity was different from plant to plant. This mutant originally had a deletion of four CAA repeats (Fig. 1). Several CAA repeats are present in the 5' non-coding region of all tobamovirus RNAs. In the first experiment (Fig. 2), one of five plants (plant 2) gave...
very severe mosaic symptoms by 17 d.p.i., three plants (plants 1, 3 and 4) showed milder symptoms by 19–27 d.p.i. and one (plant 5) did not develop symptoms at all. The levels of CP accumulation correlated directly with symptom severity. Two duplicate experiments gave similar results, with one out of four plants and one out of six plants showing severe mosaic symptoms after inoculation with 19/32. We characterized only the 19/32 progenies obtained from the first experiment.

The virus population was purified separately from each assay plant except plant 5, which did not show symptoms. The progeny virus recovered from plant 1 was named 19/32-1 and the others were named accordingly. We inoculated each of the four virus preparations onto three healthy tobacco plants at 1 µg/ml. All three plants inoculated with 19/32-2 showed severe symptoms by 19 d.p.i. Wild-type ToMV also caused mosaic symptoms by 19 d.p.i. The severity of symptoms in plants infected with 19/32-2 was almost the same as with wild-type virus, while 19/32-1, 19/32-3, 19/32-4 caused much milder symptoms around 30 d.p.i. or no symptoms at all (data not shown). The 19/32-2 virus caused more severe symptoms than the original 19/32.

We suspected that the 19/32-2 virus population included virulent variants selected during the first passage on the systemic host. RT–PCR analysis was performed to determine whether the deleted 5’ non-coding region had changed in length. Original SDM mutant 19/32 gave a band with a higher mobility than wild-type ToMV, as expected (data not shown). The mobility was consistent with a band 12 bp shorter than the wild-type. When we used 19/32-2 RNA as a template for RT–PCR, we detected a broad band migrating between the wild-type and original 19/32 markers (data not shown). The original 19/32 and 19/32-1, -3 and -4 RNA gave only the higher mobility band (data not shown). This result indicated that 19/32-2 is a length variant or a mixture of variants in 5’ leader length, but is still shorter than wild-type ToMV RNA in the 5’ non-coding region. We suspect that the appearance of length variants correlates with symptom severity.

RT–PCR analysis indicated that 19/32-2 was a mixed population. To separate several viruses for further analysis, 19/32-2 was serially passaged three times on N. tabacum cv. Xanthi nc. After three cycles of local lesion isolation, each virus population was re-inoculated onto N. tabacum cv. Samsun. Systemically diseased leaves were harvested and virus was purified. We found two types of necrotic local lesions on Xanthi nc tobacco during the first inoculation. One type comprised larger lesions (2-0–3-5 mm in diameter at 4 d.p.i.), almost equivalent in size to those of wild-type virus. The other type comprised smaller lesions (diameter 0-3–0-7 mm). The number of large lesions was greater than the number of small ones. Virus obtained from large lesions produced only large lesions when passaged. Likewise, virus from small lesions produced only smaller lesions. We designated these two variants of 19/32-2 large variant and small variant. In parallel, the original 19/32 transcripts were inoculated on Xanthi nc tobacco plants and showed no variation in lesion size. Thus, several rounds of replication in a local lesion host do not produce the lesion size variants in detectable amounts.

We analysed the RNA sequences of the variants, focusing on the 5’ non-coding region, using a specific primer and avian myeloblastosis virus reverse transcriptase. Direct RNA sequencing showed sequence homogeneity in the virus populations of 18 large variants after the single lesion isolation procedure; all had an additional CAAA sequence in place of the original deletion in the 19/32 virus sequence.

Single-lesion isolation and propagation procedures were difficult to perform on the small variants. Virus obtained from the small lesions often failed to form subsequent lesions during the three-cycle procedure and many could not be propagated through successive Samsun tobacco inoculations. Virus variants from small lesions replicated poorly in planta. Only three small variants yielded enough material for sequence analysis. Two variants had a CAAACAA sequence in place of the 19/32 deletion and one variant was found to have the original 19/32 virus sequence.

A rabbit reticulocyte lysate was used to check whether RNA from each variant virion preparation could direct synthesis of the 126 kDa replicase protein as efficiently as wild-type RNA or the original 19/32 mutant RNA. The amount of 126 kDa protein synthesized in vitro was very similar in each case (data not shown). Subtle differences apparent in the gel could not explain the variation in virulence in plants.

We next performed protoplast experiments to investigate whether the variation in sequence in the 5’ non-coding region could be correlated with differences in efficiency of autonomous replication. The large variants showed high rates of virus-coded protein synthesis, comparable to wild-type ToMV. The small variant was only faintly detectable at this time (Fig. 3). Rapid and high-level synthesis of virus-coded proteins by wild-type ToMV and the large variants, in contrast to the original 19/32 mutant, correlated well with their virulence in plants. The profile could explain the difference in virulence between variants.

To confirm that the additional sequence (CAACAAAA or CAAA) alone conferred lesser or greater virulence on the 19/32 genome, we performed site-directed mutagenesis on a full-length clone of 19/32 to create mutants with only those additional sequences and checked their phenotype. pBSTMV5 is a subclone containing a 1.85 kbp PstI (0.85 kbp upstream of TMV cDNA)-Xhol (nucleotide 1002 in wild-type ToMV) fragment of TMV cDNA in pBluescript SK (+) (Takamatsu et al., 1991). Mutagenesis was conducted on pBSTMV5 ssDNA according to the method described by Kunkel et al. (1987). A 0.5 kbp NsiI (0.25 kbp upstream of TMV cDNA)-EcoRI (nucleotide 273 in wild-type ToMV) fragment was excised
from mutant pBSTMV5 clones and used to replace the corresponding fragment of pTLW3 (Hamamoto et al., 1993). One virus construct, designated LL, which had acquired the CAAA sequences in the 19/32 deletion region, caused lesions of almost the same size as wild-type ToMV. Another construct, SL, into which the CAAACAA sequence was inserted at the 19/32 deletion site, gave smaller lesions than ToMV, as seen with the small variants. These results confirmed the above assumption that the extra leader sequences alone affected the virulence of the 19/32 genome.

A gain of CAAA or CAAACAA in place of CAACAACAACAA could restore some virulence. Thus, could wild-type ToMV become even more virulent by introduction of extra CAA sequences? We created a virus called 25/19, which had two extra CAA repeats of the region 19–25. This mutant showed less severe mosaic symptoms than wild-type ToMV in Samsun tobacco plants (data not shown). In three independent experiments, wild-type virus caused typical mosaic symptoms by 19 d.p.i., whereas 25/19 caused mosaic symptoms only after 33–36 d.p.i. We also performed single-lesion isolations on the 25/19 progeny from these plants. Direct RNA sequencing was performed to determine whether the 5’ non-coding region had changed in the progeny viruses. The results showed that the 25/19 progeny were stable (data not shown) and that extra CAA repeats do not endow ToMV RNA with enhanced virulence.

During the SDM inoculation experiments reported here, we found that deletion of CAA repeats, which are characteristic motifs among tobamoviruses (Meshi et al., 1992; Murphy et al., 1994), leads spontaneously and rapidly to length variants in the 5’ non-coding region. We are unable to judge whether elimination of the CAA sequences themselves or destruction of some RNA secondary structure leads to the production of variants during propagation in plants but involvement of the CAA motifs is clear. We did not observe similar changes in symptoms or length reversions in mutants 8/18 and 47/62; mutant 8/18 had lost two CAA repeats, like 19/32, but in contrast to 19/32 no CAA repeats lay next to the deletion. It was characteristic and significant that 19/32 variants regained either CAA or CAAACAA, similar to the deleted CAA sequence. Exactly the same sequences are found at the flanking positions. We cannot judge whether the position of the CAA sequence or the number of repeats leads to the appearance of inserted nucleotides.

These ‘redundant’ repeat sequences may have been generated by template slippage during replication. A great number of virus replication cycles occur during infection of plants. Even if template slippage occurred at a very low frequency, the resultant variant might have an advantage related to replication rate, as indicated by the protoplast experiments (Fig. 3). As the replication cycle is repeated, the replication-efficient variants would increase as a proportion of the total virus population. The appearance of virulent variants depends on chance. Thus, we observed inconsistency of symptoms from one 19/32-inoculated plant to the next. It is also likely that other variants were formed during mul-
tiplication of SDM 19/32 but were diluted out due to poor adaptation and therefore not recovered.

What is the advantage of retaining a certain number of CAA-like sequence repeats in the replication of wild-type virus? We could not determine at present whether the position of the CAA sequence, the number of repeats or the RNA structure leads to the virulence of the large lesion variants. Extra CAA motifs alone in the wild-type sequence did not lead to a more virulent virus (25/19).

Large lesion variants showed comparable virulence to wild-type ToMV. Thus we should remember that wild-type virus is not always the most virulent form, tested extensively through adaptation, evolution and survival in host plants. Virus populations could give rise to more virulent forms even within the time-span of these experiments.

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References


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