Replication of wild-type and mutant clones of satellite tobacco mosaic virus in Nicotiana benthamiana protoplasts

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RNA transcribed from cloned satellite tobacco mosaic virus (STMV) cDNA replicated in Nicotiana benthamiana protoplasts when co-inoculated with tobacco mild green mosaic virus (TMGMV) genomic RNA, but degraded when inoculated alone. STMV genomic RNA extracted from wild-type virions replicated in protoplasts when co-inoculated with TMGMV, tobacco mosaic virus (TMV) or tomato mosaic virus (ToMV). Transcripts from clones of two STMV coat protein (CP) mutants accumulated to the same level as wild-type transcripts in protoplasts when co-inoculated with TMGMV, whereas a third mutant accumulated to detectable levels in some, but not all, experiments. These results confirm that STMV RNA requires helper virus for replication, and that the helper specificity exhibited by cloned STMV reflects a specific requirement for the TMGMV replicase. It also demonstrates that the low accumulation of STMV CP mutants observed previously in whole plants cannot be attributed to inefficient RNA replication.

Satellite tobacco mosaic virus (STMV) shares, with other satellite viruses and satellite RNAs, a requirement for a helper virus, in this case one of several tobamoviruses, to establish infection. Based on studies in whole tobacco plants, the helper virus is believed to provide factors required for the replication of STMV (Valverde et al., 1986, 1987, 1991). Subclones of STMV cDNA, from which biologically active RNA can be transcribed, have been created (Mirkov et al., 1990; Kurath et al., 1993). Interestingly, these transcripts only accumulated in tobacco plants when co-inoculated with tobacco mild green mosaic virus (TMGMV; a California isolate previously referred to as TMV-U5 was used). In contrast, STMV RNA purified from virions accumulated systemically in plants when co-inoculated with any one of several tobamoviruses (Valverde et al., 1991). Deletion and frameshift mutations have been engineered into one of the original STMV cDNA clones. Approximately 80% of the internal coat protein (CP) coding sequence can be removed without entirely abolishing the ability of these mutants to accumulate in whole plants; however, these CP-deletion mutants did not accumulate in every plant that was co-inoculated with mutant STMV RNA and TMGMV RNA. Viability of these STMV CP mutants was affected by the conditions under which the plants were maintained, and when they were biologically active they accumulated to approximately 100-fold lower levels than the original clone (Routh et al., 1995).

To initiate an analysis of the replication of wild-type and mutant STMV, Nicotiana benthamiana protoplasts were inoculated with combinations of various tobamoviruses and STMV RNAs. The objectives of this study were to verify that replication of STMV was dependent upon factors provided by the helper virus, to determine if the specificity for TMGMV helper exhibited by cloned STMV RNA occurred at the level of replication, and to analyse the replication efficiency of STMV CP mutants which accumulated poorly in whole tobacco plants. The only other study of satellite virus replication in protoplasts involved satellite tobacco necrosis virus (STNV) (Andriessen et al., 1995).

Protoplasts of N. benthamiana were isolated from 3-month-old leaves as described by Carrington & Freed (1990). These protoplasts were transfected with helper virus RNA, STMV RNA, or both by using the PEG-mediated transfection protocol of Negrotiti et al. (1987). (An aliquot of each preparation was counted on a haemocytometer and $2 \times 10^6$ cells were used for each inoculation.) Protoplast preparations inoculated with STMV RNA or helper virus RNA were incubated with 2 µg RNA. Protoplasts co-inoculated with both STMV RNA and helper virus RNA were incubated with a total of 4 µg RNA (2 µg STMV RNA and 2 µg helper virus RNA). The transfected protoplast cultures were maintained at room

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Fig. 1. Maps of STMV RNAs used in this study. Type strain STMV RNA (STMV), transcribed from plasmid pSTMV, contains nearly the entire STMV sequence but lacks the 3′-terminal adenosine (1059). Type strain STMV RNA transcribed from STMVHIII contains this adenosine. The three mutant STMV RNAs were transcribed from CPFSHIII, CPUAAHIII and ∆CP407HIII, respectively, and contain the 3′-terminal A residue. The mutant CPFS contains a frameshift at position 329. Translation of this ORF results in a protein containing 55 amino acids of the STMV CP, followed by 72 out-of-frame amino acids. The mutant CPUAA contains a UAA stop codon at position 163, the site of the CP start codon. This mutant is predicted to be incapable of directing synthesis of the STMV CP. The mutant ∆CP407 contains a 407 nt deletion in the CP gene, leaving only the nucleotides which encode the CP amino-terminal 13 amino acids, followed by nine out-of-frame amino acids. The 6.8 kDa and CP ORFs (Dodds, 1991) are denoted by boxes. Shaded boxes indicate out-of-frame amino acids. The nucleotide positions of the mutations are marked below each map. All of these RNA species contain two additional non-viral guanosines (G) upstream of their 5′ terminus.

 Helper virus RNA and STMV virion RNA were extracted from purified virions, prepared according to Valverde et al. (1991). STMV genomic RNA was gel-purified according to Kurath et al. (1993). The construction of plasmid pSTMV +2ΔOP, which can be transcribed with bacteriophage T7 RNA polymerase (Ambion) to produce biologically active STMV RNA, has been described elsewhere (Mirkov et al., 1990). This transcript is 1060 nt long, contains two extra G residues at the 5′ terminus and lacks the 3′-terminal A relative to STMV RNA isolated from virions. Henceforth, this clone will be referred to as pSTMV. Two of the STMV CP mutants used, p∆CP407 and pCPFS were derived from pSTMV, as described in Routh et al. (1995), and have a deletion of 407 nt within the CP gene or a frameshift mutation predicted to disrupt the carboxy-terminal two-thirds of the CP. An additional plasmid, pCPUAA, in which the STMV CP start codon was changed to a UAA stop codon, was created by site-directed mutagenesis of internal sites within pSTMV by the method of Higuchi (1990). Mutagenic PCR was carried out for 15 cycles using Taq polymerase (Promega). To confirm the mutation, DNA sequencing was done (Sanger et al., 1977) with commercially available reagents (Sequenase; United States Biochemical). In later experiments, to avoid producing aberrant RNA species resulting from T7 transcription of templates with 3′ overhangs, all plasmids were subjected to mutagenic PCR as described above to introduce a HindIII site at the 3′ terminus of their STMV sequence. When these constructs were digested with HindIII and incubated with T7 RNA polymerase, transcripts contained the 3′-terminal A residue of the wild-type STMV sequence, which was lacking in transcripts of the PstI-
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(a) Protoplasts were inoculated with TMGMV genomic RNA, STMV RNA transcribed from pSTMV, or co-inoculated with both TMGMV and STMV. These cells were then incubated for 25 or 50 h (designated 25 or 50, respectively). As a control, protoplasts were inoculated with water and incubated for 50 h (uninfected). STMV transcript (50 ng) and TMGMV virion RNA (5 ng) were loaded directly to the gel (STMV RNA and TMGMV RNA, respectively). Hybridization was done with RNA probes complementary to (+) strand STMV genomic RNA and (+) strand TMGMV genomic and subgenomic RNAs.

(b) Protoplasts were co-inoculated with genomic RNA extracted from virions of TMV, ToMV or TMGMV, and STMV RNA extracted from either wild-type virions (STMV virion RNA) or STMV RNA transcribed from STMVHIII (STMV transcript). Control inoculations were made with TMGMV RNA (TMGMV) and water (uninfected). Protoplasts were incubated for 42 h p.i. STMV RNA (5 ng), extracted from virions, was loaded as a standard (STMV RNA). Hybridization was done with an RNA probe complementary to (+) strand STMV genomic RNA. STMV (+) strand RNA or STMVHIII (STMV), various STMV CP mutants (CPFS, CPUAA and ΔCP407), or co-inoculated with CP mutants and TMGMV RNA extracted from virions (TMGMV). Templates used for transcription were STMVHIII (STMV), CPFSHIII (CPFS), CPUAAHIII (CPUAA), or ΔCP407HIII (ΔCP407). Protoplasts were incubated for 48 h p.i. Duplicate Northern blots were hybridized with RNA complementary to STMV (+) or (-) strand RNA. The position of the STMV genomic RNA (STMV G) TMGMV genomic RNA (TMGMV G) and subgenomic RNAs (TMGMV sg) are arrowed.

(c) Protoplasts inoculated with RNA transcribed from wild-type strain STMV (STMV (+) RNA) or STMVHIII (STMV), various STMV CP mutants (CPFS, CPUAA and ΔCP407), or co-inoculated with CP mutants and TMGMV RNA extracted from virions (TMGMV). Templates used for transcription were STMVHIII (STMV), CPFSHIII (CPFS), CPUAAHIII (CPUAA), or ΔCP407HIII (ΔCP407). Protoplasts were incubated for 48 h p.i. Duplicate Northern blots were hybridized with RNA complementary to STMV (+) or (-) strand RNA. The position of the STMV genomic RNA (STMV G) TMGMV genomic RNA (TMGMV G) and subgenomic RNAs (TMGMV sg) are arrowed.

Fig. 2. Replication of STMV RNAs used in protoplasts. Northern blot analysis of nucleic acids extracted from *N. benthamiana* protoplasts. (a) Protoplasts were inoculated with TMGMV genomic RNA, STMV RNA transcribed from pSTMV, or co-inoculated with both TMGMV and STMV. These cells were then incubated for 25 or 50 h (designated 25 or 50, respectively). As a control, protoplasts were inoculated with water and incubated for 50 h (uninfected). STMV transcript (50 ng) and TMGMV virion RNA (5 ng) were loaded directly to the gel (STMV RNA and TMGMV RNA, respectively). Hybridization was done with RNA probes complementary to (+) strand STMV genomic RNA and (+) strand TMGMV genomic and subgenomic RNAs. Protoplasts were co-inoculated with genomic RNA extracted from virions of TMV, ToMV or TMGMV, and STMV RNA extracted from either wild-type virions (STMV virion RNA) or STMV RNA transcribed from STMVHIII (STMV transcript). Control inoculations were made with TMGMV RNA (TMGMV) and water (uninfected). Protoplasts were incubated for 42 h p.i. STMV RNA (5 ng), extracted from virions, was loaded as a standard (STMV RNA). Hybridization was done with an RNA probe complementary to (+) strand STMV genomic RNA. STMV (+) strand RNA or STMVHIII (STMV), various STMV CP mutants (CPFS, CPUAA and ΔCP407), or co-inoculated with CP mutants and TMGMV RNA extracted from virions (TMGMV). Templates used for transcription were STMVHIII (STMV), CPFSHIII (CPFS), CPUAAHIII (CPUAA), or ΔCP407HIII (ΔCP407). Protoplasts were incubated for 48 h p.i. Duplicate Northern blots were hybridized with RNA complementary to STMV (+) or (-) strand RNA. The position of the STMV genomic RNA (STMV G) TMGMV genomic RNA (TMGMV G) and subgenomic RNAs (TMGMV sg) are arrowed.

linearized parental clones. These constructs were designated STMVHIII, ΔCP407HIII, CPFSHIII and CPUAAHIII, respectively. Maps of the four STMV transcript RNAs are shown in Fig. 1.

To confirm the helper-dependent replication of STMV RNA, *N. benthamiana* protoplasts were inoculated with TMGMV virion RNA, STMV RNA transcribed from pSTMV (linearized with *PstI*), or co-inoculated with both RNAs. Half of the protoplasts were harvested at 25 h post-inoculation (p.i.), and the remainder at 50 h p.i. As a control, mock-inoculated
protoplasts were harvested at 50 h p.i. A Northern blot on 1 µg of total nucleic acid extracted from each of these protoplast preparations was probed with a mixture of riboprobes complementary to the (+) sense TMGMV RNA and (+) sense STMV RNA (Fig. 2a). STMV genomic RNA was detected in extracts taken from protoplasts inoculated with STMV, or co-inoculated with TMGMV and STMV. At 25 h p.i., the levels of STMV genomic RNA were lower in protoplasts inoculated with STMV transcript alone than in protoplasts co-inoculated with STMV transcript and TMGMV virion RNA. The level of STMV RNA also increased between 25 and 50 h p.i. when co-inoculated with helper virus genomic RNA, but decreased with time when protoplasts had been inoculated with STMV transcript alone. TMGMV subgenomic RNAs were easily detected in the nucleic acid extracts from protoplasts inoculated with this helper virus, indicating that it was being replicated. Neither probe hybridized with any nucleic acids in extracts taken from mock-inoculated protoplasts. These results confirm that STMV is dependent on its helper virus for the replication of its genome.

The specific requirement of STMV transcript RNA for TMGMV as a helper was determined and was found to function at the level of replication. Protoplasts were co-inoculated with STMV RNA purified from virions, and RNA purified from virions of TMGMV, tobacco mosaic virus, U1 strain (TMV-U1), or tomato mosaic virus (ToMV). Protoplasts were also co-inoculated with RNA purified from virions of each of these three helper viruses and STMV RNA transcribed from pSTMV. Protoplasts were then incubated for 42 h at room temperature before they were harvested. STMV RNA accumulated in protoplasts that had been co-inoculated with STMV virion RNA and any of the three helper viruses (Fig. 2b). However, STMV transcript RNA accumulated in protoplasts only when co-inoculated with TMGMV RNA and never in protoplasts co-inoculated with STMV transcript and either TMV-U1 or ToMV, or when inoculated with STMV transcript RNA alone.

Three STMV RNAs with mutant CP sequences were then analysed for their ability to be replicated by TMGMV helper in protoplasts. One of these mutants, ACP407 (Fig. 1), lacked most of the STMV CP coding sequence and retained only the amino-terminal 13 amino acids, followed by nine non-CP amino acids. Mutant CPFS (Fig. 1) was nearly wild-type in RNA sequence, but had the capacity to code for only the amino-terminal 55 amino acids of the 158 residue CP genome, followed by 72 out-of-frame amino acids. Mutant CPUAA had a UAA stop codon in place of the start codon. The 22 amino-terminal amino acids of this protein lacked a methionine and, with the exception of the start codon for a 6-8 kDa protein ORF, there were no other AUG sequences upstream of the CP start codon. This mutant was predicted to be incapable of directing synthesis of any CP sequences. Protoplasts were co-inoculated with TMGMV virion RNA and STMV RNA transcribed from STMVHIII, ACP407HIII, CPFSHIII or CPUAAHIII. STMV (+) and (−) sense RNAs accumulated in protoplasts co-inoculated with TMGMV genomic RNA and RNA transcribed from each of these constructs (Fig. 2c). CP407 and CPFS RNAs accumulated as efficiently as wild-type STMV. In one experiment, the (−) strand RNA of one of the mutants, CPFS, accumulated to higher levels than observed with the other clones, including the wild-type strain. In two out of four experiments, mutant CPUAA did not replicate to detectable levels.

Results obtained from protoplasts inoculated with STMV RNA alone, or co-inoculated with STMV RNA and TMGMV RNA, indicate that it is a true satellite virus as described by Roossinck et al. (1992). These results confirm those presented in a recent study of satellite tobacco necrosis virus STNV (Andriessen et al., 1995), which demonstrated conclusively that this satellite virus uses the polymerase proteins encoded by ORFs 1 and 2 of TNV helper virus. One interesting aspect of STMV biology has been that RNA purified from wild-type STMV virions is capable of accumulating in whole tobacco plants when co-inoculated with a variety of toamovirus helpers (Valverde et al., 1991), whereas transcripts derived from STMV cDNA clones could only use TMGMV as a helper virus (Mirkov et al., 1990; Kurath et al., 1993). Subsequently, it was demonstrated that this helper specificity occurred independently of sequences present at the 3′iRNA-like region of STMV and suggests that nucleotide sequences at the 5′ terminus are responsible for the phenomenon. It was hypothesized that these 5′-terminal sequences may be responsible for the adaptation of STMV RNA to a wide range of helper tobamoviruses (Kurath et al., 1993). Results presented in this paper confirm that the phenomenon of helper specificity is related to STMV RNA replication, rather than some other aspect of helper virus-assisted satellite virus accumulation in whole plants. The efficient synthesis and accumulation of both (+) and (−) strand RNA of two out of three STMV CP mutants in protoplasts parallels the results obtained for TMV, which demonstrate that CP is dispensable for efficient replication of this virus (Ishikawa et al., 1991). STMV CP is, therefore, likely to facilitate systemic satellite virus accumulation in plants simply by protecting its genomic RNA from degradation. The mutant STMV transcript CPUAA, which accumulated to detectable levels in two out of four protoplast experiments, may therefore replicate inefficiently due to changes in RNA secondary structure caused by nucleotide changes at the site of the CP start codon.

Taken together, these results confirm previous conclusions about the replication of STMV based on experiments performed in whole plants. The results extend our understanding of STMV replication by demonstrating efficient replication in protoplasts of STMV CP mutants containing only amino-terminal CP sequences. These mutants were previously reported to accumulate to reduced levels in a condition-dependent manner in whole plants (Mirkov et al., 1990; Valverde et al., 1991; Kurath et al., 1993; Routh et al., 1995).
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References


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