Tomato golden mosaic virus (TGMV) is a bipartite geminivirus with six well-characterized genes. An additional open reading frame (ORF), AL4, lies within the essential AL1 gene. Recent studies of monopartite, dicot-infecting geminiviruses have revealed that mutations in their analogous C4 ORFs have host-specific effects on infectivity, symptomatology, virus movement and/or viral DNA accumulation. We have investigated whether TGMV has a similar host-specific requirement for AL4. The phenotypes of three TGMV al4 mutants were determined in a range of hosts, which included species that revealed c4 mutant phenotypes for monopartite geminiviruses. Each TGMV al4 mutant was indistinguishable from wild-type TGMV in all hosts tested. Additional analyses of double mutants revealed no evidence for functional redundancy between AL4 and the AL3, or AR1 genes. In contrast to the putative C4 proteins of monopartite geminiviruses, TGMV AL4, if it is expressed, is either non-functional, or functionally redundant with an essential TGMV gene product.

Tomato golden mosaic virus (TGMV) belongs to the subgroup III genus of the Geminiviridae (Murphy et al., 1995). Like bean golden mosaic virus (BGMV), the type member of subgroup III, TGMV has a bipartite genome, the components of which are designated A and B (Hamilton et al., 1984). The TGMV genome contains six well-characterized genes, four located on DNA A and two on DNA B. Genes on DNA A encode products required for replication (AL1, AL3), encapsidation (AR1) and the control of viral gene expression (AL2) (Elmer et al., 1988; Gardiner et al., 1988; Sunter et al., 1990; Sunter & Bisaro, 1992). The DNA B genes (BL1, BR1) are required for the spread of infection in plants (Brough et al., 1988). Mutations in the AL1, AL2, BL1 or BR1 genes prevent TGMV from infecting Nicotiana benthamiana systemically, whereas al3 and ar1 mutants give rise to attenuated systemic infections in this host (Brough et al., 1988; Elmer et al., 1988; Gardiner et al., 1988).

In addition to the genes described above, the nucleotide sequence of TGMV DNA A revealed an open reading frame (ORF) nested within AL1, which was designated AL4 by Elmer et al. (1988) (Fig. 1a). Other dicot-infecting geminiviruses have equivalent ORFs, called AC4 or C4, that occupy an analogous position in the genome, and could potentially encode small proteins with some amino acid sequence similarity (see Stanley et al., 1992). However, a TGMV mutant (al4-I) containing a premature termination codon in AL4 was indistinguishable from wild-type TGMV A in its ability to infect transgenic N. benthamiana containing tandem copies of the TGMV B component (Elmer et al., 1988). Although the presence of al4 mutant virus progeny in infected plants was not confirmed, this result suggested that AL4 might not represent a functional gene. A similar conclusion was reached for AC4 of the Old World bipartite geminivirus, African cassava mosaic virus (ACMV) (Etessami et al., 1991). In contrast, more recent studies of dicot-infecting, monopartite geminiviruses have shown that mutations in their AL4 analogue, which is called C4, have host-specific effects on infectivity, symptomatology, viral DNA accumulation and/or virus movement (Jupin et al., 1994; Rigden et al., 1994; Stanley et al., 1992; Stanley & Latham, 1992). The authors of these studies suggested that TGMV al4 mutants might have distinct phenotypes in hosts other than N. benthamiana. To examine the possibility of a host-specific requirement for TGMV AL4, we constructed three new al4 mutants and tested them in plant species which revealed c4 mutant phenotypes for two monopartite geminiviruses, Australian tomato leaf curl virus (TLCV-Aus; Rigden et al., 1994) and tomato yellow leaf curl virus (TYLCV; Jupin et al., 1994).

Because AL4 is located entirely within the essential AL1 gene, mutations in AL4 were designed to maintain the amino acids encoded by AL1 (Fig. 1b). Site-directed mutagenesis was carried out as described previously (Schaffer et al., 1995) on pTG1.3A (Fontes et al., 1994), a plasmid which contains a partial tandem dimer of TGMV DNA A with AL4 in the

Author for correspondence: Ian T. D. Petty.  
Fax +1 919 515 7867. e-mail timpetty@mbio.ncsu.edu
duplicated sequences. For each mutant allele, a plasmid was identified with the required mutation in both copies of AL4. Like the analogous C4 ORFs of TLCV-Aus and TYLCV, TGMV al4-2 formed part of a new restriction site. TLCV-Aus was studied by Rigden et al. (1984) of wild-type TGMV, and the encoded amino acid sequences, are shown in the region where mutations were introduced into AL4. Nucleotides that were altered by site-directed mutagenesis are boxed. The encoded amino acids in AL4 which were altered are circled, and the introduced termination codon forms part of an AseI or Spel restriction site, respectively.

The infection phenotypes of TGMV al4 mutants were compared to wild-type TGMV in N. benthamiana, Nicotiana tabacum variety Petite Havana SR1, Datura stramonium and Petunia × hybrida. These plants represent a spectrum of hosts which differ in the severity of induced disease symptoms and the extent of TGMV DNA accumulation. Plants were inoculated by microprojectile bombardment (Schaffer et al., 1995) with plasmids containing either wild-type or al4 mutant DNA A, together with pTG1.4B (Fontes et al., 1994), which contains a partial tandem dimer of TGMV DNA B. All three al4 mutants were used to inoculate N. benthamiana, N. tabacum and D. stramonium, with similar results in each case. Petunia was inoculated only with the al4-2 mutant. In all hosts tested, the symptoms elicited by al4 mutants, and their rate of appearance, were not reproducibly different from those of wild-type TGMV. Large chlorotic lesions were produced on inoculated leaves in all cases, and in N. tabacum and petunia they were accompanied by vein yellowing. Systemic symptoms of TGMV infection in N. benthamiana were uniform vein chlorosis and severe epinasty and rugosity of the leaves, accompanied by stunting of the plants. In N. tabacum, systemic symptoms consisted of mild leaf distortion with uniform vein chlorosis. Systemically infected petunia leaves had only chlorotic spots, and the systemically infected leaves of D. stramonium remained asymptomatic. Total nucleic acids were

---

(a)

\[\text{WILD TYPE}\]

\[
\begin{align*}
\text{AL1} & \text{AL2} & \text{AL4} & \text{ART1} \\
\text{EagI} & \text{BamHI} & \text{AseI} & \\
\end{align*}
\]

(b)

\[\text{MUTANTS}\]

\[
\begin{align*}
\text{AseI} & \text{Spel} \\
\text{AL1} & \text{AL2} & \text{AL4} & \text{ART1} \\
\text{EagI} & \text{BamHI} & \text{AseI} & \\
\end{align*}
\]

Fig. 1. Genetic organization of TGMV DNA A and location of al4 mutations. (a) Linear map of TGMV DNA A showing the positions and orientations of the five open reading frames (open arrows). The locations of pertinent restriction sites in wild-type TGMV A are indicated. The region enclosed by dashed lines is shown expanded below. (b) The nucleotide sequence complementary to the virion-sense (coordinates 2448–2260; Hamilton et al., 1984) of wild-type TGMV, and the encoded amino acid sequences, are shown in the region where mutations were introduced into AL4. Nucleotides that were altered by site-directed mutagenesis are boxed. The encoded amino acids in AL4 which were altered are circled, and the introduced termination codon forms part of an AseI or SpeI restriction site, respectively.
extracted, treated with ribonuclease, and resolved by electrophoresis and Southern blotting, as described previously (Schaffer et al., 1995). Viral DNA was detected in both inoculated and systemically infected leaves of all types of host plants, and the accumulation of al4 mutant DNA was found to be similar to that of wild-type TGMV (Fig. 2, and data not shown). The presence of the al4 mutation in the DNA of viral progeny from infected plants was confirmed by nucleotide sequencing (al4-2), or by susceptibility to digestion with Asel (al4-3) or SpeI (al4-4) (data not shown). In addition, the stability of the al4 mutations was assessed by passing infected N. benthamiana extracts to further N. benthamiana plants. Viral DNA isolated from plants infected in this manner retained the appropriate al4 mutation (Fig. 3). Thus, TGMV al4 mutations are stably maintained through at least one passage in N. benthamiana. These results confirm and extend those of Elmer et al. (1988), and show that al4 mutants are indistinguishable from wild-type TGMV in hosts which revealed distinct phenotypes for the equivalent c4 mutants of TLCV-Aus and TYLCV.

The finding that TGMV AL4 is non-essential for infection of several host plant species might indicate functional redundancy between the putative AL4 protein and another TGMV gene product. Because mutations which disrupt either the AL1, AL2, BL1 or BR1 ORFs eliminate systemic infectivity of TGMV, AL4 cannot substitute completely for any of these gene products. However, TGMV mutants which lack the coat (AR1) or AL3 proteins produce attenuated systemic infections in N. benthamiana. To determine whether an intact AL4 ORF contributes to the viability of either ar1 or al3 mutants, we constructed double mutants of each with the al4-2 allele. The ar1 mutant was constructed by filling-in the unique XhoI site in pTG1.3A (Fig. 1a), which created a frameshift mutation within the AR1 ORF. The mutant al3 gene from pTGA45 (Sunter et al., 1990) was excised on an 838 bp EcorI-BstBI fragment, and inserted in place of the equivalent region of pTG1.3A. Double mutants were constructed using pTG1.3AL4-2, which contains the al4-2 mutation, as the recipient plasmid in each case. The ar1/al4 and al3/al4 double mutants were inoculated into N. benthamiana and compared to single mutant controls. As described previously (Gardiner et al., 1988), an ar1 mutant produced milder systemic symptoms than wild-type TGMV, and the overall level of viral DNA accumulation was lower in systemically infected leaves. The phenotypes of ar1 and ar1/al4 mutants were indistinguishable. The TGMV al3 mutant was previously reported to accumulate lower levels of viral DNA than wild-type TGMV A, and elicit only vein clearing symptoms on systemically infected leaves of agroinoculated transgenic N. benthamiana containing TGMV B (Elmer et al., 1988). In our experiments, both viral genome components were introduced into non-transgenic N. benthamiana by microprojectile bombardment. We observed very low levels of

![Fig. 2. Accumulation of wild-type and al4 mutant TGMV DNA in various hosts. Plants were infected with plasmids containing either wild-type (wt) or al4-2 mutant (al4) TGMV DNA A, together with a plasmid containing wild-type TGMV DNA B in each case. Hosts used were Nicotiana benthamiana (N.b.), N. tabacum var. Petite Havana SR1 (N.t.) and Datura stramonium (D.s.). Aliquots of total nucleic acids (2 μg from N.b., 10 μg from N.t. and D.s.) from systemically infected leaves were analysed by Southern blotting and hybridization to a 32P-labelled dsDNA probe specific for TGMV DNA A (607 bp EcorI-BstBI fragment). The positions of viral open-circular (oc) and supercoiled (sc) dsDNA, and ssDNA (ss) are indicated. The D.s. panel shows a longer exposure of the autoradiogram than those for N.b. and N.t., and therefore the signal intensities cannot be directly compared.

![Fig. 3. Stability of TGMV al4 mutations through a passage in Nicotiana benthamiana. Nucleic acids extracted from plants infected with wild-type (wt), al4-3 or al4-4 mutant TGMV were used for microprojectile bombardment of further plants. Total nucleic acids were prepared from systemically infected leaves of the latter plants at 18 days post-inoculation. Aliquots (2 μg) were left untreated (−) or incubated with restriction enzymes Asel (+A) or Spel (+S). The samples were analysed by Southern blotting and hybridization to a 32P-labelled ssDNA probe specific for the complementary-sense of TGMV DNA A (565 bp BamHI-Scal fragment). The positions of viral open-circular (oc), linear (lin) and supercoiled (sc) dsDNA forms are indicated. Viral ssDNA (ss) also cross-hybridized slightly with the probe. There are no sites for Spel, and one site for Asel, in wild-type TGMV A (see Fig. 1a). Linear TGMV A contains 2588 bp, and the expected Asel digestion products of the al4-3 mutant are 1349 bp and 1239 bp. The positions of linear dsDNA markers with the indicated sizes in bp are shown on the right.]
The AL1 protein is necessary for TGMV DNA replication (Elmer et al., 1988), and transient expression assays in N. benthamiana protoplasts suggested that TGMV AL4 may repress expression of the overlapping AL1 gene (Gröning et al., 1994). The AL1 protein is necessary for TGMV DNA replication (Elmer et al., 1988), so if \( \text{AL}4 \) mutants have a reduced ability to repress AL1 expression, they might be expected to exhibit elevated levels of DNA replication. However, we observed no effect of \( \text{AL}4 \) mutations on TGMV DNA accumulation in infected plants, even in the replication-deficient \( \text{AL}3 \) mutant background (Sunter et al., 1990). Thus, if TGMV AL4 represses AL1 gene expression in planta, its effect must be slight, or readily compensated for by AL1 itself (Gröning et al., 1994). Taken together, our data support the conclusion that TGMV AL4, if it is expressed in infected plants, is either non-functional, or functionally redundant with one or more of the essential TGMV-encoded proteins.

Our failure to observe a distinct phenotype for TGMV \( \text{AL}4 \) mutants is similar to the results obtained in studies of ACMV in plants (Etessami et al., 1991) and in protoplasts (Hong & Stanley, 1995). Potato yellow mosaic virus \( \text{AL}4 \) mutants were also found to be indistinguishable from the wild-type virus in both N. benthamiana and potato plants (Sung & Coutts, 1995). In addition, it is noteworthy that two of the bipartite geminiviruses for which genome sequences have been determined, pepper huasteco virus (Torres-Pacheco et al., 1993) and Indian tomato leaf curl virus (Padidam et al., 1995), each naturally contains a truncated \( \text{AL}4 \) ORF which is equivalent to the TGMV \( \text{AL}4-4 \) mutant allele. Thus, as previously suggested by others, \( \text{AL}4 \) in bipartite geminiviruses may represent a vestigial gene which remains conserved largely due to sequence constraints imposed by the essential overlapping \( \text{AL}1 \) gene (Elmer et al., 1988; Etessami et al., 1991). Monopartite whitefly-transmitted geminiviruses are currently assigned to the subgroup III genus (Murphy et al., 1995), even though they differ from the type member, BGMV, in having non-segmented genomes. The apparent functional difference between the \( \text{C4} \) and \( \text{AL}4 \) (AC4) ORFs provides additional support for the removal of monopartite, whitefly-transmitted geminiviruses from subgroup III to a separate genus.

We thank Carla Randall, Jim Moyer, Chris Taylor and Mark Conkling for seeds. Arthur Weissinger for the use of his gene gun, and David Bisaro for kindly providing pTGA45. We are grateful to Eric Miller and Steve Lommel for their comments on the manuscript. Geminivirus-infected plants were maintained in the South Eastern Plant Environment Laboratory at North Carolina State University. W.P. thanks the Thai government for a scholarship. This research was supported in part by the North Carolina Agricultural Research Service, and by Public Health Service Grant no. GM-48067.

References


Received 22 January 1996; Accepted 19 April 1996