Tomato golden mosaic virus open reading frame AL4 is genetically distinct from its C4 analogue in monopartite geminiviruses

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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 (al4-1) 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 gemivirus, 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
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 was part of a new restriction site formed part of the restriction site in each case, al4-2 is likely to be a null mutation. A similar c4 initiation codon mutant of TLCV-Aus was studied by Rigden et al. (1994). The other TGMV al4 mutants were constructed by introducing premature termination codons into AL4 after 10 (al4-3) or 60 (al4-4) encoded amino acids (full-length AL4 could encode 87 amino acid residues). In each case, the termination codon formed part of a new restriction site (AseI in al4-3, SpeI in al4-4), and the amino acid sequence of AL1 was unaltered. The al4-3 mutant contains the same transversion as the al4-1 mutant described previously (Elmer et al., 1988), but has an additional Cys → Tyr substitution in the putative truncated AL4 product. A similar TYLCV mutant, expected to encode only the N-terminal 10 residues of C4, was studied by Jupin et al. (1994). The TGMV al4-4 mutant also has an amino acid substitution at the final residue of the putative truncated AL4 product, in this case Leu → His. Because the termination codons in TGMV al4-3 and al4-4 form part of the restriction site in each case, reversion of the al4 mutations cannot occur without concomitant loss of the site (Fig. 1b).

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
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 passaging 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 ar4 mutant was constructed by filling-in the unique XhoI site in pTG1.3A (Fig. 1a), which created a frameshift mutation within the AL1 ORF. The mutant al3 gene from pTGA45 (Sunter et al., 1990) was excised on an 838 bp Eagl-BamHI 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/ar4 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
al3 mutant DNA accumulation, and infected plants remained asymptomatic. However, the phenotypes of al3 and al3/al4 mutants were indistinguishable. Thus, the viable phenotypes of TGMV ar1 and al3 mutants are not due to partial redundancy between the functions of the AR1 or AL3 proteins and AL4.

Mutations in the C4 genes of monopartite geminiviruses which infect dicots have host-specific effects on infectivity, symptomatology and viral DNA accumulation in infected plants. These phenotypes were observed not only for the whitefly-transmitted viruses TLCV-Aus and TYLCV, but also for beet curly top virus, a leafhopper-transmitted, monopartite geminivirus which is the type member of the subgroup II genus (Stanley et al., 1992; Stanley & Latham, 1992). In contrast, our experiments showed that TGMV al4 mutants with genotypes similar to those of the c4 mutants of TLCV-Aus (al4-2) or TYLCV (al4-3) had phenotypes which were not discernibly different from wild-type TGMV when tested in a range of plant species. It still remains possible that AL4 participates in whitefly transmission of TGMV. Previous studies have shown that TGMV-infected N. benthamiana plants contain relatively abundant transcripts which could act as AL4 mRNAs (Sunter & Bisaro, 1989), 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 al4 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 al4 mutations on TGMV DNA accumulation in infected plants, even in the replication-deficient al3 mutant background (Sunter et al., 1990). Thus, if TGMV AL4 represses AL1 gene expression in planta, its effect must be slight, or readily compensatable 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 al4 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 al4 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 AL4 ORF which is equivalent to the TGMV al4-4 mutant allele. Thus, as previously suggested by others, AL4 in bipartite geminiviruses may represent a vestigial gene which remains conserved largely due to sequence constraints imposed by the essential overlapping AL1 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 C4 and AL4 (C4) ORFs provides additional support for the removal of monopartite, whitefly-transmitted geminiviruses from subgroup III to a separate genus.

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References


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