A Single Amino Acid Change in Tobacco Mosaic Virus Replicase Prevents Symptom Production

Dennis J. Lewandowski and William O. Dawson

Department of Plant Pathology, University of California, Riverside 92521 U.S.A.
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A tobacco mosaic tobamovirus (TMV) mutant that produced a symptomless systemic infection in tobacco and other plants was found to have a single nucleotide change resulting in an amino acid substitution in both the 126- and 183-kDa proteins. Virus accumulation was reduced in both inoculated and systemically infected leaves of tobacco relative to TMV U1.

Tobamovirus mutants that produce attenuated symptoms have been isolated, including a masked strain of tobacco mosaic tobamovirus (TMV) that was isolated from the parental strain that causes a severe distorting mottle on tobacco (Holmes 1934) and $L_{11}A$, a spontaneous mutant of tomato mosaic tobamovirus (ToMV) that produces attenuated symptoms in tobacco and tomato (Oshima *et al.* 1965). A mutant with a similar phenotype was isolated from the wild-type population of TMV strain U1 by selection at elevated temperature (Dawson, unpublished) with the regimen used for selection of temperature-sensitive, replication-deficient mutants (Dawson and Jones 1976). Unlike the parental TMV strain U1, which produces mosaic symptoms on leaves above those inoculated, mutant V-36 did not produce any symptoms in inoculated or upper leaves of tobacco (*Nicotiana tabacum* L. 'Xanthi') plants at 25 °C.

Mutant V-36 was revived from a single local lesion of *N. tabacum* 'Xanthi-nc' stored at −80 °C and used to inoculate Xanthi tobacco that was maintained at 25 °C. In upper leaves of Xanthi tobacco plants, TMV U1 caused typical mosaic symptoms (Fig. 1A) but V-36 produced no symptoms (Fig. 1B). To examine whether the symptomless phenotype of V-36 was unique to tobacco, we inoculated *N. sylvestris* Speg. & Comes and *N. benthamiana* Domin. In upper leaves of *N. sylvestris*, TMV U1 caused mosaic symptoms (Fig. 1C), but V-36 produced no visible symptoms (Fig. 1D). TMV U1 induced necrotic rings in inoculated leaves and stem necrosis in *N. benthamiana* 1–2 wk after inoculation (Fig. 1E). V-36 did not elicit stem necrosis in *N. benthamiana* (Fig. 1F) but caused a mild chlorosis in some upper leaves. Less virus was present in the uppermost leaves of *N. sylvestris* infected with V-36 than with TMV U1, as measured by local lesion assay. Due to the stem necrosis induced by TMV U1, relative levels of V-36 and TMV U1 present in upper uninoculated leaves of *N. benthamiana* were not quantified.

To localize the mutations involved in the symptomless phenotype, cDNA was synthesized to defined regions of the V-36 genome and substituted into derivatives of an infectious wild-type TMV U1 cDNA clone (Dawson *et al.* 1986; Donson *et al.* 1991). pTMV118 contained the 7.3-kb *Pst*I/*Kpn*I fragment from pTKU1 (Donson *et al.* 1991) that encodes the complete cDNA of TMV strain U1 behind the λ phage promoter from pPM1 (Ahlquist and Janda 1984) in pUC118. pΔASBL1, pΔSB2, and pΔBN2 were the wild-type TMV deletion vectors for substitution cloning of V-36 cDNA into the wild-type TMV genome. pΔASBL1 had deletions of nt 256–1675 and 3347–4929 (numbering according to Goelet *et al.* 1982). pΔSB2 had a deletion of nt 1675–3332, and pΔBN2 had a deletion of nt 3332–5459. Three independent V-36 first-strand cDNA reactions encompassing nt 256–5459 were amplified with the polymerase chain reaction and independently substituted into the wild-type TMV deletion vectors (Fig. 2B).

Xanthi-nc tobacco was independently inoculated with *in vitro* RNA transcripts derived from 5 μg of *Kpn*I linearized plasmid DNA of the V-36/TMV hybrids or pTMV118 and maintained at 25 °C (Dawson *et al.* 1986; Donson *et al.* 1991). To identify which genomic fragment was responsible for the symptomless phenotype, virus from single local lesions induced by each hybrid was used to inoculate Xanthi tobacco that was maintained at 25 °C. V-36as and V-36bn (Fig. 2B) produced typical mosaic symptoms in Xanthi tobacco. In contrast, V-36sb (Fig. 2B), which contained the V-36 *Stu*I/*Bam*HI (1675–3332) cDNA fragment produced a symptomless infection in Xanthi tobacco.

Mutation(s) involved in the symptomless phenotype were localized further by subcloning the V-36 *Stu*I/*Bam*HI cDNA fragment from pV36sb and screening the hybrids. In Xanthi tobacco, V-36sab (Fig. 2C) produced mosaic symptoms, but V-36ssa (Fig. 2C), which contained the V-36 *Stu*I/*Sac*II (1675–2650) cDNA fragment, produced a symptomless infection. From DNA sequence analysis (Zagursky *et al.* 1985), we found two C→U transitions relative to pTMV118. The nucleotide change at 2249 was translationally silent, but the nucleotide change at 1996 resulted in a Ser *643*→Phe substitution within the 126- and 183-kDa proteins. This alteration occurred between

Present address of first author: University of Florida, Citrus Research and Education Center, Lake Alfred 33850.

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domains 1 and 2 (Haseloff *et al.* 1984; Ahlquist *et al.* 1985), adjacent to the N-terminal border of replicase domain 2. The two single nucleotide changes were separated, and the phenotype of each hybrid was tested (Fig. 2C). On Xanthi tobacco, V-36csa, which contained the silent nucleotide change at 2249, produced mosaic symptoms, but V-36sc, which contained Phe *643*, produced a symptomless infection indistinguishable from the original mutant. Thus, a single nucleotide change controlling a single amino acid substitution within the 126- and 183-kDa proteins was sufficient to change the phenotype of TMV from virulent to symptomless on tobacco as well as on *N. sylvestris* and *N. benthamiana*.

By comparison, Holmes' masked strain of TMV has 55 nucleotide changes that result in 11 amino acid substitutions (Holt *et al.* 1990), and the ToMV mutant $L_{11}A$ has 10 nucleotide changes that result in three amino acid substitutions (Nishiguchi *et al.* 1985). Holt *et al.* (1990) were able to localize the mutations responsible for the attenuated phenotype of the Holmes' masked strain of TMV to the 126- and 183-kDa open reading frame but were unable to localize the symptomless phenotype to less than the original eight amino acid substitutions within the 126- and 183-kDa proteins or the 23 silent changes within this region. It was not determined whether all three amino acid substitutions within the 126- and 183-kDa proteins are required for the attenuated phenotype of $L_{11}A$.

To examine how the mutation(s) within the V-36 genome prevented symptom induction in tobacco, we examined several viral properties. Cell-to-cell movement was compared by inoculating opposite half leaves of Xanthi-nc tobacco with TMV 118, V-36, V-36ssa, or V-36sc. In plants maintained continuously at 25 °C, V-36, V-36ssa, and V-36sc produced local lesions that were indistinguishable from wild-type virus (TMV 118). Another set of Xanthi-nc tobacco plants was maintained at 32 °C for 3 days, allowing virus to spread without inducing necrosis. After the shift to 25 °C, there was a rapid induction of necrosis at the boundary of viral spread, allowing an estimate of cell-to-cell movement. Under these conditions, V-36, V-36ssa, V-36sc, and TMV 118 moved similarly from cell to cell.

To compare virus accumulation in inoculated leaves, Xanthi tobacco was inoculated with TMV 118 or V-36ssa, the symptomless hybrid containing the smallest fragment of V-36 cDNA then available, and the infectivity of sap samples was measured. Like the parental mutant V-36, V-36ssa accumulated to low levels (in comparison with TMV 118) in upper uninoculated leaves of tobacco plants. The specific infectivities of purified virions (number of

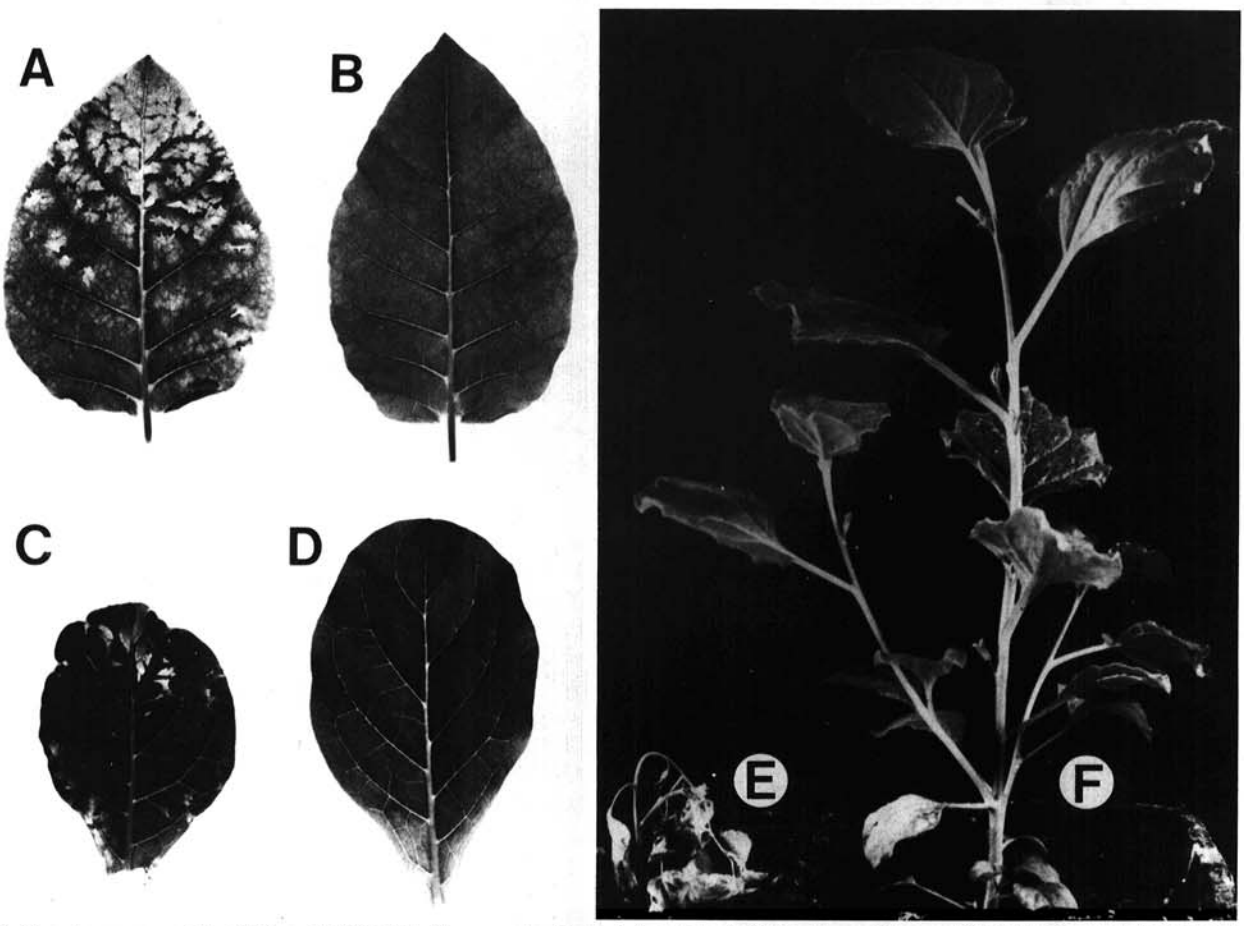

Fig. 1. Symptoms caused by V-36 and TMV U1. Upper systemically infected leaf of *Nicotiana tabacum* 'Xanthi' tobacco infected with **A,** TMV U1, or **B,** V-36. Upper systemically infected leaf of *N. sylvestris* infected with **C,** TMV U1, or **D,** V-36. *N. benthamiana* infected with **E,** TMV U1, or **F,** V-36. Ten local lesions were ground and used to inoculate 6-wk-old Xanthi tobacco, *N. sylvestris*, and *N. benthamiana* plants that were maintained in the greenhouse. Photographs were taken 14 days postinoculation.

lesions per microgram of virus) of TMV 118 and V-36ssa were the same (data not shown). The relative infectivity of TMV 118 in inoculated leaves of Xanthi tobacco rapidly increased between 3 and 6 days postinoculation (DPI) and leveled off between 6 and 14 DPI (Fig. 3). In contrast, the levels of V-36ssa in inoculated leaves of Xanthi tobacco were one-tenth to one-fifth the wild-type virus levels at comparable times. Also, the maximal level of V-36ssa infectivity (9 DPI) was below the lowest measured infectivity of TMV 118 (3 DPI), suggesting that V-36 was defective in replication at 25 °C. In contrast, the accumulation of Holmes' masked strain of TMV was similar to the U1 strain levels in inoculated leaves of tobacco (Nelson *et al.* 1993).

To compare the long-distance movement and systemic infection of V-36ssa with that of TMV 118, the relative infectivity from samples of randomly sampled upper leaves of the same Xanthi tobacco plants used above was determined by assaying opposite half leaves of Xanthi-nc tobacco (Table 1). Infectivity was lower in essentially all comparable upper leaves of Xanthi tobacco inoculated with V-36ssa than with TMV 118. Levels of virus from most TMV 118-infected, uninoculated upper leaves were five to 10 times higher than the equivalent leaves infected with V-36ssa, although both had measurable infectivity at all times tested. These data suggest that V-36ssa has a defect in replication rather than in movement.

Although the mutations responsible for the symptomless phenotypes of V-36, Holmes' masked strain of TMV, and ToMV mutant $L_{11}A$ have all been localized to the replicase open reading frames, these mutants are functionally different. Cell-to-cell and long-distance movement of V-36 was similar to that of wild-type virus, but levels of virus were reduced in both inoculated and upper leaves, suggesting a defect in replication rather than in transport. In contrast, Holmes' masked strain of TMV accumulates at or above wild-type virus levels in inoculated leaves but has reduced accumulation in upper leaves of tobacco (Nelson *et al.* 1992), suggesting that this mutant is defective in long-distance movement rather than in replication. ToMV mutant $L_{11}A$ also multiplies to levels similar to the parental virus in tobacco protoplasts (Nishiguchi *et al.* 1985) but has reduced cell-to-cell movement (Nishiguchi and Oshima 1977) and reduced levels of movement protein and of movement protein subgenomic mRNA synthesis in tobacco protoplasts (Watanabe *et al.* 1987).

Although the primary role of the 126- and 183-kDa proteins is believed to be in replication, certain mutations

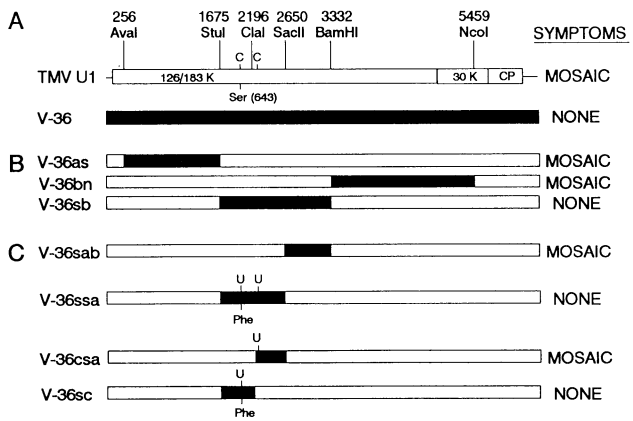

Fig. 2. Localization of mutations responsible for the symptomless phenotype of V-36 in *Nicotiana tabacum* 'Xanthi.' **A,** Genome organization of TMV U1. **B,** Diagrams of original V-36/TMV hybrids. V-36as contained the V-36 *AvaI*/*StuI* (256–1675) cDNA fragment synthesized with a primer complementary to nt 2246–2264, amplified with primers complementary to nt 1922–1942 and corresponding to nt 78–97, digested with *AvaI*/*StuI* and ligated into pΔASBL1. V-36bn contained the V-36 *Bam*HI/*Nco*I (3332–5459) cDNA fragment synthesized with a primer complementary to nt 5673–5692, amplified with primers complementary to nt 5541–5565 and corresponding to nt 3324–3345, digested with *Bam*HI/*Nco*I and ligated into pΔBN2. V-36sb contained the V-36 *StuI*/*Bam*HI (1675–3332) cDNA fragment synthesized with a primer complementary to nt 3563–3583, amplified with primers complementary to nt 3357–3376 and corresponding to nt 1667–1688, digested with *StuI*/*Bam*HI and ligated into pΔSB2. **C,** Diagrams of hybrids produced by subcloning. V-36sab and V-36ssa contained nt 2650–3332 and 1675–2650, respectively, derived from pV36sb. V-36csa and V-36sc contained nt 2196–2650 and 1675–2196, respectively, derived from pV36ssa. Relevant restriction sites are indicated above the TMV U1 diagram. Amino acid and nucleotide changes determined by DNA sequence analysis of pV36ssa are shown above and below the diagrams, respectively, relative to pTMV118. Numbering according to Goelet *et al.* (1982). Open bars = TMV U1 cDNA. Shaded bars = V-36 cDNA.

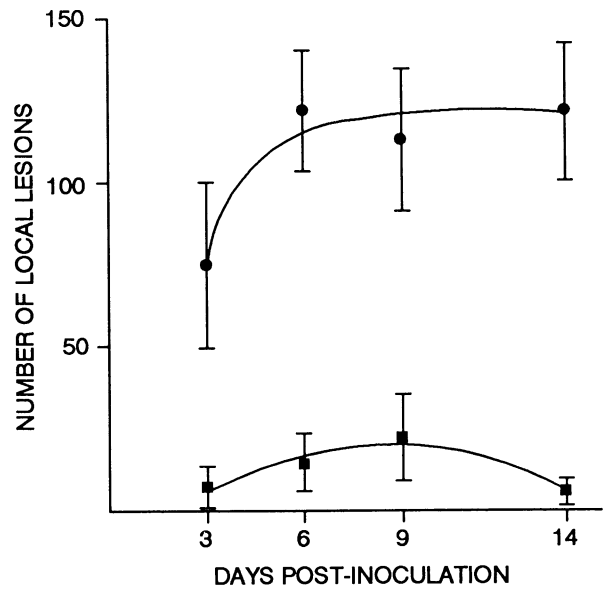

Fig. 3. Relative accumulation of V-36ssa and TMV 118 in inoculated leaves of *Nicotiana tabacum* 'Xanthi.' Relative infectivity of V-36ssa (■) and TMV 118 (●) (average number of lesions on seven half leaves of *N. tabacum* 'Xanthi-nc'). Two expanded leaves (~8 cm long) of each of 10 6-wk-old Xanthi tobacco plants were inoculated with sap from TMV 118 or V-36ssa infected tobacco at concentrations that gave confluent local lesions on Xanthi-nc tobacco. Plants were maintained in a growth chamber for 14 days at 25 °C under 16-h days at 3,000 lux. One 16-mm diameter disk was randomly punched from each of the 20 inoculated leaves at 3, 6, 9, and 14 days postinoculation. Samples were stored at −20 °C until all samples had been taken. Leaf tissue was ground and diluted 1:1,000 (w/v) in 50 mM glycine, 30 mM K_2HPO_4, pH 9.2, containing 1% Celite. The 1:1,000 dilution was assayed in a random block design on opposite half leaves of *N. tabacum* 'Xanthi-nc,' with each sample replicated seven times.

Table 1. Relative accumulation of V-36ssa and TMV 118 in systemically infected upper leaves of *Nicotiana tabacum* 'Xanthi'

	Relative infectivity[a] ($\bar{x}$ local lesions)							
	Leaf + 2[c]		Leaf + 3		Leaf + 4		Leaf + 5	
DPI[b]	V-36[d]	TMV[e]	V-36	TMV	V-36	TMV	V-36	TMV
3	1	0	...[f]	...	...	...	...	...
4	0	29	...	...	...	...	...	...
5	7	4	...	...	...	...	...	...
6	30	54	4	26	...	...	...	...
7	1	16	3	33	...	...	...	...
8	3	10	3	25	...	...	...	...
9	5	24	17	98	1	31	3	39
10	4	33	23	59	1	29	1	10
13	6	52	20	103	2	43	0	10
14	...	...	20	105	2	36	1	26

[a] Average number of local lesions per half leaf of Xanthi-nc tobacco. Equivalent V-36ssa and TMV 118 samples from two randomly selected Xanthi tobacco half leaves were ground, diluted 1:500, and directly compared on four opposite half leaves of Xanthi-nc tobacco.
[b] Days postinoculation (of two consecutive leaves of 10 6-wk-old Xanthi tobacco plants).
[c] Leaf number above uppermost inoculated leaf.
[d] Hybrid V-36ssa, containing the V-36 *Stu*I/*Sac*II cDNA fragment.
[e] Virus previously derived from pTMV118.
[f] Not determined.

in these proteins can alter other virus functions that affect virus-host interactions and influence symptom expression. Although the single amino acid change at position 643 affected both virus accumulation and symptom induction, we do not know if reduced virus accumulation or disruption of other viral functions was responsible for the symptomless phenotype.

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