A Missense Mutation in *Tomato mosaic virus* L11A-Fukushima Genome Determines Its Symptomless Systemic Infection of Tomato

Hideki YAMAMOTO1*, Takeshi ABE2, Kenji UEDA3, Masayasu INOUE3 and Tsutomu MATSUMOTO3

**ABSTRACT**

The genome of an attenuated isolate L11A-Fukushima (L11A-F) of *Tomato mosaic virus* was cloned, its complete nucleotide sequence was determined, and full-length clone of L11A-F was then assembled. Its transcripts systemically infected tomato without causing any symptom development. When a missense back mutation at the nucleotide corresponding to nucleotide 2350 of the genome was introduced into the clone, its transcripts produced distinct mosaic on the upper leaves of the inoculated tomato, and virus accumulation in the upper leaves increased about five times. The missense mutation in the L11A-F genome was thus confirmed to be sufficient to attenuate its virulence on tomato.

(Received June 10, 2002; Accepted October 30, 2002)

**Key words**: attenuated isolate, complete nucleotide sequence, infectious clone, L11A-Fukushima, site-directed mutagenesis, *Tomato mosaic virus*.

An attenuated isolate L11A derived from virulent isolate L of *Tomato mosaic virus* (ToMV) has been used widely in Japan to protect tomato (*Lycopersicon esculentum* Mill.) from virulent ToMV over 30 years without any problems6). We demonstrated that L11A-Fukushima (L11A-F) derived from L11A gives much higher cross protection against virulent ToMV than L11A and its derivative isolates L11A-237 and L11A-Chiba (L11A-C)6). A comparison of the complete nucleotide sequence between L11A and L suggested that three missense mutations at nucleotides (nts) 1117, 2349 and 2754 on the gene for the 130-Kd protein of L11A are responsible for the attenuation of its virulence6). Our previous study12) showed that, of these, only the missense mutation at nt 2349 was conserved in all L11A-derived attenuated isolates, which suggested that the missense mutation most contributes to the attenuation of the virulence in L11A and its derivative isolates. Molecular techniques have made possible the production of infectious cDNA clones from *Tobacco mosaic virus* (TMV)4-9, and clones from attenuated isolates were manipulated to identify the region responsible for their symptom attenuation4,5,10: Symptomless systemic infection of a mutant was proved to be caused by a single amino acid change in both the 130- and 180-Kd proteins10. Although infectious clones of L11A were also constructed10, missense mutation(s) responsible for the phenotypic change from virulent to symptomless infectivity has not yet been identified. In this paper, we directly confirmed that the missense mutation at nt 2349 most contributes to the symptomless infection of L11A-F by introducing a back mutation into infectious clones of its genome.

To clone the L11A-F genome as a first step, its genomic RNA was extracted as described previously5, followed by polyadenylation at the 3′ termini with poly(A) polymerase (Takara, Japan). Double-stranded cDNAs were synthesized using a Marathon cDNA Amplification Kit (Clontech, USA) and then ligated to an EcoRI-NotI-BamHI Adapter (Takara), which was cloned into EcoRI-digested pBluescript II SK(+) (Stratagene, USA). Cloning of the 5′ ends was performed using a FirstChoice RLM-RACE Kit (Ambion, USA) and then ligated to an EcoRI-NotI-BamHI Adapter (Takara), which was cloned into EcoRI-digested pBluescript II SK(+) (Stratagene, USA). Cloning of the 5′ ends was performed using a FirstChoice RLM-RACE Kit (Ambion, USA) and then inserted into pGEM-T Easy Vector (Promega, USA). These clones were sequenced using an Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia)

1 Akita Prefectural Center for Biological Resources Development, Ohgata, Akita 010-0442, Japan
2 Akita Prefectural College of Agriculture, Ohgata, Akita 010-0444, Japan
3 Akita Prefectural University, Ohgata, Akita 010-0444, Japan
* Corresponding author (E-mail: yamamoto@pref.akita.jp)
The L,1A-F genome was shown to be composed of 6385 nts, two bases longer than the L,1A genome, because it consists of one more residue at the oligo(U) and oligo(A) stretches located in the 5'- and 3’-noncoding regions, respectively (Fig. 1). The nt number of the corresponding position thereby differs between the two isolates; for instance, nt 2349 in L,1A corresponds to nt 2350 in L,1A-F. Thus, nt number described hereafter is that of the L,1A-F genome. The nucleotide sequence has been deposited in DDBJ under accession No. AB083196. As compared with the L,1A genome11, five base substitutions were found, all of which were located within the gene for the 130-Kd protein. Two were missense mutations, one of which at nt 1118 was identical to that of the L genome12. It was reported that the back mutation was also found on the L,1A-C genome12. The other was at nt 1969, which was unique to the L,1A-F genome among ToMV isolates L, L,1A, L,1A237, L,1A-C and L,1A-F (Yamamoto, H., unpublished results).

A full-length clone of L,1A-F was constructed on the basis of the following plasmids: p11 is pGEM-T Easy Vector containing an insert corresponding to nts 1-277 of the L,1A-F genome, and p80 and p212 are pBluescript SK(+) carrying cDNA corresponding to nts 126-383 and 3696-6385, respectively, with an adapter sequence containing a BamHI site at both ends (Fig. 2). The insert of p212 was transferred to pUC18 as follows: p212 was subjected to double digestion with BamHI and BspHI and electrophoresed on 1% agarose gel. The insert was excised, purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and cloned into the BamHI site of pUC18. The resulting clone was named p212UC18. To construct the 5’ terminus cDNA clone, p11 as template and a primer set of L-257 and T7-ToMV were used. L-257 is complementary to nts 258-277 of the genome; and T7-ToMV, 5’ TCGCGATGGGCCCACAACCGGGGTTTC3’, contains a terminal KpnI site (double underlined), which was not used in this study, and the T7 promoter (underlined) followed by a sequence corresponding to the 5’ terminus of L,1A-F (bold). The clone containing the 3’ terminal region was amplified with p212 as template and a primer set of U5989.1, corresponding to nts 5990-6009 of the genome and ToMV-NruI, 5’ TCGCGATGGGCCCACAACCGGGGTTTC3’, which contains sequences complementary to the 3’ terminus of the genome (bold) and 3’ flanking NruI site (underlined). Thermal conditions for the polymerase chain reaction (PCR) using Advantage-HF 2 PCR Kit (Clontech) were: 94°C for 1 min; 35 cycles of 94°C, 50°C, 72°C, each for 1 min; followed by 72°C for 5 min. The two PCR-amplified fragments were inserted into pGEM-T Easy Vector and sequenced. The two clones containing the 5’ and 3’ termini were named pT7 and pNRI, respectively.

These plasmids were then assembled as follows. The insert excised from pNRI by double digestion with NsiI and ScaI was ligated into p212UC18 digested with the two restriction enzymes, and the resulting plasmid was named p3FN. Similarly, the insert excised from pT7 by double digestion with StuI and SacI was ligated into p80 restricted with the two enzymes, and the resultant plasmid was named pT5’F. Finally, the insert excised from pT5’F by digestion with SacI and BspEI was ligated between the two enzyme sites of p3FN. The full-length cDNA clone of L,1A-F, which was named pTFN, was not sequenced but was confirmed to produce the expected restriction patterns with the three enzymes used and the

Fig. 1. Schematic representation of the ToMV genome organization and a comparison of the complete nucleotide sequence among L,1A-F, L,1A and L. The nucleotide position of silent (1) and missense (1) mutations is shown for the L,1A-F sequence, and base substitutions and amino acid substitutions in the missense mutations are shown in parentheses and brackets, respectively. Numerals at the oligo(U) and oligo(A) stretches indicate the number of their constitutive residues.