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# *Pepper mild mottle virus* pathogenicity determinants and cross protection effect of attenuated mutants in pepper

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**Abstract**

To determine the pathogenicity domain and to apply cross protection, *Pepper mild mottle virus* (PMMoV) point-mutations in the replicase (REP) gene between the methyl-transferase and helicase domains, and deletions truncating pseudoknots in the 3′ non-coding region (NCR), were constructed. Some mutants substituting a single amino acid in REP residue 348 exhibited mild symptoms in *Nicotiana benthamiana* or pepper plants. Accumulation of these mutants was higher than that of other REP mutants or wild-type PMMoV. Deletion mutants in the 3′ NCR pseudoknot showed the lowest virus replication and accumulation among the mutants tested. Six attenuated mutants, which combined 3′ NCR deletions and single or double REP substitution mutations were constructed to investigate cross protection effects on pepper plants. All six of the attenuated mutants showed milder symptom development than wild-type virus. These results suggest that REP and the pseudoknot in the 3′ NCR are major pathogenicity determinants of the virus, and engineered PMMoV attenuated mutants can be useful for protection against the virus in pepper plants.
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## 1. Introduction

The genome of *Pepper mild mottle virus* (PMMoV), a species in the *Tobamovirus* genus, consists of a single-strand positive-sense RNA of 6357 nucleotides (Garcia-Luque et al., 1990; Kirita et al., 1997). PMMoV causes severe viral diseases on bell, hot and ornamental peppers (Choi et al., 2005; Garcia-Luque et al., 1990; Kirita et al., 1997). The virus contains four open-reading frames encoding for five proteins: the 126-kDa protein having the methyl-transferase (MT) and, RNA helicase (HEL) domains; the read-through 183-kDa protein containing RNA replicase (REP) domain; the 30-kDa movement protein; and the 17.5-kDa coat protein (CP) (Garcia-Luque et al., 1990; Tenllado et al., 1996; Lewandowski and Dawson, 2000).

PMMoV-S and PMMoV-J have been categorized into $P_{1,2}$ by their responses to four allelic tobamoviral resistance genes: $L^1$, $L^2$, $L^3$ and $L^4$ in *Capsicum* spp. (Boukema, 1984; Garcia-Luque et al., 1990; Tsuda et al., 1998). To date, two isolates, an Italian isolate (PMMoV-I) and a Japanese isolate (PMMoV-Ij), which are able to overcome $L^3$ resistance, have been described. In both of these isolates, changes in the CP amino acid sequence are responsible for this resistance-breaking pathotype (Garcia-Luque et al., 1993; Tsuda et al., 1998).

Nucleotide sequence analysis of the *Tomato mosaic virus* (ToMV) $L_{11}$ strain revealed that an amino acid substitution at residue 348 in the middle of the 126-kDa replicase is responsible for the loss of symptom expression (Nishiguchi et al., 1985). By amino acid substitution at this position, virus accumulation of $L_{11}$A was reduced to less than 20% in tomato compared to wild-type ToMV (Goto and Nemoto, 1971). The 3′ noncoding region (NCR) of the tobamoviruses possesses five pseudoknot structures divided into two areas: two pseudoknots from the 3′ tRNA-like structure and three pseudoknots upstream from the tRNA-like structure (Takamatsu et al., 1990; Tanguay and Gallie, 1996). The very 3′ terminal pseudoknot serves as the starting point for synthesis of minus-strand RNA, in *Tobacco mosaic virus* (TMV) (Takamatsu et al., 1990).

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When serial deletions into the cluster of three pseudoknots labeled PK1–PK3 were introduced, viral multiplication was drastically reduced, and accompanied by a loss of systemic mosaic symptoms on tobacco (Takamatsu et al., 1990). These results suggest that the development of systemic tobamovirus symptoms on tobacco plants is associated with the degree of virus multiplication in host plants.

Cross protection is a procedure that has been utilized to protect crops against virulent strains of viruses by pre-treatment with closely related attenuated strains of the virus (Fulton, 1986; Kurihara and Watanabe, 2003; Sherwood, 1987). Cross protection has been used for reducing yield loss in several crops including tomato, sweet orange, grapefruit, and pepper (Broadbent, 1976; Costa and Muller, 1980; Goto and Nemoto, 1971; Oshima, 1981).

For this purpose, attenuated strains of viruses were studied to obtain those effective in protecting crops (Goto and Nemoto, 1971; Ichiki et al., 2005; Kurihara and Watanabe, 2003). In Japan, the first attenuated strain of ToMV, $L_{11}$, was applied to protect tomato (Oshima et al., 1978). In a previous study, Goto and Nemoto (1971) isolated a more stable attenuated strain $L_{11}A$ from $L_{11}$-infected plants. This attenuated strain has been used to protect greenhouse-grown tomatoes (Oshima, 1981).

In this study, the viral factors controlling systemic mosaic symptoms were investigated using engineered PMMoV mutations. We introduced amino acid substitutions into the 126-kDa replicase based on similarly located substitutions in the $L_{11}A$ mutation, and we also deleted the double-helical segments in the pseudoknots located in the 3′ NCR of the PMMoV genome. In order to determine if there was sufficient ability to protect pepper plants in the field, the effects of attenuated strains in combination with the challenging wild-type PMMoV strain were studied and discussed.

## 2. Materials and methods

### 2.1. Plant culture and virus inoculation

Four-week-old green pepper plants (*Capsicum annuum* L. var. *angulosum* Mill) were used as the host plants in all experiments. These were maintained at 22–28 °C with a 16 h light/8 h dark photoperiod. Green pepper and *Nicotiana benthamiana* plants were mechanically inoculated with crude sap extracted from the progeny virus of PMMoV and attenuated mutants in inoculation buffer (10 mM potassium phosphate buffer, pH 7.6).

To investigate cross protection efficiency, the first inoculations were conducted on four leaves of eight leaf-stage pepper plants. The second inoculations were performed 4 weeks later on two young upper leaves.

### 2.2. In vitro transcription for inoculation of host plants

The full-length cDNA clone of PMMoV, pPMFEX1, used as a transcription template, was digested with *Xba* I for run-off transcription (Yoon et al., 2001). The transcription reaction was performed in the presence of cap analog ($m^7G[5']ppp[5']G$) using T7 RNA polymerase according to previously described methods (Yoon et al., 2001). Synthesized transcripts were directly inoculated onto carborundum-dusted leaves of *N. benthamiana* and pepper plants at the four-to-five leaf stage. Virus symptoms on the transcript-inoculated plants were compared with those of the wild-type virus in the greenhouse.

### 2.3. Construction of PMMoV substitution, deletion and combination mutants

The plasmid pTPC4350, harboring the full-length PMMoV cDNA, with the substituted PMMoV-Ij CP gene responsible for overcoming the $L^3$ tobamovirus resistance gene of pepper plants (Tsuda et al., 1998), was used as a template and positive control.

To determine the pathogenicity of the virus on the host plant, the consecutive pseudoknot structures in the 3′ NCR of the PMMoV genome were partially truncated according to the previously described ToMV mutants (Takamatsu et al., 1990), resulting in construction of four 3′ NCR serial deletion mutants (Fig. 1).

To create mildly pathogenic viruses, a series of PMMoV mutants derived from pTPC4350 by site-directed mutagenesis according to the sequence information of the ToMV-$L_{11}A$ genome (Nishiguchi et al., 1985) were constructed. The first mutation, pTPJ, substituted methionine 348 in the PMMoV 126 kDa replicase with a tyrosine to correspond to $L_{11}A$. For the second substitution, pTPA, glycine 762 was substituted with aspartic acid to again correspond to aspartic acid 760 in $L_{11}A$ (Fig. 2).

To select attenuated mutants for cross-protection in pepper, six combinations of PMMoV deletion and substitution mutants derived from pTPJ, pTPA, pTPD3-6207, and pTPD3-6219 were constructed (Fig. 2). After vector construction of pTPC4350, pTPJ-6207, pTPJ-6219, pTPA-6207, pTPA-6219, pTPD-6207, and pTPD-6219, in vitro synthesized transcripts derived from each linearized plasmid were inoculated onto *N. benthamiana* and green pepper plants in order to detect their infectivity and resulting symptoms.

### 2.4. Western blot and ELISA analysis

Total protein was extracted from systemically diseased leaves of all plants. Sample preparation, Western blotting, and ELISA were all conducted as previously described by Yoon et al. (2001). For Western blotting, the probe was antiserum against PMMoV CP diluted 1:1000 (v/v), followed by incubation with alkaline phosphatase conjugated goat anti-rabbit IgG diluted in 1:7500 (Promega Corp.) as the secondary antibody.

For ELISA, leaves were harvested 2 weeks post-inoculation (wpi) and ground in carbonate buffer ($Na_2CO_3$ 1.59 g, $NaHCO_3$ 2.93 g, polyvinylpyrrolidone 20 g, pH 9.6, 1 L) (Shim et al., 2005). Anti-PMMoV CP antibodies, diluted to 1:300 in PBS–BSA, were used as the probe, and reactions were measured at 405 nm with an ELISA reader (Metertech S 960). For the quantitation of virus concentration, a series of dilutions of known amounts of PMMoV were assayed for each experiment to establish a standard curve.

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