Arch Virol (2005) 150: 2009–2020
DOI 10.1007/s00705-005-0551-8

Archives of Virology
Printed in Austria

Integration of mutations responsible for the attenuated phenotype of *Pepper mild mottle virus* strains results in a symptomless cross-protecting strain

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Received November 16, 2004; accepted April 7, 2005
Published online June 22, 2005

Summary. An enhanced attenuated strain of *Pepper mild mottle virus* (PMMoV) was constructed by incorporating mutations that affect viral attenuation from three reported attenuated strains of PMMoV, which causes serious economic losses in the production of green pepper in Japan. The new strain caused no symptoms on pepper plants and protected them from infection by a wild-type strain. The mutations responsible for viral attenuation were located in the intervening region (IR) of the 126-kDa/183-kDa proteins. The mutations had synergistic effects in terms of the attenuation of symptoms and decreased the accumulation of the viral coat protein in infected pepper plants. In this paper, we propose an efficient method for the improvement of attenuated viruses by reverse genetics in plant viruses.

Introduction

The genome of pepper mild mottle virus (PMMoV), a member of the genus *Tobamovirus*, consists of positive-sense single-stranded RNA that encodes at least four proteins, namely, 126-kDa and 183-kDa replicases, the movement protein (MP) and the coat protein (CP). The 126-kDa replicase encoded by the 5′-proximal region of the viral genome includes methyltransferase (MT) and helicase-like (Hel) domains, with an intervening region (IR) between their domains, while the 183-kDa protein is a read-through protein, derived from the open reading frame

The nucleotide sequence data reported in this paper for PMMoV Pa18 and TPO-2-19 will appear in the DDBJ/EMBL/GenBank nucleotide sequences databases with the accession numbers AB113116 and AB113117.

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for the 126-kDa protein, that contains, in addition to the above two domains, an RNA polymerase (Pol) domain [2, 12, 13].

PMMoV causes serious economic losses in green pepper production in Japan, because it is a soil-borne virus and eradication is very difficult once a greenhouse is contaminated. One of the most natural and effective measures used to control plant viral diseases is the use of attenuated viral strains [10]. Among tobamoviruses, an attenuated srain of tomato mosaic virus [19] and cucumber green mottle mosaic virus [18] have been successfully applied to protect tomato and melon plants, respectively, from infection by severe virus strains without showing symptoms on plants. For the protection of pepper plants from infection by PMMoV, three attenuated strains of PMMoV, namely, Pa18, C-1421 and TPO-2-19 have been studied in Japan [7, 8, 21]. Pa18 and C-1421 were selected after heat treatment and TPO-2-19 was isolated from a field of pepper plants. Pepper seedlings planted to the heavily contaminated field after pre-inoculation with these strains increased 20–30% of crop yield over non-inoculated controls [7, 8, 20]. However, production of strains with greater practical utility is desirable because these three strains still cause mild mosaic symptoms in green peppers at higher temperatures. Furthermore, these strains cannot infect popular commercial cultivars of green peppers that carry the L^3 resistance gene.

Selection or improvement by traditional methods of attenuated strains with desirable phenotypes requires long periods of time because mutations introduced by such methods are random and not site-directed. In an earlier study, we found that a single amino acid substitution in C1421 resulted in attenuation of symptoms in green peppers [9]. In the present study, we analyzed the mutations in Pa18 and TPO-2-19 and found mutations responsible for attenuation.

We then inserted the mutations that were responsible for attenuation from all three strains into a wild-type infectious cDNA clone that overcomes the L^3 resistance gene [27] to get an enhanced attenuated virus. We then examined its ability to cross-protect plants from infection with a virulent wild type strain.

Materials and methods

Infectious cDNA clones and viral strains

Plasmid pTPW1 [12], a full-length cDNA clone of PMMoV-J (the $P_{1.2}$ pathotype; DDBJ Accession no. AB000709) and pTPC4350 [27], a full-length cDNA clone that overcomes the L^3 resistance gene (the $P_{1.2.3}$ pathotype), were used as wild-type strains. Recently, a mistake in the reported sequence of PMMoV-J was noted at the 2610th nucleotide, without a change in the encoded amino acid. That nucleotide was T, not G, as described in both pTPW1 and pCh2015 [12]. The sequence of PMMoV-J that has been registered in DDBJ, as indicated above, has been corrected. Pa18, C1421 and TPO-2-19 were used as attenuated strains.

Sequencing analysis and construction of cDNA infectious clones

We determined the complete nucleotide sequences of cDNA from genomic RNAs of strains Pa18 and TPO-2-19 using a Dye Terminator Cycle Sequence Kit (Perkin-Elmer Applied Biosystems, Foster City), as described previously [9]. To analyze mutations that caused viral attenuation, we constructed clones with site-directed mutagenesis and recombinant clones, as shown in Fig. 2. Oligonucleotides that directed single amino acid substitutions at positions

549, 556 and 760 in pTPR, pTPT and pTPL respectively, were 1699:5′-GTACTGGACGTT AAAAGGAGCTTGGAAGAAGC-3′,1719:5′-CTTGGAAGAAGCAGAAACGATGTACA ATGCTTTG-3′ and 2334:5′-GGTGAAACCTCTATTAAAAGGACATGCTTG-3′. Nucleotides different from the sequence of pTPW1 are underlined and the number of each oligonucleotide name indicates the base position in the genomic sequence of PMMoV-J [12]. pTPRT, pTPTL and pTPRL were constructed using several combinations of the oligonucleotides described above. To construct recombinant cDNA clones, we used the following reverse (R) and forward (F) pairs of primers: (R)1670:5′-TTAACCAATCTATCGTGGAA-3′ and (F)1:5′-GCGAATTCTAATACGACTCACTATAGTAAATTTTTCACAATTTAACAACAAC AAC-3′, with an *Eco*RI site (underlined) for pPa5′; (R)2570:5′-TCAACAAGTGTAACCT TTGC-3′ and (F)831:5′-CGATATGTTGAACTTTTCTT-3′ for pTPa18; and (R)6358:5′-GCT CTAGAGGATCCTGGGCCGCTACCCGCGGTTC-3′, with a *BamH*I site (underlined) and (F)2331:5′-GTTGGTGAAACCTCTATCAAAAGGACATGCTTGGGGTGTG-3′ for pTPa3′ (2784) and pTPa3′(3823). pTPa18ch, which contained both the attenuating mutation of Pa18 and that of C-1421 at nucleotide position 2015 [9], was constructed by insertion of the single mutation of C-1421 into pTPa18 by using the oligonucleotide (F):2000 5′-CCTC AATCAAGGAAGCGGTTCGAAAATCAGAG-3′. The underlined nucleotide is different from that in the sequence of pTPW1. After digestion by appropriate restriction enzymes, each amplified fragment was cloned into the pCRII vector (Invitrogen Corp., Carlsbad, CA, USA) and used to transform *E. coli* DH5α or XL1-Blue competent cells. DNA sequencing was performed to confirm that each mutation was located correctly in each fragment and restriction fragments were subcloned into pTPW1 or pTPC4350 at the appropriate positions.

Symptomatology

Transcripts from the various cDNA infectious clones were first inoculated to *Nicotiana benthamiana*. Infected leaves were collected 5 days after inoculation as described elsewhere [9] and homogenized in 0.1 M sodium phosphate buffer (pH 7.0) that contained 0.01 M Na_2SO_3. The concentration of modified/wild type virus in each sample of sap was adjusted to 1 μg/ml. Seedlings of the green pepper (*Capsicum annuum* var. *angulosum*) cultivar New tosahikari or Miogi, which has the L^3 resistance gene, were inoculated with the sap and grown in an air-conditioned greenhouse at $27 \pm 3\,^{\circ}C$. The relative intensity of symptoms on each inoculated plant was scored visually 35 days after inoculation. We selected viruses that only induced attenuated symptoms in the pepper plants.

Accumulation of CP in green pepper plants

Viruses were inoculated into cotyledons of pepper plants (cv. New tosahikari) at a concentration of 1 μg/ml. Fourteen days after inoculation, all the leaves, apart from the inoculated leaves, were collected and homogenized in 0.1 M phosphate buffer that contained 0.1% BSA, 10 mM EDTA, 0.15% PVP-40, 0.1% skimmed milk and 0.05% NaN_3. A calibration curve was prepared for a DAS-ELISA in terms of absorbance at 405 nm, A_{405}, using a purified preparation of virus, which had been diluted serially in the same buffer, and then levels of CP were determined from the calibration curve.

Evaluation of an enhanced attenuated strain

An enhanced attenuated strain of virus was partially purified from *N. benthamiana* previously inoculated with transcripts from the cDNA infectious clone, pTPa18ch. Pepper plants (cv. New tosahikari) at the six- to eight-leaf stage were then preinoculated with 10 μg/ml of this partially purified virus. Additional plants either healthy or inoculated with the partially purified parental viruses Pa18 and C-1421 were used as controls. These plants were grown under green house conditions ($27 \pm 3\,^{\circ}C$) and natural lightning. Three weeks following

pre-inoculation, the plants were separated into 2 sets. The two upper leaves from each plant in set 1 were challenge inoculated with 1 μg/ml of partially purified wild type virus (PMMoV-J). To compare differences in cross-protection at the roots, plants from the second set were removed from pots 3 weeks after pre-inoculation, and their roots dipped into 0.1 M sodium phosphate buffer containing 1 μg/ml of wild type virus. After dipping, the inoculated plants were repotted back into the same pots. All challenge inoculated plants from both sets were grown in the greenhouses as before and symptoms were observed continuously.

Results

Nucleotide sequences of attenuated strains

Pa18 had eight nucleotide mutations in the 126-kDa/183-kDa replicase, one in MP and one nucleotide deletion in the non-coding region (Fig. 1). Among these mutations, those at positions 1715, 1735, 1736 and 2348 resulted in three amino acid changes. All amino acid changes were located in the IR in the 126-kDa/183-kDa protein: residues 549 (Lys to Arg), 556 (Val to Thr), and 760 (Ser to Leu). TPO-2-19 had the same substitutions as Pa18 with the addition of mutations at nucleotides 831 and 4257.

Mutations responsible for attenuation of symptoms

Since TPO-2-19 contained all amino acid substitutions found in Pa18, we analyzed Pa18 to narrow down these mutations responsible for viral attenuation. Green peppers infected with recombinant viruses designated Pa5′, TPa3′(2784) and TPa3′(3823), which were recovered from pPa5′, pTPa3′(2784), pTPa3′(3823),

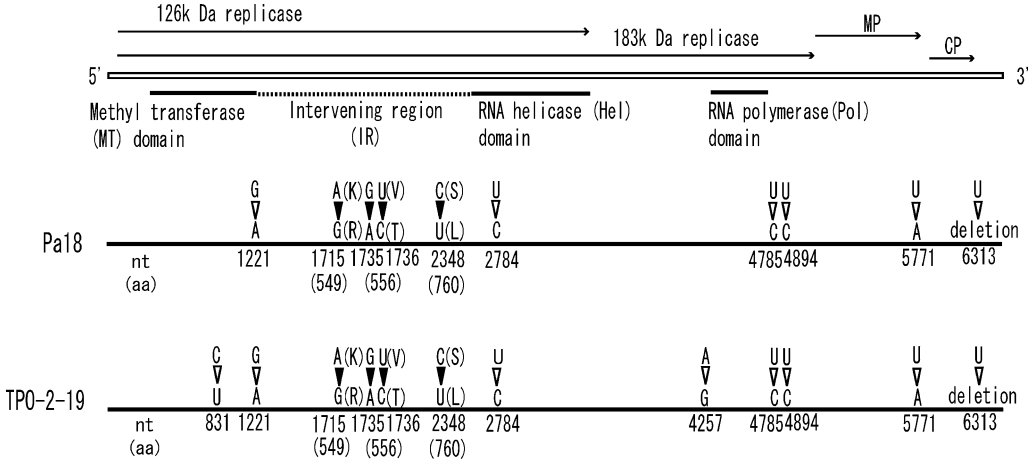

Fig. 1. Mutations in the genome of strains of PMMoV Pa18 and TPO-2-19. Closed arrowheads indicate nucleotide mutations that caused amino acid changes, while open arrowheads indicate nucleotide changes that caused silent mutations. Upper letters show nucleotides and amino acids (in parentheses) in PMMoV-J (wild type) and lower letters show those in the indicated attenuated strains. Positions of mutated nucleotides and amino acids (parentheses) are shown below horizoned lines. The structure of the genome of PMMoV is shown at the top. *MP*, movement protein; *CP*, coat protein

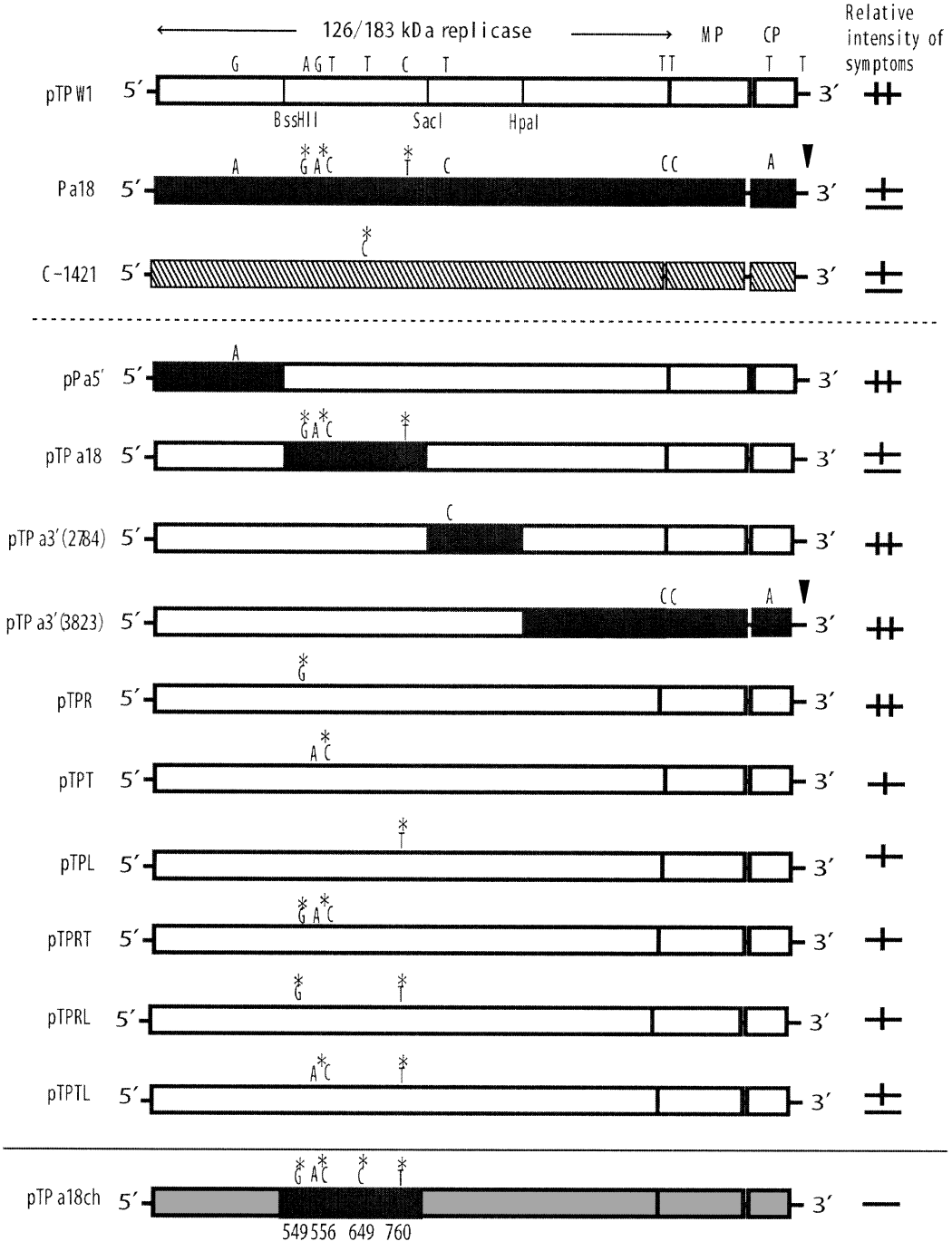

Fig. 2. Construction of infectious cDNA clones, with relative intensities of symptoms on green pepper plants induced by the resultant viruses. *MP*, Movement protein; *CP*, coat protein. Asterisks (∗) indicate nucleotide mutations that caused changes in amino acids. Arrowheads indicate nucleotide deletions. Open, closed, diagonal lined, and shaded bars indicate restriction fragments obtained from pTPW1 cDNA, Pa18 cDNA, C-1421 cDNA and pTPC4350 cDNA, respectively. Numbers below the representation of pTPa18ch, show numbering of amino acid residues in the 126-kDa/183-kDa protein. Viruses derived from cDNA clones were inoculated to green pepper plants (cv. New tosahikari or cv. Miogi). Plants were scored by eye for the intensity of symptoms 35 days after inoculation, as shown in Fig. 3

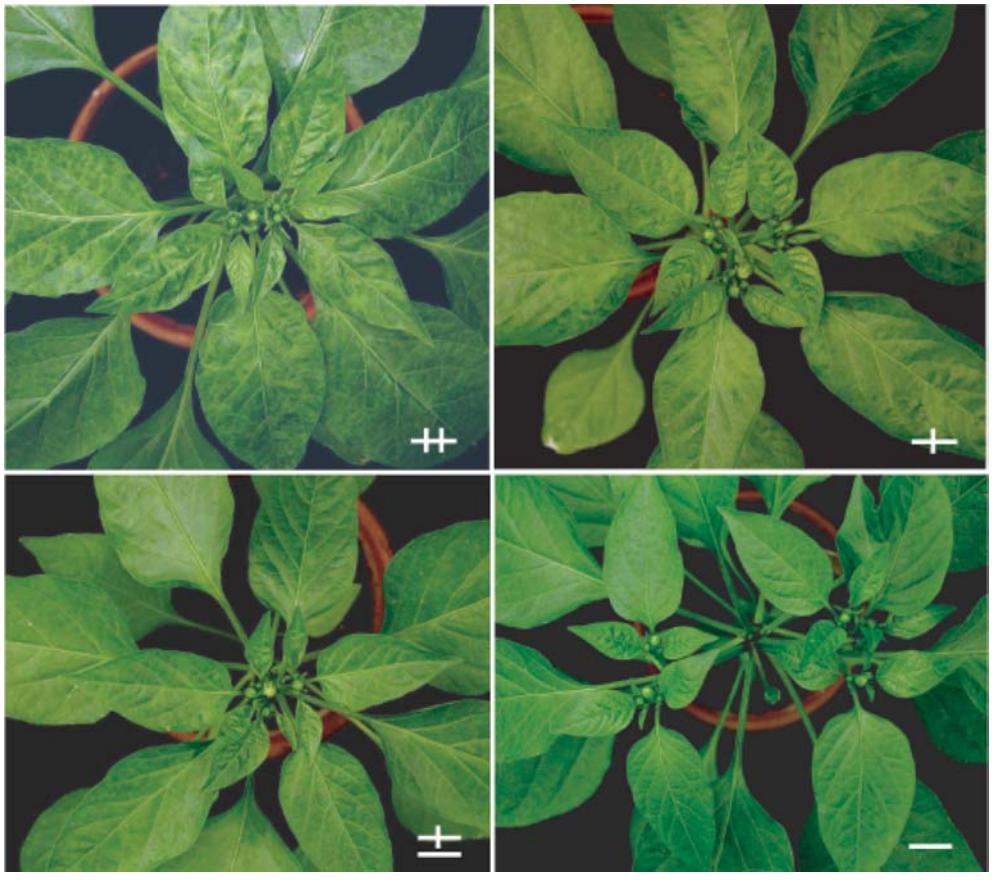

Fig. 3. Green pepper plants, photographed 35 days after inoculation with various strains of PMMoV. ++, Severe light and dark green mosaic; +, mosaic; ±, mild mosaic; −, no symptoms

respectively, exhibited severe mosaic symptoms (Figs. 2 and 3), accompanied with rough and crooked midribs on developing leaves, as TPW1 infected plants. This result showed that silent mutations did not result in any attenuation of symptoms. By contrast, modified virus TPa18, which included all the amino acid changes in Pa18, caused only very slight mosaic symptoms and no malformation of leaves of plants, as in the case of plants inoculated with the parental Pa18 strain. Symptoms on pepper plants infected with TPT and TPL, which encoded single amino acid changes at residues 556 and 760, respectively, were much milder than those on plants infected with TPW1: TPT and TPL caused mosaic and slightly rough leaves, but the midribs of developing leaves were not crooked. TPR, which encoded a single amino acid substitution at residue 549, induced severe symptoms in plants, as did the three recombinant viruses Pa5′, TPa3′(2784), TPa3′(3823) and, the wild-type virus TPW1.

The above results suggested that nucleotide mutations that resulted in amino acid changes were associated with differences in severity in the appearance of symptoms. We then constructed clones that included two amino acid changes in combination and examined their pathogenicity. TPRT and TPRL, which contained amino acid mutations at residues 549 and 556, and at residues 549 and 760,

respectively, caused identical visible symptoms on plants to those caused by TPT and TPL. TPTL, which contained two mutations, at residues 556 and 760, was more strongly attenuated than TPRT and TPRL, and the inoculated plants exhibited very slight mosaic symptoms, resembling plants inoculated with TPa18. Examination of the pathogenicity of viruses suggested that the two amino acid changes at residues 556 and 760 were mainly responsible for the attenuation of TPW1.

Construction of an enhanced attenuated strain with the $P_{1.2.3}$ pathotype that infects green peppers carrying the L^3 resistance gene

To produce enhanced attenuated viruses, we simultaneously inserted the amino acid mutation at residue 549, in addition to those at residues 556 and 760 of Pa18, and the mutation at residue 649 of C-1421 (9) into TPC4350 with the $P_{1.2.3}$ pathotype. The substitution at residue 549 did not induce attenuation of symptoms, but was included in view of the earlier result that an attenuated strain of ToMV, L_{11}A, which contained mutations at two amino acid residues, in addition to the critical single amino acid change in L_{11}, was phenotypically more stable than L_{11} itself [23, 24]. The newly constructed cDNA clone was named pTPa18ch. Plants infected with TPa18ch did not show any symptoms, and we had to perform DAS-ELISA to detect viral infection.

Accumulation of CP in plants infected with various viruses

The accumulation of CP in plants infected with Pa5′, TPa3′ (2784), TPa3′(3823) and TPR, respectively, was similar to that in plants infected with wild type TPW1 (Fig. 4). TPT, TPL, TPRT and TPRL induced symptoms of medium intensity,

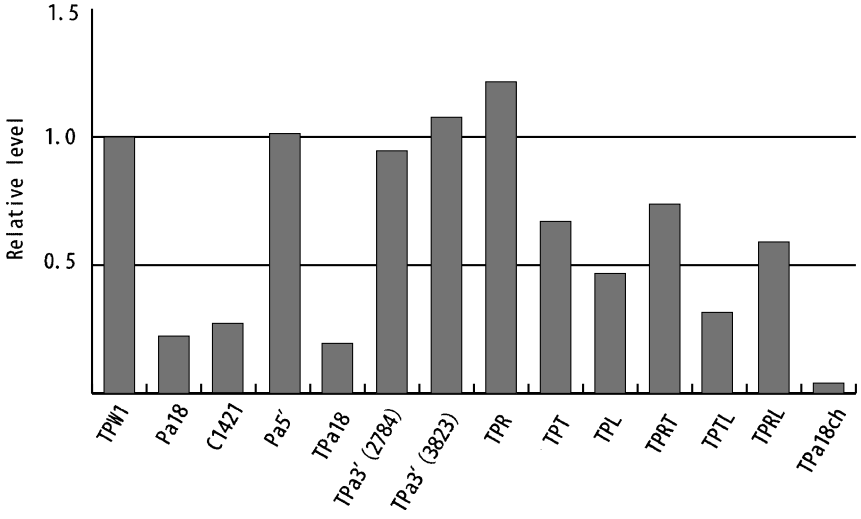

Fig. 4. Relative levels of coat protein (*CP*) of PMMoV in infected green pepper plants. Cotyledons of green pepper plants (cv. New tosahikari) were inoculated with the indicated viruses. Fourteen days later, all the leaves, apart from the inoculated leaves, were collected and the accumulation of CP was examined by DAS-ELISA. Values shown are relative to the level of CP in leaves infected with TPW1 and are the means of results from three experiments

Table 1. Cross-protection of pepper plants by an enhanced-attenuated strain of PMMoV against wild type strain

Pre-inoculum	Site of challenge-inoculation			
	Upper leaves		Whole roots	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
TPa18ch	0/8[a]	0/6	0/5	0/8
Pa18	0/8	0/6	0/6	0/2
C-1421	0/8	0/4	1/5	0/8
None	8/8	6/6	6/6	8/8

[a]No. of plants with obvious mosaic symptoms/no. of plants tested

and accumulated intermediate amounts of CP. Pepper plants infected with TPTL accumulated less CP than plants infected with TPRT or TPRL. The amount of CP in TPa18-infected pepper was almost the same as that in plants infected with the parental viruses Pa18 and C1421, and it was one-fifth of that in plants infected with TPW1. Relative levels of CP in plants infected with TPa18ch were lower than those in plants infected with Pa18 or with C-1421.

Cross-protection provided by individual attenuated viruses and an enhanced attenuated strain

All the plants pre-inoculated to leaves with Tpa18ch did not show any symptoms 6 weeks after challenge inoculation to leaves and roots (Table 1). On the other hand, the wild type virus, PMMoV-J inoculated on leaves of healthy plants, induced severe mosaic symptoms on them, 3 weeks after challenge inoculation. Pepper plants pre-inoculated with Pa18 or C-1421 and challenge inoculated with the PMMoV-J also did not show any notable symptoms, except for one plant with severe mosaic for C-1421 in root challenge inoculation test.

Discussion

A reverse genetic system with *in vitro* transcription, established in the 1980's [1, 16], allows identification of mutations responsible for the attenuation of plant viruses. In this study, we first demonstrated that amino acid changes at residues 556 and 760 in the IR of Pa18 126-kDa protein were responsible for attenuation. In a previous study, it was shown that one amino acid change at position 649 in the C-1421 strain of PMMoV caused the attenuation of symptoms [9]. In other tobamoviruses, symptoms were affected by changes at residues 360 and 601 in the IR among 12 amino acid changes examined in the M strain of TMV [25]; by one change at residue 643 in the V36 strain of TMV [15], and by one change at position 760 among three amino acid changes in the L_{11}A-Fukushima

strain of ToMV [29]. These results indicated that the amino acid changes responsible for symptom attenuation are located in the IR of the 126-kDa/183-kDa polymerases.

Recent studies on the functions of proteins expressed by tobamoviruses suggest that CP is involved in the systemic movement of viruses and in host responses, while MP contributes to viral movement between cells, and the 126-kDa/183-kDa proteins play roles in replication of viral RNA and suppressing posttranscriptional gene silencing [3, 4, 14, 17]. As described above, all attenuated strains of tobamoviruses selected by traditional methods have amino acid changes in the IR of the 126-kDa/183-kDa protein. In addition, only amino acid changes that locate in IR were responsible for viral attenuation [9, 11, 15, 25, 26]. Mutations in domains with crucial functions, such as MT, Hel and Pol in the 126-kDa/183-kDa protein, and in MP and CP can induce remarkable, and in some cases, fatal changes [6, 27]. Therefore, mutations in attenuated strains are probably concentrated in the IR as a consequence of the milder functional changes that they induce in the replicase.

The various mutations responsible for attenuation did not seem to affect viral pathogenicity equally. The single amino acid changes in C-1421, L_{11}A-Fukushima (ToMV) and V-36 (TMV) were effective enough to induce attenuation [9, 15, 29], whereas several changes were needed for attenuation of the M strain of TMV [11, 25] and Pa18. Thus, we investigated mutations responsible for attenuation of C-1421, Pa18 and TPO-2-19 and combined these mutations. The new strain, TPa18ch did not show any symptoms on inoculated pepper plants without losing its cross-protection ability at roots and leaves similar to its parental viruses when challenge inoculation was made 3 weeks after pre-inoculation.

The study on viral accumulation in pepper plants inoculated with Tpa18ch showed that viral concentration in such plants were much lower than those in plants inoculated with their parental strains. This result suggested that inefficient functioning of the 126-kDa/183-kDa proteins due to the mutation in the IR region is the cause of the reduction of viral replication, which eventually resulted in the symptom attenuation. Therefore, it is likely that integration of several mutations associated with attenuation, found in independent attenuated strains, effect attenuation of symptoms and reduction in the accumulation of CP in inoculated pepper plants synergistically. Thus, this method possibly allows the efficient construction of enhanced and useful attenuated strains that can protect plants from infection by virulent wild type virus. This is the first report, to our knowledge, on the proposal for development of an attenuated plant virus by the incorporation of some mutations from different attenuated strains by plasmid-based reverse genetics.

The plants challenge-inoculated on leaves with wild-type virus, 2 weeks after pre-inoculation with TPa18ch, exhibited severe mosaic symptom in a preliminary examination in contrast to the protection of the plants challenge-inoculated 3 weeks after the pre-inoculation. Pa18 and C-1421, the parental viruses of TPa18ch, can both protect plants from infection of wild type strain 9–14 days from pre-inoculation [8, 21]. Our results suggest that to protect plants from infection of wild type virus by inoculation with TPa18ch, early pre-inoculation of plants

is necessary because a longer time is required for TPa18ch than for its parental strains, to exhibit its ability of cross-protection. These results suggest that pre-inoculated plants are not able to be protected from the challenge viral infection before the enough accumulation of the pre-inoculated virus in the plants.

TPa18ch could spread throughout a whole pepper plant and caused no symptoms even at higher temperatures (data not shown). Therefore, TPa18ch will possibly be useful in warmer districts, however more studies for the timing of pre-inoculations and this strain's stabilities are needed.

Interestingly, respective phenotypic features of attenuated strains, such as cell-to-cell or systemic movement, the accumulation of CP or MP in protoplasts or infected leaves, and the replication of genomic or subgenomic RNA, differ considerably, even though all the amino acid mutations responsible for attenuation in the individual strains were located in the IR [5, 15, 22, 28]. Further analysis of correlations among sequences of viral genomes, viral phenotypes, and the appearance of symptoms on infected plants will provide information to design more effective attenuated strains for the protection of crops from viral damage.

Acknowledgments

The authors thank Mr. H. Goto for providing the attenuated strain TPO-2-19 and Dr. Y. Watanabe, Dr. A. Tymon and Dr. M. J. Adams for helpful suggestions for this manuscript.

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