Single amino acid substitution in the methyltransferase domain of *Paprika mild mottle virus* replicase proteins confers the ability to overcome the high temperature-dependent *Hk* gene-mediated resistance in *Capsicum* plants

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Abstract

Capsicum plants harboring the *Hk* gene (*Hk*) show resistance to *Paprika mild mottle virus* (PaMMV) at 32 °C but not 24 °C. To identify the viral elicitor that activates the *Hk*-mediated resistance, several chimeric viral genomes were constructed between PaMMV and *Tobacco mosaic virus*–L. Infection patterns of these chimeric viruses in *Hk*-harboring plants revealed responsibility of PaMMV replicase genes for activation of the *Hk*-mediated resistance. The comparison of nucleotide sequence of replicase genes between PaMMV and PaHk1, an *Hk*-resistance-breaking strain of PaMMV, revealed that the adenine-to-uracil substitution at the nucleotide position 721 causes an amino acid change from Threonine to Serine at the 241st residue in the methyltransferase domain. Introduction of the A721U mutation into the replicase genes of parental PaMMV overcame the *Hk* resistance at 32 °C. The results indicate that *Hk*-mediated resistance is induced by PaMMV replicase proteins and that methyltransferase domain has a role in this elicitation.

The genus *Tobamovirus* includes devastating viral pathogens in solanaceous plants. A tobamovirus, *Paprika mild mottle virus* (PaMMV) encodes two replicase proteins from overlapping open reading frames: the 126 kDa protein and the 183 kDa read-through protein (Hamada et al., 2003). The 126 kDa replicase protein contains domains with methyltransferase and putative helicase activities, whereas the 183 kDa protein contains an additional polymerase domain (Buck, 1999). In addition, tobamoviruses encode a movement protein involved in cell-to-cell movement of the viruses and a coat protein (CP) involved in the encapsidation of the viral RNA into virions. For control of tobamovirus diseases, tobamovirus resistance genes, such as *N* gene and *N'* gene in tobacco plants and *Tm-1* gene in tomato plants, were incorporated into commercial cultivars. Virus elicitors for these tobamovirus-resistance genes differ from each other: features of replicase proteins and CP are recognized by *N* gene-haboring tobacco plants (Padgett and Beachy, 1993; Erickson et al., 1999) and *Tm-1* gene-harboring tomato plants (Hamamoto et al., 1997; Meshi, et al., 1988; Strasser and Pfitzner, 2007), and *N'*gene-harboring tobacco plants (Saito et al., 1987), respectively. A common problem with these tobamovirus resistance genes is that most of them lose their function at high temperatures (eg. 30ºC).

Four allelic genes at *L* locus, L^1, L^2, L^3 and L^4, provide increased protection against different kinds of tobamovirus pathotypes P_0, P_1, $P_{1,2}$ and $P_{1,2,3}$ in *Capsicum* plants (Boukema, 1980 and 1982; Rast and Th, 1988). The tobamovirus CP is the elicitor of the *L* genes-mediated hypersensitive response in the genus *Capsicum* (Berzal-Herranz et al., 1995; Dardick et al., 1999; de la Cruz et al., 1997; Gilardi et al., 1998 and 2004; Hamada et al., 2002; Tsuda et al. 1998). The *L* genes-mediated resistance also loses its function at high temperatures. However, we previously identified the L^{1a} gene, a new allele of *L* genes, which confers temperature-insensitive resistance against tobamovirus

P_0 pathotype (Sawada et al., 2004). L^{1a} gene-mediated resistance shares a common viral elicitor with temperature-sensitive *L* genes (Matsumoto et al., 2008).

In addition to the temperature-insensitive L^{1a} gene, we identified a new tobamovirus resistance gene in *Capsicum* plants, *Hk* (Sawada et al., 2005), which conferred resistance to PaMMV but not to other tobamoviruses tested so far, including *Tobacco mosaic virus*-Ob (TMV-Ob), *Tobacco mosaic virus*–L (TMV-L), *Tobacco mild green mosaic virus* (TMGMV), and *Pepper mild mottle virus* (PMMoV). A remarkable feature of *Hk* gene-mediated resistance is high temperature-dependent: it functions at high temperatures such as 32°C but not at lower temperatures, (eg. 24°C), under which other tobamovirus resistance genes work. The *Hk* gene is a single incompletely dominant gene located in a chromosome differed to that of *L* genes. In this study we aimed to identify the viral elicitor involved in the induction of *Hk* gene-mediated resistance in *Capsicum* plants cultivated at high temperatures.

We constructed chimeric viral genomes between TMV-L and PaMMV-J, a Japanese *Capsicum* strain of PaMMV (PaMMV-J, Hamada et al. 2003), investigated in a previous study (Matsumoto et al., 2008). Chimeric tobamovirus genomes were constructed by recombining DNA fragments from cDNA clones, pTLW3 (Hamamoto et al., 1997) and pPAJ (Hamada et al., 2003), from which infectious virus RNA genomes are transcribed *in vitro*. The resulting recombinant DNAs were used as templates for transcription by T7 RNA polymerase (TaKaRa). RNA transcripts were mechanically inoculated onto *Nicotiana benthamiana* with inoculation buffer (Tris-EDTA buffer, pH. 8.0 and 0.25% bentonite). Infected leaves harvested 6 to 7 days post inoculation (dpi) were ground with 10 mM sodium phosphate buffer (pH 7.4), and the leaf sap was used as the inoculum to mechanically inoculate pepper plants cultivated in growth chambers at 25 °C, with a 16 h photoperiod and a light intensity of 10,000 lux. For each inoculum,

five plants were used and experiments were performed in triplicate. Chimeric tobamoviruses used in this study were named as follows: the first letter was an abbreviation of the background virus (L or Pa for TMV-L or PaMMV-J, respectively), followed by an abbreviation of the virus from which the recombined gene derives (L or Pa), and the name of the gene (Rep, MP or CP, for replicase, movement protein or coat protein, respectively). For example, L-CPPa is a TMV-L mutant whose CP gene is replaced by the CP gene of PaMMV-J.

Virus infections in inoculated and uninoculated upper leaves at both 24 and 32 °C were assessed by press blot immunoassay (Srinivasan and Tolin, 1992). In press blot immunoassay, blots of inoculated leaves and uninoculated upper leaves were prepared at 5 and 9 dpi, respectively, and the viral CP was detected using an appropriate antibody. The press blot immunoassay showed that PaMMV-J systematically infected *C. annuum* L. cv. Nanbu-Ohnaga (*Hk*/*Hk,* Sawada et al., 2005) at 24 °C (Fig. 1); with vein necrosis and systemic necrosis in inoculated leaves and uninoculated upper leaves, respectively (Table 1). On the other hand, the virus induced necrotic lesions in the inoculated leaves at 32 °C (Table 1) and no virions were detected in uninoculated upper leaves (Fig. 1): ie, *Hk* gene inhibits systemic infectivity of PaMMV-J at 32 but not 24 °C. In contrast, TMV-L systemically infected the *Hk*/*Hk* plants (Fig. 1) with no symptoms in inoculated leaves and mosaic symptoms in uninoculated upper leaves at both 32 and 24 °C (Table 1).

All chimeric viruses caused systemic infection with mosaic symptoms in *C. annuum* cv. Shosuke (L^+/L^+) at both 24 and 32 °C (Data not shown). When *Hk* plants were inoculated with the chimeric viruses having the replicase genes of TMV-L (Pa-RepL, L-MPPa, L-CPPa), no symptoms were induced in inoculated leaves and mosaic symptoms were induced in uninoculated upper leaves at both 24 and 32 °C (Table 1).

Furthermore, the virions were detected in both inoculated leaves and uninoculated upper leaves (Fig. 1). Therefore, the chimeric viruses having the replicase genes of TMV-L systematically infected *Hk* plants at any temperature, like TMV-L.

In contrast, the chimeric viruses having the replicase genes of PaMMV-J (Pa-MPL, Pa-CPL and L-RepPa) failed to cause systemic infection (Fig. 1), and the viruses induced necrotic lesions in the inoculated leaves at 32 °C (Table 1). On the other hand, these chimeric viruses succeeded in the systemic infection of *Hk* plants at 24 °C (Fig. 1), and vein necrosis and systemic necrotic symptoms were induced in inoculated leaves and uninoculated upper leaves, respectively (Table 1). These results suggest that the replicase genes of PaMMV-J are responsible for the induction of high temperature-dependent *Hk* gene-mediated resistance to PaMMV-J.

A spontaneous mutant strain of PaMMV-J that overcame *Hk* resistance and was designated as PaHk1, was isolated from a PaMMV-J-inoculated *Capsicum* plants holding the *Hk* gene. The press blot immunoassay analysis showed that PaHk1 systemically infected *Hk* plants at 32 °C as well as 24 °C (Fig. 1). The chimeric viruses between PaMMV-J and TMV-L showed that the replicase genes of PaMMV-J are responsible for the elicitation of high temperature-dependent *Hk* gene-mediated resistance to PaMMV-J. Therefore, we compared nucleotide sequences of replicase genes between PaMMV-J and PaHk1 and found one nucleotide substitution at position 721 of PaHk1 replicase genes from adenine (PaMMV-J) to uracil (PaHk1). This mutation causes an amino acid substitution from threonine at position 241 of replicase protein to serine. The mutation resided in the methyltransferase domain.

To analyze the involvement of A721U in replicase genes in overcoming *Hk* gene-mediated resistance, we constructed a mutant PaRepT241S, in which A721U mutation alone was introduced. Two DNA fragments, fragment 721-1 and fragment

721-2, were PCR-amplified using pPAJ as a template and M4 (5'-GTTTTCCCAGTCACGAC-3') and PaA789T- (5'-GCTCCACGGAGCTTGCCTCAAG-3'), and RV (5'-GTCCTTTGTCGATACTG-3') and a primer complementary to primer PaA789T-, respectively. A cDNA clone of PaRepT241S was then created by recombinant PCR-amplification using fragment 721-1 and fragment 721-2 as templates and M4 and RV as primers. The nucleotide sequences of recombinant DNA were analyzed using an Automated DNA Sequencer Model 373 (Applied Biosystems). The mutant, PaRepT241S, systemically infected *Hk* plants at both 24 and 32 °C, as did PaHk1 (Fig. 1). These results suggest that replacement of A-721 by U in replicase genes is sufficient to overcome *Hk* resistance in *Capsicu*m plants.

Viral RNA accumulation was examined by northern hybridization analysis in PaMMV-J, PaHk1- and PaRepT241S-inoculated leaves at 5 dpi and uninoculated upper leaves at 9 dpi, using a sequence corresponding to nucleotide position 6008-6508 of PaMMV-J RNA as a 5'-DIG-labelled RNA probe. The targets were detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and the CDP-Star Detection Reagent (Amersham Biosciences) according to the manufacturer's protocol. Northern analysis showed that RNA of PaMMV-J was detected in inoculated leaves at both 24 and 32 °C and in uninoculated upper leaves at 24 but not 32 °C (Fig. 2). On the other hand, RNA of PaHk1 and PaRepT241S was detected not only in inoculated leave but also in uninoculated upper leaves at both 24 and 32 °C. Importantly, viral RNA accumulated to a similar extent at 24ºC in both inoculated and uninoculated upper leaves infected with one of PaMMV-J, PaHk1 and PaRepT241S, suggesting that A721U mutation does not affect virus multiplication in plants.

In *Hk* plants cultivated at 32 °C but not 24 °C, systemic infectivity of both PaMMV

and chimeric viruses harboring the replicase gene of PaMMV was inhibited. The results allowed us to identify the replicase genes of the PaMMV as the viral factor required for activation of *Hk* gene-mediated resistance in *Capsicum* plants cultivated at high temperatures. Furthermore, T241S substitution in the methyltransferase domain resulted in the breaking of *Hk* resistance at 32 °C, suggesting that this domain of PaMMV replicase proteins is responsible for activating the effect of a *Capsicum* resistance gene, *Hk,* at high temperatures. Knapp et al. (2005 and 2007) demonstrated using the bipartite *Tobacco mosaic virus*-defective RNA system that methyltransferase domain is involved in both cell-to-cell movement and long distance movement in *N. benthaminana*. However, all chimeric viruses and PaRepT241S tested in this study systemically infected L^+/L^+ plants and *Hk* plants at 24 °C, suggesting that A721U substitution does not affect virus systemic infectivity in *Capsicum* plants.

Among plant virus resistance genes elicitors that have been identified, tobacco *N* and tomato *Tm-1* genes are also known to be elicited by tobamovirus replicase proteins. Unlike *Hk*, however, their elicitor activity was mapped to helicase domain of the replicase proteins. Indeed, all parental and chimeric viruses used in this study induced necrotic local lesions on tobacco plants containing *N* gene, regardless of the reaction of *Hk*-plants at 32 ℃ to the viruses (data not shown). These facts highlighted the unique property of *Hk* gene that the methyltransferase domain is involved in pathogen recognition by resistance gene product, in addition to its high temperature dependence.

It has been reported that mutations of the methyltransferase domain in replicases is one of major pathogenicity determinants of PMMoV. 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 (Hagiwara et al., 2002; Ichiki et al., 2005; Yoon et al., 2006). In contrast, though The T241S substitution in replicases of PaMMV

led in change of disease symptom in the inoculated leaves of *Hk* plants at 24 ºC (Table 1), the Northern blotting analysis showed PaHk1 and PaRepT241S retained their ability to replicate in infected leaves of *Hk* plants at 24 ºC, like PaMMV-J (Fig. 2). Furthermore, the press blotting immunoassay also showed equal systemic infectivity of PaHk1 and PaRepT241S to PaMMV-J in *Hk* plants at 24 ºC (Fig. 1). These results collectively suggest that the T241S mutation, which breaks the *Hk* resistance, does not affect the infectivity function of methyltransferase domain, defect of which leads to viral attenuation. On the other hand, it remains possibility that the avirulence motif could reside in the RNA of replicase genes. The efficient inoculation method with the transcript from cDNA clones of tobamoviruses into *Capsicum* plants has not yet developed. Furthermore, though we developed the transit expression system of replicase genes using *Agrobacterium tumefaciens*, the bacteria nonspecially induced necrotic lesion in infiltrated area of *Capsicum* plants (data not shown). Therefore, the development of experiments involving the creation of stop codon mutants in the replicase genes is required for analysis on elicitor activity of RNA of replicase genes.

Cucumber mosaic virus (CMV) RNA1 codes for the 1a protein, which bears putative methyltransferase and helicase domains (Palukaitis and Garcìa-Arenal, 2003). The amino acid substitution at position 461 in the methyltransferase domain of the 1a protein of CMV Ns strain is reportedly involved in HR elicitation on *N. tabacum* cv. Xanthi-nc and on *N. glutinosa* (Salánki et al., 2007). The C461A and C461S substitutions led to a lack of necrosis induction on the inoculated leaves while the resistance phenotype did not change. The resistance pathway and the cell death pathway of *Cauliflower mosaic virus*-induced HR on *Nicotiana* spp. are reportedly uncoupled (Cawly et al., 2005) and induced selectively (Cole et al., 2001). Kim and Palukaitis (1997) also described the presence of an inhibition response distinct from the HR in

cowpea infected by CMV. In *Hk* plants at 24 ºC, PaMMV methyltransferase domain may induce cell death pathway but not resistance pathway, resulting in systemic PaMMV infection in the *Hk* plants with systemic necrosis. It is possible that viral methyltransferase domain, as a common and intrinsic nature, activate differentially the cell death and resistance signaling pathways in host plants that harbor resistance genes perceiving the viral protein. The A721U mutation abrogated the viral protein function to induce both cell death and resistance pathways in *Hk* plants regardless of the temperature.

The replacement of Thr-241 by Ser, which lies in the methyltransferase domain of the proteins, but not in the consensus sequence motif in type I methyltransferase domain of positive-strand RNA virus (Koonin and Dolja, 1993), was sufficient to overcome *Hk* resistance in *Capsicu*m plants. Identities of deduced amino acid sequences of replicase proteins between PaMMV-J and TMV-Ob showed 86.8 %, and amino acid at position 241 of both strains was Thr. Moreover, amino acids at position 241 of ToMV, a Japanese strain of PMMoV (Kirita et al., 1997) and a Japanese strain of TMGMV (Morishima et al., 2003) were His, Tyr and Glu, respectively. These evidences suggest that substituted Ser itself is not involved in the interaction with host machinery for virus perception and that the mutation likely affects conformation of replicase proteins. Since it evident that the amino acid substitution at position 241 of replicase proteins do not affect RNA replication in *Hk* plants, it is more likely that T241S mutation slightly affects the conformation and/or the interaction with other protein(s) of the replicase proteins.

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Table 1.

Infectivity of *Paprika mild mottle virus* Japanese strain (PaMMV-J) and *Tobacco mosaic virus*–L (TMV-L) and their chimeric viruses on *Capsicum annum* haboring *Hk* gene

Virus	Incubation temperature (°C)	
	24	32
TMV-L	SM[1]	SM
PaMMV-J	SN	LN
L-RepPa	SN	LN
L-MPPa	SM	SM
L-CPPa	SM	SM
Pa-RepL	SM	SM
Pa-MPL	SN	LN
Pa-CPL	SN	LN
PaHk1	SM	SM
PaRepT241S	SM	SM

[1] SM, no symptoms in the inoculated leaves, mosaic symptoms in the uninoculated upper leaves, and virus systemically infected the inoculated plants; SN, vein necrosis in the inoculated leaves, systemic necrosis symptoms in the uninoculated upper leaves, and virus systemically infected the inoculated plants; LN, necrotic local lesion and vein necrosis in the inoculated leaves, no symptoms in the uninoculated upper leaves, and virus locally infected the only inoculated leaves.

Figure legend

Fig. 1. Distribution of the coat protein (CP) of the *Paprika mild mottle virus* Japanese strain (PaMMV-J), *Tobacco mosaic virus*–L (TMV-L) and their chimeric viruses in *Capsicum annum* plants harboring *Hk* gene. In press blot immunoassay, blots of inoculated leaves (Ino.) and uninoculated upper leaves (Upp.) were prepared at 5 and 9 days post inoculation (dpi), respectively, and the viral CP was detected using an appropriate antibody.

Fig. 2. Accumulation of genomic RNA of *Paprika mild mottle virus* Japanese strain (PaMMV-J), PaRepT241S and PaHk1 in *Capsicum annum* plants harboring *Hk* gene. Total RNA (1 μg) extracted from the viruses-inoculated leaves (I) and uninoculated upper leaves (U) of *C. annum* at 5 and 9 days post-inoculation, respectively, was used for northern blot analysis using a sequence corresponding to nucleotide position 6008-6508 of PaMMV-J RNA as a 5'-DIG-labelled RNA probe. Ethidium bromide staining of rRNA is shown as a load control.

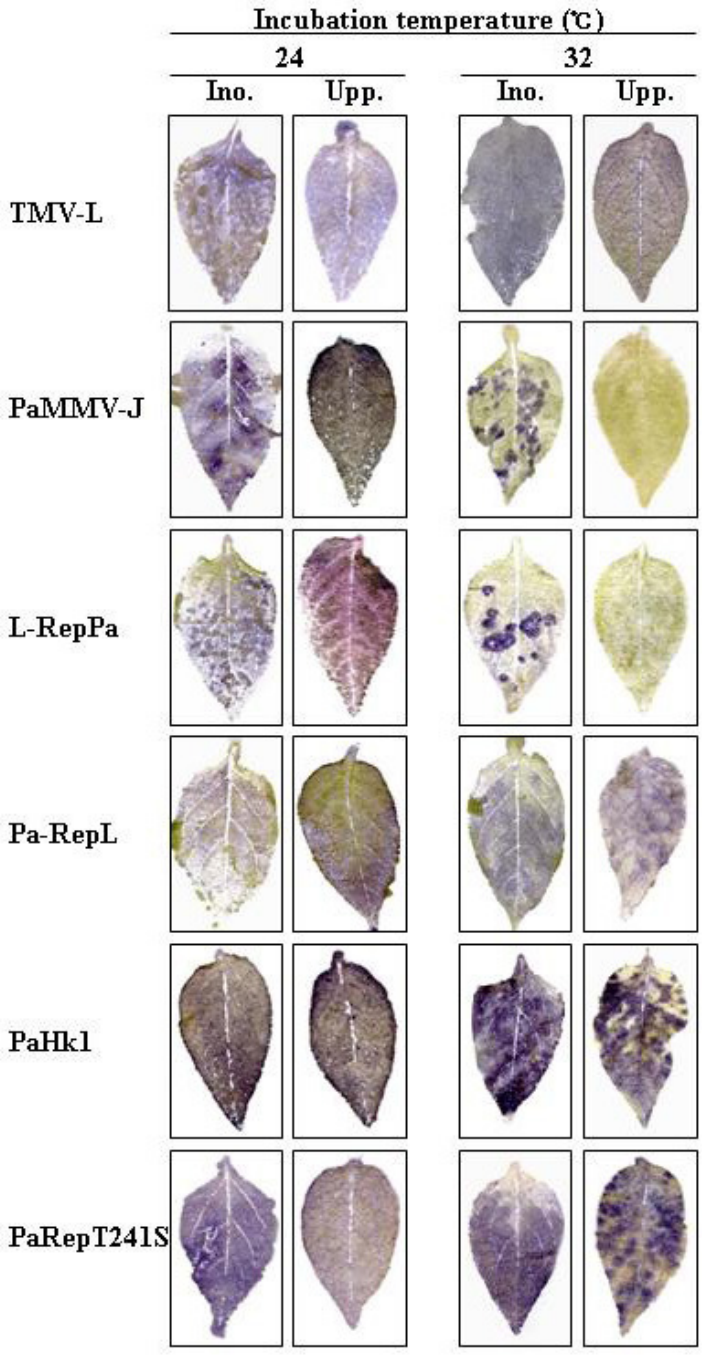

Fig. 1, Matsumoto et al., Virus Research

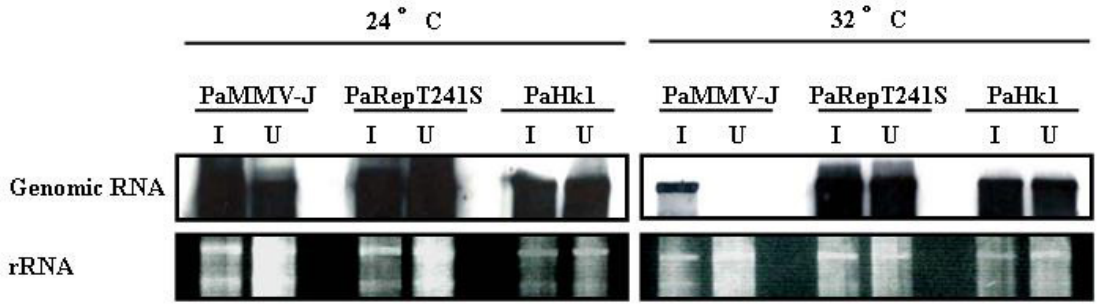

Fig. 2, Matsumoto et al., Virus Research