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## **An Analysis of Spontaneous Deletion Site in RNA2 of a New Variant of *Soil-borne wheat mosaic virus* Systemically Infects Plants at High Temperatures**

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**Abstract:** Experiments were conducted to analyze the occurrence of deletion mutation in RNA2 of new variants of *Soil-borne wheat mosaic virus* (SBWMV) Japanese strain-JT isolate, which have the ability of replication and systemic infection of plants at higher temperature of 25°C. Barley plants infected by SBWMV were grown in a growth cabinet of 17°C for 1 month, followed by shifting temperature to 22°C and finally to 25°C, each for 1 month, respectively. New variants emerged with the ability of systemic infection of barley plants at 25°C and spontaneous deletion mutation occurred in RNA2 by retaining same nucleotide positions of 5' and 3' terminal region of RT gene among individual plants. C-terminal region of RT protein which had already been shown to be restored in-frame, was truncated after out-frame deletion of RT gene leading to a stop codon (TGA, nt2497-2499) suggesting that C-terminal region of RT protein is not necessarily translated after deletion of nucleotide positions 867-2445 downstream of the capsid protein gene. The virus maintains 3'-terminal of RT gene due to act as a promoter for transcription of p19 subgenomic RNA, which is required for systemic infection of plants.

**Key words:** Deletion mutation, sequence analysis, readthrough protein, temperature sensitivity

### **INTRODUCTION**

*Soil-borne wheat mosaic virus* (SBWMV) is a type member of the genus *Furovirus* (Shirako and Wilson, 1993), causing disease on wheat in Japan, Italy, United States, China, France, Brazil and Egypt (Chen *et al.*, 1994). Green or yellow mosaic on the leaves associated with stunted forms of plants, are typical symptoms of natural hosts, wheat and barley. The RNA1 contains 7226 nt, coding for two N-terminally overlapping putative replicase proteins 152 and 211 kDa in the 5'-terminal region and a 37 kDa cell-to-cell movement protein (MP) in the 3'-terminal region. The RNA2 contains 3574 nt, coding for 19 kDa Capsid Protein (CP) and a 83 kDa readthrough (RT) product of CP (CP-RT protein). The optimum temperature for the propagation of this virus is 17°C and systemically infected plants can recover from the virus if the temperature increases (Ohsato *et al.*, 2003). The inoculation of infectious *in vitro* transcripts of RNA1 and RNA2 into barley mesophyll protoplasts showed that the Capsid Protein (CP) mainly accumulated at 17°C, but was undetectable at 25°C (Ohsato *et al.*, 2003). Spontaneous deletion mutation occurs in CP-RT region of RNA2, which is hypothesized, to be required for virus transmission in the soil by a vector, *Polymixa graminis* (Shirako and Brakke, 1984).

Here, the occurrence of deletion mutation after prolonged growth in 25°C, was investigated and compared with earlier studies on RT region of RNA2 carried out by Chen *et al.* (1994) and Yamamiya *et al.* (2005), in lower than 25°C condition. The importance of 5' and 3'-terminal region of RT gene, which is retained by the virus and the necessity of C-terminal region of CP-RT protein, is discussed.

### **MATERIALS AND METHODS**

This study was conducted between 2004-2007 in the graduate school of Agriculture and Life Sciences, The University of Tokyo, Japan.

**Plants and infectious *in vitro* transcripts of RNA1 and RNA2:** Four barley plants (cultivar Mokusekko provided by Barley and Wild Plant Resource Center, Research Institute for Bioresources, Okayama University, Kurashiki, Japan) were seeded into pots followed by mechanical inoculation of SBWMV Japanese strain-JT isolate at 17°C. Temperature was shifted from 17°C to 22°C and finally to 25°C, respectively, by growing plants at least one month at each temperature. All plants (No. 6, 7, 8 and 9) showed disease symptoms at 25°C were used for further analysis.

**Virus purification and Western blot analysis:** Leaf tissues (100-300 mg) was ground with a mortar and pestle in 5 mL of 0.5M sodium borate buffer (pH = 9.0) containing 1 mM EDTA, followed by centrifugation at 4000 rpm for 5 min. To the supernatant 1 mL of 20% Triton X-100 was added and centrifuged at 38000 rpm for 2 h. The pellet was resuspended in 250  $\mu$ L of water to prepare the virion solution. Ground plant tissue or purified virus in sample buffer (0.05 M Tris-HCl, pH 9.0, 2% SDS, 15% sucrose, 0.05% bromo-phenol blue, 5% 2-mercaptoethanol), heated at 95°C for 3 min and used for SDS-PAGE and Western blots, as described earlier (Ohsato *et al.*, 2003; Yamamiya *et al.* 2005).

**RNA extraction and RT-PCR amplification of total RNA2:** Purified virion was used for RNA extraction. A solution containing 50  $\mu$ L of the virus suspension, 350  $\mu$ L of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), 20  $\mu$ L of 25X STE (2.4 M NaCl, 0.48 M Tris-HCl, pH 7.5, 25 mM EDTA, pH 8.3) and 80  $\mu$ L of 10% SDS was prepared and heated at 70°C for 5 min followed by placing on ice. This solution was then treated twice with phenol/chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol. RNA was then prepared by ethanol precipitation.

Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA2 of SBWMV Japanese strain-JT wild type (JTWT) was performed using one step RNA PCR kit (AMV; Takara Bio). To amplify the sequences, appropriate forward and reverse primers to produce segments A1, A2, A3, B, C1, C2 and C3 were used (Fig. 1, Table 1). In the case of No. 6, 7, 8 and 9 only readthrough region were amplified using primers TP80 and TP9 to produce segment B (Fig. 1, 2). Thermal conditions for amplification were 50°C for 15 min and one cycle at 94°C for 2 min followed by 28 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1.5 min and 25°C for 1 min. The products were sequenced directly.

**Sequence analysis of total RNA 2:** RT-PCR products of total RNA2 of JTWT and RT region of No. 6, 7, 8 and 9 were sequenced using BigDye Terminator v3.1 Reaction mix (ABI). The primers used for sequencing of total RNA2 of JTWT were: TP27, TP8, TP40, TP4, TP80, TP9, TP42, TP81, TP70 and TP46. For RT region of No. 6, 7, 8 and 9, primers TP80 and TP9 were used (Fig. 1, Table 1). Sequence data were analyzed by the ABI PRISM sequence analysis program and assembled using the ABI Auto Assembler (Perkin Elmer).

## RESULTS AND DISCUSSION

It was earlier found a new variant of SBWMV Japanese strain, which substitutes Thr172 (ACT) with Ala

(GCT) by changing A (nt6205) to G in the movement protein gene which is hypothesized to give the virus the ability of movement and systemic infection at higher temperature (25°C) (Unpublished data). To examine the effect of higher temperature on occurrence of spontaneous deletion mutation in RNA2, RT-PCR products of total RNA2 of JTWT were amplified using primers TP27 and TP8 for segment A; TP27 and TP40 for segment A2; TP27 and TP4 for segment A3; TP80 and TP9 for segment B; TP42 and TP81 for segment C1; TP42 and TP81 for segment C2 and TP 46 and TP81 for segment C3 (Fig. 1, Table 1). In case of RT region of No. 6-9, only primers TP80 and TP9 were used to produce segment B. RT-PCR products of primers TP80 and TP9 were amplified and compared in all JTWT and #6-9 samples (Fig. 2). Due to deletion mutation in the RT region, all samples No. 6-9 produced a 0.5 kb fragment, whereas JTWT produced 2.2 kb fragment, as indicated in Fig. 2. The RT-PCR products were sequenced directly and sequence alignment was carried out using CLC Bio multiple alignment tool (<http://www.clcbio.com>), by comparing RNA2 of SBWMV-JT gene bank, SBWMV-JTWT and RT region of No. 6-9 (data not shown). The results showed that deletion occurred at the same positions of nucleotides 867-2445 in RT region of No. 6, 7, 8 and 9. Each plant contained the RNA2 with deleted nucleotides from 867 to 2445, which occurred out-frame. In previous studies, inoculated plants with the same wild-type U.S. strain (Nebraska isolate) resulted in different sizes of RNA2 after 4 months propagation at 17°C in a growth cabinet (Shirako and Brakke, 1984). Similarly, when the viruses were serially passaged in wheat, different sizes of RNA2 dominated in population (Chen *et al.*, 1994; Shirako and Ehara, 1986; Yamamiya *et al.*, 2005).

Out-frame deletion led to the truncation of CP-RT protein by introducing a stop codon at nucleotide positions 2445-2497 (Fig. 3, JT). Out-frame deletion of RNA2 was already reported by Chen *et al.* (1994), who showed the deletion mutation of nt1469-2526 led to introduction of UAG stop codon two nucleotides downstream of deletion site in Lab1 (Fig. 3). In another study by Yamamiya *et al.* (2005), from the four sets of serial passages of Japanese strain, 19 deletion mutant RNA2s were detected, in which 13 had in-frame and 6 had out-frame deletions. None of the out-frame deletion mutants became dominant at the end of each set of experiment.

Spontaneous deletion mutation, which occurred in RNA2 RT region of Okl-7 and Lab-1 of Chen *et al.* (1994) studies, compared with deleted region in JT obtained in this study (Fig. 3). Present results indicated that the dominated RNA2 population in individual plants was similar in deletion site. This is in contrast with Lab-1 and

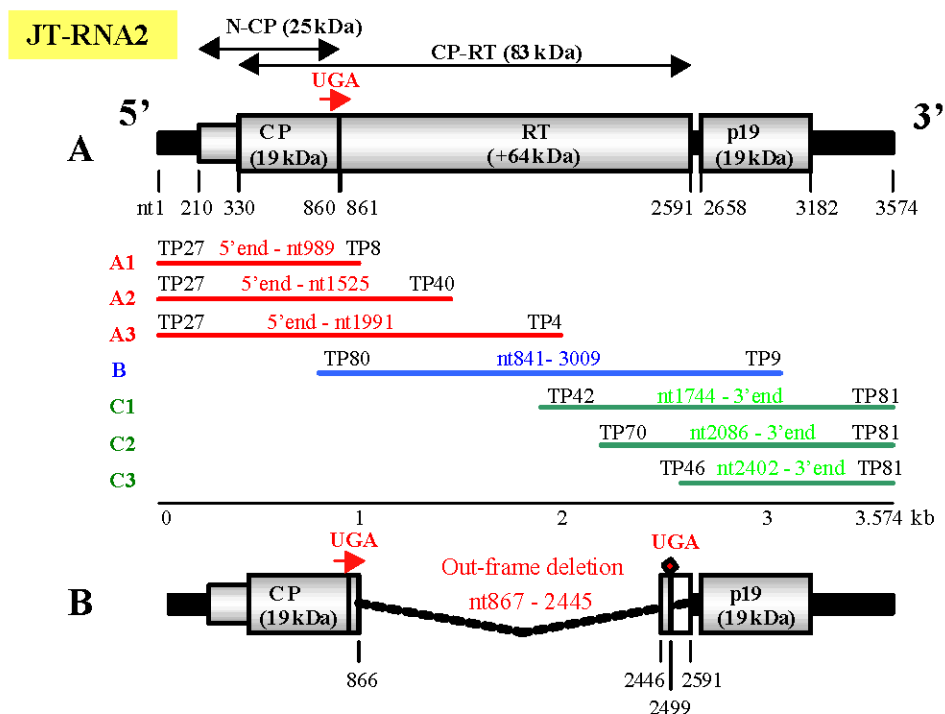


Fig. 1: Schematic diagram of RNA2 of SBWMV Japanese strain-JT isolate and the primers used for RT-PCR and sequencing, (A) Out-frame deletion led to the introduction of UGA stop codon at nt 2497-2499 and the truncation of the protein between nt 2500-2591 and (B) The leaky UGA codon of readthrough is indicated by a red arrow and the UGA stop codon is indicated by filled red diamond

Table 1: Primers. RNA2 RT-PCR amplification and sequencing analysis primers of total RNA2

TP27	5'/3+Nhe+SP6+18/+	AGA/GCT AGC/ATT TAG GTG ACA CTA TA/G TAT TAT TAT CAC ATT AC
TP8	/973-989/-	CGG CTC ATC ACG TAT CA
TP40	/1509-1525/-	ACT GCT CTG ACG AAC AT
TP4	/1975-1991/-	CTG TCC TGC CAT GAG TA
TP80	/841-857/+	AGT GGG AAG GTA CAA GT
TP9	/2993-3009/-	TAG CAC TAT CAC TGA GC
TP42	/1744-1760/+	TAG GCT GTG ACG TCG AG
TP81	3'-terminal 20 nt/-	TGG GCC GGA TAA CCC TCC GG
TP70	/2086-2102/+	AGA TGC TGC CAT CTG TA
TP46	/2402-2418/+	TAG TGC AGA CAC GTC TA

Ok1-7 from Chen *et al.* (1994) experiments and JT isolate from Yamamiya *et al.* (2005) experiments, in which the position of deletion mutation differed in each set of experiment and the stable deletion mutant, which was dominated in the final RNA2 population differed from plant to plant. Yamamiya *et al.* (2005) showed that deletion of nucleotide 2504-2533 led to the reduced level of p19 subgenomic RNA, whereas deletion of 2504-2563 caused undetectable amounts of p19 subgenomic RNA, which was not infectious to plants. Taken together with present result, implies that retaining 3' region of RT gene is required for p19 transcription and systemic infection of plants. p19, which is involved in regulation of RNA

replication and transcription or as a RNA silencing suppressor has been shown in other viruses like *Pecluvirus*, *Hordeivirus*, *Benyvirus*, *Pomovirus* and *Tobravirus* (Bragg and Jackson, 2004; Dunoyer *et al.*, 2002; Liu *et al.*, 2002).

It has been shown that CP-RT protein contributes in virion formation in *Beet necrotic yellow vein virus* (BNYVV) and *Potato mop-top virus* (Cowan *et al.*, 1997; Haerberle *et al.*, 1994; Schmitt *et al.*, 1992); however, the necessity of C-terminal part is still unknown. C-terminal region was restored in-frame even after introducing a stop codon for manually truncating the CP-RT protein (Yamamiya *et al.*, 2005) and retained in

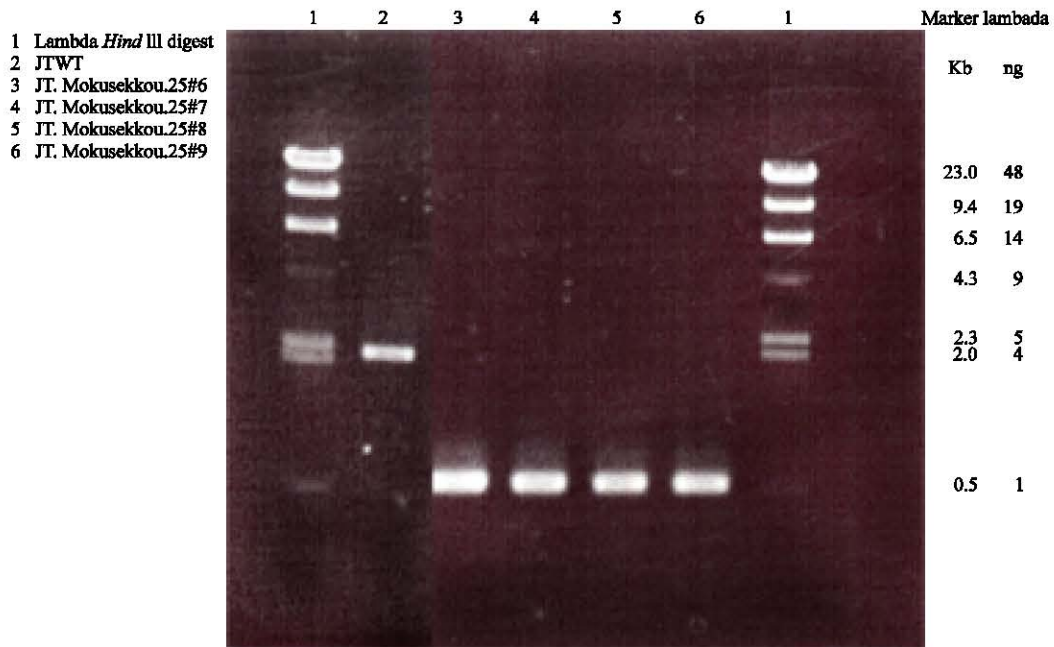


Fig. 2: PCR amplification of RT-PCR products of segment B; containing RT region in RNA 2 of JTW, #6-9 using primers TP80(+) and TP9(-)

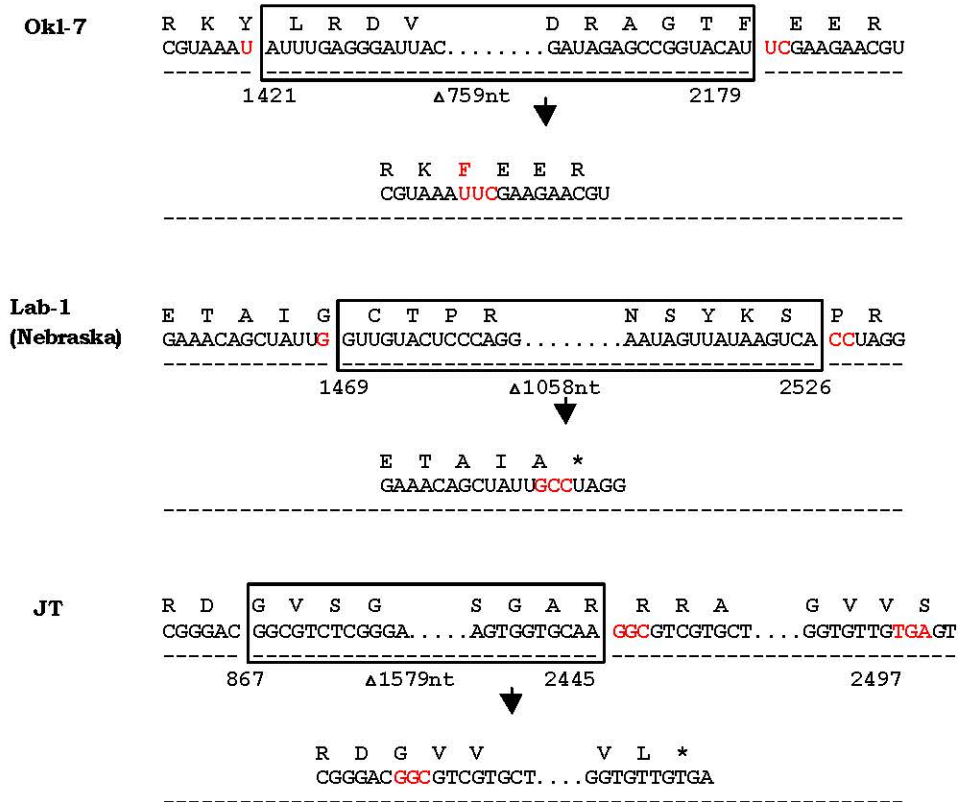


Fig. 3: Partial sequence of RNA2 readthrough region. Comparison of deletions in Ok1-7, Lab1 (Chen *et al.*, 1994) and JT (present results). Ok1-7 had an in-frame deletion, whereas Lab-1 and JT had frameshift leading to introduction of termination stop codon and a truncated CP-RT protein

Ok1-7 after in-frame deletion of nt 1421-2179 (Fig. 3) (Chen *et al.*, 1994). Also in frame deletion of CP-RT region have been found in *Potato mop top virus* (Sandgren *et al.*, 2001; Torrance *et al.*, 1999). Present results implied that CP-RT protein would be truncated and C-terminal part is not essential for infecting plants in a stable deletion mutant. The same happened in Lab-1 by an out-frame deletion, in which a stop codon (UAG) was introduced two nucleotides downstream of the deletion site and led to a CP-RT truncated protein (Fig. 3, Lab-1). Taken together the results suggest that the mutation of CP-RT gene is not directed to produce conserved amino acids of CP-RT protein. The effect of temperature on producing same population of deletion mutants is yet to be understood.

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