論 文 の 内 容 の 要 旨

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論文題目　Genome characterization of *Soil-borne wheat mosaic virus* variants causing systemic infection at higher temperatures（高温条件下で全身感染するムギ類萎縮ウイルス変異株の遺伝因子に関する研究）

Soil-borne wheat mosaic virus (SBWMV), the type species of the genus *Furovirus*, contains a bipartite positive-stranded RNA genome (RNA1 and RNA2) encapsidated in separate rod-shaped particles. The vector that transmits this virus in soil is a plasmodiophoraceous protozoan, *Polymyxa graminis* (Shirako and Wilson, 1993). The RNA1 of genomic RNA, which is 7099 nucleotides (nt) in the U.S. strain-Nebraska isolate (SBWMV-NE) and 7226 nt in the Japanese strain- JT isolate (SBWMV-JT), codes two N-terminally overlapping putative replicase proteins in the 5'-terminal region (150 and 209 kDa for SBWMV-NE, and 152 and 211 kDa for SBWMV-JT) and a 37-kDa cell-to-cell movement protein (MP) in the 3'-terminal region. The C-terminal 59-kDa regions of the 209- or 211-kDa proteins are expressed by translational readthrough. In the 3'-terminal region of RNA1, the 37-kDa MP is probably expressed from subgenomic RNA. RNA2 (3593 nt in SBWMV-NE and 3574 nt in SBWMV-JT) codes the 19-kDa capsid protein (CP) in the 5'-terminal region; an 83-kDa protein that is a readthrough product of the 19-kDa protein, which is hypothesized to be required for transmission of the virus by the vector *P. graminis* (Shirako and Brakke, 1984); and a 19-kDa cysteine-rich protein in the 3'-terminal region. In addition to these proteins, RNA2 codes a 24-kDa protein that has 40 amino acids extension towards the N-terminus of the CP. The initiation codon of the 24-kDa protein was identified as a CUG (Shirako, 1998).

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 (Rao and Brakke, 1970). The inoculation of infectious *in vitro* transcripts of RNA1 and RNA2 into barley mesophyll protoplasts showed that the CP most abundantly accumulated at 17°C, but was undetectable at 25°C (Ohsato *et al.*, 2003), suggesting that replication of the virus is temperature sensitive. However, little is known about the involvement of the movement protein in the temperature sensitivity of SBWMV. Considering that cell-to-cell movement of *Tobacco mosaic virus* (TMV) RNA is temperature-dependent (Boyko *et al*. 2000) and mutations in the MP gene of TMV have been

reported to be involved in temperature sensitivity (Boyko *et al.*, 2000; 2007), thus, it could be possible that both replication and cell-to-cell movement of the virus are implicated in SBWMV temperature sensitivity.

Under laboratory conditions, when the infected plants are subjected to a shift in temperature (17°C to 22°C to 25°C), both plants without symptoms (i.e., plants recovered from the viral infection) and plants retaining the symptoms appear (Shirako, 2005). The latter plants are thought to harbor SBWMV variants with altered temperature sensitivity. Mutations were examined that could occur in the MP gene of variants that can propagate under higher temperatures in order to determine the possible involvement of mutations in the MP gene of SBWMV in temperature sensitivity. Two isolates, SBWMV-NE and SBWMV-JT, which are genetically related and belonging to a single species (Miyanishi *et al*. 2002) have been used in this study.

Plant materials and temperature shifting

Wheat cv. Fukuho (65 plants) and barley cv. Ryoufu (80 plants) were seeded into pots, kept at 17°C for 10 days until the two-leaf stage, and then used for mechanical inoculation of SBWMV-NE. Temperature was shifted from 17°C to 25°C as described by Shirako (2005) with minor modification by keeping the plants at each temperature for one month. Inoculated plants were kept at 17°C for 1 month, transferred to and kept at 22°C for 1 month, and finally transferred to and grown at 25°C for 1 month. The plants that still had disease symptoms at 25°C were used for virion purification. In the case of SBWMV-JT, 4 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 and temperature shifting as described for SBWMV-NE.

Western blotting

Ground plant tissue or purified virus in sample buffer was treated at 95°C for 3 min and used for SDS-PAGE and Western blots. A 10-µl aliquot of sample per lane was loaded onto 12.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane. The CP of SBWMV was detected using anti-SBWMV CP as the primary antibody (raised against SBWMV-NE or SBWMV-JT) and goat anti-rabbit IgG-alkaline phosphatase-conjugated antibody as the secondary antibody and visualized by BCIP (bromo-chloro-indryl phosphate) and NBT (nitro blue tetrazolium).

Virus purification

Leaf tissue (100–300 mg) were ground with a mortar and pestle in 5 ml of 0.5 M sodium borate buffer (pH = 9.0) containing 1 mM EDTA, followed by centrifugation at 4000 rpm for 5 min. One-tenth volume of 20% Triton X-100 was added to the supernatant and centrifuged at 38000 rpm for 2 h. The pellet was resuspended in 250 µl of water to prepare the purified virion solution.

RNA extraction and RT-PCR

Purified virion was used for RNA extraction followed by reverse transcription-polymerase chain reaction (RT-PCR) of the 3'-terminal 1.8-kb region of RNA1. To amplify the sequences, primers TN16 and TN202 were used for SBWMV-NE, and primers TP3 and TP25 were used for SBWMV-JT (Fig. 1), followed by PCR and cloning the PCR products into pGEM-T (Promega) to examine the sequence of the MP gene of the independent variant or were sequenced directly.

Preparation of independent sequence clones

RT-PCR products were cloned into the pGEM-T vector according to the manufacturer's instructions. The ligation products were introduced to strain MC1061 of *Escherichia coli* to obtain clones of the sequence of the MP gene from the independent mutants.

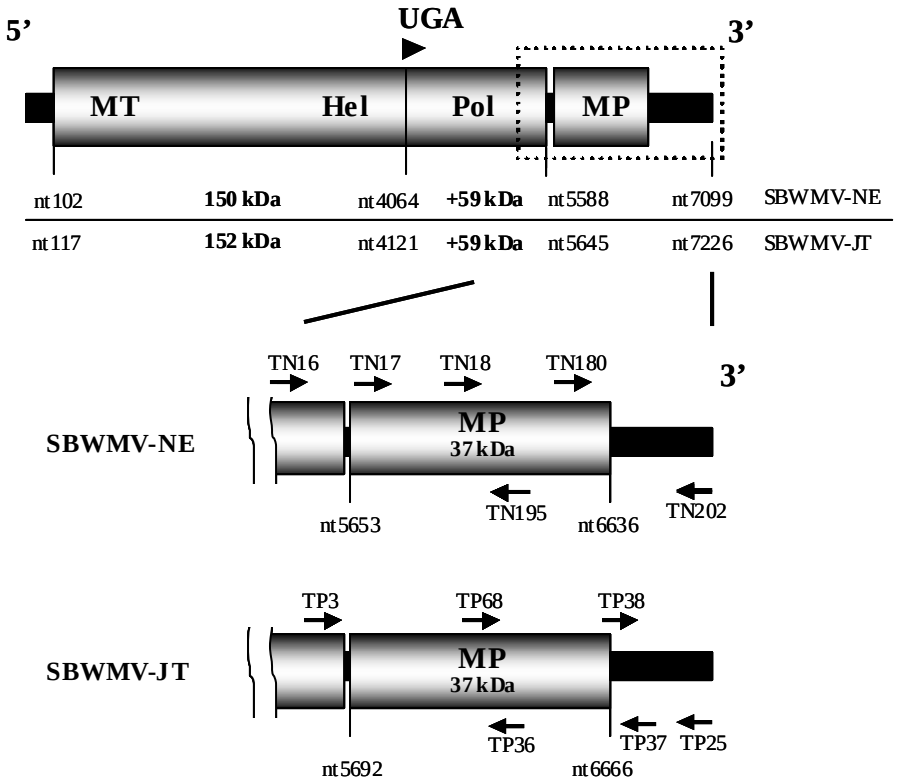

Fig. 1. RNA 1. Schematic diagram of the position of the MP gene and the primers used for sequencing. The leaky UGA codon of readthrough is indicated by arrowhead.

Plasmid DNA extraction and sequence analysis

Plasmids were extracted and used for direct sequencing using the BigDye Terminator v3.1 Reaction mix (ABI). The primers used for sequencing are shown in Fig. 1. Sequence data were analyzed by the ABI PRISM sequence analysis program and assembled using the ABI Auto Assembler (Perkin Elmer).

RESULTS AND DISCUSSION

Symptom development of SBWMV by temperature shifting

Sixteen out of 65 wheat cv. Fukuho plants and 3 out of 80 barley cv. Ryoufu were systemically infected with SBWMV-NE at 17°C among which, 3 wheat plants (F22, F30, and F63) and 1 barley plant (R80) kept the symptoms at 25°C and were selected for viral purification followed by SDS-PAGE and Western blotting. Symptom severity was increased at higher temperatures (such as yellow mosaic of the leaves and severe stunting of the plants at 25°C comparing to the green mosaic and minimal stunting at 17°C) (data not shown). In the case of the JT isolate, all of the 4 inoculated barely plants that showed disease symptoms at 25°C were further analyzed.

Determination of the sequence of the 3'-terminal region of RNA 1 containing the MP gene of SBWMV-NE

In the case of SBWMV-NE, purified RT-PCR products from variants, F22, F30, F63 and R80 were sequenced directly. Amino acid substitutions were observed in the variants propagated in wheat F30 and F63 and in barley R80, whereas only a silent mutation was observed in the variants in F22 (Fig. 2). In the variants in F30, nucleotide 5831, which was originally adenine (A), was changed to a mixture of A and guanine (G). These changes contained a mixed population of the variants that

have glutamine or arginine as the 60th amino acid (Gln-60 or Arg-60) in the MP (Fig. 2). Also, a mutation of G to A at nt 6483 was observed, which is a silent mutation. In the variants in F63, C at nt 6173 (C6173) was mutated to T, causing amino acid substitution of threonine at position 174 (Thr-174) to methionine (Met) (Fig. 2). In R80, the variants had a mixture of A and G at nt 5759, which leads Gln-36 to become a mixture of Gln and Arg. Also, a mixture of A and T at nt 6172 was observed, indicating the presence of a mixture of variants harboring Ser or Thr at position 174. The amino acid substitutions observed in these variants may play a role in changing the temperature sensitivity of SBWMV-NE, especially the substitution at amino acid position 174, which occurred in variants of two independent plants (F63 and R80). However, considering that the variants in F22 only had a silent mutation in the MP gene, mutations of another gene(s) of SBWMV-NE may also be involved in changing the temperature sensitivity of this virus.

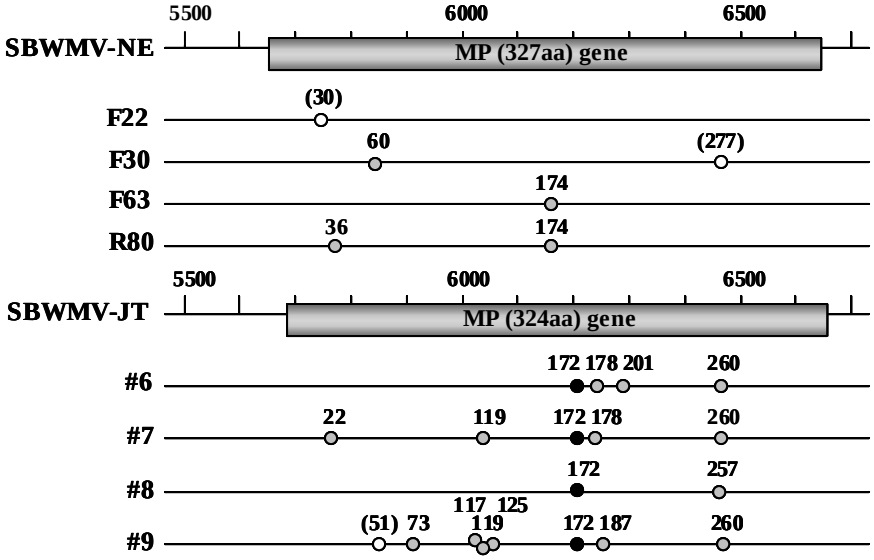

Fig. 2. Comparison of amino acid mutations among variants of two SBWMV isolates. Closed gray circles: Mutations causing changes in amino acids; Open circles: Silent mutations; Closed black circles: Amino acid 172 that 63–90% of the cloned sequences had the mutation.

Determination of sequence of the 3'-terminal region of RNA 1 containing the MP gene of SBWMV-JT

For mutations in SBWMV-JT, the sequences of independent clones (10–11 clones for each virus sample) of the RT-PCR products of the MP region of each virus sample (#6–9) were analyzed. Among the various mutations observed in the independent clones, most clones showed a mutation from A to G at nt 6205, which caused a Thr-172 to Ala substitution (Fig. 2). Seven out of 11 clones of #6, 8 out of 10 clones of #7, 9 out of 10 clones of #8, and 7 out of 10 clones of #9 had this mutation. These results strongly suggest the possible role of the amino acid substitution of Thr-172 to Ala-172 of the MP in the change in temperature sensitivity of SBWMV-JT.

In conclusion, it was shown that mutation of Thr174 (in SBWMV-NE) or Thr172 (in SBWMV-JT) occurred probably due to adaptation of virus to the new environment (higher temperature) for cell-to-cell and long-distance movement as well as complete systemic infection, and possible involvement of the movement protein in the temperature sensitivity of SBWMV was revealed.