論 文 の 内 容 の 要 旨

生産・環境生物学　専攻
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氏　名　　尤　園
指導教員　東京大学教授　白子幸男

論文題目　Establishment of a reverse genetics system for *Barley yellow mosaic virus* and the studies on functions of P1, P2 and VPg proteins in systemic infection
（オオムギ縞萎縮ウイルスのリバースジェネティクス法の確立とP1、P2およびVPgタンパク質の全身感染性における機能に関する研究）

Barley yellow mosaic virus (BaYMV), the type species of the genus *Bymovirus* in the family *Potyviridae,* is one of the major viral pathogens of barley in Europe and Easy Asia. It is transmitted by *Polymyxa graminis* in soil and causes a yellow mosaic disease of winter barley with significant yield losses. The virus has a bipartite positive-sense RNA genome. RNA1 (7.6 kb) encodes a large polyprotein which is processed into eight products (P3/6K1/CI/6K2/VPg/NIa-Pro/NIb/CP). RNA2 (3.6 kb) also encodes a polyprotein which is cleaved into two products (P1/P2). Breeding resistant cultivars using *rym* resistant genes from barley landlaces is the only practical way to avoid crop losses due to BaYMV infection in fields. However, resistance-breaking BaYMV variants are continuing to emerge. To study molecular biological aspects of interactions among the virus, host plants and the transmission vector, establishment of reverse genetics of BaYMV is a prerequisite.

In this study, full-length cDNA clones of RNA1 and RNA2 of a Japanese K05 isolate of BaYMV (BaYMV-JK05) were constructed. *In vitro* transcripts were systemically infectious. Using the *in vitro* transcripts system, functions of P1, P2 and VPg in systemic infection and resistance and susceptibility of barley cultivars were studied.

1. Establishment of reverse genetics of BaYMV

Based on the genome information previously studied during my Master's course, full-length cDNA inserts for RNA1 and RNA2 were individually cloned into a pBR322-derived plasmid and propagated in *Escherichia coli* MC1061. Multiple cDNA clones for RNA1 and RNA2 were examined by infectivity assay in barley mesophyll protoplasts (cvs. Minori and Ryofu). Using the highest expression level of CP in transfected protoplasts as a criterion, one each clone for RNA1 and RNA2 were selected and designated as pBY1 and pBY2, respectively.

RNA1, which encodes eight proteins including a viral proteinase NIa-Pro, the RNA-dependent RNA polymerase NIb, a genome-linked protein VPg and the capsid protein CP, replicated autonomously in protoplasts optimally at 15° C, a temperature similar to the optimum for causing disease in barley fields. P1 was detected from protoplasts transfected with RNA1 and RNA2 transcripts, but not from those transfected with RNA2 transcripts alone, indicating that RNA2 replication depends on RNA1. The accumulation level of CP increased several folds in the presence of RNA2, indicating that RNA2 or RNA2-encoded proteins enhance RNA1 replication and gene expression in host cells.

Barley plants (cv. Ryofu) were mechanically inoculated with RNA1 and RNA2 transcripts and grown at 15° C. Three weeks after inoculation, upper uninoculated leaves of 53.5% inoculated plants started showing yellow mosaic symptoms. From these leaves, CP, VPg, VPg-containing polyproteins and P1 were detected by Western blot analysis. The progeny viral RNA1 and RNA2 had identical nucleotide sequences as in cDNA inserts of pBY1 and pBY2, respectively. Thus *in vitro* transcripts of RNA1 and RNA2 were proven to be systemically infectious in barley. None of the plants inoculated with RNA1 transcripts alone were infected, indicating that RNA2 is essential for systemic infection.

2. Analysis of RNA2-encoded proteins in systemic infection

Four pBY2-derivative mutants were constructed, in which the P1-coding region was deleted (pBY2.ΔP1), the P2-coding region was deleted (pBY2.ΔP2), a termination codon was inserted after the P1-coding region (pBY2.$P1_{TAA}$) and the -1 amino acid at the P1/P2 boundary (Gly/Ser) was mutated to abolish cleavage (pBY2.P12V). The expected protein products from these mutant constructs were confirmed by cell-free translation of *in vitro* transcripts.

Protoplasts transfected with RNA1 and WT RNA2 or ΔP2 RNA2 transcripts gave similar strong bands of CP and P1 on Western blots. However, from protoplasts transfected with

RNA1 together with ΔP1 RNA2, $P1_{TAA}$ RNA2, or P12V RNA2 transcripts, CP was only weakly detectable, similar to the situation when protoplasts were transfected with RNA1 transcripts alone. P12 protein was readily detected in the case of P12V RNA2. P1 was not detected in the case of $P1_{TAA}$ RNA2, probably due to the additional P2-coding sequence in the 3' UTR having a deleterious effect in replication. These results indicated that expression of P1 from RNA2 leads to efficient RNA1 replication and the gene expression in host cells.

Barley plants (cv. Ryofu) were inoculated with RNA1 and ΔP2 RNA2 transcripts. Five out of 29 plants showed small chlorotic spots on upper uninoculated leaves at 5 weeks after inoculation, and CP and P1 were weakly detectable from these leaves, indicating that the plants were systemically infected. Sequencing analysis confirmed that the original ΔP2 mutation was retained in progeny virus genome. On the other hand, plants inoculated with RNA1 transcripts alone or together with ΔP1 RNA2 or P12V RNA2 transcripts were not infected. One out of 28 plants inoculated with RNA1 and $P1_{TAA}$ RNA2 transcripts was systemically infected, in which the inserted UAA termination codon was mutated to a UCA serine codon, regenerating a Gly/Ser cleavage site. Therefore, both P1 and P2 are required for efficient systemic infection: P1 is essential whereas P2 may facilitate virus movement and WT symptom development.

3. Analysis of VPg132 RNA1 mutants in virus replication and systemic infection

In the case of European isolates of BaYMV, a substitution of Lys at the position 132 of VPg with Asn or His was suspected to be responsible for breaking *rym*4-resistance. The Japanese BaYMV JK05 isolate has a His at the same position of VPg. To investigate the importance of the VPg132 amino acid in BaYMV replication and systemic infection, four VPg132 mutant RNA1 constructs were prepared with His codon replaced with Ala, Lys, Asn or Tyr codons. Protoplasts prepared from susceptible cvs. Hadaka1 and New Golden and a *rym*4-posessing Franka were transfected with WT or VPg132 mutant RNA1 together with WT RNA2 transcripts. From Hadaka1 and New Golden protoplasts, CP and P1 were detected strongly with WT His RNA1, lesser with Tyr RNA1, much lesser with Asn RNA1, and almost undetectable with Lys or Ala RNA1. From Franka (*rym*4) protoplasts, CP and P1 could not be detected in all cases. Thus, using the Japanese BaYMV JK05 isolate, none of His, Asn, Lys, Ala or Tyr at the position 132 of VPg could break the *rym*4 resistance.

When Hadaka1 and New Golden plants were inoculated with the Tyr RNA1 transcripts with WT RNA2 transcripts, one Hadaka1 plant and two New Golden plants developed the wild-type symptoms in five weeks. Sequencing analysis of the progeny viral genome showed that the mutated Tyr codon was retained or reverted to the WT His codon partially or completely in the progeny virus population. Hadaka1 and New Golden plants inoculated with Asn RNA1 and RNA2 transcripts were not infected. These results indicated that VPg is one of the essential viral proteins in replication and systemic infection and that the amino acid at the position 132 is important for the function of VPg.

4. Analysis of resistance and susceptibility of barley cultivars using transcripts

The infectious *in vitro* transcripts system enables analysis of resistance and susceptibility of barley cultivars against BaYMV infection at the molecular level. Barley cultivars with *rym*1,2, 3, 4 ,5 or 6 genes as well as those without known *rym* genes were subjected to infectivity assay at the cellular level and at the whole plant level using the *in vitro* transcripts. The result showed that cultivars with *rym*1, 2, 4, 5 or 6 were immune to BaYMV JK05 at the cellular level. Two cultivars with *rym*3, Haganemugi and Ishukushirazu, were susceptible at the cellular level but not at the whole plant level, indicating that these cultivars are resistant at either cell-to-cell or long-distance movement levels. Haruna Nijo without known resistance genes was shown to be immune at the cellular level, implying that Haruna Nijo has an unidentified resistance gene. Kashimamugi without known resistance genes was susceptible at the cellular level but did not develop recognizable symptoms. However, CP was detected from symptomless leaves as well as from roots, indicating that Kashimamugi is systemically infected with JK05 BaYMV. Thus, the *in vitro* transcripts system was proven to enable precise analysis of the virus-host compatibility and finding resistant cultivars at the cellular level.

This is the first report of establishment of a reverse genetics system for a virus in the genus *Bymovirus* and the first studies on biology of bymoviruses and host resistance and susceptibility at the molecular level. The obtained infectious cDNA clones of BaYMV open new avenues for the studies on bymovirus-plant host-transmission vector interactions, leading to development of efficient control measures for bymovirus diseases.