博士論文

Studies on Bymovirus Infectivity and Host Resistance

（*Bymovirus* 属ウイルスの感染性と宿主植物の抵抗性に関する研究）

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Contents

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Chapter 1

General introduction

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1.1 Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare* L.) is one of the first domesticated cereal crops originating in the area of Middle East known as the Fertile Crescent over 10,000 years ago by archaeological evidence (Badr et al., 2000) and now widely grown in Europe, Middle East, America and Asia. In 2007, Barley production ranked fourth, after wheat, maize and rice, among the cereal crops globally. The total barley yield in the world for 2013 is 144,755,038 tons in 49,781,046 hectares that accounts for 5.2% of the total yield of cereals (FAOSTAT data, 2014).

Barley is a member of the genus *Hordeum* belonging to the tribe Triticeae of the grass family Poaceae (also known as Gramineae). The Triticeae tribe comprises the major economically important temperate cereals (such as wheat, rye and barley) and forages (such as the crested wheatgrasses and Russian wildrye). Barley varieties are classified based on different factors such as feed or malting barley, winter or spring growth habit, six-, four- or two-rowed varieties, covered of naked/hulled barley, and starch amylose/amylopectin ratio (OECD, 2004).

Barley was originally cultivated and used for human food. Nowadays, barley is used for a number of purposes, primarily for animal feed. Approximately 85% of the barley production worldwide is destined for feeding animals (such as beef cattle, dairy cattle, swine, poultry and fish). The rest is used for malt production, human food consumption and starch production for the food use or chemical industry. Barley is also used for non-alcoholic drinks such as ‘barley tea’, which is very popular in Japan (OECD, 2004).

The barley genome is composed of seven pairs of chromosomes designated 1H to 7H according to the homologous relationship with those of other Triticeae species (such as wheat and rye) (Pedersen et al., 1996). It is diploid with a large haploid genome of 5.1 gigabases, twice the size of human genome. The genome of barley is abundant in repetitive elements, with 84% of the genome comprised of mobile elements or other repeat structures. Total 79,379 transcript clusters have been identified, including 26,159 'high-confidence' genes that have homology with the genomes of other cereals *Sorghum*, rice, *Brachypodium* and *Arabidopsis* (International Barley Genome Sequencing Consortium, 2012). The diploid characteristic makes barley the important material for genome research in the Triticeae crops such as wheat and rye.

Barley is a crop of temperate climate, although it can be also grown in many sub-tropical countries. The crop prefers the areas neither cold nor hot with higher relative humidity. The best climate condition for barley agriculture is air temperature between 0 °C and 18-20 °C with 70-80% relative humidity. Barley can be sown in autumn or spring due to the local climate conditions and classified into winter and spring barley, respectively. Winter barley is more common around the world. Spring barley is grown in the regions having harder winter conditions and if possible, spring sowing should be done as early as possible (The MARS Crop Yield Forecasting System, 2014).

1.2 Wheat (*Triticum aestivum* L.)

Wheat (*Triticum aestivum* L.) is another important domesticated cereal grain originating in the Fertile Crescent (Mori et al., 2003). Southeast Turkey is believed to be the earliest center for emmer (a tetraploid wheat) and einkorn (a diploid wheat) domestication dated 10,000 years ago (Nesbitt and Samuel, 1998; Özkan et al., 2002). Nowadays, wheat is widely grown throughout the world and has become the second most-produced cereal after maize. The total wheat yields in the world for 2013 is 713,182,914 tons in 218,460,701 hectares accounts for a quarter of the total cereal productions (FAOSTAT data, 2014).

Common wheat is referred to the species *Triticum aestivum*, belonging to the genus Triticum of the grass family Poaceae. *T. aestivum* has a complex hexaploid genome (AABBDD, 2n=6x=42), derived from a hybridization between cultivated emmer (*T. turgidum*, AABB, 2n=4x=28), an ancestor of durum wheat grown today, and the grass species, *T. tauschii* (DD, 2n=2x=14) (GRAMENE, 2014; McIntosh et al., 1995). Wheat cultivars are classified by growing season, gluten content, color and the shape of kernels into six classes, hard red winter, hard red spring, soft red winter, durum (hard), hard white wheat and soft white wheat (Small Grains: The Internet Source for Small Grain Growers, 2014).

Wheat is primarily consumed by humans and produces flour for human nutrition. Wheat grain is also used in the production of paste, alcohol, oil and gluten. Wheat straw is used for livestock and poultry feed, and in the industrial manufacture of newsprint, paperboard, and other products (Gibson and Benson, 2002).

Wheat is hexaploid, with a genome size estimated at 17 gigabases, six times of the human genome (Langridge, 2012). Wheat genetics is complicated with three complete subgenomes and 7 pairs of chromosomes in each subgenome in the nucleus of each cell. The chromosome-based draft sequence of wheat genome has been published in 2014, and 124,201 genes were annotated on individual chromosome arm sequences (International Wheat Genome Sequencing Consortium, 2014). Moreover, the sequence assembling of the largest chromosome 3B of wheat provide a template for analysis of the other chromosomes (Choulet et al., 2014).

Wheat is also a temperature crop and can be sown in autumn or spring like barley. It prefers cool climate with moderate rainfall for growing and comparatively high temperatures before harvesting. The air temperature between 7 °C to 20 °C with 750-1600 mm/year rainfall is suitable for wheat growth (The MARS Crop Yield Forecasting System, 2014).

1.3 The genus *Bymovirus* in the family *Potyviridae*

Barley yellow mosaic virus (BaYMV) and *Barley mild mosaic virus* (BaMMV) are the causal agents of the economically important yellow mosaic disease of winter barley in Europe and East Asia. Similarly, *Wheat yellow mosaic virus* (WYMV) and *Wheat spindle streak mosaic virus* (WSSMV) cause significant yield losses of wheat in North America and Asia (Kühne, 2009). All of these four viruses are members of the genus *Bymovirus* in the family *Potyviridae*.

1.3.1 Taxonomy

The genus *Bymovirus*, designated from its type species **B**arley **y**ellow **mo**saic **virus**, belongs to the family *Potyviridae*, the second largest plant virus family after Geminiviridae (Roossinck, 2012). It contains six known species: BaYMV, WYMV, BaMMV, *Oat mosaic virus* (OMV), *Rice necrosis mosaic virus* and WSSMV (Adams et al., 2012). Many of the viruses are related serologically within the genus *Bymovirus* (Kanyuka et al., 2003).

1.3.2 Virion properties

The genus *Bymovirus* in the family *Potyviridae* consists of viruses with bipartite plus-sense RNA genomes, with 7.3–7.6 kb RNA1 and 3.5–3.7 kb RNA2. Each RNA is encapsidated by the coat protein (CP) to form filamentous virions averaging 250–300 nm and 500–600 nm in length, respectively, and 13 nm in diameter. Both RNAs are believed to have a genome-linked viral proteins (VPg) covalently bound to the 5' end and are polyadenylated at the 3' end (Fig. 1-1) (Adams et al., 2012).

1.3.3 Genome organization

RNA1 encodes one large polyprotein that is cleaved by a nuclear inclusion protein a-protease (NIa-Pro) into eight mature proteins: from the 5' proximal region, the gene order is P3, 6K1, cylindrical inclusion protein (CI), 6K2, VPg, NIa-Pro, nuclear inclusion protein b (NIb) and CP genes (Adams et al., 2005) (Fig.1-1A). An additional overlapping open reading frame termed '*pipo*' has been predicted to be expressed as a fusion protein with the N-terminal region of P3 by ribosomal frameshifting (Fig.1-1A) (Chung et al., 2008). The gene organization of bymovirus RNA1 is homologous to that of monopartite virus RNA in other genera in the family *Potyviridae*, expect for the lack of P1 and helper component-protease (HC-Pro) genes in the 5'-terminal region. Instead, RNA2 encodes a polyprotein that is cleaved into P1 and P2 (Fig.1-1B) (Adams et al., 2005). Neither P1 nor P2 has significant amino-acid sequence similarity with the P1 and HC-Pro proteins of other viruses in the family *Potyviridae*.

1.3.4 Functions of viral proteins

Functions encoded on bymovirus genome are postulated by analogy with the genus *Potyvirus* and predicted to have equivalent functions to their counterparts (Adams et al., 2005).

1.3.4.1 RNA1-encoded proteins

P3: It is one of the least conserved proteins and not well characterized even in the genus *Potyvirus*. Potyvirus P3 is required for virus replication (Klein et al., 1994). It also plays a role in host range and symptom development (Adams et al., 2012; Sáenz et

al., 2000; Urcuqui-Inchima et al., 2001).

6K1: The function of this protein is unknown. The counterpart in the genus *Potyvirus* localizes to the cell periphery of infected plant leaves and may be responsible for cell-to-cell movement (Hong et al., 2007).

CI: Potyvirus CI protein has helicase activity for virus replication. It is suggested to be involved in cell-to-cell movement and symptom development (Sochor et al., 2012; Urcuqui-Inchima et al., 2001).

6K2: Potyvirus 6K2 protein is believed to be a small membrane-associated protein anchoring the replication complex to the endoplasmic reticulum (ER). It may affect viral long distance movement and symptom induction (Adams et al., 2012; Sochor et al., 2012; Spetz and Valkonen, 2004).

VPg: VPg is thought to be covalently linked to the 5' end of two genomic RNAs of the bymoviruses. This protein is predicted to be an intrinsic disordered protein (Adams et al., 2012). Incomplete cleavages by NIa-Pro can produce different forms of VPg such as 6K2-VPg, VPg, VPg-NIa-Pro (NIa), 6K2-NIa (You and Shirako, 2010). Potyvirus VPg performs multiple functions in virus infection cycle such as replication, translation and RNA silencing by interacting with host factors (Adams et al., 2012; Urcuqui-Inchima et al., 2001). Bymovirus VPg has been also referred as a virus determinant in overcoming the eukaryotic translation initiation factor 4E (eIF4E)-mediated recessive resistance (Kanyuka et al., 2005; Stein et al., 2005).

NIa-Pro: The NIa-Pro protein is a serine-like cystein proteinase that catalyses

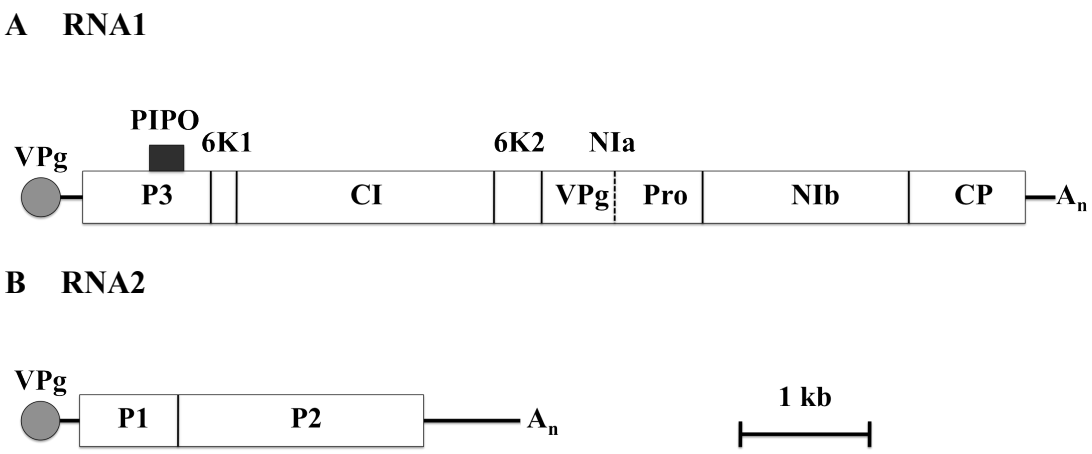

Fig. 1-1 Schematic representation of the genome organization of the genus *Bymovirus*. **(A)** RNA1:RNA1-encoded polyprotein is processed by NIa-Pro into eight mutrue proteins. A small box above the long ORF indicates the *pipo* ORF. **(B)** RNA2: RNA2-encoded polyproteins cleaved into two proteins by P1 proteinase. Both RNA1 and RNA2 have VPg attached to the 5' end and are polyadenylated at the 3' end. Map scale, 1 kilobase (kb).

proteolytic processes at the seven sites in RNA1-encoded polyprotein (Adams et al., 2012). Moreover, potyvirus NIa-Pro contributes another function in host specificity (Chen et al., 2008; Sochor et al., 2012).

NIb: Potyvirus NIb protein is the only RNA-dependent RNA polymerase and essential for virus genome replication (Adams et al., 2012).

CP: CP is the only structural protein of the viruses in the family *Potyviridae*. Potyvirus CP also plays a role in cell-to-cell movement and long distance movement and aphid transmission (Sochor et al., 2012; Urcuqui-Inchima et al., 2001).

PIPO: *pipo* gene is also predicted in the genus *Bymovirus*. It is embedded within the P3 gene and translated as a fusion protein P3N-PIPO by +2 frameshifting. Potyviral P3N-PIPO facilitates the cell-to-cell movement (Chung et al., 2008; Vijayapalani et al., 2012; Wei et al., 2010; Wen and Hajimorad, 2010).

1.3.4.2 RNA2-encoded proteins

P1: P1 protein is a cysteine autoproteinase that cleaves RNA2-encoded polyprotein into two mature proteins P1 and P2. P1 contains a proteinase domain homologous to the C-terminal region of the potyviral HC-Pro (Adams et al., 2005; Kashiwazaki et al., 1991). Study on BaYMV reveals that P1 facilitates the accumulation of CP in barley cells (You and Shirako, 2010).

P2: P2 protein is unique to bymoviruses without homology with any protein in the genus *Potyvirus*. P2 may play a role in vector transmission by *Polymyxa graminis* as judged by amino acid homology with the capsid protein readthrogh (CP-RT) domains of

the viruses in the genera *Furovirus*, *Pomovirus* and *Benyvirus*, which are transmitted by the zoospores of plasmodiophorids. In addition, the P2 protein of BaYMV has been proved to be required for efficient systemic spread in the host plant (You and Shirako, 2010).

1.3.5 Host range

The host range of bymoviruses is restricted to the family *Gramineae*. Each species of the genus *Bymovirus* can infect only one single type of host in the fields. For instance, the barley-infecting viruses do not infect wheat and the wheat-infecting viruses do not infect barley (Adams et al., 2012; Kanyuka et al., 2003).

1.3.5 Transmission

One feature of bymoviruses distinguished from other genera of the family *Potyviridae* is transmission in soil by the root-inhabiting *Polymyxa graminis*, a plasmodiophoraceous protest (Adams et al., 2012). The life cycle of *P. graminis* can be separated into two phases resulting in the production of zoospores and resting spores. Virus acquisition and transmission by *P. graminis* on cereal crops are still unknown. However, *P. graminis* acquires the viruses inside the zoospores and resting spores from the infected plants. The viruliferous resting spores survive in soil for many decades, and they can cause virus spread to the plants under suitable environmental conditions (Kanyuka et al., 2003). Bymoviruses can be also transmitted by mechanical inoculation in the laboratory condition (Adams et al., 2012).

1.4 *Barley yellow mosaic virus* (BaYMV)

BaYMV is the type species of the genus *Bymovirus*. It was first reported in 1940 in Japan (Ikata and Kawai, 1940). BaYMV causes a yellow mosaic disease in winter barley, sometimes by mixed infection with BaMMV, resulting in serious yield reductions in Europe and East Asia (Chen et al., 1999; Huth and Adams, 1990; Lee et al., 2006; Nishigawa et al., 2008).

1.4.1 Classification

In Japan, BaYMV was classified into eight strains, named I-1, I-2, I-3, II-1, II-2, III, IV and V, based on their pathogenicity to different barley cultivars (Kashiwazaki et al., 1989; Nishigawa et al., 2008; Sotome et al., 2010; Usugi et al., 1985). However, the current BaYMV classification system is not applicable for all the Japanese stains of BaYMV and new strains have been continually occurring in the barley fields (Sotome et al., 2011; You and Shirako, 2013). In Europe, BaYMV are classified into two strains BaYMV-1 and BaYMV-2 by the ability to overcome the *rym4*-mediated resistance in barley plants (Kühne et al., 2003). Otherwise, the other strains isolated from China and Korea are not systemically classified.

1.4.2 Resistance to BaYMV

Because *P. graminis* can survive in soil for many decades, chemical measures to prevent *P. graminis*–transmitted virus diseases are either ineffective or unacceptable for ecological and economical reasons. Therefore, the only way to control these diseases is to use resistant cultivars (Ordon et al., 2009).

At present, total eighteen resistance genes, including fifteen recessive genes termed *rym* (**r**esistance to **y**ellow **m**osaic) and three dominant genes termed *Rym*, have been identified in barley resistant to different isolates of BaYMV and BaMMV. By molecular analysis, recessive resistance genes *rym1–13*, *rym15* and *rym18* were mapped on six chromosomes 1H, 3H, 4H, 5H, 6H and 7H, and dominant resistance genes $Rym14^{Hb}$, $Rym16^{Hb}$ derived from *Hordeum bulbosum* and *Rym17* were mapped on chromosome 6H, 2H and 4H, respectively (Kai et al., 2012; Kanyuka et al., 2003; Ordon et al., 2009). Of these resistance genes, *rym4*, *rym5* and *rym6* genes are recognized as allelic genes coding for eIF4E on chromosome 3H (Kanyuka et al., 2005; Stein et al., 2005).

The resistance genes of *rym1–6* have been used for breeding of barley cultivars resistant to BaYMV and BaMMV, but none of these genes confers complete resistance to any isolate of BaYMV by their own and to make the situation worse, new BaYMV virulent strains continually emerging (Kühne, 2009; Ordon et al., 2005; Sotome et al., 2010; Sotome et al., 2011; You and Shirako, 2013). In Europe, resistant cultivars harboring *rym4* have been grown for a long period, but the resistance was overcome by BaYMV-2 (Kühne et al., 2003). Afterwards, *rym5* instead of *rym4* was utilized to barley breeding in Europe but again overcome by BaMMV (Habekuss et al., 2008; Hariri et al., 2003; Kanyuka et al., 2004). In Japan, resistant cultivars carrying *rym5* has been bred and widely grown in fields, but the resistance was soon overcome by BaYMV-III (Kashiwazaki et al., 1989). However, cultivars harboring *rym5* and *rym3* are resistant to all strains of BaYMV currently identified in Japan (Sotome et al., 2010; Sotome et al., 2011). Moreover, Mokusekko 3, a Chinese landrace of barley, carrying

rym1, *rym5* and another unidentified resistance gene, is completely resistant against all identified strains of BaYMV and BaMMV and used for breeding barley cultivars with better suitable resistance in Japan (Konishi et al., 1997; Miyazaki et al., 2001). Nowadays, in addition to identification of new resistant genes, combination of two or more resistance genes in barley breeding has been a feasible strategy to prevent virus infection (Kai et al., 2012; Ordon et al., 2005).

1.5 *Wheat yellow mosaic virus* (WYMV)

WYMV is another agriculturally important species in the genus *Bymovirus* and causes a yellow mosaic disease in wheat. WYMV first described in 1927 in Japan (Sawada, 1927) and has continually spread in Japan and China (Han et al., 2000; Namba et al., 1998; Ohki et al., 2014).

1.5.1 Classification

The WYMV was classified into three pathotypes I, II and III in Japan on the basis of responses to different wheat cultivars (Ohto et al., 2006).

1.5.2 Resistance to WYMV

It has been reported that a number of wheat cultivars confer resistance to WYMV. Because WYMV resistance was controlled by one to three genes, the genetics and molecular mechanisms are complex and poorly understood (Zhu et al., 2012). WYMV resistance genes were mapped to chromosome 2D in the European wheat cultivar Ibis (Nishio et al., 2010) and the Chinese cultivar Yangfu 9311 (Liu et al., 2005a), respectively, to 2A in the Chinese cultivar Ningmai 9 (Liu et al., 2005b) and to 3B, 5A and 7B in the variety Xifeng originally introduced from Japan into China (Zhu et al., 2012).

1.6 Eukaryotic translation initiation factor 4E (eIF4E)

Of all known resistance genes against potyviruses, a large fraction is recessive (Provvidenti and Hampton, 1992). These recessive genes have been identified to encode translation initiation factors, eIF4E, eIF4G or their isoforms, of which eIF4E is the best-studied one (Fraile and García-Arenal, 2012). Fifteen out of eighteen *rym* genes resistant to BaYMV are recessively inherited and *rym4*, *rym5*, and *rym6* are eIF4E-mediatd resistance genes (Kai et al., 2012; Kanyuka et al., 2005; Ordon et al., 2005; Stein et al., 2005).

1.6.1 Roles of eIF4E in plants

The majority of translation in eukaryotic cells is cap-dependent and mediated by eIF4F, a multisubunit protein complex composed of eIF4E, eIF4G and eIF4A. eIF4E is an essential translation initiation factor that recognizes of the mRNA cap structure. eIF4E binding to the 7-methylguanosine cap structure is considered as the first step in translation initiation (Malys and McCarthy, 2011). In plants, two isoforms of eIF4F are present and seem to have complementary role: eIF4F composed eIF4E and eIF4G, and eIF(iso)4F composed eIF(iso)4E and eIF(iso)4G (Browning, 2004).

1.6.2 Possible roles of eIF4E in the virus infection

The role of eIF4E in virus infection has not been fully understood. eIF4E is hypothesized to be involved in several processes of virus life cycle, including translation, replication and cell-to-cell movement (Truniger and Aranda, 2009; Robaglia and Caranta, 2006).

It has been speculate that the interaction of VPg and eIF4E facilitates viral RNA translation (Lellis et al., 2002; Thivierge et al., 2005). It is possible that the VPg functions as a cap mimic and recruits the translation initiation factors for viral RNA translation (Dreher and Miller, 2006; Robaglia and Caranta, 2006).

The interaction of VPg and eIF4E may also participate in viral genome replication (Gao et al., 2004; Schaad et al., 2000). The uridylated VPg as a primer might be positioned near the viral poly(A) by interaction with eIF4E for complementary strand synthesis (Herold and Andino, 2001; Robaglia and Caranta, 2006).

In addition, the VPg–eIF4E interaction may play a role in cell-to-cell movement of viral RNA(Gao et al., 2004). It can be explained by the interaction of the cytoskeletal structures with the translational machinery facilitating cell-to-cell movement of viral genome (Bokros et al., 1995; Robaglia and Caranta, 2006).

1.6.3 Virus counterparts of eIF4E

VPg is a key virus counterpart of eIF4E in resistance has been proved in many cases of potyviruses. Recently, some other viral factor, such as HC-Pro, CI and P1 are also identified to interact with eIF4E.

VPg: VPg associated with eIF4E is essential for viral infection as mentioned above. The interaction has been proved by yeast-two hybrid (Y2H) assay in potyviruses (Léonard et al., 2000; Schaad et al., 2000).

P1: *Clover yellow vein virus* (ClYVV) with a single mutation introduced to P1 was able to break eIF4E–mediated resistance in pea (Nakahara et al., 2010).

HC-Pro: Potyvirus HC-Pro contains an eIF4E-binding motif. Introduced mutations in the motif affected the virulence of *Potato virus A* (PVA), suggesting an unknown role for HC-Pro and eIF4E in virus infection (Ala-Poikela et al., 2011).

CI: CI of *Lettuce mosaic virus* (LMV) also functions as a virulence determinant responsible for overcoming eIF4E–mediated recessive resistance. The interaction of CI–eIF4E and CI–VPg were confirmed *in vivo* by bimolecular fluorescence complementation (BiFC) assays (Abdul-Razzak et al., 2009; Tavert-Roudet et al., 2012).

1.7 Aim and significance of this study

Viruses in the genus *Bymovirus* are widespread around the world and threaten the production of cereal crops. They are known for long but still poorly understood with difficulty in mechanical transmission. Establishment of a reverse genetics system for BaYMV provides a convenient approach to study these bymoviruses in the laboratory. The objective of the present study is to determine infectivity of two bymoviruses BaYMV and WYMV and evaluate the host resistance using infectious cDNA clones.

In this study, complete genome sequences of a Tochigi isolate of BaYMV from *rym5* plants were determined and analysed by comparison with the other published isolates. Then, the infectious full-length cDNA clones of this isolate were constructed and used for infectivity assay. A set of susceptible and resistant barley varieties was utilized to evaluate the virus pathogenicity at both cellular and whole plant levels. Subsequently, both viral and host factors involved in overcoming *rym5* resistance were examined. On the one hand, mapping analysis of several chimeric constructs between Tochigi isolate (JT10, *rym5*–breaking) and Kurashiki isolate (JK05, *rym5*–nonbreaking) revealed that VPg played a key role in overcoming *rym5* resistance. Further mutagenetic analysis showed Ser at the position 118 and His at the position 142 of VPg are the most important amino acids responsible for overcoming *rym5* resistance. On the other hand, *rym5* is indeed an eIF4E gene was proved. Co-expression of susceptible eIF4E enabled *rym5*–nonbreaking virus to replicate in *rym5* barley cells. Additionally, viral determinant in overcoming a non-eIF4E-mediated resistance *rym2* was also studied at the cellular level. Moreover, replicative capability of BaYMV and WYMV were investigated in barley and wheat protoplasts using infectious cDNA clones.

BaYMV replicated in both barley and wheat protoplasts, but WYMV replicated only in wheat protoplasts. Further analysis indicated both VPg and the host eIF4E are involved in the host tropism of BaYMV and WYMV at the replication level.

Infectivity assays carried out in both protoplast cells and whole plants were helpful for resistance study. It is clear to understand which step of replication or movement was blocked during resistance occurred. The interaction between VPg an eIF4E required for viral infection has been proved for many potyviruses. It is the first time to testify this hypothesis for bymoviruses. The studies here provided functional evidence for the importance of VPg and eIF4E for bymovirus infection. This research broads our understanding in overcoming resistance against bymoviruses and sets a useful model for understanding other resistance mechanisms.

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Chapter 2

Analysis of a *Barley yellow mosaic virus* Tochigi isolate JT10 and the host resistance

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2.1 Genome characterization

2.1.1 Introduction

BaYMV causes a yellow mosaic disease of winter barley in East Asia and Europe, resulting in significant yield losses in barley production. So far, dozens of BaYMV isolates from different countries have been sequenced, fifteen of which have been published with the full-length sequences of RNA1 and RNA2 in the Genbank database (Chen et al., 1999; Davidson et al., 1991; Kashiwazaki et al., 1990; Kashiwaziki et al., 1991, Kühne et al., 2003; Lee et al., 2006; Nishigawa et al., 2008; Peerenboom et al, 1992; You and Shirako, 2010). BaYMV has been classified into eight strains (I-1, I-2, I-3, II-1, II-2, III, IV and V) in Japan based on the pathogenetic responses of different cultivars (Kashiwazaki et al., 1989; Nishigawa et al., 2008; Sotome et al., 2010; Usugi et al., 1985) and two strains in Europe based on the pathogenecity of *rym4*–mediated resistance (Kühne et al., 2003). BaYMV is still not well studied especially in resistance research. It remains unknown that how the resistance is broken by the virus and what kinds of viral and host factors are required for breaking resistance. Currently, only one group in Germany predicted that VPg was a viral determinant in breaking *rym4* by analysis of amino acid sequences of *rym4*–breaking and –nonbreaking isolates. There is no experimental evidence for this prediction yet. Studies on BaYMV in Japan are still limited to sequence determination and no further analysis was performed for breaking resistance.

Here, a *rym5*–breaking strain from Tochigi Prefecture in Japan was isolated and used for study on *rym5*–mediated resistance. The viral and host factors were examined

using *in vitro* transcripts from cDNA clones of this isolate in this study. At the beginning of this study, the complete sequence of this isolate was determined and analysed by comparing with the other published isolates.

2.1.2 Materials and methods

2.1.2.1 Virus isolation

BaYMV Tochigi isolate JT10 was collected from a *rym5*–resistant barely cultivar Mikamo Golden grown at Tochigi Prefecture, Japan. Virions were purified from 2 g infected leaves by a procedure described previously (Shirako and Brakke, 1984) and suspended in RNase-free dH_2O. Virus suspension was treated with 20 μg Proteinase K in 20 mM Tris-HCl (pH 7.5), 20 mM EDTA and 0.5% SDS at 37 °C for 30 min, then immediately chilled on wet ice (mixture of ice and water). Subsequently, the reaction mixture was mixed with STE buffer (120 mM NaCl, 24 mM Tris-HCl, pH7.5 and 1.25 mM EDTA), then transferred to room temperature, and treated with 20 mM DTT and 1% SDS. The mixture was then incubated at 95 °C for 1 min and again chilled on wet ice. Extracted RNA was precipitated with 7.5 M NH_4Ac, 100% ethanol and glycogen at -70 °C for 10 min, spun at 13,000 rpm for 3 min, washed with 80% ethanol. Viral RNA was resuspended in RNase-free dH_2O and used as a template for cDNA synthesis.

2.1.2.2 Nucleotide sequence determination

First-strand cDNA was synthesized using PrimeScript® Reverse Transcriptase (Takara Bio, Japan) and then amplified by overlapping RT-PCR using PrimeSTAR® HS DNA Polymerase. All amplification products were purified using the FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Japan) and then directly submitted to a commercial sequencing service (Eurofins Operon, Japan). All the primers used for this purpose were listed in the Appendix Table S1.

2.1.2.3 Sequence assembly and phylogenetic analysis

After sequencing, all the sequences were assembled by Factura software (ABI) to obtain the full-length nucleotides of Tochigi isolate JT10. Multiple sequence alignments were performed using the translated amino acid sequences by Genetyx-Mac (Genetyx, Japan) software, and phylogenetic trees were constructed using UPGMA method implemented in the software.

2.1.3 Results

2.1.3.2 Genome organization analysis of BaYMV Tochigi isolate JT10

Complete nucleotide sequences of RNA1 and RNA2 of BaYMV Tochigi isolate JT10 were determined by direct sequencing of RT-PCR products amplified from extracted virus RNA. Genome sequences and organization were analysed based on the predication by Adams et al. (2005). As shown in Fig. 1-1 and Table 2-1, RNA1 was 7,642 nucleotides in length excluding the poly(A) tail at the 3' end, containing a long ORF of 7,239 nucleotides. It encoded a 271-kDa polyprotein, which is subsequently cleaved into eight mature proteins (P3/6K1/CI/6K2/VPg/NIa-Pro/NIb/CP) by NIa-Pro. RNA1 also contained a +2 shift reading frame (nt 684-941) encoding a small protein PIPO. RNA2 was 3,585 nucleotides in length, containing an ORF of 2,673 nucleotides. It encoded a 98-kDa polyprotein, which is cleaved into two mature proteins (P1/P2) by P1 proteinase. RNA1 and RNA2 nucleotide sequences of Tochigi isolate JT10 were deposited in the Genbank database with the accession number AB920780 for RNA1 and AB920781 for RNA2.

2.1.3.2 Phylogenetic analysis of BaYMV isolates

Up to date, sixteen of full-length RNA1 and twelve of full-length RNA2 sequences have been published in BaYMV, including Tochigi isolate JT10. To elucidate the phylogenetic relationships of this isolate in the BaYMV group, both RNA1- and RNA2-encoded polyprotein sequences were subjected to phylogenetic analysis with those of the other known isolates. The phylogenetic trees were constructed using the UPGMA method. For RNA1-encoded polyprotein 1 analysis (Fig. 2-1A), two subgroups,

Table 2-1 Genome organization of BaYMV Tochigi isolate JT10.

RNA1				**CDS[a] (nt 172-7410)**						
Long ORF	**5'-UTR**	**P3**	**6K1**	**CI**	**6K2**	**VPg**	**NIa-Pro**	**NIb**	**CP**	**3'-UTR**
Position	1-171	172-1155	1156-1353	1354-3330	3331-3696	3697-4257	4258-4932	4933-6516	6517-7407	7411-7642
nt length	171	984	198	1977	366	561	675	1584	891	232
aa length		328	66	659	122	187	225	528	297	
+2 F/S[b]	**PIPO**									
Position	684-941									
nt length	258									
aa length	86									

RNA2		**CDS (nt 155-2827)**		
	5'-UTR	**P1**	**P2**	**3'-UTR**
Position	1-154	155-919	920-2824	2828-3585
nt length	154	765	1905	758
aa length		255	635	

[a], CDS, coding DNA sequence.
[b], +2 F/S, +2 shift reading frame

separating Asian isolates from European isolates were clearly identified. Within the Asian isolates, polyproteins 1 of Tochigi isolate JT10 was closest to that of a BaYMV-III isolate. Analysis of RNA2-encoded polyprotein 2 (Fig. 2-1B) consistently revealed a division of two subgroups of Asian and European isolates. Unlike polyprotein 1 analysis, polyprotein 2 of Tochigi isolate JT10 showed a closer phylogenetic relationship to Kurashiki isolate JK05 than the other isolates.

2.1.3.3 Sequence comparison of Tochigi isolate JT10 with two other Japanese isolates

As Tochigi isolate JT10 was closer to the BaYMV-III and Kurashiki JK05 isolates in evolution, comparisons of these three isolates were performed at both nucleotide and amino acid levels (Table 2-2 and Fig. 2-2). In the case of polyprotein 1, Tochigi isolate JT10 shared higher sequence identity with that of the BaYMV-III isolate (98.3% and 99.1% at nucleotide and amino acid levels, respectively) than with that of Kurashiki isolate JK05 (98.4% and 98.8% at nt and aa levels, respectively). The polyprotein 1 of Tochigi isolate JT10 was different at 22 positions from that of the BaYMV-III isolate and at 29 positions from that of Kurashiki isolate JK05 among the whole polyprotein 1. Moreover, the NIb and PIPO of Tochigi isolate JT10 were identical to those of the BaYMV-III isolate and the CP was identical to that of Kurashiki isolate JK05. In the case of polyprotein 2, Tochigi isolate JT10 was closely related to Kurashiki isolate JK05 with an amino aicd identity of 98.9% and 10 amino acid differences among the polyprotein 2. The polyproteins 2 of Tochigi isolate JT10 showed 98.1% similarity to and 17 amino acid differences with that of the BaYMV-III isolate.

A

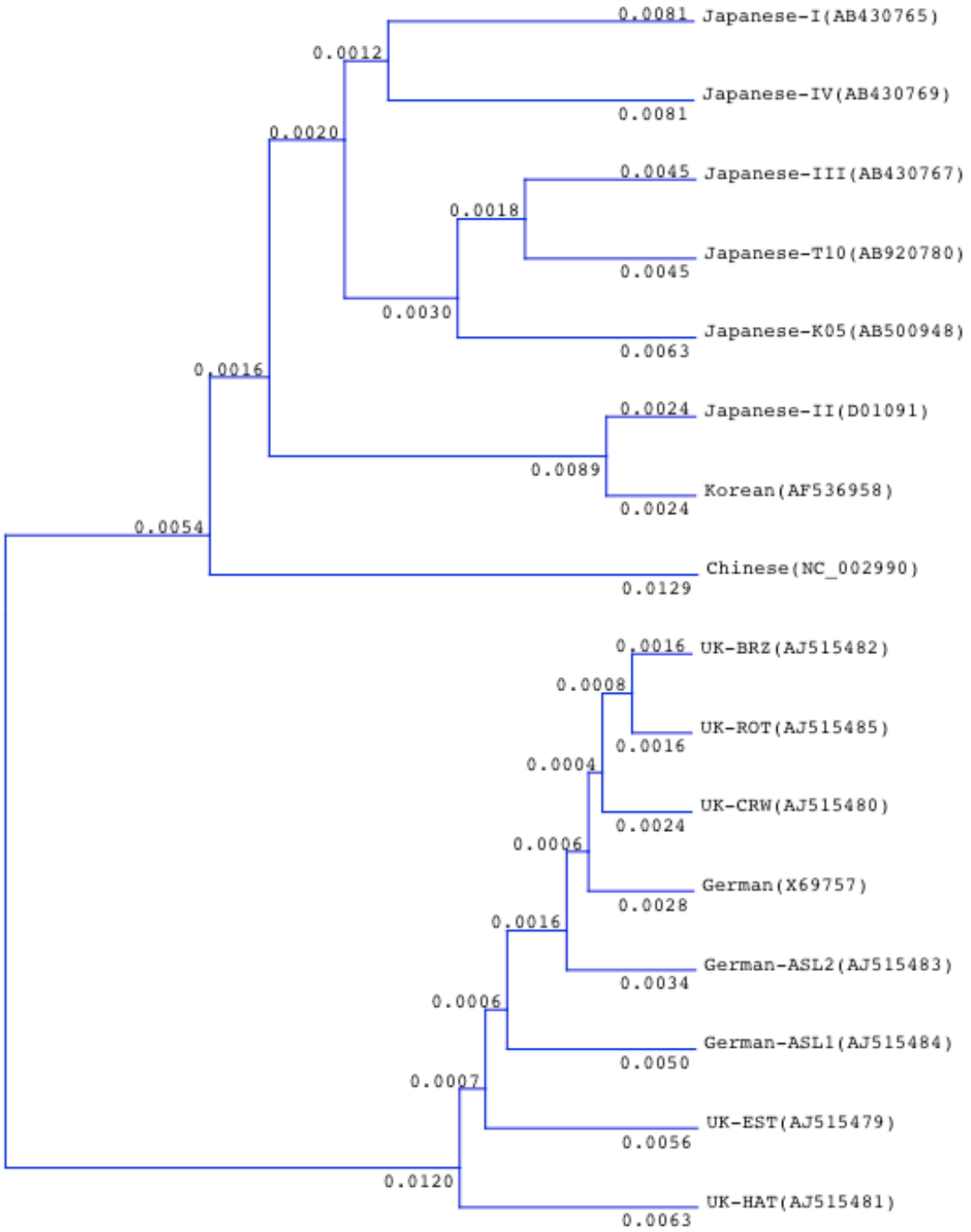


Fig. 2-1 Phylogenetic trees based on alignments of amino acid sequences of BaYMV RNA1- **(A)** and RNA2- **(B)** encoded polyproteins. **(A)** UPGMA phylogenetic tree constructed from the alignments of the translated amino acid sequences of the BaYMV RNA1 CDSs from 16 isolates. The numbers over the branches represented the distance data. The selected strains were from the Genbank database. The numbers in parentheses represented the accession number in the database.

B

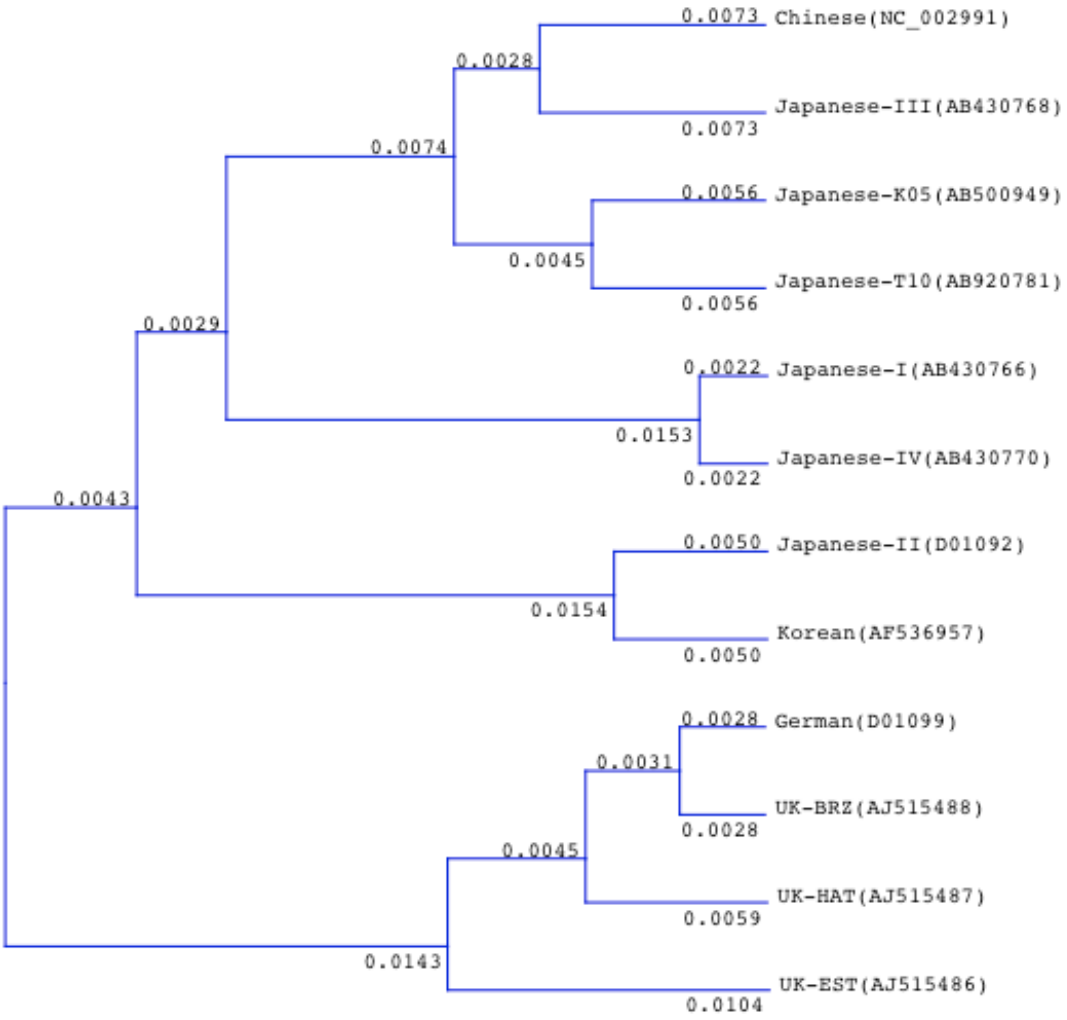


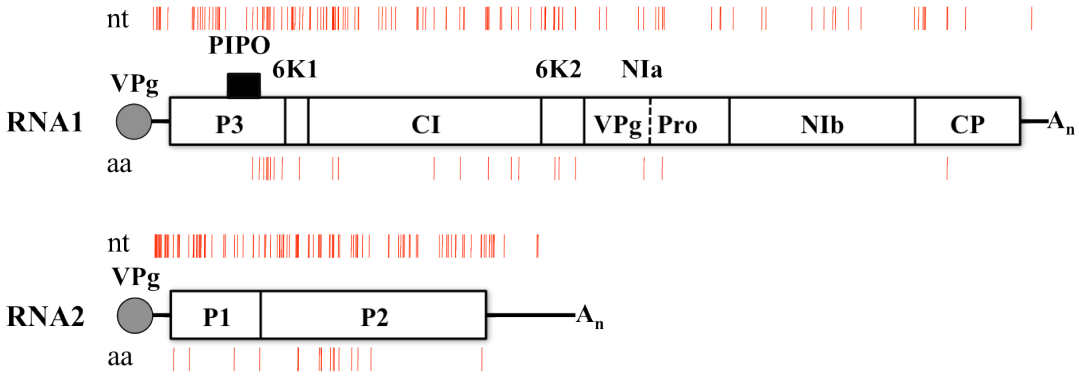
Fig. 2-1 Phylogenetic trees based on alignments of amino acid sequences of BaYMV RNA1- **(A)** and RNA2- **(B)** encoded polyproteins. **(B)** UPGMA phylogenetic tree constructed from the alignments of the translated amino acid sequences of the BaYMV RNA2 CDSs from 12 isolates. The numbers over the branches represented the distance data. The selected strains were from the Genbank database. The numbers in parentheses represented the accession number in the database.

Table 2-2 Nucleotide and amino acid sequence identities between Tochigi isolate JT10 and two other isolates (the BaYMV-III and Kurashiki JK05 isolates).

		BaYMV–III isolate (AB430767 and AB430748) [a]		**Kurashiki isolate JK05** (AB500948 and AB500949)	
		nt	**aa**	**nt**	**aa**
RNA1	**Full length**	7512/7642(98.3%)	—	7518/7642 (98.4%)	—
	Long ORF	7117/7239 (98.3%)	2390/2412 (99.1%)	7119/7239 (98.3%)	2383/2412 (98.8%)
	5' UTR	164/171 (95.9%)	—	167/171 (97.7%)	—
	P3	959/984 (97.5%)	320/328 (97.6%)	958/984 (97.4%)	317/328 (96.6%)
	6K1	187/198 (95.4%)	65/66 (98.5%)	190/198 (96.0%)	64/66 (97.0%)
	CI	1930/1977 (97.6%)	652/659 (98.9%)	1933/1977 (97.8%)	655/659 (99.4%)
	6K2	355/366 (97.0%)	119/122 (97.5%)	354/366 (96.7%)	118/122 (96.7%)
	VPg	557/561 (99.3%)	186/187 (99.5%)	553/561 (98.6%)	181/187 (96.8%)
	NIa-Pro	668/675 (99.0%)	224/225 (99.6%)	668/675 (99.0%)	224/225 (99.6%)
	NIb	1574/1584 (99.4%)	528/528 (100%)	1574/1584 (99.4%)	527/528 (99.8%)
	CP	884/891 (99.2%)	296/297 (99.7%)	886/891 (99.4%)	297/297 (100%)
	3' UTR	231/232 (99.6%)	—	232/232 (100%)	—
	PIPO	255/258 (98.8%)	86/86 (100%)	254/258 (98.4%)	85/86 (98.8%)
RNA2	**Full length**	3476/3585 (97.0%)	—	3504/3586 (97.7%)	—
	ORF	2587/2673 (96.8%)	873/890 (98.1%)	2611/2673 (97.7%)	880/890 (98.9%)
	5' UTR	139/154 (90.3%)	—	144/154 (93.5%)	—
	P1	739/765 (96.7%)	251/255 (98.4%)	740/765 (96.9%)	251/255 (98.4%)
	P2	1845/1905 (96.9%)	622/635 (98.0%)	1868/1905 (98.1%)	629/635 (99.1%)
	3'UTR	750/758 (98.9%)	—	749/759 (98.6%)	—

[a] The numbers in parentheses represented the accession number in the database.

A BaYMV Tochigi isolate JT10 and BaYMV-III isolate



B BaYMV Tochigi isolate JT10 and Kurashi isolate JK05

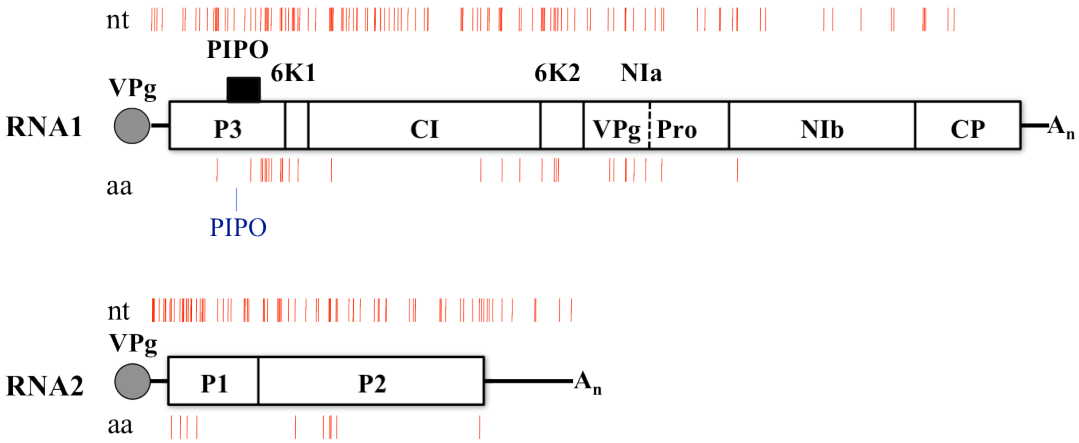


Fig. 2-2 Nucleotide and amino acids differences of BaYMV Tochigi isolate JT10 compared with BaYMV-III **(A)** and Kurashiki isolate JK05 **(B)**. The upper red lines of each RNA indicate the differences at the nucleotide level and the lower red lines indicate the differences at the amino acid level. The blue lines indicate the amino acid difference in the PIPO protein between Tochigi JT10 and Kurashiki JK05 isolates.

2.1.4 Discussion

Phylogenetic analysis was carried out between Tochigi isolate JT10 and the other published BaYMV isolates. Two clusters representing Asian isolates and European isolates were consistently identified in phylogenetic trees for RNA1-encoded polyprotein 1 (Fig. 2-1A) and RNA2-encoded polyprotein 2 (Fig. 2-1B), suggesting that these two subgroups of BaYMV followed different evolutionary paths. Tochigi isolate JT10, as a Japanese isolate, had a closer relationship to the Kurashiki JK05 and BaYMV-III isolates. Analyses of polyprotein 1 and polyproteins 2 of Tochigi isolate JT10 showed similar but distinct phylogenetic relationship to the other isolates. The polyprotein 1 of Tochigi isolate JT10 was closer to that of the BaYMV-III isolate than Kurashiki isolate JK05, whereas the polyprotein 2 was closer to that of Kurashiki isolate JK05. In agreement with this result, the polyprotein 1 of Tochigi isolate JT10 shared higher amino acid identity with the BaYMV-III isolate than Kurashiki isolate JK05, whereas the polyprotein 2 had higher amino acid identity with Kurashiki isolate JK05. Tochigi JT10 and the BaYMV-III isolates were geographically close in the source. They were obtained from the BaYMV infected *rym5* plants of Mikamo Golden grown at the different barley fields in the Tochigi Prefecture in Japan (Nishigawa et al., 2008). Even so, they were still distinct isolates with 22 amino acid differences in polyprotein 1, 17 amino acid differences in polyprotein 2.

Tochigi isolate JT10 can break *rym5* resistance and Kurashiki isolate JK05 was non-infectious in *rym5* cells and plants (You and Shirako, 2013). Examination of viral determinants involved in breaking *rym5* resistance could be performed by comparative analysis between these two isolates. This will be studied in detail in 2.3.

2.2 Infectivity assay at the cellular and whole plant levels using infectious *in vitro* transcripts

2.2.1 Introduction

Breeding of resistant cultivars that harbor one or more resistance gene(s) is believed to be the only approach to control the *P. graminis*–transmitted virus disease and to prevent severe losses of both yield and quality of the crop in field (Kanyuka et al., 2003; Kühne, 2009). At present, total eighteen resistance gene against yellow mosaic disease caused by BaYMV and BaMMV have been identified, fifteen of which were described as recessively inherited genes (Kai et al., 2012; Kanyuka et al., 2003; Ordon et al., 2009). Of these recessive resistance genes, *rym1–6* are commonly used for breeding of barley resistant cultivars, but none of these related genes could confer a complete resistance to any BaYMV isolate by their own (You and Shirako, 2013). Moreover, resistance genes were also challenged by the constant emergence of new resistant breaking strains (Kühne, 2009; Ordon et al., 2005; Sotome et al., 2010; Sotome et al., 2011). Combining of respective resistance genes in barley breeding has become an effective solution to these situations. The key issue is how to screen suitable resistance genes for combining, which is relay on the understanding of viral pathogenecity and resistance mechanisms (Kai et al., 2012; Ordon et al., 2005).

Previous reports showed that barley varieties with *rym1* gene were susceptible to BaYMV-III, -IV and -V; barley varieties with *rym2* gene were susceptible to BaYMV-II; barley varieties with *rym3* gene was susceptible to BaYMV-IV and -V; barley varieties with *rym4* gene was susceptible to BaYMV-III, -IV and European strain

BaYMV-2; barley varieties with *rym5* gene was susceptible to BaYMV-III and with *rym6* gene was only resistant to BaYMV-II (Table 2-3) (Kashiwazaki et al., 1989; Konishi et al., 1997; Ordon et al., 2005; Sotome et al., 2010; Sotome et al., 2011; You and Shirako, 2013). In the past, barley cultivars carrying *rym5* resistance against all the strains of BaYMV and BaMMV were widely grown in Japan until this resistance gene was broken by BaYMV BaYMV-III isolate (Kashiwazaki et al., 1989). To solve this problem, it requires combining *rym5* and one and more other resistance gene(s) in barley breeding. Mokusekko 3, a Chinese landrace of barley, carrying *rym1*, *rym5* and another unidentified resistance gene, is completely resistant against all the strains of BaYMV and BaMMV and used for breeding barley cultivars with durable resistance in Japan (Konishi et al., 1997; Miyazaki et al., 2001).

Construction of infectious full-length cDNA clone is an important approach to investigate the viral infection both at the cellular and whole plant levels. Until recently, a reverse genetics system for BaYMV was established and used for infectivity assay in the protoplasts and plants (You and Shirako, 2010, 2012, 2013). The feature of BaYMV, viral pathogenicity and resistance mechanisms were well studied by using *in vitro* transcripts.

In this subchapter, the full-length cDNA clones of Tochigi isolate JT10 were constructed and used for infectivity assay at the cellular and whole plant levels.

Table 2-3 Responses of barley cultivars with different resistance genes (*rym* genes) to BaYMV strains in field tests [a,b].

Barely cultivars	*rym* genes	Japanese strains					European strains	
		I	**II**	**III**	**IV**	**V**	**1**	**2**
Nittakei 68	***rym1***	R	R	S	S	S	R	R
Mihori Hadaka 3	***rym2***	R	S	R	R	R	R	R
Ishuku Shirazu /Haganemugi	***rym3***	R	R	R	S	S	R	R
Franka	***rym4***	R	R	S	S	R	R	S
Mikamo Golden	***rym5***	R	R	S	R	R	R	R
Amagi Nijo	***rym6***	S	R	S	S	S	S	S

[a] The responses of barley cultivars to BaYMV strains were referred to Kashiwazaki et al. (1989); Konishi et al. (1997); Ordon et al. (2005); Satome et al. (2010, 2011) and You and Shirako (2013).
[b] S, susceptible; R, resistant.

2.2.2 Materials and methods

2.2.2.1 Plants

Twelve varieties of barley were chosen to evaluate the pathogenecities of Tochigi JT10 and Kurashiki JK05 isolates of BaYMV: Ryofu and KoA used as susceptible controls, Nittakei 68 (*rym1*), Mihori Hadaka 3 (*rym2*), Ishuku Shirazu (*rym3*), Haganemugi (*rym3*), Express (*rym4*), Mikamo Golden (*rym5*), Misato Golden (*rym5*), Haruna Nijo (*rym6*), Amagi Nijo (*rym6*) and Miho Golden (*rym6*). Ryofu seeds were purchased from a local JA (Japan Agriculture Cooperative) branch. Seeds of Nittakei 68 were provided by the Bioresources Research and Development Department of Sapporo Breweries Ltd, Japan. Barley Seeds of Express was kindly granted by Dr. Thomas Kühne in the Institute of Resistance Research and Pathogen Diagnostics, Germany. The other barley seeds were obtained from the Institute of Plant Science and Resources at Okayama University, Japan.

2.2.2.2 Construction of full-length cDNA clones of Tochigi isolate JT10

Construction of infectious full-length cDNA clones of BaYMV Tochigi isolate JT10 RNA1 and RNA2 (Fig. 2-3) were performed as described for Kurashiki isolate JK05 isolate (You and Shirako, 2010). The infectious cDNA clones for RNA1 and RNA2 of Kurashiki isolate JK05 were redefined as pBY-K1 and pBY-K2 instead of pBY1 and pBY2 used for previous study. To generate a full-length cDNA clone of JT10 RNA1, designated as pBY-T1, I did RT-PCR using TB162 (Fig. 2-3 and Appendix Table S2) for first-strand cDNA synthesis and TB87 (Fig. 2-3 and Table S2) and TB162 for PCR procedure. The amplified 7.8-kb product was purified and digested with *Xba*I

and *Bam*HI, then cloned into the *Xba*I–*Bam*HI sites modified from pBY-K1 constuct (infectious cDNA clone of RNA1 of Kurashiki JK05 isolate) (You and Shirako, 2010). For construction of cDNA clone of RNA2, designated as pBY-T2, I did RT-PCR using TB166 (Fig. 2-3 and Table S2) for first-stand cDNA synthesis and TB163 (Fig. 2-3 and Table S2) and TB166 for PCR amplification. The purified 3.7-kb PCR product was digested with *Bam*HI and *Spe*I, then cloned into the *Bam*HI–*Spe*I sites of pBY-K2 (infectious cDNA clone of Kurashiki JK05 isolate) (You and Shirako, 2010). Both of the recombinant DNA procedures were carried out by standard methods using *Escherichia coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts. All the primers used for cloning were listed in Table S2.

2.2.2.3 Construction of GFP-expressed mutant RNA2

The GFP-expressed RNA2 mutant pBY-T2.GFP (Fig. 2-3) was derived from pBY-T2 in the same manner as pBY-K2.GFP with a GFP gene replacement of the P1 and P2 genes between the 5'- and 3'-untranslated regions (UTRs) of RNA2 (You and Shirako, 2010). The primers used for construction were listed in Appendix Table S3.

2.2.2.4 *In vitro* transcription

The RNA transcripts were synthesized *in vitro* from the liberalized plasmids by a T7 RNA polymerase using an mMESSAGE mMACHINE® T7 Kit (Ambion, USA). pBY-T1 and pBY-T2 of Tochigi isolate JT10 were linearized using *Bam*HI and *Spe*I, respectively, and pBY-K1 and pBY-K2 of Kurashiki isolate JK05 were linearlized using *Xho*I and *Spe*I, respectively. A 10 μL reaction mixture contained 2 μL of

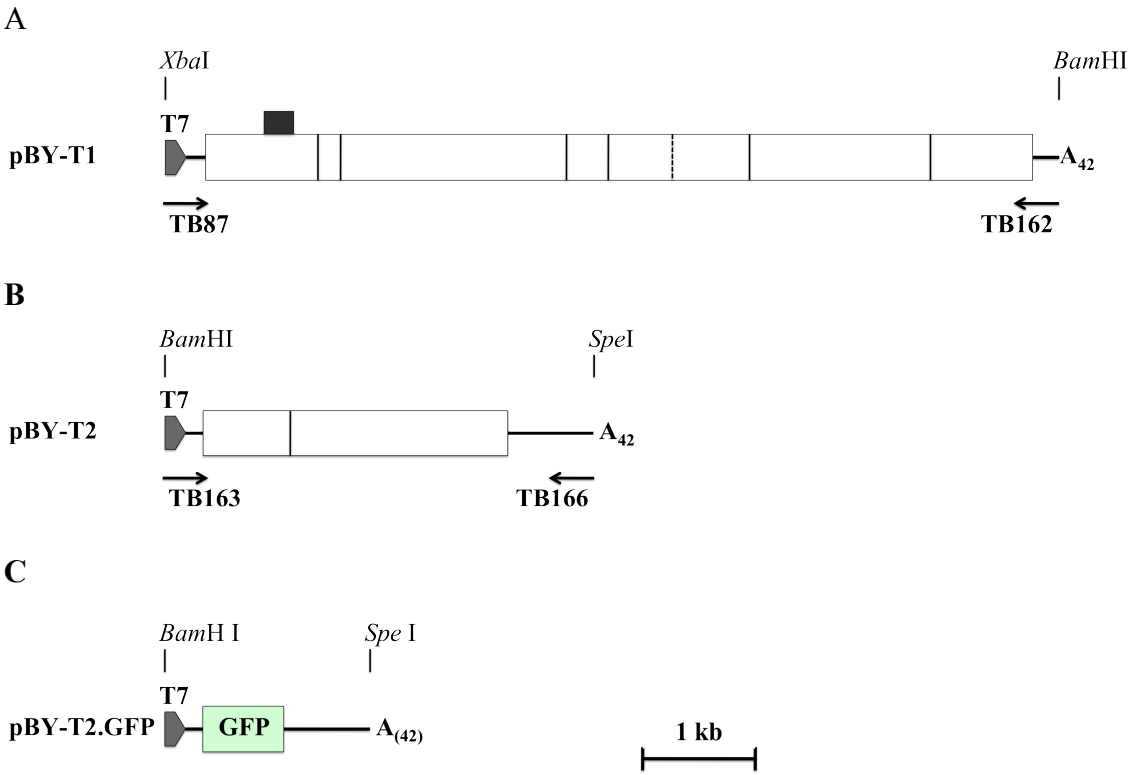


Fig. 2-3 Schematic representation of infectious full-length cDNA clones of BaYMV Tochigi isolate JT10. **(A)** pBY-T1: the infectious full-length cDNA clones of RNA1 of BaYMV Tochigi isolate JT10. The primers TB87 and TB162 containing the *Xba*I and *Bam*HI sites, repectively, were used for contruction of pBY-T1. **(B)** pBY-T2: the infectious full-length cDNA clone of RNA2 of BaYMV Tochigi isolate JT10. The primers TB163 and TB166 containing the *Bam*HI and *Spe*I sites, repectively, were used for contruction of pBY-T2. **(C)** pBY-T2.GFP: pBY-T2 derivative mutants with a GFP gene in the place of the P1/P2 polyprotein gene. The rightward filled pentagons at the 5' end indicate the T7 promoter. The 3' UTR sequences of all cDNA clones were followed by 42 adenylates. Map scale, 1 kilobase (kb).

RNase-free water, 5 μL of 2X NTP/CAP, 1 μL of 10X Reaction Buffer, 1 μL of Enzyme Mix and 100 ng of linearized DNA as the template. Transcription reaction was accomplished at 37 °C for 1 h.

2.2.2.5 Mesophyll protoplast preparation and RNA transfection

Barley seeds were sown in plastic pots containing a mixture of peat moss and vermiculite at a 1:1 ratio, and the pots were placed in a growth cabinet maintained at 22 °C under a 16-h light:8-h dark. The seedlings were fertilized with half-strength Hoagland solution (PhytoTechnology Laboratories, USA). Barley seedlings at the 6~7-day-old stage were harvested for protoplast preparation according to the procedure described previously (Ohsato et al., 2003), except that 0.2% Cellulase Onozuka RS (Yakult Pharmaceutical, Japan) was used instead of 2% Cellulase Onozuka R-10. Approximately 5 x 10^5 cells were transfected with capped *in vitro* transcripts of WT or mutant RNA1 and RNA2 (approximately 2 μg transcript for each) using a PEG method and incubated for 66 h at 15 °C in the dark.

2.2.2.6 Viral infection to plants using *in vitro* transcripts

Inoculum applied to plant seedlings was prepared by mixing 5 μg of *in vitro* transcripts for each with 500 μL of inoculation buffer containing 50 mM glycine, 30 mM K_2HPO_4 (pH 9.2). Each plant at 2~3-leaf stage was first dusted with carborundum powder (600 mesh) and then mechanically inoculated with 100 μL of this mixture by an electric toothbrush (Omron, Japan). Inoculated plants wrapped with a plastic bag were placed in dark box for 2 days at 15 °C, and then kept in a growth cabinet at 15 °C with 16 h of illumination.

2.2.2.7 Western blot analysis

Transfected protoplast cells and plants were harvested for Western blot analysis. Extracted samples were suspended in 100 µL 1X Sample Buffer containing 100 mM Tris-HCl (pH 9.0), 4% SDS, 30% sucrose and 5% 3-Mercapto-1, 2-propanediol. The proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto 0.45 µm nitrocellulose membranes (Protran BA85, Whatman, Germany) with a Trans-Blot® Turbo™ Transfer system (Bio-Rad, USA). Western blot analysis was performed with polyclonal antibodies against BaYMV CP (diluted 1:5000) (You and Shirako, 2010) as the primary antibody. Antibody binding was visualized by alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research, USA). The specific blots were detected using a NBT/BCIP method. Images were taken with a scanner, and ImageJ software (Wayne Rasband National Institutes of Health, USA) was used to measure the strength of CP bands (You and Shirako, 2013).

2.2.2.8 Fluorescent light microscopy

After incubation in the dark, protoplasts transfected with GFP RNA2 transcripts were monitored for the detection of GFP fluorescence under a fluorescent light microscope. Images were obtained with a VB-7010 cooled CCD camera (Keyence, Japan) equipped with an IX70 fluorescence microscope (Olympus, Japan) using the following settings: one second exposure time and 200 ISO film.

2.2.3 Results

2.2.3.1 Construction of full-length cDNA clones of Tochigi isolate JT10

For construction of full-length cDNA clone of RNA1, first strand synthesis was performed with a primer TB162 (Fig. 2-3 and Table S2) containing the most 3'-terminal 18 nucleotides, 42T and a *Bam*HI site. PCR fragment covering the whole RNA1 sequence was generated with TB162 and another primer TB87 (Fig. 2-3 and Table S2) containing 3 protect nucleotides, a *Xba*I site, T7 promoter sequence, a G and the most 5'-terminal 20 nucleotides. The 7.8-kb PCR products were cloned into modified plasmid vectors derived from pBR322 and then propagated in *E. coli* MC1061 cells. The same strategy was also employed to construct full-length cDNA clone of RNA2 except that *Spe*I and *Bam*HI sites were used instead of *Bam*HI and *Xba*I, respectively. Finally, two clones confirmed by sequencing were chosen for further study and designated as pBY-T1 and pBY-T2, respectively. Both these two constructs could be transcribed *in vitro* by T7 RNA polymerase (Fig. 2-4). These transcripts should contain the full-length sequences of RNA1 and RNA2 with a cap structure at the 5' ends and a poly(A) tail of 42 nucleotides at the 3' ends.

2.2.3.2 Infectivity of cDNA clones in protoplast cells using *in vitro* transcripts

The infectivity of cDNA clones was first examined in mesophyll protoplasts using *in vitro* transcripts. Previous study proved that BaYMV RNA1, which encodes a set of replication proteins, autonomously replicated in barley protoplasts and replication of RNA2, with a lack of the replicase gene, relied on RNA1 (You and Shirako, 2010). Here, the mixed transcripts from pBY-T1 and pBY-T2 were transfected to protoplasts

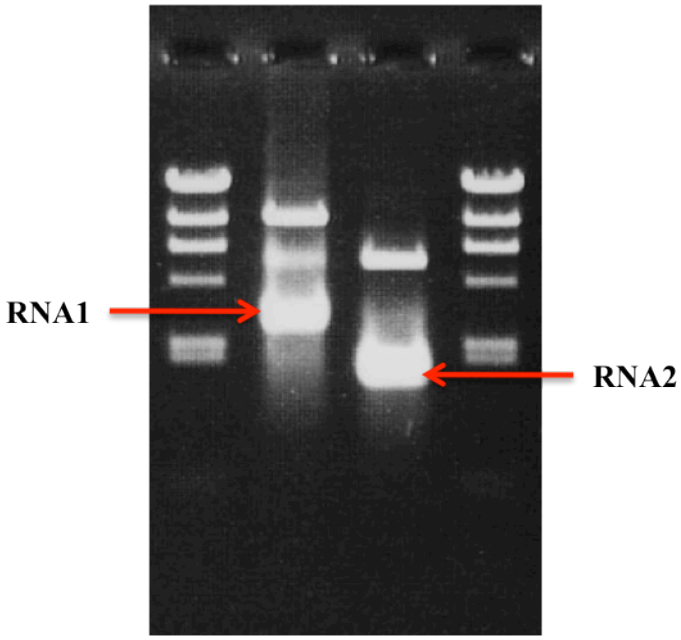


Fig. 2-4 *In vitro* transcripts of full-length cDNA clones of RNA1 (pBY-T1) and RNA2 (pBY-T2) of Tochigi isolate (JT10). The red arrowheads indicated the transcripts of RNA1 and RNA2. The other bands are the template DNA (upper band) and byproducts (lower band). The λ DNA-*Hin*dIII digested marker was applied here.

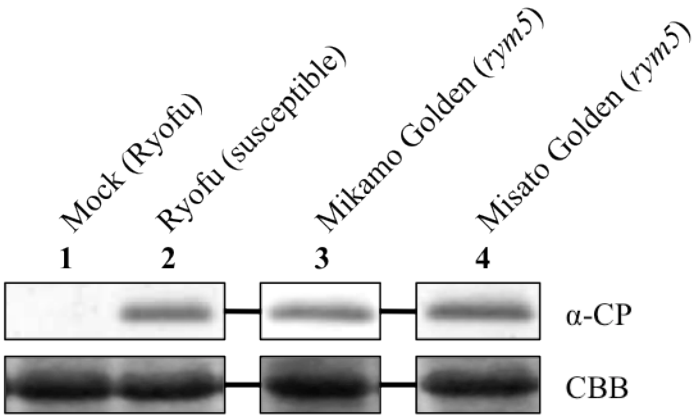
isolated from Ryofu, Mikamo Golden (*rym5*) and Misato Golden (*rym5*), respectively. After incubation at 15 °C for 66 h under dark, total protoplast cells were harvested and subjected to Western blot analysis. Detection of the 32-kDa band was used as a criterion for RNA1 replication. The result showed that CP was detected in the protoplast cells from either susceptible or *rym5* plants transfected with the transcripts from pBY-T1 and pBY-T2 (Fig. 2-5). It suggested that *in vitro* transcripts replicated in susceptible and *rym5* protoplasts.

I also utilized a GFP microcopy method to evaluate the replicative ability of *in vitro* transcripts in cells. Both susceptible and *rym5* protoplast cells were transfected with the transcripts from pBY-T1 and pBY-T2.GFP. In this assay, GFP could be expressed from replicating RNA2 using active replication proteins from the RNA1. Transfected protoplasts were monitored at 24 hpi, 48 hpi and 66 hpi under the microscope with a GFP filter. Almost no GFP fluorescence was observed in transfected cells at 24 hpi and GFP fluorescence could be visualized at 48 hpi (data not shown) and achieved to a higher level at 66 hpi (Fig. 2-5A). Approximately 30% of the transfected cells from either susceptible or *rym5* plants emitted GFP fluorescence at 66 hpi (Fig. 2-5B).

2.2.3.3 Systemic infectivity of cDNA clones using *in vitro* transcripts

To analyze the infectivity of the full-length cDNA clones *in planta*, *in vitro* transcripts from pBY-T1 and pBY-T2 of Tochigi isolate JT10 were used to systemic infection assay in susceptible (cv. Ryofu) and *rym5* (cvs. Mikamo Golden and Misato Golden) plants. Here, Ryofu plants transfected with transcripts from pBY-K1 and

A



B

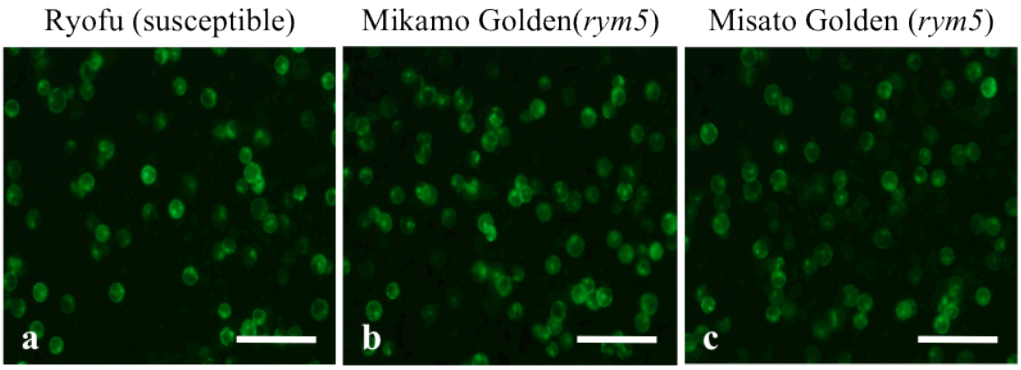


Fig. 2-5 Infectivity assay of cDNA clones of Tochigi isolate JT10 in susceptible (cv. Ryofu) and *rym5* (cvs. Mikamo Golden and Misato Golden) mesophyll protoplasts. **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts form pBY-T1 and pBY-T2. **α-CP**: Total proteins subjected to Western blot analysis using anti-BaYMV CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** GFP fluorescence observed in transfected protoplasts with *in vitro* transcripts from pBY-T1 and pBY-T2.GFP. Bars, 200 μm. The transfected protoplasts were incubated at 15 °C for 66 h.

pBY-K2 of Kurashiki isolate JK05 were used as controls to estimate the systemic infectivity of Tochigi isolate JT10. Barley plants at 2~3-leaf stage were mechanically inoculated with *in vitro* transcripts. The typical yellow mosaic systemic symptoms were observed in upper non-inoculated leaves of susceptible and *rym5* plants inoculated with transcripts from pBY-T1 and pBY-T2 at 5 weeks post-inoculation (wpi), whereas systemic symptoms were shown in Ryofu inoculated with transcripts of pBY-K1 and pBY-K2 at 3 wpi. Tochigi isolate JT10 caused a weak systemic infection than Kurashiki isolate JK05 *in planta* (Fig. 2-6). The transcripts of pBY-T1 and pBY-T2 were infectious in 30% (6 out of 18) of Misato Golden (*rym5*) plants, a lower efficiency than those of Kurashiki isolate JK05 in Ryofu plants (>80%). Viral infection was also confirmed by Western blot analysis using CP antiserum and sequencing of RT-PCR products of virus progenies. Therefore, the RNA1 and RNA2 transcripts from cDNA clones of Tochigi isolate JT10 were infectious to both susceptible and *rym5* plants.

2.2.3.4 Infectivity of Tochigi isolate JT10 in protoplasts

Twelve different barley varieties were used for infectivity assay of Tochigi isolate JT10 compared with Kurashiki isolate JK05. The accumulation of CP was analysed in mesophyll protoplasts transfected with *in vitro* transcripts from cDNA clones of Tochigi JT10 and Kurashiki JK05 isolates, respectively. In protoplasts transfection experiments, CP expression of JK05 isolate in Ryofu was considered as 1.00.

In protoplast transfection tests of JT10 isolate (Fig. 2-7 and Table 2-4), a comparable relative CP expression was detected in Ishuku Shirazu (*rym*3), a 0.6~0.8-fold relative CP expression in Amagi Nijo (*rym6*), a 0.4~0.6-fold relative CP

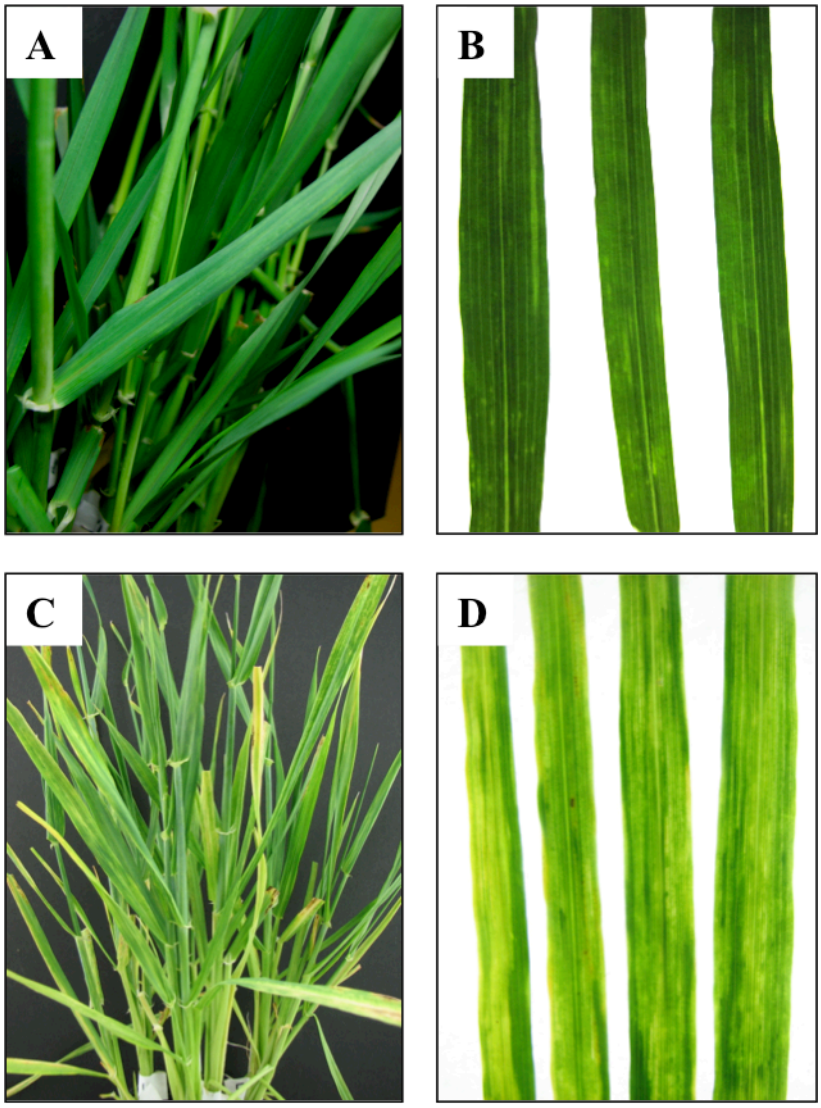


Fig. 2-6 Symptoms in plants inoculated with *in vitro* transcripts**. (A)** and **(B)** Misato Golden plants inoculated with *in vitro* transcripts from pBY-T1 and pBY-T2 of Tochigi isolate JT10. **(C)** and **(D)** Ryofu plants inoculated with *in vitro* transcripts from pBY-K1 and pBY-K2 of Kurashiki isolate JK05.

expression in Ryofu, KoA, Haruna Nijo (*rym6*), Haganemugi (*rym3*), Express (*rym4*), Misato Golden (*rym5*) and Miho Golden (*rym6*), a 0.2~0.4-fold relative CP expression in Mikamo Golden (*rym5*) and Nittakei 68 (*rym1*), almost undetectable CP expression in Mihori Hadaka 3 (*rym2*). BY-T1 RNA1 and BY-T2 RNA2 transcripts of Tochigi isolate JT10 replicated in susceptible, *rym1*, *rym3*, *rym4*, *rym5* and *rym6* but not in *rym2* cells.

In protoplasts tests of Kurashiki isolate JK05 (Fig. 2-7 and Table 2-4), a much higher relative CP expression was detected in var. Haganemugi (*rym3*), a 0.6~0.8-fold relative CP expression in KoA, a 0.4~0.6-fold relative CP expression in Express (*rym4*), a 0.2~0.4-fold relative CP expression in Mihori Hadaka 3 (*rym2*), Ishuku Shirazu (*rym3*), Amagi Nijo (*rym6*) and Miho Golden (*rym6*), a less than 0.2-fold relative CP expression in Haruna Nijo and Nittakei 68 (*rym1*), and no CP expression in Mikamo Golden (*rym5*) and Misato Golden (*rym5*). BY-K1 RNA1 and BY-K2 RNA2 transcripts of Kurashiki isolate JK05 replicated in susceptible and *rym1*, *rym2*, *rym3*, *rym*4, *rym5* but not in *rym5* cells.

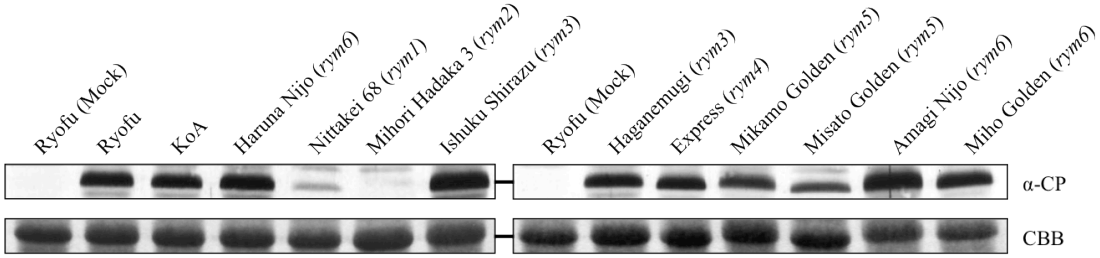
2.2.3.5 Systemic infectivity of Tochigi isolate JT10

Systemic infectivity assay of JT10 isolate was also examined in the above twelve varieties except Mihori Hadaka 3 (Table 2-4). The yellow mosaic symptoms were shown in susceptible Ryofu and KoA, *rym4*-harboring Express, *rym5*-harboring Mikamo Golden and Misato Golden and also *rym6*-harboring Haruna Nijo, Amagi Nijo and Miho Golden. Systemic infection was also confirmed by Western blot with CP antiserum. No symptoms were shown in *rym1*-harboring Nittakei 68 and

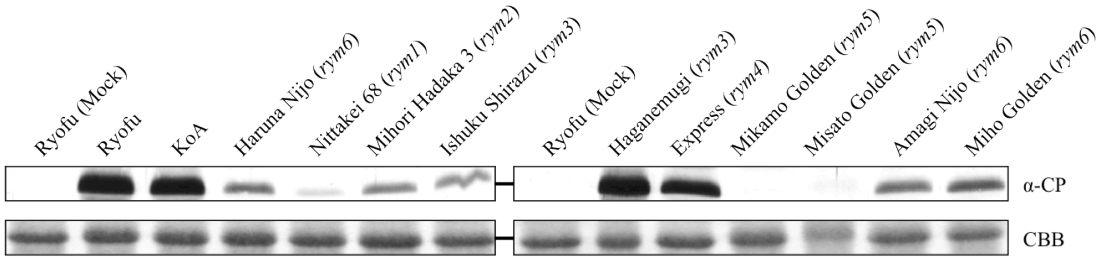
rym3-harboring Ishuku Shirazu and Haganemugi and no detectable CP expression was observed in these plants. Collectively, Tochigi isolate JT10 caused systemic infection in susceptible, *rym4*, *rym5* and *rym6* plants but not in *rym1*, *rym2* and *rym3* plants.

Here, systemic infectivity of JK05 isolate was only examined in *rym4* plants Express. The yellow mosaic symptom was shown on upper non-inoculated leaves of Express and viral infection was confirmed by Western blot analysis using CP antiserum.

A Tochigi isolate JT10



B Kurashiki isolate JK05



C

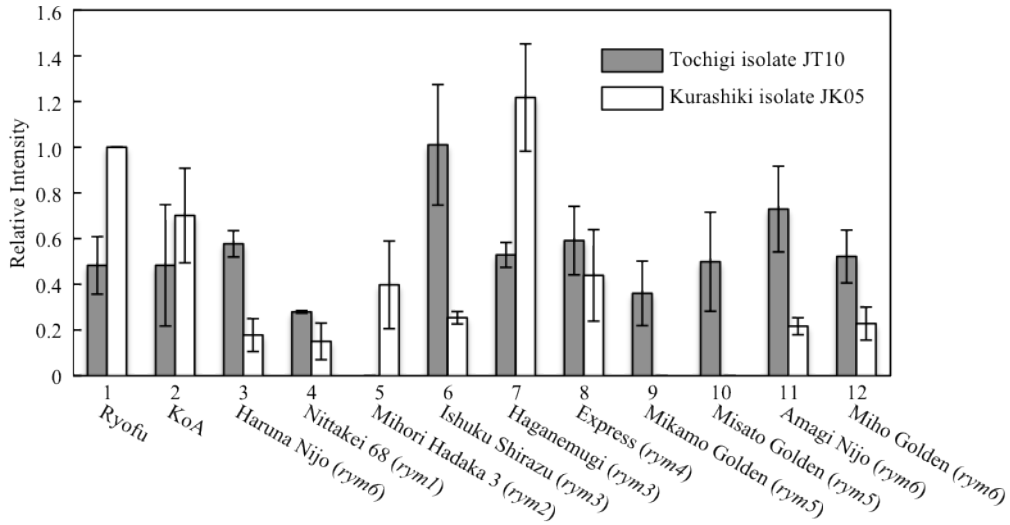


Fig. 2-7 Infectivity assay of *in vitro* transcripts of cDNA clones of Tochigi JT10 and Kurashiki JK05 solates in protoplasts from tweleve barley varieties. **(A)** and **(B)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(C)** Relative intensity of CP accumulation in transfected protoplasts. Experiments were at least triplicated . The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Ryofu transfected with transcripts from pBY-K1 and pBY-K2 of Kurashiki isolate JK05 was used as a control and set as 1.00. The transfected protoplasts were incubated at 15 °C for 66 h.

Table 2-4 Infectivity of BaYMV JT10 and JK05 isolates to different barley varieties.

Barley varieties	Resistant genes		Tochigi isolate JT10		Kurashiki isolate JK05	
	rym genes	Locations[a]	Protoplasts[b]	Plants[c]	Protoplasts	Plants[d]
Ryoufu	—	—	0.48	**S**	1.00	**S**
KoA	—	—	0.48	**S**	0.70	**S**
Haruna Nijo	***rym6***	3H	0.58	**S**	0.18	**R**
Nittakei 68	***rym1***	4H	0.27	**R**	0.15	**R**
Mihori Hadaka 3	***rym2***	7H	0	**R**	0.40	**R**
Ishuku Shirazu	***rym3***	5H	1.01	**R**	0.26	**R**
Haganemugi	***rym3***	5H	0.53	**R**	1.22	**R**
Express	***rym4***	3H	0.59	**S**	0.44	**S**
Mikamo Golden	***rym5***	3H	0.36	**S**	0	**R**
Misato Golden	***rym5***	3H	0.50	**S**	0	**R**
Amagi Nijo	***rym6***	3H	0.73	**S**	0.22	**R**
Miho Golden	***rym6***	3H	0.52	**S**	0.28	**R**

[a] *rym4/5/6* resistance genes are allelic and encode for eIF4E.
[b] The amount of CP accumulation in protoplasts from Ryofu transfected with BY-K1 RNA1 and BY-K2 RNA2 transcripts of Kurashiki isolate JK05 was used as a control and set as 1.00.
[c] **S**, susceptible **R**, resistant.
[d] Systemic infectivity assay of Kurashiki isolate JK05 in Express (*rym4*) was done in this study and in other varieties were referred to You and Shirako (2013).

2.2.4 Discussion

In this study, I constructed the full-length cDNA clones of RNA1 and RNA2 of Tochigi isolate JT10 designated as pBY-T1 and pBY-T2, respectively and used for infectivity assay both at the cellular and whole plant levels. Transcribed RNA1 and RNA2 replicated in protoplasts and caused systemic infection to plants of susceptible barley (cv. Ryofu). Then, the infectivity of this isolate using *in vitro* transcripts was examined. The transcripts from pBY-T1 and pBY-T2 also replicated in *rym5* protoplasts and caused systemic infection to *rym5* plants, although with a weak yellow mosaic symptom observed at 5 wpi. Thus, *in vitro* transcripts from pBY-T1 and pBY-T2 were infectious to susceptible and *rym5* plants and could be used for further analysis.

To well characterize Tochigi isolate, I further testify the infectivity of Tochigi isolate JT10 in tweleve varieties carrying different resistance or no resistance genes both at the cellular and whole plant levels. The responses of these varieties to Tochigi isolate JT10 were summarized in Table 2-4. Ryofu, KoA, Express (*rym4*), Mikamo Golden (*rym5*), Misato Golden (*rym5*), Haruna Nijo (*rym6*), Amagi Nijo (*rym6*) and Miho Golden (*rym6*) were systemically infected with Tochigi isolate JT10. Ishuku Shirazu (*rym3*) and Haganemugi (*rym3*) supported virus replication at the cellular level but were not systemically infected, indicating that the Ishuku Shirazu (*rym3*) and Haganemugi (*rym3*) were resistant to Tochigi isolate JT10 at the movement level. Nittakei 68 (*rym1*) supported limited replication at the cellular level and could be considered as partial resistance to Tochigi isolate JT10 at the cellular level. No virus replication was detected in Mihori Hadaka 3 (*rym2*), indicating that Mihori Hadaka 3

(*rym2*) was completely resistant to Tochigi isolate JT10. The responses of different resistant varieties to BaYMV Kurashiki isolate JK05 were previously examined at the cellular and whole plant levels by You and Shirako (2013). Here, the infectivity of Kurashiki isolate at the cellular level was tested again and systemic infectivity in Express (*rym4*) was also examined using high-quality *in vitro* transcripts. The responses of these varieties to Kurashiki isolate JK05 were also summarized in Table 2-4. To supplement previous data, Express (*rym4*) is systemically infected with Kurashiki isolate JK05. Haruna Nijo (*rym6*), Amagi Nijo (*rym6*) and Miho Golden (*rym6*) supported limited replication of Kurashiki isolate JK05 at the cellular levels. Collectively, susceptible cultivars Ryofu and KoA and Express (*rym4*) were susceptible to Kurashiki isolate JK05. Barley varieties with *rym1*, *rym2*, *rym3* or *rym6* gene were resistant only at the whole plant level. Barley varieties with *rym5* gene were completely resistant to Kurashiki isolate JK05. The mechanisms of *rym2*, *rym5* and *rym6* resistances could be studied by comparative analysis of Tochigi JT10 and Kurashiki JK05 isolates.

Previously, BaYMV was classified into eight strains in Japan, named I-1, I-2, I-3, II-1, II-2, III, IV and V, based on their pathogenicity to different barley cultivars (Kashiwazaki et al., 1989; Nishigawa et al., 2008; Sotome et al., 2010; Usugi et al., 1985). Tochigi isolate JT10, as a *rym5*-breaking strain, was most closely related to BaYMV-III. However, it could be not simply classify Tochigi isolate JT10 as a strain of pathotype III. Tochigi JT10 and BaYMV-III isolates are distinct in pathogenecity to *rym1* variety. *rym1* variety is resistant to Tochigi isolate at the whole plant level, but susceptible to BaYMV-III (Sotome et al., 2010; Sotome et al., 2011). The current

BaYMV classification system is not applicable for Tochigi isolate JT10 as some other new strains (Sotome et al., 2011; You and Shirako, 2013). Moreover, there is another defect that only whole plant responses were considered in this classification system. It requires a new BaYMV classification system suitable for all the strains, molecular characterization and resistance responses both at the cellular and whole plant levels should be taken into account for this purpose, which was also discussed by You and Shirako (2013).

2.3 Viral genome mapping analysis and amino acids identification of VPg to break *rym5* resistance

2.3.1 Introduction

The *rym5* BaYMV resistance gene originated from a Chinese barley landrace Mokusekko 3. Barley cultivars carrying *rym5* gene were widely used grown in fields in Japan until the resistance was overcome by BaYMV-III (Kashiwazaki et al., 1989). In Europe, growing *rym5* resistant cultivars was a useful approach to prevent virus infection by BaYMV-2, but *rym5* resistance was again overcome by BaMMV (Habekuss et al., 2008; Hariri et al., 2003; Kanyuka et al., 2004).

The resistance mechanisms in barley-BaYMV system are poorly understood. *rym4*, *rym5* and *rym6* located on Chromosome 3HL are recognized as allelic genes coding for eIF4E (Kanyuka et al., 2005; Stein et al., 2005). eIF4E also confers resistance to many potyviruses and the virulence genes involved in eIF4E–mediated resistance have been largely identified as VPg (Bruun-Rasmussen et al., 2007; Léonard et al., 2000; Ruffel et al., 2002; Schaad et al., 2000). Direct interaction between potyvirus VPg and eIF4E has been proved by yeast-two hybrid assay (Léonard et al., 2000; Schaad et al., 2000). In case of bymoviruses, mutations in VPg were also considered to be responsible for breaking of *rym4*– and *rym5*–mediated resistance in barley by amino acid comparison of breaking and non-breaking strains (Kanyuka et al., 2004; Kühne et al., 2003). However, there is no experiment proof for this hypothesis in bymoviruses.

In this subchapter, mapping analysis of viral determinant involved in *rym5*–mediated resistance was proceeded between BaYMV Tochigi (JT10,

rym5–breaking) and Kurashiki (JK05, *rym5*–nonbreaking) isolates. VPg was identified as the key factor responsible for breaking *rym5* resistance. Furthermore, the key amino acid(s) of VPg involved in breaking *rym5* resistance were also studied here.

2.3.2 Materials and Methods

2.3.2.1 Plant and virus

Barley cultivar Misato Golden (*rym5*) was used as plant material in this study. Two BaYMV isolates from Tochigi JT10 and Kurashiki JK05 were used in this study.

2.3.2.2 Construction of RNA1 mutants

For mapping analysis of viral determinant for overcoming *rym5*, eight chimeric infectious cDNA clones were constructed for infectivity analysis: pBY-K1/T-VPg, pBY-K1/T-MsNs, pBY-K1/T-KpNs, pBY-K1/T-XbNs, pBY-K1/T-MsNd, pBY-K1/T-KpMs, pBY-K1/T-XbMs and pBY-K1/T-NsNd (Fig. 2-8). These constructs were modified from pBY-K1 by replacements of designated regions of Kurashiki isolate JK05 with those of Tochigi isolate JT10. To generate pBY-K1/T-VPg, PCRs were performed in the presence pBY-K1 and pBY-T1 as the templates. The resultant PCR products were purified and served as a mixed template to generate the fragment containing a VPg gene from Tochigi isolate JT10 at the background of Kurashiki isolate JK05. The final amplified fragments were purified and cloned into pBY-K1 with *Afl*II and *Sal*I sites using an In-Fusion HD Cloning System CE kit (Clontech Laboratories, USA). The primers used for this construction were listed in the Appendix Table S4. To generate pBY-K1/T-MsNs construct, a *Msc*I-*Nsi*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate pBY-K1/T-KpNs construct, a *Kpn*I-*Nsi*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate pBY-K1/T-XbNs construct, a *Xba*I-*Nsi*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate

pBY-K1/T-MsNd construct, a *Msc*I-*Nde*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate pBY-K1/T-KpMs construct, a *Kpn*I-*Msc*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate pBY-K1/T-XbKp construct, a *Xba*I-*Kpn*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1. To generate pBY-K1/T-NsNd construct, a *Nsi*I-*Nde*I fragment was released from pBY-T1 and ligated into similarly digested pBY-K1.

To introduce point mutations into VPg gene on pBY-T1, PCR-based mutagenesis was used for constrcutions of T-VPg_F73Y, T-VPg_E85D, T-VPg_S118T, T-VPg_T120K, T-VPg_H142Y, T-VPg_V175A. The primers used for each construct were listed in Table S4. The final amplified fragments were purified and cloned into pBY-T1 with *Afl*II and *Sal*I sites using an In-Fusion HD Cloning System CE kit (Clontech Laboratories, USA). Site-directed mutagenesis of VPg gene on pBY-K1 was done similarly for these constructs pBY-K1/VPg_118S/142H and pBY-K1/VPg_118S/120T/142H. The primers used for each construct were listed in the Table S4.

All the recombinant DNA procedures were carried out by standard methods using *E. coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts.

2.3.2.3 *In vitro* transcription

It was done as described in 2.2.2.4.

2.3.2.4 Mesophyll protoplast preparation and RNA transfection

It was done as described in 2.2.2.5.

2.3.2.5 Viral infection to plants using in vitro transcripts

It was done as described in 2.2.2.6.

2.3.2.6 Western blot analysis

It was done as described in 2.2.2.7.

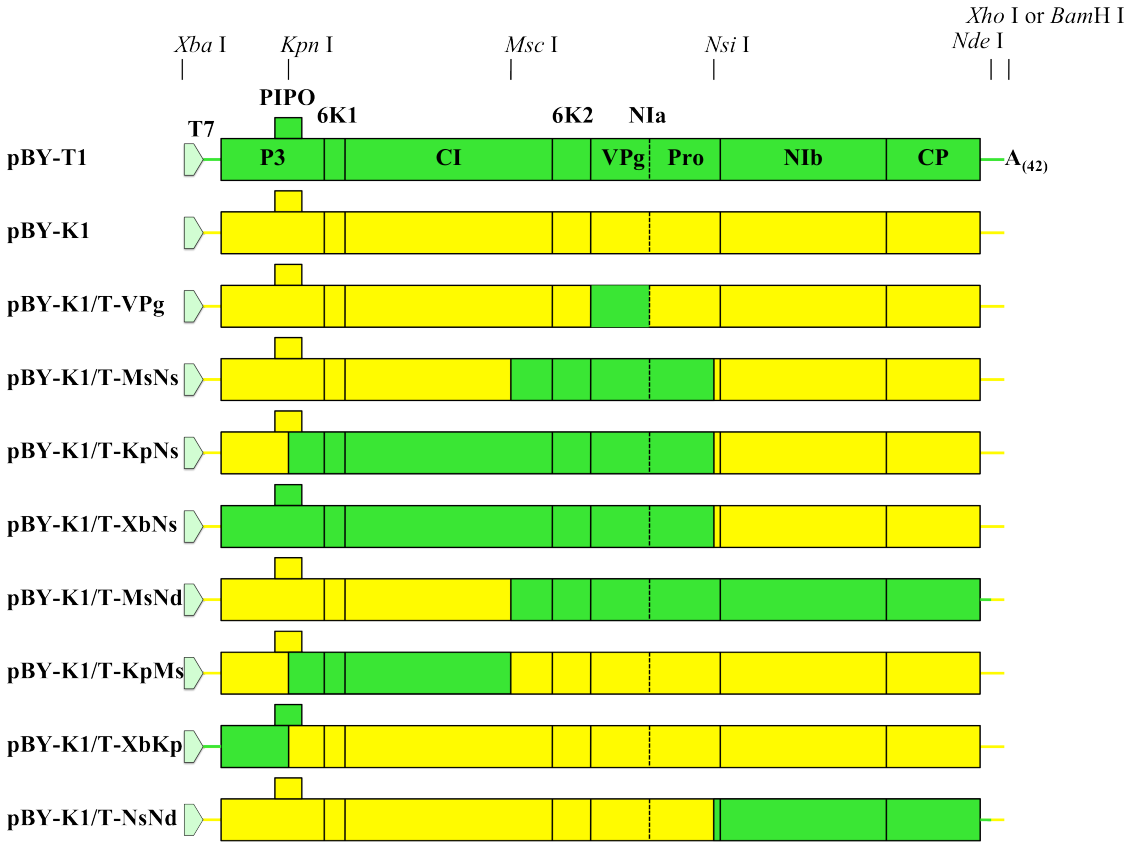


Fig. 2-8 Schematic representation of chimeric cDNA clones between Tochigi JT10 and Kurashiki JK05 isolates.

2.3.3 Results

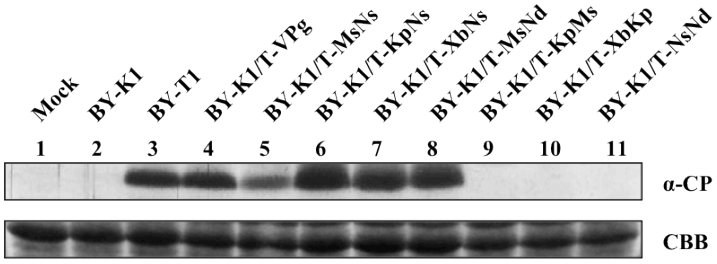
2.3.3.1 Mapping analysis between Tochigi JT10 and Kurashiki JK05 isolates in breaking *rym5* resistance

To identify the viral determinants involved in breaking *rym5*, I made eight chimeric constructs between pBY-K1 and pBY-T1 by exchanging reciprocal regions (Fig. 2-8). Mesophyll protoplasts were transfected with *in vitro* transcripts from pBY-K1, pBY-T1 or mutant pBY-K1 in combination with pBY-K2 or pBY-T2 transcripts. After incubation at 15 °C for 66 h, the protoplasts were harvested for Western blot analysis using CP antiserum. The results showed that CP could be similarly detected in *rym5* protoplasts transfected with transcripts from chimeric mutants of T-VPg, T-KpNs, T-XbNs and T-MsNd as that from pBY-T1, less CP was detected in *rym5* protoplasts transfected with T-MsNs transcript, and CP was undetectable in *rym5* protoplasts transfected with transcripts from constructs of T-KpNs, T-XbKp and T-NsNd as non-infectious transcripts from pBY-K1 (Fig. 2-9). The common nature of those constructs causing viral replication in *rym5* protoplast, including T-VPg, T-KpNs, T-MsNs, T-XbNs and T-MsNd mutants, was that they all contained the T-VPg region, indicating that T-VPg was the critical determinant in breaking *rym5* at the cellular level.

2.3.3.2 Systemic infectivity assay of T-VPg mutant in *rym5* plants

To testify the ability of T-VPg mutant in breaking *rym5* at the whole plant level, Misato Golden (*rym5*) plants were inoculated with transcripts from pBY-K1/T-VPg and pBY-K2. Symptom development was monitored for 2 months post-inoculation. At 4

A



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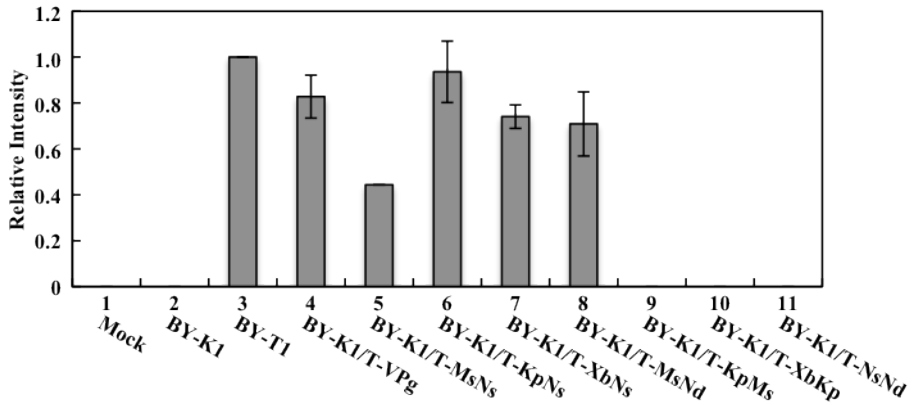


Fig. 2-9 Infectivity assay of *in vitro* transcripts of chemeric constructs between Tochigi JT10 and Kurashiki JK05 isoaltes in protoplasts from Misato Golden (*rym5*). **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** Relative intensity of CP accumulation in transfected protoplasts. Experiments were at least triplicated . The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Misato Golden (*rym5*) transfected with transcripts from pBY-T1 and pBY-T2 was used as a control and set as 1.0. The transfected protoplasts were incubated at 15 °C for 66 h.

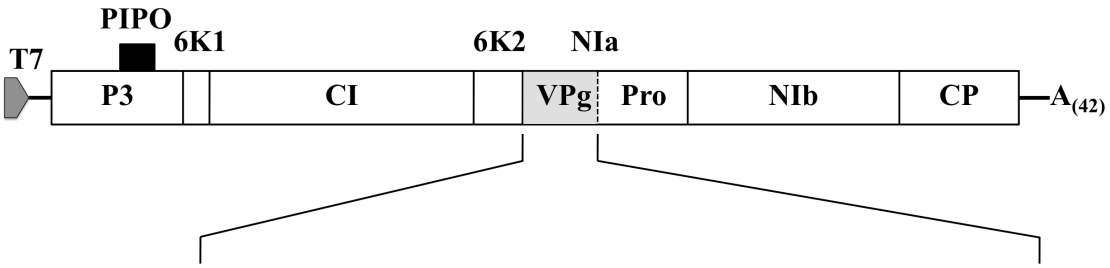
wpi, mild mosaic symptoms could be observed in *rym5* plants and the symptom was similar to that in *rym5* plants inoculated with WT RNA1 and RNA2 transcripts of Tochigi isolate JT10. Systemic infection was also confirmed by Western blot analysis using CP antiserum and sequencing of RT-PCR products of virus progenies. The identities of progenies derived from upper non-inoculated leaves were confirmed by sequencing of RT-PCR products of all regions. Therefore, T-VPg mutant of Kurashiki isolate JK05 could cause systemic infection in *rym5* plants.

2.3.3.3 Infectivity assay of K-VPg mutants in *rym5* protoplasts

Alignment of amino acid sequences of K-VPg (VPg of Kurashiki isolate JK05) and T-VPg showed that there are six amino acid differences out of 187 amino acids; Tyr in K-VPg and Phe in T-VPg at the position 73; Asp in K-VPg and Glu in T-VPg at the position 85; Thr in K-VPg and Ser in T-VPg at the position 118; Lys in K-VPg and Thr in T-VPg at the position 120; Tyr in K-VPg and His in T-VPg at the position 142; Ala in K-VPg and Val in T-VPg at the position 175 (Fig. 2-10). I hypothesized that amino acid substitutions in one or more positions of VPg resulted in pathogenic differences between Tochigi JT10 and Kurashiki JK05 isolates in overcoming *rym5*.

Thus, to identify the critical amino acids of VPg in overcoming *rym5*, I constructed six T-VPg mutants with a single amino acid substitution in each construct (T-VPg_F73Y, T-VPg_E85D, T-VPg_S118T, T-VPg_T120K, T-VPg_H142Y and T-VPg_V175A). Infectivity of these mutants was examined in mesophyll protoplasts from Misato Golden (*rym5*). Western blot analysis showed that comparable expression of CP was detected with transfection of T-VPg_F73Y, T-VPg_E85D and

A



B

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JT10      1 GKGNKYRPREDARLMYSTREDATFDAWNEKAKERRKKVTDKSEPELRRAYEKRPYFNFYD  60
JK05      1 ............................................................  60

JT10     61 LQTDSNILEAIFYTTEGDEFFRTADPNKDMNLVADKLRSFLDTKLVVGHHQRQMLEETAK 120
JK05     61 ............F...........E..................................S.T 120

JT10    121 VIIKDTKGTAHHMDISQHDPDYLKQNGSGKIGYPEHRGQFRQEGSAKTTDYDLGAEFGTD 180
JK05    121 ....................H.................................V..... 180

JT10    181 TDDITLE                                                       187
JK05    181 .......                                                       187
```

Fig. 2-10 Amino acid sequence alignment of VPgs from JT10 and JK05 isolates. **(A)** Schematic representation of BaYMV RNA1. VPg was shown in gray rectangle. (B) Alignment of amino acid sequences of VPg from JT10 and JK05 isolates.

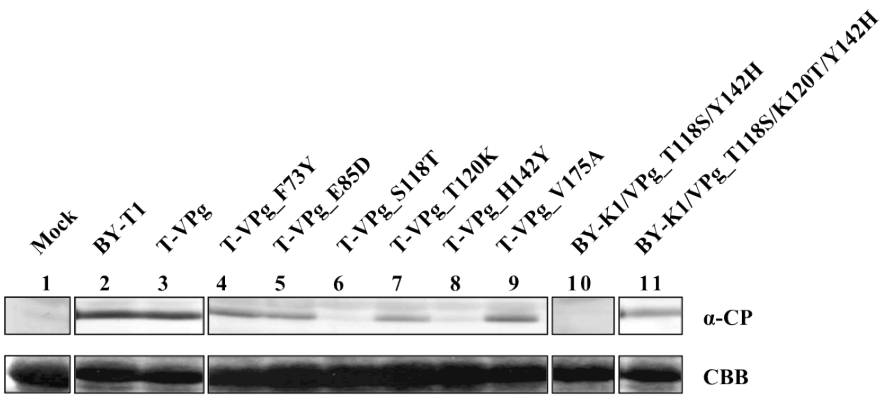
T-VPg_V175A mutant transcripts as that of wide-type Tochigi isolate JT10 (Fig. 2-11A lanes 2, 4, 5 and 9, and 2-12B bars 2, 4, 5 and 9), a less expression of CP with transfection of T-VPg_T120K mutant transcript (Fig. 2-11A lane 7, 2-10B bar 7), and almost undetectable CP with transfection of T-VPg_S118T and T-VPg_H142Y mutant transcripts (Fig. 2-11A lanes 6 and 8, and 2-10B bars 6 and 8). This result clearly demonstrated that the determinant of virulence at VPg, involved in the ability of breaking *rym5* resistance, resided on Ser at the position 118 and His at the position 142.

To confirm the role of Ser at the position 118 and His at the position 142 of VPg in breaking *rym5*, these two amino acid mutations of VPg were introduced into Kurashiki isolate JK05 to produce a VPg_T118S/Y142H mutant of pBY-K1. However, no detectable expression of CP was shown in Western blot analysis (Fig. 2-11A lane 10, 2-10B bar 10), indicating that only Ser at the position 118 and His at the position 142 of VPg were not enough and some other amino acid mutations may be also required for establishment of virulence in *rym5* cells. The next test was carried out with another pBY-K1 mutant with VPg_T118S/K120T/Y142H. In this case, a comparable expression of CP was detected in *rym5* protoplasts transfected with VPg_T118S/K120T/Y142H mutant transcript (Fig. 2-11A lane 11, 2-10B bar 11). Non-infectious Kurashiki isolate JK05 was granted the ability of replication in *rym5* cells by the triple mutations T118S/K120T/Y142H in VPg. Altogether, these results showed that Ser at the position 118 and His at the position 142 of VPg were the key amino acid but insufficient for breaking *rym5*, and Tyr at the position 120 was also required for virus replication in *rym5* protoplast cells.

2.3.3.4 Systemic infectivity assay of VPg_T118S/K120T/Y142H mutant in *rym5* plants

Next, systemic infectivity of VPg_T118S/K120T/Y142H mutant was examined in Misato Golden (*rym5*). Plants were inoculated with transcript from VPg_T118S/K120T/Y142H mutant in combination with that from pBY-K2, which replicated well in *rym5* protoplast cells. No systemic infection could be observed during two months post-inoculation (data not shown).

A



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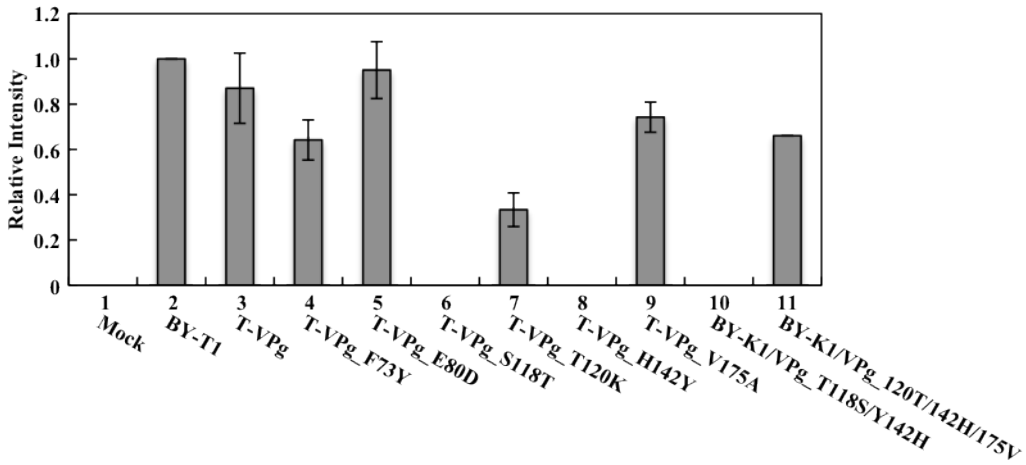


Fig. 2-11 Infectivity assay of VPg mutants in protoplasts from Misato Golden (*rym5*). **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-BaYMV CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** Relative intensity of CP accumulation in transfected protoplasts. The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Misato Golden (*rym5*) transfected with transcripts from pBY-T1 and pBY-T2 was used as a control and set as 1.0. The transfected protoplasts were incubated at 15 °C for 66 h.

2.3.4 Discussion

Mapping analysis of virus infectivity between Tochigi JT10 and Kurashiki JK05 isolates showed that VPg is the virulence determinant for breaking *rym5*. Introduction of T-VPg gene into pBY-K1 enabled the mutant to replicate in *rym5* protoplasts and cause systemic infection in *rym5* plants. Amino acid sequences of T-VPg were confirmed by sequencing virus progenies derived from upper non-inoculated leaves in three independent experiments. Virus progenies also contained one another amino acid mutation with valine mutated from alanine at position 95 of P2 protein, which was observed only once but did not consistently occur in three tests, indicating that it is not involved in breaking *rym5*. The role of VPg in breaking eIF4E–mediated resistance is first proved in bymoviruses.

Introduced mutations into VPg at the positions 118 and 142 were lethal to Tochigi isolate JT10 in *rym5* protoplasts, indicating that these two amino acids of VPg, Ser at the position 118 and His at the position 142, were critical for breaking *rym5* resistance. VPg_T118S/Y142H mutant of Kurashiki isolate JK05 could not replicate in *rym5* protoplasts. These two mutations are not efficient for breaking *rym5* and more amino acid mutations are required. Furthermore, another mutation in VPg was also introduced into Kurashiki isolate JK05 to produce VPg_T118S/K120T/Y142H mutant. *rym5* cultivars supported replication of this mutant at the cellular level but was resistant to whole plant level. More mutations in VPg are required for systemic infection in *rym5* plants. In total, Ser at the position 118 and His at the position 142 of VPg are essential but insufficient for breaking *rym5* and the other amino acids at positions 73, 85, 120 and 175 may have cumulative effect to finally achieve systemic infection in *rym5* plants.

2.4 Expression of eIF4E gene from susceptible barley in *rym5* protoplasts

2.4.1 Introduction

Plant-potyvirus system provides a useful model to investigate recessive resistance mechanisms. Current known recessive resistance genes against potyviruses were identified to encode translation initiation factors, eIF4E, eIF4G or their isoforms, of which eIF4E is the best-studied one (Fraile and García-Arenal, 2012). eIF4E–mediated resistance genes confer qualitative, genotype-specific resistance to viruses: *pvr1/2* and *pot-1* from pepper resistant to *Tobacco each virus* (TEV) and *Potato virus Y* (PVY) (Kang et al., 2005b; Ruffel et al., 2002; Ruffel et al., 2005), *mol1/2* from lettuce resistant to LMV (Nicaise et al., 2003; Roudet-Tavert et al., 2007), *sbm1*, *cyv2* and *wlv* from pea resistant to *Pea seed-borne mosaic virus* (PSbMV), ClYVV and *Bean yellow mosaic virus* (BYMV), respectively (Andrade et al., 2009; Bruun-Rasmussen et al., 2007; Gao et al., 2004), and *bc-3* from bean resistant to *Bean common mosaic virus* (BCMV) (Naderpour et al., 2010). The eIF4E–mediated resistances resulted from amino acid substitutions in the eIF4E proteins encoded by the recessive resistance alleles (Ruffel et al., 2006; Yeam et al., 2007). The *Potato virus X*-mediated transient expression of eIF4E from susceptible cultivars by mechanical inoculation rendered resistant cultivars susceptible to the certain virus pathotype, which has been proved for PVY and *Pepper veinal mottle virus* (Ruffel et al., 2002; Ruffel et al., 2006).

eIF4E in the host cell is a well-known cellular cap-binding protein that is essential for initiating protein translation (Yeam et al., 2007). However, the function of eIF4E

involved in virus infection was not fully understood. eIF4E has been hypothesized to play multiple roles in several processes of potyvirus infection, including translation, replication and cell-to-cell movement (Robaglia and Caranta, 2006; Truniger and Aranda, 2009).

Recessive resistance genes *rym4/5/6* against BaYMV were also mapped to barley chromosome 3HL and encode barley eIF4E gene (Kanyuka et al., 2005; Stein et al., 2005). In this subchapter, the role of eIF4E in breaking *rym5* was examined.

2.4.2 Materials and Methods

2.4.2.1 Plant and virus

Barley cultivar Misato Golden (*rym5*) was used as plant material in this study. Two BaYMV isolates JT10 and JK05 were used in this study.

2.4.2.2 Construction of eIF4E expressed RNA2 mutants

Three eIF4E expressed RNA2 mutants, pBY-K2.P12/KoA_eIF4E(P1/P2), pBY-K2.P12/KoA_eIF4E(NIb/CP) and pBY-K2.P12/*rym5*_eIF4E(NIb/CP), were constructed with a P1/P2 or NIb/CP cleavage site sequence between P2 and eIF4E genes (Fig. 2-12). PCRs were performed in the presence of pBY-K2 and the KoA_eIF4E or *rym5*_eIF4E gene as separate templates. The resultant PCR products were purified and served as a mixed template to generate the P12/KoA_eIF4E(P1/P2), P12/KoA_eIF4E(NIb/CP) and P12/*rym5*_eIF4E(NIb/CP) fragments, respectively. Subsequently, the fragments were digested and ligated into pBY-K2 using *Bss*HII and *Nhe*I. All the recombinant DNA procedures were carried out by standard methods using *E. coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts. The primers used for each construct were listed in Appendix Table S5.

2.4.2.3 *In vitro* transcription

It was done as described in 2.2.2.4.

2.4.2.4 Mesophyll protoplast preparation and RNA transfection

It was done as described in 2.2.2.5.

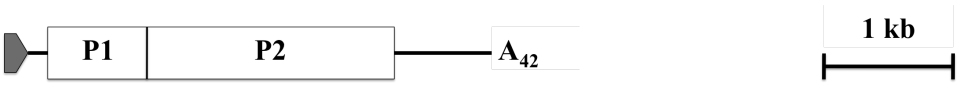
2.4.2.5 Viral infection to plants using in vitro transcripts

It was done as described in 2.2.2.6.

2.4.2.6 Western blot analysis

It was done as described in 2.2.2.7.

A WT RNA2: pBY-K2



B eIF4E-expressed RNA2 mutant: pBY-K2.P12/KoA_eF4E(P1/P2), pBY-K2.P12/KoA_eF4E(NIb/CP) and pBY-K2.P12/*rym5*_eF4E(NIb/CP).

Fig. 2-12 Schematic representation of wild-type (WT) and eIF4E-expressed RNA2 mutants. Map scale, 1 kilobase (kb).

2.4.3 Results

2.4.3.1 Expression of eIF4E from mutant RNA2 in protoplasts

To examine the expression of eIF4E from mutant RNA2, protoplasts were transfected with BY-K1 RNA1 and BY-K2.P12/KoA_eIF4E RNA2 transcripts. After incubation, P2 and KoA eIF4E could be detected in protoplasts transfected with BY-K1 RNA1 and BY-K2.P12/KoA_eIF4E(NIb/CP) RNA2 transcripts (Fig. 2-13A lane 1 and B lane 1), indicating that the polyprotein product of mutant RNA2 with a NIb/CP cleavage site was efficiently cleaved by NIa-Pro proteinase and produced P1, P2 and eIF4E proteins. Protoplasts transfected with BY-K1 RNA1 and BY-K2.P12/KoA_ eIF4E(P1/P2) RNA2 transcripts were used as controls. Only fused KoA_eIF4E was detected in these transfeced protoplasts (Fig. 2-13A lane 2 and B lane 2), which is because P1 is a proteinase *in cis* and could not cleave the fused P2/KoA_eIF4E. Here, it should be noticed that KoA_eIF4E protein was difficult to detect with a faint band (Fig. 2-13B lane 1) or sometimes undetectable, whereas fused P2/KoA_eIF4E protein was always strongly detected (Fig. 2-13B lane 2), indicating that KoA_eIF4E protein was quite unstable in cells.

2.4.3.2 Effects of co-expression with eIF4E in *rym5* cells

To test the role of eIF4E in overcoming *rym5* resistance, two mutants of pBY-K2 co-expressed with a eIF4E gene derived from susceptible KoA (KoA_eIF4E) were constructed with different cleavage sites [P12/KoA_eIF4E(NIb/CP) and p12/KoA_eIF4E(P1/P2)] (Fig. 2-12) and applied to the following tests. Mesophyll protoplasts were transfected with transcript from pBY-K1 in combination with that

from pBY-K2 mutants P12/KoA_eIF4E or P12/*rym5*_eIF4E, respectively. After incubation at 15 °C for 66 h, the protoplasts were harvested for Western blot analysis with CP antiserum. As shown in Fig. 2-14, CP was abundantly detected in *rym5* protoplasts transfected with transcript from pBY-K1 in combination with that of pBY-K2.P12/KoA_eIF4E(NIb/CP) as transfected with transcript from pBY-T1 and pBY-T2 of Tochigi isolate JT10 (Fig. 2-14A lanes 4 and 3, and 2-12B bars 4 and 3), much less detected in *rym5* protoplasts transfected with transcript from pBY-K1 in combination with that of pBY-K2.P12/KoA_eIF4E (P1/P2) (Fig. 2-14A lane 5, and 2-12B bar 5). In contrast, no detectable CP was shown in *rym5* protoplasts transfected with transcript from pBY-K1 in combination with that from pBY-K2 or *rym5*_eIF4E-expressed mutant pBY-K2.12/*rym5*_eIF4E (Fig. 2-14A lanes 6 and 2, and 2-12B bars 6 and 2). These results indicated that co-expression of KoA_eIF4E enabled non-infectious Kurashiki isolate JK05 replicate in *rym5* protoplasts. The susceptible KoA_eIF4E neutralized the resistance conferring by *rym5* gene to Kurashiki isolate JK05.

2.4.3.3 Systemic infectivity assay of eIF4E-expressed mutant in *rym5* plant

To analyse systemic infectivity of eIF4E-expressed mutants in *rym5* plants, Misato Golden (*rym5*) plants were inoculated with transcripts from pBY-K1 and eIF4E-expressed pBY-K2 construct pBY-K2.P12/KoA_eIF4E(NIb/CP). During the whole monitor period, no systemic infection was observed that was confirmed by Western blot analysis (data not shown).

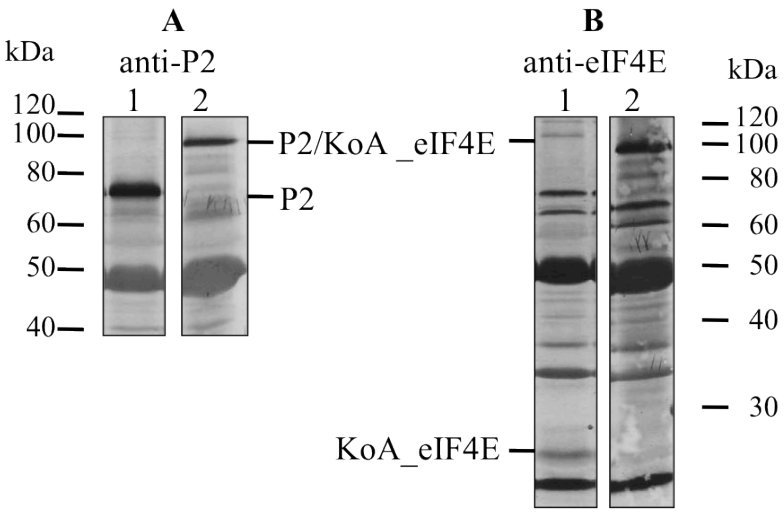
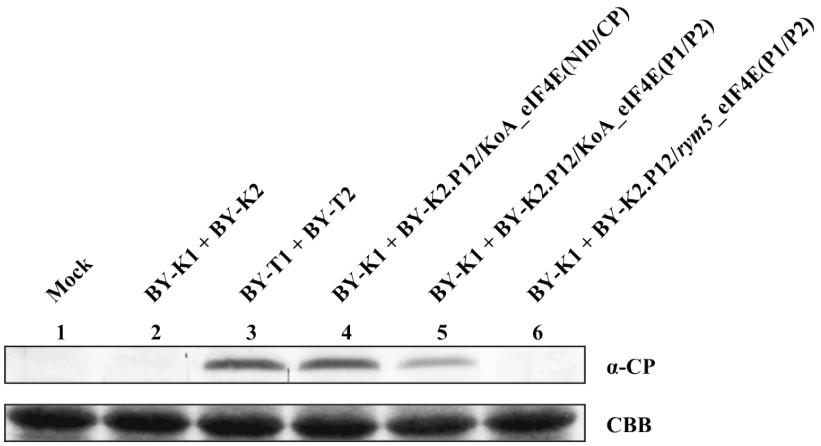


Fig. 2-13 Expression of eIF4E from mutant RNA2 in protoplasts from susceptible barley (cv. Ryofu). Lane 1, the protoplasts transfected with BY-K1 RNA1 + BY-K2.P12/KoA_eIF4E(NIb/CP); Lane 2, the protoplasts transfected with BY-K1 RNA1 + BY-K2.P12/ KoA_eIF4E(P1/P2). The transfected protoplasts were incubated at 15 °C for 66 h. Total proteins subjected to Western blot analysis using P2 and eIF4E antibodies.

A



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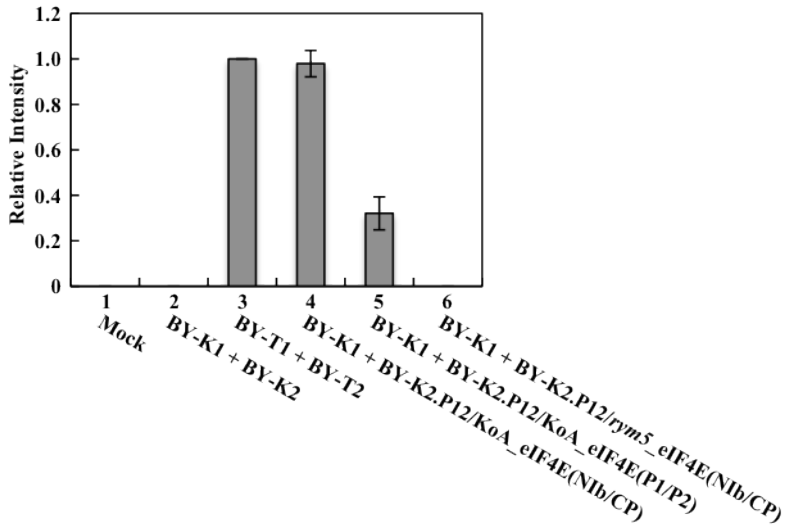


Fig. 2-14 Infectivity assay of co-expression of eIF4E with JK05 isolate in protoplasts from Misato Golden (*rym5*). **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-BaYMV CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** Relative intensity of CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Misato Golden (*rym5*) transfected with transcripts from pBY-T1 and pBY-T2 was used as a control and set as 1.0. The transfected protoplasts were incubated at 15 °C for 66 h.

2.4.4 Discussion

Co-expression of susceptible eIF4E (KoA_eIF4E) enabled Kurashiki isolate JK05 to replicate in *rym5* protoplasts, indicating that the resistance by *rym5* is indeed conferred by eIF4E gene. However, co-expression of KoA_eIF4E with Kurashiki isolate JK05 could not break *rym5* at the whole plant level. There should be another resistance gene affecting virus movement in *rym5* plants. The *rym5* resistance gene in Mikato Golden and Misato Golden could be traced back to the Chinese landrace Mokusekko 3, carrying *rym1*, *rym5* and another unidentified resistance gene on 7HS (Konishi et al., 1997; Miyazaki et al., 2001). Some other resistance genes may also be introduced into *rym5* cultivars when they were bred.

Study on the mechanisms of *rym5* resistance showed both VPg and eIF4E proteins are required for virus replication. However, a direct or indirect interaction of the two proteins has not been observed by a yeast two-hybrid assay, as was shown with potyviruses (Léonard et al., 2000; Schaad et al., 2000) , or by a co-immunoprecipitation assay (data not shown). A low temperature requirement for replication or the presence of other intermediate factors (Abdul-Razzak et al., 2009; Ala-Poikela et al., 2011; Nakahara et al., 2010) may be the reasons for negative results by yeast two hybrid assays. In the present study, it has also been proved that eIF4E is quite unstable in cells and has a short half-life, making detection of its possible interaction with VPg even more difficult by a co-immunoprecipitation assay.

There are six amino acid differences within 215 amino acids between eIF4E proteins derived from KoA and *rym5* plants (Fig. 2-15). These differences in

*rym5*_eIF4E protein may change its confirmation, resulting in incapability of interaction with replication-related proteins such as VPg in this study and establishment of resistance.

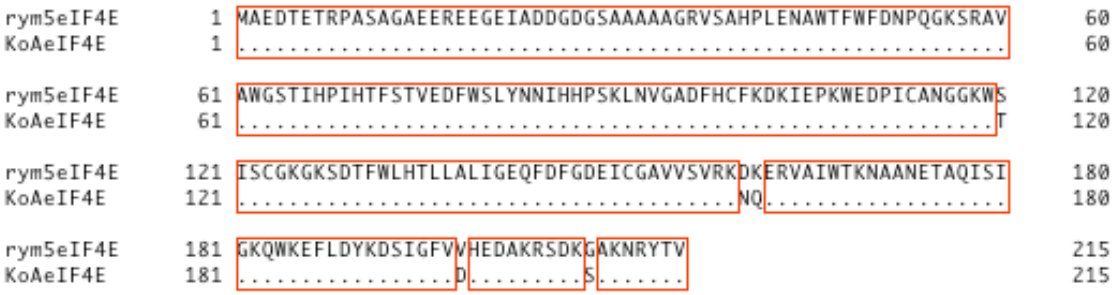


Fig. 2-15 Alignments of amino acid sequences of eIF4Es isolated from *rym5* plants (both Mikamo Golden and Misamo Golden) and KoA using Genetyx-Mac software (Genetyx, Japan).

2.5 Viral genome mapping analysis to break *rym2* barley resistance

2.5.1 Introduction

The resistance gene *rym2* derived from Mihori Hadaka 3 was mapped on chromosome 7H (McGrann and Adams, 2004) but the host factor mediating *rym2* resistance has not been identified. Among the current BaYMV strains identified in Japan and Germany, Mihori Hadaka 3 (*rym2*) was only susceptible to BaYMV-II-2 (Konishi and Kaiser-Alexnat, 2000; Sotome et al., 2010; Sotome et al., 2011). In this study, BaYMV Tochigi JT10 and Kurashiki JK05 isolates have different pathogenicity to Mihori Hadaka 3 (*rym2*), which supported virus replication for Kurashiki isolate JK05 but resistant to Tochigi isolate JT10 at the cellular level as mentioned in 2.2.

In this subchapter, virulence determinant in breaking *rym2* at the cellular level was studied by mapping analysis between Tochigi JT10 and Kurashiki JK05 isolates.

2.5.2 Materials and Methods

2.5.2.1 Plant and virus

Mihori Hadaka 3 (*rym2*) was used as plant materials in this study. Two BaYMV isolates from Tochigi JT10 and Kurashiki JK05 were used in this study.

2.5.2.2 Construction of RNA1 mutants

Total fifteen chimeric infectious cDNA clones were utilized for mapping analysis of viral determinant for replication in *rym2* protoplast cells. Among these constructs, eight out of fifteen, pBY-K1/T-VPg, pBY-K1/T-MsNs, pBY-K1/T-KpNs, pBY-K1/T-XbNs, pBY-K1/T-MsNd, pBY-K1/T-KpMs, pBY-K1/T-XbMs and pBY-K1/T- NsNd (Fig. 2-8), was modified from pBY-K1 and constructed in 2.3.2.2. The other seven chimeric infectious cDNA clones pBY-T1/K-KpNs, pBY-T1/K-MsNs, pBY-T1/K-KpMs, pBY-T1/K-CI, pBY-T1/K-Kp/6K1, pBY-T1/K-6K1 and pBY-T1/K-Kp/P3 were modified from pBY-T1 (Fig. 2-16). To generate pBY-T1/K-KpNs, a *Kpn*I-*Nsi*I fragment was released from pBY-K1 and ligated into similarly digested pBY-T1. To generate pBY-T1/K-MsNs construct, a *Msc*I-*Nsi*I fragment was released from pBY-K1 and ligated into similarly digested pBY-T1. To generate pBY-T1/K-KpMs construct, a *Kpn*I-*Msc*I fragment was released from pBY-K1 and ligated into similarly digested pBY-T1. To generate pBY-T1/K-CI pBY-T1/K-Kp/6K1, pBY-T1/K-6K1 and pBY-T1.T-Kp/P3 constructs, PCRs were performed in the presence pBY-T1 and pBY-K1 as the templates. The resultant PCR products were purified and served as a mixed template to generate the fragment containing the expected region from pBY-K1 and cloned into pBY-T1 with *Kpn*I and *Nru*I sites for pBY-T1/K-CI

using an In-Fusion HD Cloning System CE kit (Clontech Laboratories, USA), and *Kpn*I and *Msc*I sites for pBY-T1/K-Kp/6K1 pBY-T1/K-6K1 and pBY-T1.T-Kp/P3 constructs. The primers used for each construct were listed in Appendix Table S6.

All the recombinant DNA procedures were carried out by standard methods using *E. coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts.

2.5.2.3 *In vitro* transcription

It was done as described in 2.2.2.4.

2.5.2.4 Mesophyll protoplast preparation and RNA transfection

It was done as described in 2.2.2.5.

2.5.2.5 Western blot analysis

It was done as described in 2.2.2.7.

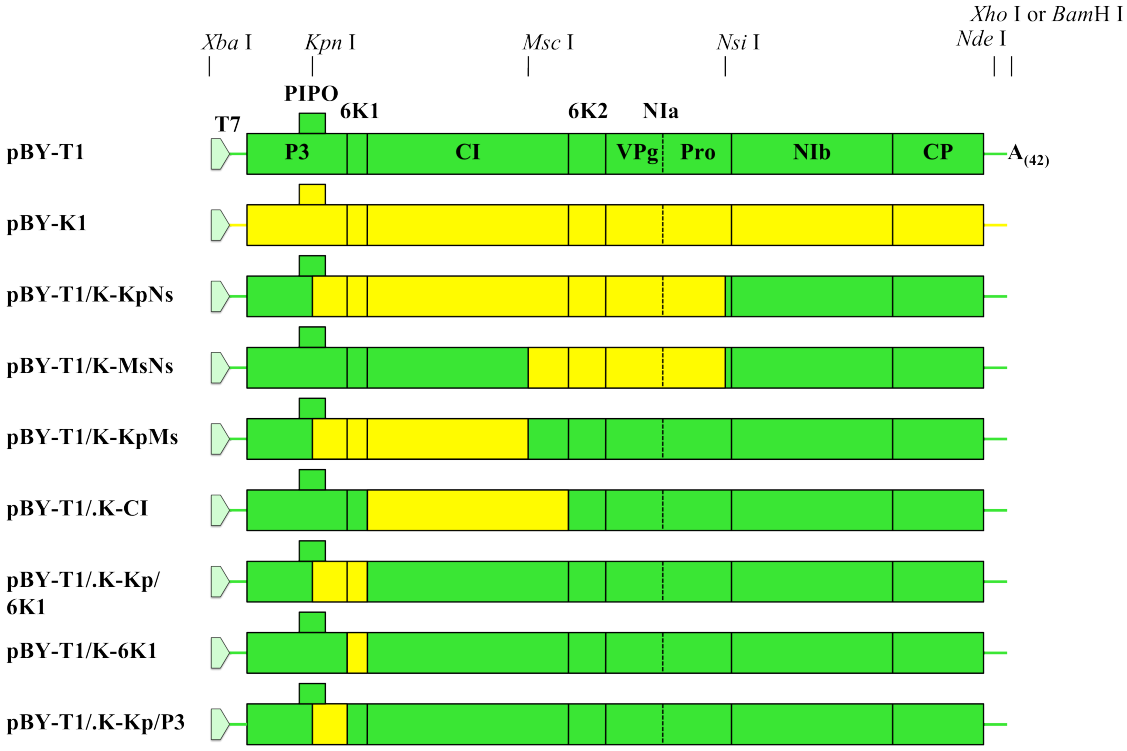


Fig. 2-16 Schematic representation of chimeric cDNA clones between Tochigi JT10 and Kurashiki JK05 isolates.

2.5.3 Results

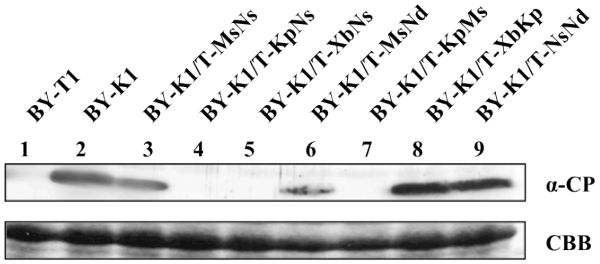
2.5.3.1 Infectivity assay of pBY-K1 mutants in *rym2* protoplasts

To identify the viral determinants involved in overcoming *rym2* resistance, I constructed several chimeras utilizing common available restriction enzyme sites between pBY-K1 and pBY-T1 for exchanging reciprocal regions (Fig. 2-8). Mesophyll protoplasts were transfected with *in vitro* transcripts of wide-type or mutant RNA1 in combination of RNA2 transcripts. After incubation at 15 °C for 66 h, the protoplasts were harvested for Western blot analysis using CP antiserum. Compared with the wild-type RNA1 of Kurashiki isolate JK05, a comparable CP accumulation was detected in *rym2* cells transfected with transcript from T-XbKp mutant (Fig. 2-17A lanes 2 and 8, and 2-15B bars 2 and 8), suggesting that the *Xba*I-*Kpn*I region of Tochigi isolate JT10 had no effect on replication of Kurashiki isolate JK05 in *rym2* cells. A less CP accumulation was detected in *rym2* cells transfected with transcripts from T-NsNd, T-MsNs and T-MsNd mutants (Fig. 2-17A lanes 2, 3, 6 and 9, and 2-15B bars 2, 3, 6 and 9), suggesting that *Msc*I-*Nsi*I and *Nsi*I-*Nde*I regions of Tochigi isolate JT10 negatively affected the infectivity of Kurashiki isolate JK05 in *rym2* cells. In contrast, CP was undetectable in *rym2* cells transfected with transcripts from T-KpNs, T-XbNs and T-KpMs mutants as that from pBY-T1 (Fig. 2-17A lanes 1, 4, 5 and 7, and 2-15B bars 1, 4, 5 and 7). The non-infectious mutants of T-KpNs, T-XbNs and T-KpMs contain the same region of *Kpn*I-*Msc*I from Tochigi isolate JT10. Thus, the viral determinant responsible for replication in *rym2* cells was minimized to the *Kpn*I-*Msc*I region of Kurashiki isolate JK05.

2.5.3.2 Infectivity assay of pBY-T1 mutants in *rym2* protoplasts

To identify the precise viral factors involved in replication in *rym2* resistance, I constructed another set of chimeras in the background of pBY-T1 by exchanging reciprocal regions between pBY-K1 and pBY-T1 (Fig. 2-16). Mesophyll protoplasts were transfected with *in vitro* transcripts and incubated at 15 °C for 66 h. After incubation, the protoplasts were harvested for Western blot analysis with CP antiserum. As expected, K-KpNs mutant replicated in *rym2* cells, although with a low infectivity efficiency in CP accumulation (Fig. 2-18A lane 3, 2-16B bar 3). Furthermore, CP could be also detected in *rym2* cells transfected with transcripts from K-KpMs, K-Kp/6K1 and K-Kp/P3 mutants, respectively (Fig. 2-18A lanes 5, 7 and 9, 2-16B bars 5, 7 and 9). Of these chimeras, pBY-T1/K-Kp/P3 contains the limited region of *Kpn*I-P3 from pBY-K1. In contrast, the other three mutants of K-MsNs, K-CI and K-6K1, lack of the *Kpn*I-P3 region of Tochigi isolate JT10, did not replicate in *rym2* cells (Fig. 2-18A lanes 4, 6 and 8, 2-16B bars 4, 6 and 8). Thus, *Kpn*I-P3 region of Kurashiki isolate JK05 was the key factor for replication in *rym2* cell.

A



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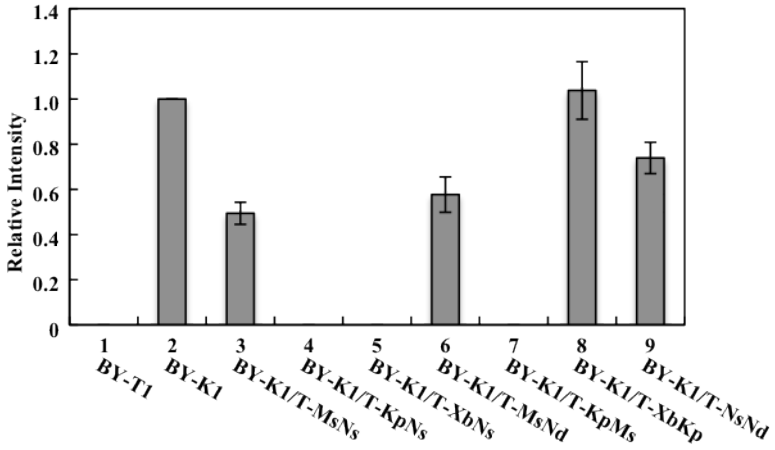
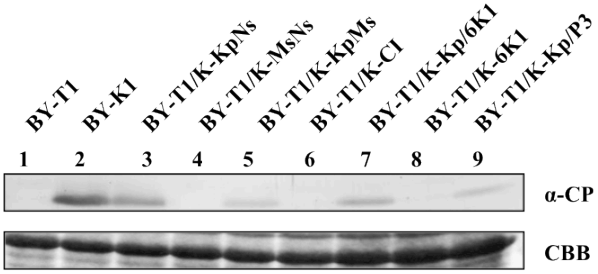


Fig. 2-17 Infectivity assay in protoplasts from Mihori Hadaka 3 (*rym2*) with *in vitro* transcripts of chemeric constructs between JT10 and JK05 isoaltes in the backfround of pBY-K1. **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-BaYMV CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** Relative intensity of CP accumulation in transfected protoplasts. Experiments were at least triplicated . The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Mihori Hadaka 3 (*rym2*) transfected with transcripts from pBY-K1 and pBY-K2 was used as a control and set as 1.0. The transfected protoplasts were incubated at 15 °C for 66 h.

A



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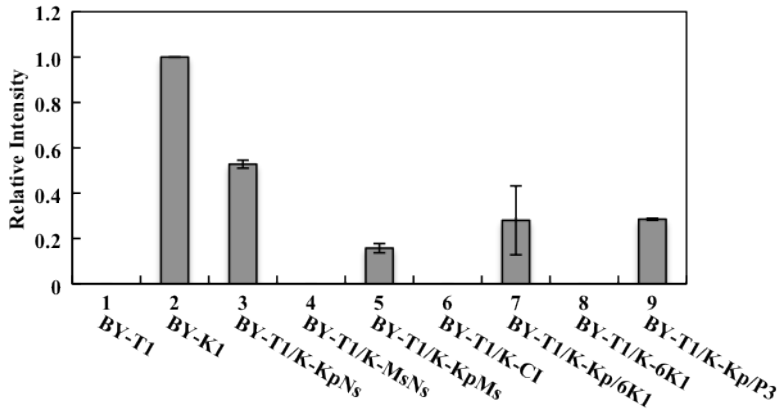

Fig. 2-18 Infectivity assay in protoplasts from Mihori Hadaka 3 (*rym2*) with *in vitro* transcripts of chemeric constructs between JT10 and JK05 isoaltes in the backfround of pBY-T1. **(A)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **α-CP**: Total proteins subjected to Western blot analysis using anti-BaYMV CP (α-CP). **CBB**: The Rubisco large subunit stained by Coomassie Brilliant Blue G-250. **(B)** Relative intensity of CP accumulation in transfected protoplasts. Experiments were at least triplicated . The CP bands on Western blots were scanned using ImageJ software. The amount of CP accumulation in protoplasts from Mihori Hadaka 3 (*rym2*) transfected with transcripts from pBY-K1 and pBY-K2 was used as a control and set as 1.0. The transfected protoplasts were incubated at 15 °C for 66 h.

2.5.4 Discussion

The C-terminal region of P3 (*Kpn*I-P3) of Kurashiki isolate JK05 was identified as virulence determinant of BaYMV replication in *rym2* cells. There are 12 nucleotide acids differences in the *Kpn*I-P3 regions between Tochigi JT10 and Kurashiki JK05 isolates, resulting in 10 amino acid differences in P3 protein and no amino acid difference between PIPO proteins (Fig. 2-19).

The P3 protein is one of the least conserved proteins in the family *Potyviridae* not well characterized even in the genus *Potyvirus*. Previous reports showed that the mutation in potyvirus P3 affected virus replication in protoplasts (Klein et al., 1994). It also plays a role in host range and symptom development (Adams et al., 2012; Sáenz et al., 2000; Urcuqui-Inchima et al., 2001). BaYMV P3 was first identified to play a role in BaYMV replication as previously shown in *Tobacco vein mottling virus* (Klein et al., 1994).

As discussed in 2.2.4, Mihori Hadaka 3 (*rym2*) is resistant to Kurashiki isolate JK05 at the whole plant level, whereas it was completely resistant to Tochigi isolate JT10 even at the cellular level, indicating that the *rym2* resistance gene encodes a host factor involved in both virus replication and movements. However, it could not rule out the possibility that the resistance in Mihori Hadaka 3 may be controlled by multiple resistance genes including *rym2* gene as in *rym5* plants.

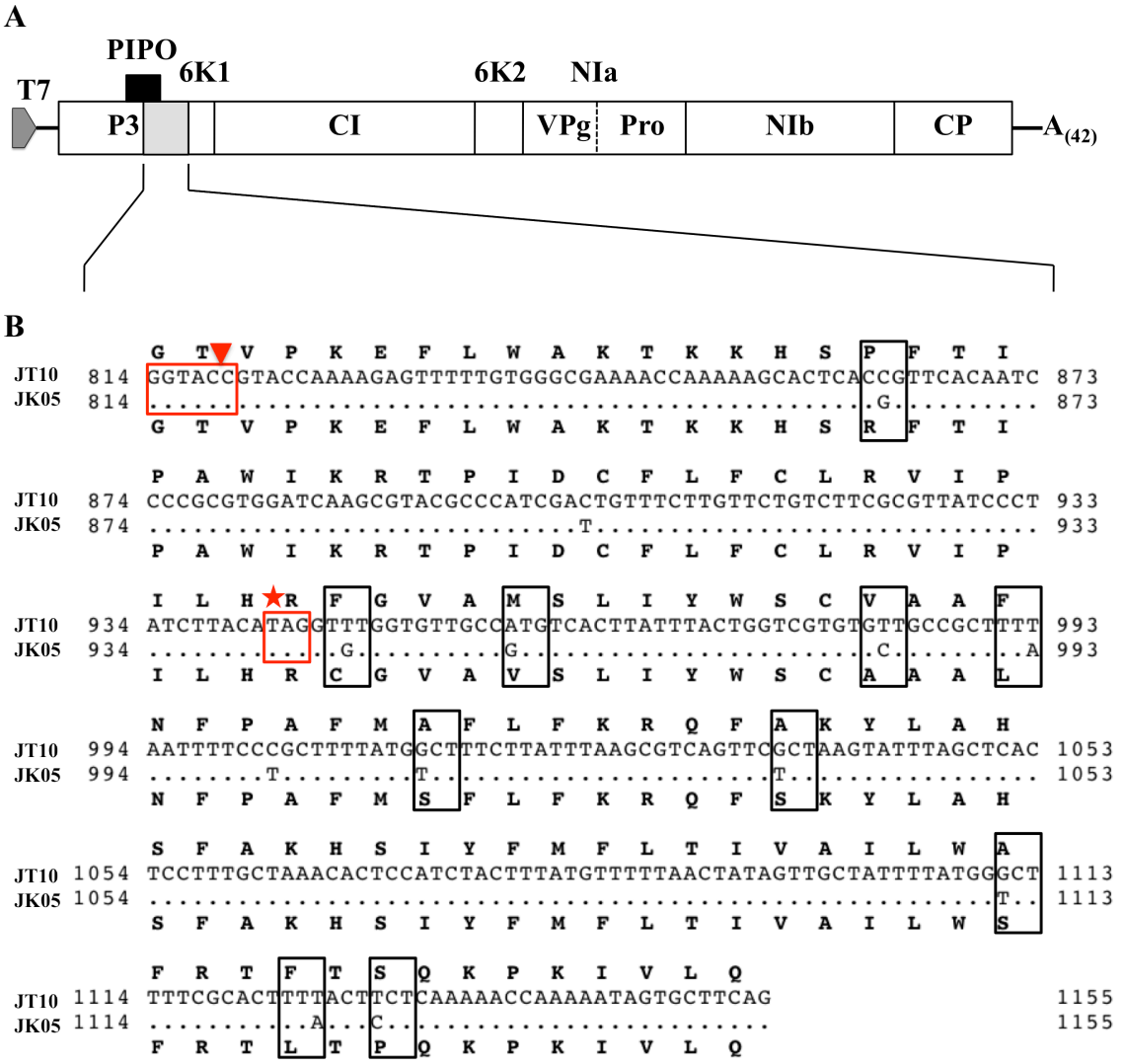

Fig. 2-19 Amino acid sequence alignment of *Kpn*I-P3 regions from JT10 and JK05 isolates. **(A)** Schematic representation of BaYMV RNA1. VPg was shown in gray rectangle. (B) Alignment of amino acid sequences of VPg from JT10 and JK05 isolates.

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Chapter 3

Association of VPg and eIF4E in the host tropism at the cellular level of *Barley yellow mosaic virus* and *Wheat yellow mosaic virus*

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3.1 Introduction

Bymoviruses have relatively narrow host ranges, and each species can infect one single type of host. The only known natural host of BaYMV is barley, and that of WYMV is wheat. The distinct host ranges of BaYMV and WYMV could also be due to host resistance and susceptibility. For systemic infection in plants, viruses must accomplish several distinct steps: virus entry, replication, cell-to-cell movement, long-distance movement (Narayanasamy, 2008), and transmission. Among the 11 viral proteins, NIb (Li et al., 1997), VPg (Puustinen and Mäkinen, 2004) and 6K2 (Restrepo-Hartwig and Carrington, 1994) are predicted to have a function in replication, and CP (Dolja et al., 1995), P3N-PIPO (Vijayapalani et al., 2012; Wei et al., 2010; Wen and Hajimorad, 2010) and CI (Carrington et al., 1998) may be involved in cell-to-cell movement. These predictions are based on amino acid sequence similarities with proteins of viruses in the genus *Potyvirus*. Furthermore, P2 is required for sufficient systemic infection (You and Shirako, 2010) and is predicted to be involved in vector transmission (Dessens and Meyer, 1996). For each step, plant viruses may also utilize additional host proteins to accomplish these functions. For example, host translation initiation factor 4E (eIF4E) and its isoform eIF(iso)4E interact with viral VPg (Léonard et al., 2000; Roudet-Tavert et al., 2007; Schaad et al., 2000; Wittmann et al., 1997). Systemic infection can be disturbed at the entry, replication and movement steps in host plants. The blocked steps that result in the host restriction of BaYMV and WYMV are unknown.

As the first step in understanding the mechanisms determining the host range of BaYMV and WYMV in fields, I examined their replication at the cellular level using

infectious *in vitro* transcripts in isolated barley and wheat protoplasts. I also delivered barley or wheat eIF4E, a common host factor for potyviruses (Charron et al., 2008), to the reciprocal host and examined the effect of exchanging VPg genes between BaYMV and WYMV on their replication using *in vitro* transcripts from mutant cDNA clones. Our results demonstrate that both host eIF4E and viral VPg are involved in the host tropism of BaYMV and WYMV at the replication level.

3.2 Materials and Methods

3.2.1 Plants and viruses

Barley (*Hordeum vulgare*, cv. Ryofu) and wheat (*Triticum aestivum*, cv. Shiranekomugi) were used as plant materials in this study. BaYMV was isolated from a barley field in Kurashiki in 2005 (JK05 isolate), and WYMV was isolated from a wheat field in Nagano in 2009.

3.2.2 Infectious full-length cDNA clones of BaYMV and WYMV

The infectious full-length cDNA clones of BaYMV RNA1 and RNA2 were constructed and referred to as pBY-K1 and pBY-K2 in this study (You and Shirako, 2010), respectively. Similarly infectious full-length cDNA clones of WYMV RNA1 and RNA2 were constructed and designated as pWY1 and pWY2, respectively (Shirako, 2012). An SP6 promoter was used in both pWY1 and pWY2 for *in vitro* transcription.

3.2.3 cDNA isolation of *Hv* eIF4E and *Ta* eIF4E genes

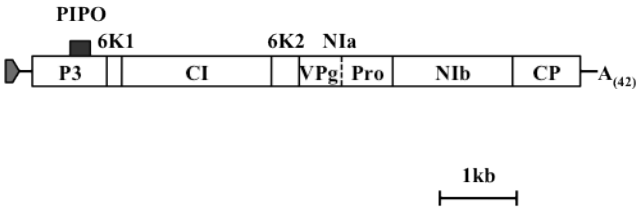
The *Hv* eIF4E gene was isolated from barley (cv. KoA), and the *Ta* eIF4E gene was isolated from wheat (cv. Shiranekomugi). For cDNA cloning of the eIF4E gene, total RNA was extracted from the leaves of barley or wheat using QuickGene-800 (Fuji Film, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA using PrimeScript™ Reverse Transcriptase (Takara Bio, Japan), and PCR was performed using PrimeSTAR® HS DNA Polymerase (Takara Bio, Japan). PCR fragments were purified using a FastGene™ Gel/PCR Extraction Kit (Nippon Genetics, Japan) and directly submitted for sequencing. The following GenBank nucleotide

sequences were used as references: AJ699059 for barley cv. Morex, AB592973 for barley cv. KoA and Z12616 for wheat cv. unknown.

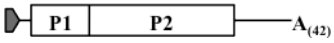
3.2.4 RNA1 and RNA2 mutant cDNA constructs of BaYMV and WYMV

Three types of mutant cDNA constructs were prepared from pBY-K1, pWY1, pBY-K2 and pWY2 (Fig. 3-1): (i) GFP-expressed mutant RNA2 constructs pBY-K2.GFP and pWY2.GFP. The mutant construct pBY-K2.GFP was modified from pBY-K2 with a GFP gene replacement of the P1 and P2 genes between the 5'- and 3'-untranslated regions (UTRs) of RNA2 (You and Shirako, 2010). The pWY2.GFP plasmid was constructed from pWY2 in the same manner. (ii) eIF4E-expressed mutant RNA2 constructs pBY-K2.P1/*Hv* eIF4E, pWY2.P1/*Ta* eIF4E and pBY-K2.P1/*Ta* eIF4E. These three mutant constructs were modified from RNA2 constructs of pBY-K2 or pWY2 by an insertion of the eIF4E gene to replace the P2 gene. To generate pBY-K2.P1/*Hv* eIF4E, PCRs were performed in the presence of pBY-K2 and the *Hv* eIF4E gene as separate templates. The resultant PCR products were purified and served as a mixed template to generate the P1/*Hv* eIF4E fragment. Subsequently, the fragment was digested and ligated into pBY-K2 using *Mlu*I and *Spe*I sites. To generate pWY2.P1/*Ta* eIF4E, PCRs were performed in the presence pWY2 and the *Ta* eIF4E gene as the templates. The resultant PCR products were purified and served as a mixed template to generate the fragment P1/*Ta* eIF4E. The final PCR product was digested with *Nde*I and *Spe*I and ligated into pWY2. To generate pBY-K2.P1/*Ta* eIF4E, PCRs were performed in the presence of pBY-K2 and the *Ta* eIF4E gene as separate templates. The resultant PCR products were purified and served as a mixed template to generate BaYMV P1/*Ta* eIF4E. The final amplified fragments were purified and cloned into pBY-K2 with *Nsi*I

A WT RNA1 constucts: **pBY-K1 and pWY1**



B WT RNA2 constucts: pBY-K2 and pWY2



C GFP-expressed RNA2 mutants: **pBY-K2.GFP and pWY2.GFP**

D eIF4E-expressed RNA2 mutants: **pBY-K2.P1/*Hv* eIF4E, pWY2.P1/*Ta* eIF4E and pBY-K2.P1/*Ta* eIF4E**

E RNA1 mutant: **pBY-K1.WY-VPg**

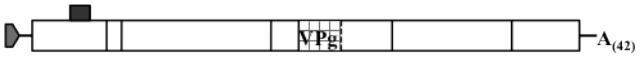


Fig. 3-1 Schematic representation of wild-type (WT) and mutant RNA1 and RNA2 constructs of BaYMV and WYMV. **(A)** pBY-K1 and pWY1: the infectious full-length cDNA clone of WT RNA1 of BaYMV or WYMV. **(B)** pBY-K2 and pWY2: the infectious full-length cDNA clone of WT RNA2 of BaYMV or WYMV. **(C)** pBY-K2.GFP and pWY2.GFP: pBY-K2- or pWY2-derivative mutants with a GFP gene in the place of the P1/P2 polyproteiin gene. **(D)** pBY-K2.P1/*Hv* eIF4E, pWY2.P1-*Ta* eIF4E and pBY-K2.P1/*Ta* eIF4E: pBY-K2- or pWY2-derivative mutants with the eIF4E gene from barley (*Hv* eIF4E) or wheat (*Ta* eIF4E) in the place of the BaYMV P2 or WYMV P2 gene. **(E)** pBY-K1.WY-VPg: A chimeric construct derived from pBY-K1 by replacing the BaYMV VPg gene with the WYMV VPg gene. The rightward filled pentagons at the 5' end indicate the T7 or SP6 promoter. Map scale, 1 kilobase (kb).

and *Eco*RI sites using an In-Fusion HD Cloning System CE kit (Clontech Laboratories, USA). (iii) WYMV VPg-expressed chimera BaYMV RNA1 construct pBY-K1.WY-VPg. The construct was modified from pBY-K1 by a replacement of the BaYMV VPg gene with the WYMV VPg gene using pBY-K1 and pWY1 as the templates.

All the recombinant DNA procedures were performed according to standard methods using *Escherichia coli* strain MC1061 and the clones were verified by sequencing the plasmid inserts. The primers used for the construction of these mutants are listed in Appendix Table S7.

3.2.5 *In vitro* transcription

Infectious cDNA clones were linearized and subsequently used as templates for *in vitro* transcription. BaYMV RNA1 (pBY-K1) and its derivative (pBY-K1.WY-VPg) were linearized using *Xho*I and *Bam*HI, respectively, and BaYMV RNA2 (pBY-K2), WYMV RNA1 (pWY1), WYMV RNA2 (pWY2) and their derivatives (pBY-K2.GFP, pBY-K2.P1/*Hv* eIF4E, pBY-K2.P1/*Ta* eIF4E, pWY2.GFP and pWY2.P1/*Ta* eIF4E) were linearized using *Spe*I. *In vitro* transcription reactions were performed with the T7 RNA polymerase for the pBY-K1, pBY-K2, pBY-K2.GFP, pBY-K2.P1/*Hv* eIF4E, pBY-K2.P1/*Ta* eIF4E, pBY-K1.WY-VPg, and pWY2.GFP constructs or SP6 RNA polymerase for the pWY1, pWY2 and pWY2.P1/*Ta* eIF4E constructs. T7 transcription reactions were performed using an mMESSAGE mMACHINE® T7 Kit (Ambion, USA) as described in 2.2.2.4. SP6 transcription reactions were performed as described previously (Yamamiya and Shirako, 2000) in a 10 μL reaction mixture containing 40 mM of Tris-HCl (pH 8.0), 8 mM of $MgCl_2$, 2 mM of spermidine, 1 mM of rNTP Mix,

2.5 mM of DTT, 20 units of RNase inhibitor (Takara Bio, Japan), 0.5 mM of m7G(5')ppp(5')G RNA Cap Structure Analog (New England Biolabs, USA), 10 units of SP6 RNA polymerase (New England Biolabs, USA) and 100 ng of linearized DNA as the template. Both T7 and SP6 transcription reactions were performed at 37 °C for 1 h.

3.2.6 Mesophyll protoplast preparation and RNA transfection

Barley and wheat seedlings at the 6-7-day-old stage were harvested for protoplast preparation as described in 2.2.2.5. Transfected protoplast cells were incubated for 66 h at 15 °C for BaYMV and 90 h at 12 °C for WYMV in the dark.

3.2.7 Viral infection to plants using in vitro transcripts

It was done as described in 2.2.2.6. Inoculated plants wrapped with a plastic bag were placed in dark box for 2 days at 15 °C (in case of BaYMV RNA1) or 12 °C (in case of WYMV RNA1), and then kept in a growth cabinet at 15 °C or 12 °C with 16 h of illumination.

3.2.8 Western blot analysis

It was done as described in 2.2.2.7. Western blot analysis was performed with polyclonal antibodies against BaYMV CP (diluted 1:5000) (You and Shirako, 2010) and against WYMV WYCP (diluted 1:1000) (this study) as the primary antibody.

3.2.9 Fluorescent light microscopy

It was done as described in 2.2.2.8.

3.3 Results

3.3.1 Comparison between BaYMV and WYMV genomes

As members of the same genus *Bymovirus* in the family *Potyviridae*, BaYMV (isolate JK-05, Japanese Pathotype II; GenBank: AB500948 for RNA1 and AB500949 for RNA2) and WYMV (isolate Nagano-B, Japan; GenBank: AB948222 for RNA1 and AB948223 for RNA2) share amino acid sequence identities between 56.0% within the 6K2 protein and 87.9% within the 6K1 protein among all 11 mature proteins (Table 3-1).

3.3.2 BaYMV replication in barley and wheat protoplasts

I first examined the ability of BaYMV to replicate in barley and wheat mesophyll protoplasts. Protoplasts transfected with *in vitro* transcripts from pBY-K1 for RNA1 (Fig. 3-1A) and from pBY-K2 for RNA2 (Fig. 3-1B) (You and Shirako, 2010) were incubated at 15 °C for 66 h. After incubation, total protoplast extracts were subjected to Western blot analysis. Detection of a 32-kDa CP band was used as a criterion for RNA1 replication and quantitative analysis. In our previous study, BaYMV RNA1, which encodes a set of replication proteins, autonomously replicated in barley protoplasts. The additional presence of RNA2, replication of which relies on RNA1, resulted in a high level of CP accumulation by a function of the expressed P1 protein (You and Shirako, 2010); the mechanism is not known at this moment. As shown here again, a very low level of BaYMV CP accumulation was detected in barley protoplasts transfected with only BaYMV RNA1 transcript in the absence of RNA2, and a high level of BaYMV CP accumulation was detected in the presence of RNA2 (Fig. 3-2A lanes 1–3, and 3-2B bars 1–3). I further examined the transfection of barley protoplasts with *in vitro*

Table 3-1 Identities of amino acied sequences between BaYMV and WYMV.

RNA1								
Long ORF	**P3**	**6K1**	**CI**	**6K2**	**VPg**	**NIa-Pro**	**NIb**	**CP**
Identities of amino acids	234/328 (71.3%)	58/66 (87.9%)	530/639 (80.4%)	70/125 (56.0%)	136/187 (72.7%)	166/225 (73.8%)	441/528 (83.5%)	213/297 (71.7%)
+2 F/S[a]	**PIPO**							
Identities of amino acids	60/86 (69.8%)							

RNA2		
ORF	**P1**	**P2**
Identities of amino acids	152/255 (59.6%)	369/649 (56.9%)

[a], +2 F/S, +2 shift reading frame.

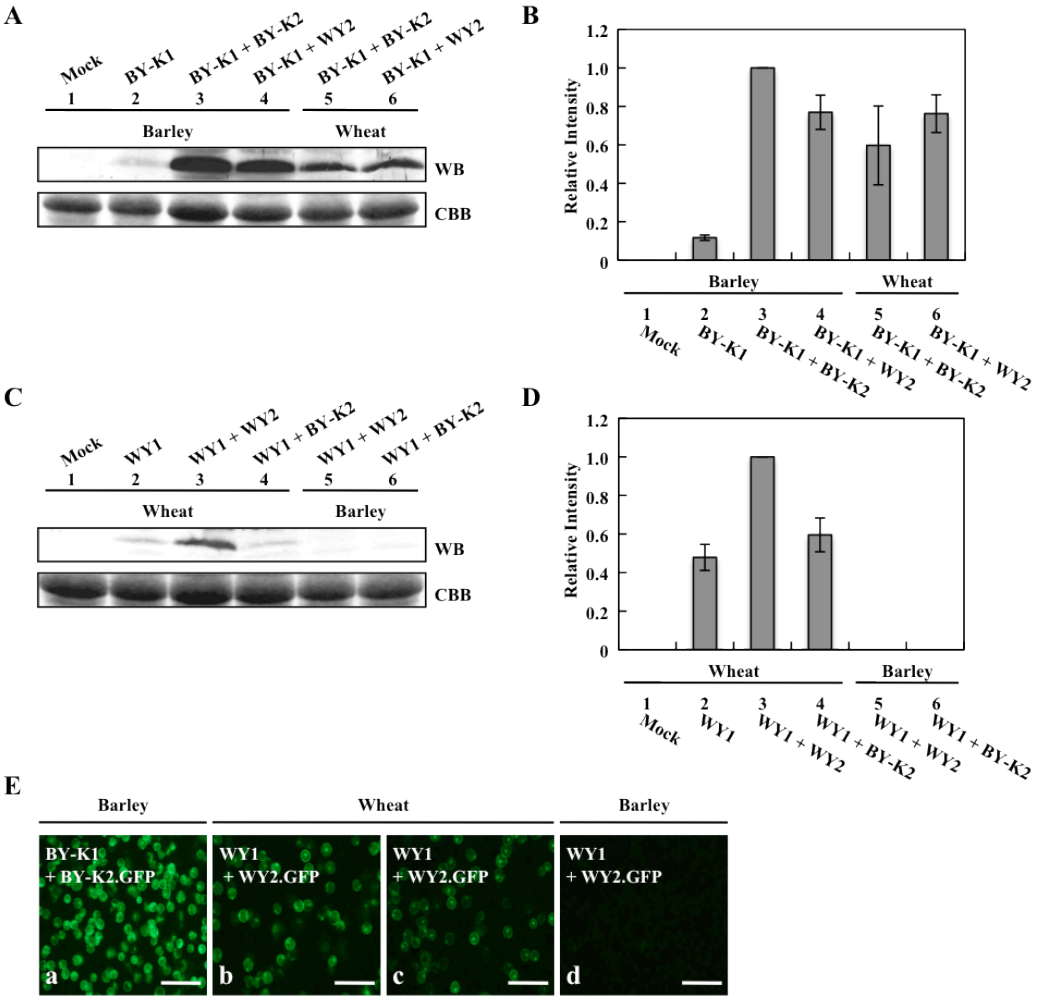


Fig. 3-2 Infectivity assay of BaYMV and WYMV in mesophyll protoplasts. Western blot analyses showed **(A)** BaYMV CP and **(C)** WYMV CP accumulation in protoplasts transfected with *in vitro* transcripts. Relative intensities were estimated according to **(B)** BaYMV CP and **(D)** WYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1.0 for the analysis of BaYMV CP accumulation. WYMV CP accumulation in wheat protoplasts transfected with WYMV RNA1 and RNA2 transcripts was also used as a control and set as 1.0 for the analysis of WYMV CP accumulation. **(E)** GFP fluorescence observed in protoplasts transfected with RNA1 and GFP RNA2 transcripts. Bars, 200 μm.

transcripts from pBY-K1 and pWY2, the latter of which is a full-length cDNA clone of WYMV RNA2 (Fig. 3-1B). The result showed a high level of BaYMV CP accumulation in the barley protoplasts, although the accumulation was slightly reduced compared to transfection with BaYMV RNA1 and BaYMV RNA2 (Fig. 3-2A lane 4 and 3-2B bar 4).

To examine the replicative ability of BaYMV in wheat protoplasts, I transfected them with BaYMV RNA1 and BaYMV RNA2 transcripts. A reduced BaYMV CP accumulation was observed in wheat protoplasts compared to in barley protoplasts (Fig. 3-2A lane 5 and 3-2B bar 5). Next, wheat protoplasts were transfected with BaYMV RNA1 and WYMV RNA2 transcripts. The result showed that CP was detected at a similar level as observed after BaYMV RNA1 and BaYMV RNA2 transfection in wheat protoplasts (Fig. 3-2A lane 6 and 3-2B bar 6). These results indicated that both barley and wheat protoplasts supported replication of BaYMV RNA1 together with BaYMV or WYMV RNA2, although in wheat protoplasts BaYMV replication efficiency was reduced than in barley protoplasts.

3.3.3 Mechanical inoculation of BaYMV to barley and wheat plants

I further examined systemic infectivity using the *in vitro* transcripts (6 to 8 plants at a time, repeated three times). Inoculation with BaYMV RNA1 and BaYMV RNA2 transcripts to barley plants resulted in systemic infection as shown previously (You and Shirako, 2010). On the contrary, inoculation with BaYMV RNA1 and WYMV RNA2 transcripts to barley plants, BaYMV RNA1 and BaYMV RNA2 transcripts to wheat plants, and BaYMV RNA1 and WYMV RNA2 transcripts to wheat plants resulted in no infection as confirmed with Western blot analysis of upper uninoculated leaf extracts

using anti-BaYMV CP antiserum (data not shown).

3.3.4 WYMV replicated in wheat protoplasts but not in barley protoplasts

Next, I examined the replicative ability of WYMV in barley and wheat mesophyll protoplasts. Because this is the first study to use a set of WYMV infectious *in vitro* transcripts from pWY1, a full-length cDNA clone of WYMV RNA1 (Fig. 3-1A), and pWY2 (Fig. 3-1B) to wheat protoplasts, I first determined the optimal incubation temperature and time length after transfection. Incubation of transfected wheat protoplasts at 12 °C for 90 h resulted in the highest level of CP accumulation (data not shown); thus, I chose this condition for transfection experiments using WYMV RNA1 transcript. Transfection of wheat protoplasts with WYMV RNA1 transcript resulted in a low level of WYMV CP accumulation in the absence of RNA2 transcript and a higher level of WYMV CP accumulation in the presence of WYMV RNA2 transcript (Fig. 3-2C lanes 1–3, and 3-2D bars 1–3), indicating that WYMV RNA1 replicated autonomously in wheat protoplasts and that a high level of WYMV CP accumulation was achieved when transfected in combination with the WYMV RNA2 transcript similar to the result with BaYMV (You and Shirako, 2010). I further examined the transfection of wheat protoplasts with a heterologous combination of WYMV RNA1 and BaYMV RNA2 transcripts. In contrast to the results using BaYMV RNA1 in barley protoplasts (Fig. 3-2A lanes 2 and 4 and 3-2B bars 2 and 4), BaYMV RNA2 enhanced WYMV CP accumulation in wheat protoplasts only slightly (Fig. 3-2C lane 4 and 3-2D bar 4) compared to WYMV RNA1 only (Fig. 3-2C lane 2 and 3-2D bar 4). To examine whether WYMV RNA1 supported the replication of BaYMV RNA2 in wheat cells, I transfected wheat protoplasts with WYMV RNA1 transcript and BaYMV GFP (green

fluorescence protein) RNA2 transcript from pBY-K2.GFP (Fig. 3-1C) (You and Shirako, 2010), using WYMV GFP RNA2 transcript from pWY2.GFP (Fig. 3-1C) as a control. In this assay, GFP was expressed from replicating RNA2 using active replication proteins from the wild-type RNA1. Positive control experiments using BaYMV RNA1 and BaYMV GFP RNA2 transcripts in susceptible barley protoplasts always gave more than 50% in transfection efficiency with strong GFP fluorescence (Fig. 3-2E-a). Mock-inoculated negative controls gave no fluorescence, making this assay a quite sensitive and confirmative method over RNA1-mediated CP detection on Western blots. Wheat protoplasts transfected with WYMV RNA1 and WYMV GFP RNA2 transcripts emitted GFP strongly (Fig. 3-2E-b). Strong GFP fluorescence was also detected in wheat protoplasts transfected with WYMV RNA1 and BaYMV GFP RNA2 transcripts (Fig. 3-2E-c), indicating that BaYMV RNA2 replicated with WYMV RNA1 in wheat protoplasts although an elevated level of CP accumulation was not detected (see Discussion).

When barley protoplasts were transfected with WYMV RNA1 and RNA2 transcripts, no WYMV CP band was detectable (Fig. 3-2C lane 5 and 3-2D bar 5) on Western blots. The same results were obtained in barley protoplasts transfected with WYMV RNA1 and BaYMV RNA2 transcripts. No WYMV CP accumulation was observed in these barley protoplasts (Fig. 3-2C lane 6 and 3-2D bar 6). Therefore, I also examined GFP fluorescence microcopy, which is more sensitive than CP band detection on Western blots as mentioned above. No GFP fluorescence was detected in barley protoplasts transfected with WYMV RNA1 and WYMV GFP RNA2 transcripts (Fig. 3-2E-d). No GFP fluorescence was detected in barley protoplasts transfected with

WYMV RNA1 and BaYMV GFP RNA2 either (data not shown), indicating that WYMV RNA1 can not replicate in barley protoplasts.

3.3.5 Barley eIF4E enhanced BaYMV replication in wheat protoplasts, and wheat eIF4E enabled WYMV to replicate in barley protoplasts

eIF4E and *eIF(iso)4E* are well-identified recessive resistance genes against virulent strains of many virus species in the genus *Potyvirus*. Here, I examined whether eIF4E could be involved in the host tropism of BaYMV and WYMV at the replication level. For this purpose, I constructed two derivative mutant constructs pBY-K2.P1/*Hv* eIF4E and pWY2.P1/*Ta* eIF4E from pBY-K2 and pWY2, respectively (Fig. 3-1D). In pBY-K2.P1/*Hv* eIF4E, the BaYMV P2 gene whose product is not required for replication (You and Shirako, 2010) was replaced with a barley eIF4E (*Hv* eIF4E) gene; that is, a serine codon was placed after the P1 gene, followed by the full-length barley eIF4E gene, so that the translated P1/*Hv* eIF4E polyprotein would be cleaved into the mature P1 autoproteinase and the barley eIF4E protein with an additional serine at the N terminus. Similarly, in pWY2.P1/*Ta* eIF4E, the WYMV P2 gene was replaced with a wheat eIF4E (*Ta* eIF4E) gene. Using *in vitro* transcripts from these mutant RNA2 constructs, barley and wheat eIF4E could be expressed in wheat and barley protoplasts. There are 16 amino acid differences within the 215 amino acids between the two eIF4E proteins (Fig. 3-3A).

Barley protoplasts transfected with BaYMV RNA1 and BaYMV P1/*Hv* eIF4E RNA2 transcripts resulted in the same level of BaYMV CP accumulation as with the BaYMV RNA1 and BaYMV RNA2 transcripts (Fig. 3-3B lanes 1 and 2 and 3-3C bars 1

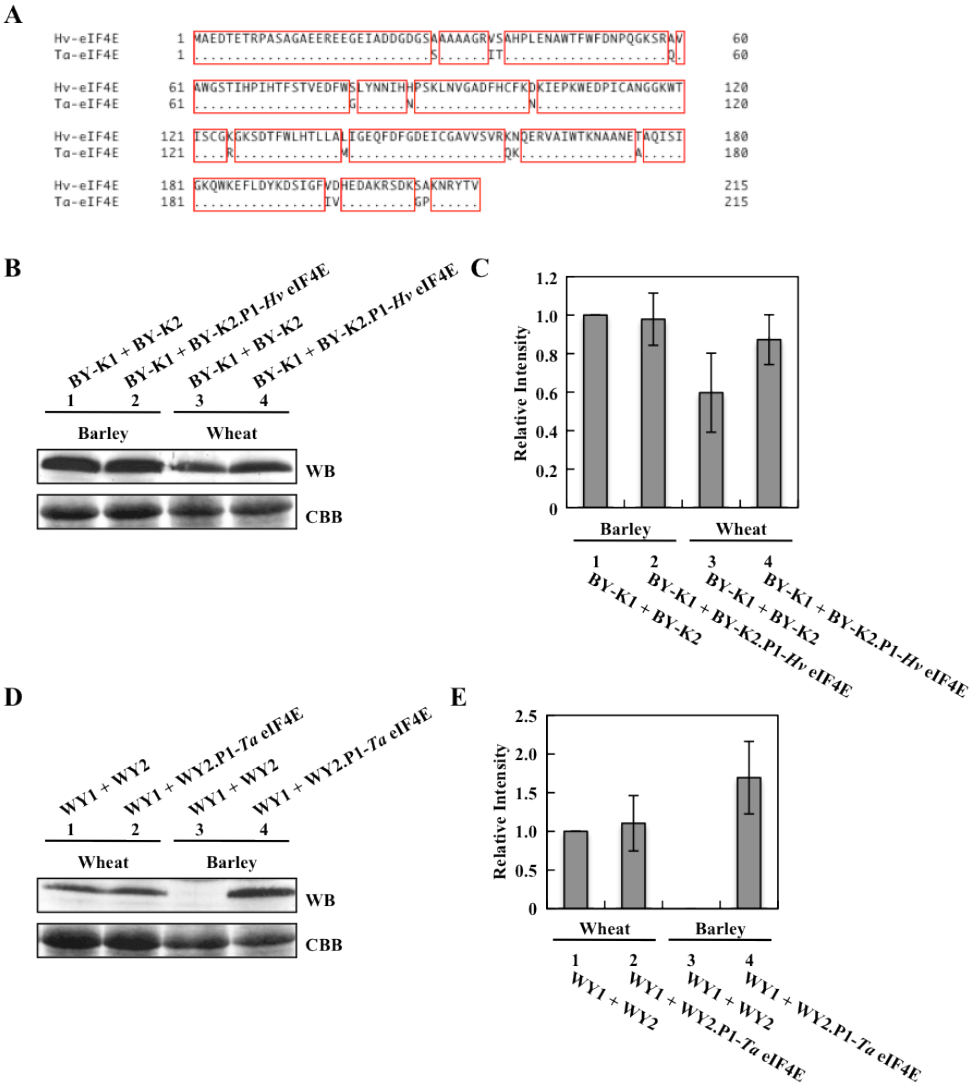


Fig. 3-3 Infectivity assay of BaYMV and WYMV co-expressed with eIF4E in barley and wheat mesophyll protoplasts. **(A)** Alignments of amino acid sequences of eIF4Es isolated from barley (cv. KoA) and wheat (cv. Shiranekomugi) using Genetyx-Mac software (Genetyx, Japan). Western blot analyses showed **(B)** BaYMV CP and **(D)** WYMV CP accumulation in protoplasts transfected with RNA1 and RNA2 or eIF4E-expressed RNA2 transcripts. Relative intensity was estimated according to **(C)** BaYMV CP and **(E)** WYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1.0 for the analyses of BaYMV CP accumulation. WYMV CP accumulation in wheat protoplasts transfected with WYMV RNA1 and RNA2 transcripts was also used as a control and set as 1.0 for the analysis of WYMV CP accumulation.

and 2). Thus, exogenous delivery of the barley eIF4E gene and its expression in transfected barley protoplasts (confirmed by Western blot analysis using P1 and eIF4E antisera, data not shown) did not appear to have an additional effect on BaYMV replication in barley cells using the intrinsic host eIF4E function. In wheat protoplasts, transfection with BaYMV RNA1 and BaYMV P1/*Hv* eIF4E RNA2 transcripts resulted in higher BaYMV CP accumulation than with the BaYMV RNA1 and BaYMV RNA2 transcripts (Fig. 3-3B lanes 3 and 4 and 3-3C bars 3 and 4). This result indicated that the exogenous barley eIF4E protein expressed from the transfected mutant BaYMV RNA2 transcript enhanced BaYMV CP accumulation in wheat cells.

Wheat protoplasts transfected with WYMV RNA1 and WYMV P1/*Ta* eIF4E RNA2 transcripts showed a slightly higher level of CP accumulation than cells transfected with the WYMV RNA1 and WYMV RNA2 transcripts (Fig. 3-3D lanes 1 and 2 and 3-3E bars 1 and 2). Barley protoplasts did not support WYMV replication, as was previously shown (Fig. 3-3D lane 3 and 3-3E bar 3; Fig. 3-2C lane 5 and 3-2D bar 5). However, in barley protoplasts transfected with WYMV RNA1 and WYMV P1-*Ta* eIF4E RNA2 transcripts, a highly elevated level of WYMV CP accumulation was detected (Fig. 3-3D lane 4, and 3-3E bar 4). This result indicated that the exogenous wheat eIF4E expressed from the transfected mutant WY2 RNA2 was functional for WYMV RNA replication in barley cells.

3.3.6 The combination of WYMV VPg and wheat eIF4E enables the replication of a BaYMV mutant in barley protoplasts

Potyvirus VPg is a virulence factor that breaks down eIF4E-mediated recessive

resistance, mostly caused by mutations in the VPg proteins (Bruun-Rasmussen et al., 2007; Kanyuka et al., 2004; Léonard et al., 2000; Ruffel et al., 2002; Schaad et al., 2000). Therefore, I also examined the role of VPg in the replication of BaYMV and WYMV RNA in barley and wheat protoplasts. BaYMV VPg and WYMV VPg differ at 51 out of 187 amino acid positions throughout the proteins (Fig. 3-4A).

For this purpose, I constructed a BaYMV RNA1-derived chimeric mutant cDNA clone with the BaYMV VPg gene replaced by the WYMV VPg gene (pBY-K1.WY-VPg) (Fig. 3-1E). Transfection of barley protoplasts with BaYMV WY-VPg RNA1 and BaYMV RNA2 transcripts resulted in no BaYMV CP accumulation (Fig. 3-4B lane 2, and 4C bar 2) compared to a high level of BaYMV CP accumulation after BaYMV RNA1 and BaYMV RNA2 transfection (Fig. 3-4B lane 1 and 4C bar 1; Fig. 3-2A lane 3 and 2B bar 3; Fig. 3-3B lane 1 and 3-3C bar 1). Transfection of barley protoplasts with BaYMV WY-VPg RNA1 and BaYMV GFP RNA2 transcripts also resulted in no GFP fluorescence (Fig. 3-4D-a), indicating that BaYMV WY-VPg RNA1 did not replicate in barley protoplasts at all.

Next I tested the replication of BaYMV WY-VPg RNA1 in wheat protoplasts. Wheat protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV RNA2 transcripts demonstrated a high level of BaYMV CP accumulation (Fig. 3-4B lane 3 and 4C bar 3). Wheat protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV GFP RNA2 transcripts also showed strong GFP fluorescence (Fig. 3-4D-b), indicating that BaYMV WY-VPg RNA1 replicated in wheat protoplasts, although this mutant RNA1 did not replicate in barley protoplasts.

Together with the previous result that WYMV RNA1 and WYMV P1/*Ta* eIF4E RNA2 replicated well in barley protoplasts (Fig. 3-3D lane 4, and 3-3E bar 4), I hypothesized that a pair of WYMV VPg and wheat eIF4E may form a part of functional replication complex for BaYMV-derivative RNA in barley cells. To test this hypothesis, I constructed a pBY-K2 mutant (pBY-K2.P1/*Ta* eIF4E) in which the dispensable P2 gene was replaced by a wheat eIF4E gene (Fig. 3-1D) in pBY-K2 background. Barley protoplasts transfected with BaYMV WY-VPg RNA1 and BaYMV P1/*Ta* eIF4E RNA2 transcripts exhibited a high level of BaYMV CP accumulation (Fig. 3-4B lane 4 and 3-4C bar 4), indicating that the replication of BaYMV WY-VPg RNA1 occurred in barley cells in the presence of RNA2-derived wheat eIF4E.

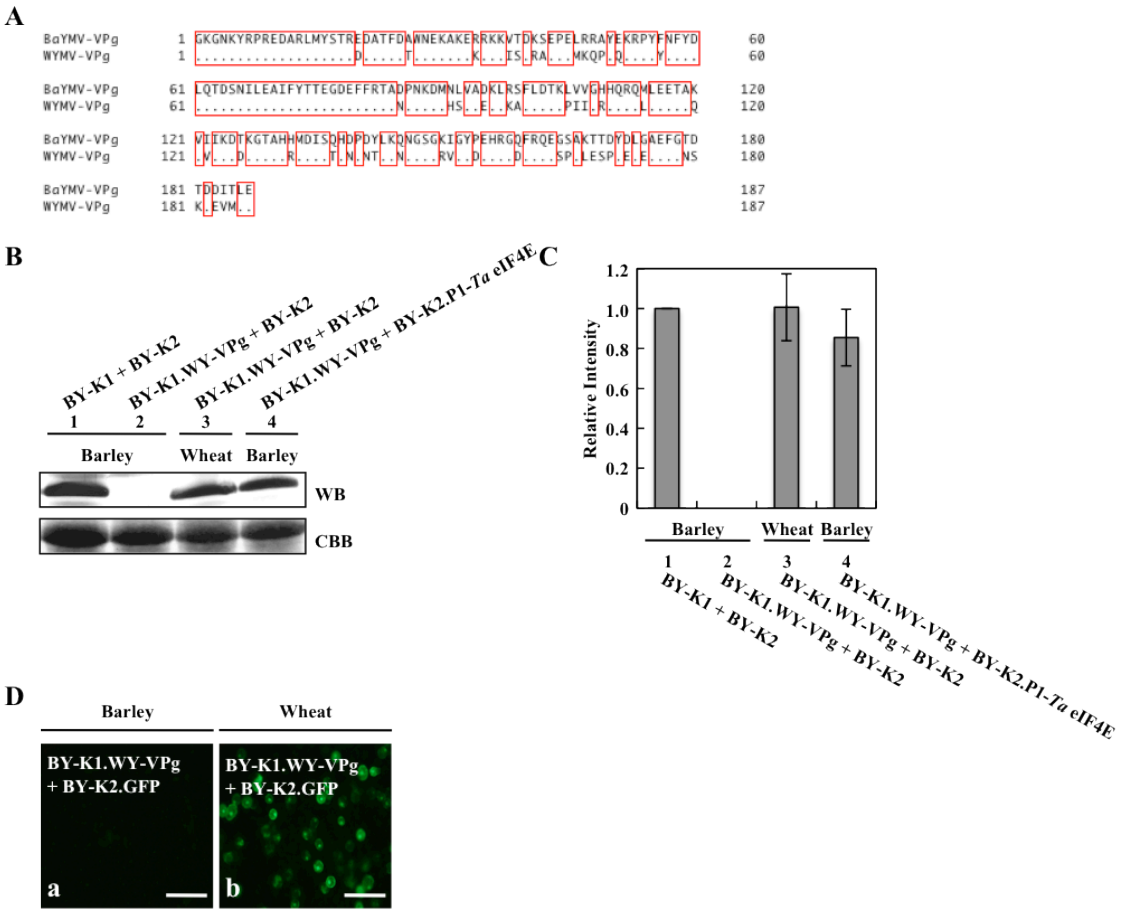


Fig. 3-4. Infectivity assay of WYMV VPg-expressed BaYMV mutant in barley and wheat mesophyll protoplasts. **(A)** Alignments of amino acid sequences of VPgs from BaYMV and WYMV using Genetyx-Mac software. **(B)** Western blot analysis of CP accumulation in protoplasts transfected with *in vitro* transcripts. **(C)** Relative intensity of BaYMV CP accumulation in transfected protoplasts. Experiments were at least triplicated. The CP bands on Western blots were scanned using ImageJ software. The amount of BaYMV CP accumulation in barley protoplasts transfected with BaYMV RNA1 and BaYMV RNA2 transcripts was used as a control and set as 1. **(D)** GFP fluorescence observed in transfected protoplasts with BaYMV WY-VPg RNA1 and GFP RNA2 transcripts. Bars, 200 μm.

3.4 Discussion

Agriculturally, BaYMV and WYMV cause similar yellow mosaic diseases in barley and wheat, respectively. However, the hosts of these two bymoviruses are distinct in fields. BaYMV is detected in barley plants, whereas WYMV is detected only in wheat plants. The objective of this study was to examine the host tropism of BaYMV and WYMV at the cellular level.

BaYMV replicated not only in barley protoplasts but also in wheat protoplasts albeit less efficiently. In contrast, WYMV replicated only in wheat protoplasts but not in barley protoplasts, as determined by CP band appearance on Western blots and GFP fluorescence. Same results were obtained regardless of the source of RNA2 (either BaYMV or WYMV), indicating that the replication efficiency and the host tropism at the cellar level are determined by factors residing on RNA1 rather than on RNA2. On the other hand, mechanical inoculation to wheat plants with BaYMV RNA1 transcript together with BaYMV or WYMV RNA2 transcripts did not cause systemic infection (repeated 3 times), indicating that a lack of field wheat infection by BaYMV is determined at the movement level as well. Previously BaYMV P2 was shown to be required for systemic infection of BaYMV in barley plants (You and Shirako, 2010). In addition to BaYMV P2, P3N-PIPO (Vijayapalani et al., 2012; Wei et al., 2010; Wen and Hajimorad, 2019), CI (Carrington et al., 1998) and CP (Dolja et al., 1995) may also be functional in systemic infection of BaYMV in wheat plants as in the cases of viruses in the genus *Potyvirus*. Further studies on the host tropism at the whole plant level are required.

In this study to detect replication of BaYMV or WYMV in transfected protoplasts, I used two criteria, CP detection on Western blots and GFP fluorescence. Intensities of GFP fluorescence directly reflected efficiency of RNA2 replication. On the other hand, intensities of CP bands on Western blots were significantly reduced in the absence of RNA2-expressed P1 protein (this study; You and Shirako, 2010). A question is whether P1 facilitated efficient replication of RNA1 to elevate CP expression level or P1 stabilized CP and protected it from degradation. With our additional mutant construct with a GFP gene inserted between the NIb gene and the CP gene in pBY-K1, similar intensities of GFP fluorescence were observed in the presence and absence of BaYMV RNA2 transcript in barley protoplasts (data not shown), suggesting that P1 stabilized expressed CP rather than that P1 enhanced RNA1 replication. Recently an interaction between P1 and CP has been also shown by a bimolecular fluorescence complementation assay (Sun et al., 2014).

To date, approximately 200 resistance genes against virus infections have been reported in plants, and half of them are recessively inherited (Kang et al., 2005; Truniger and Aranda, 2009). All the recessive resistance genes against viruses in the family *Potyviridae* encode translation initiation factors - either eIF4E (Charron et al., 2008; Kanyuka et al., 2005; Stein et al., 2005), eIF4G (Lee et al., 2010), or their isoforms (Albar et al., 2006; Gallois et al., 2010; Hébrard et al., 2008). Because BaYMV and WYMV are members in the family *Potyviridae*, I investigated whether eIF4E is also involved in their host tropism at the cellular level. Co-expression of wheat eIF4E enabled WYMV to replicate in barely protoplasts, and co-expression of barley eIF4E increased BaYMV replication in wheat mesophyll protoplasts. These results

suggest that eIF4E is also an important cellular factor that determines the host range of BaYMV and WYMV at the cellular level.

Virus counterparts that interact with eIF4E family proteins to induce resistance include VPg or P1 of *Clover yellow vein virus* (Nakahara et al., 2010), HC-Pro of *Potato virus A*, PVYand *Tobacco each virus* (Ala-Poikela et al., 2011) and CI of *Lettuce mosaic virus* (Abdul-Razzak et al., 2009). Here, I also investigated whether VPg is involved in host range determination at the cellular level. A BaYMV RNA1 mutant with a WYMV VPg gene did not replicate in barley protoplasts in the presence of BaYMV RNA2 expressing the P1 and P2 proteins. However, transfection together with a BaYMV RNA2 mutant expressing P1 and wheat eIF4E allowed the BaYMV RNA1 mutant with the WYMV VPg gene to replicate efficiently in barley cells. This result clearly indicates that the WYMV VPg located in the BaYMV RNA1 mutant was associated with wheat eIF4E expressed from the BaYMV RNA2 mutant, forming a functional replication complex with other essential viral proteins such as the NIb RNA polymerase and unidentified host factors.

It should be emphasized that even if there is no sign of replication as judged by the absence of a CP band by Western blot and a lack of GFP fluorescence proteins encoded on the input transcripts should be translated using the host translational machinery and exist for a certain period of time depending on the stability of individual proteins. BaYMV WYMV-VPg RNA1 in barley cells was one case and did not replicate unless wheat eIF4E was initially provided from the RNA2 transcript. BaYMV WYMV-VPg RNA1 in wheat cells could replicate by itself, using host wheat eIF4E, not only as in the translation machinery but also for BaYMV WYMV-VPg RNA1 replication. WYMV

RNA1 in barley cells was another case and could replicate only when wheat eIF4E was initially expressed from the RNA2 transcript.

It has been hypothesized that host eIF4E might play a role in potyvirus genome expression and replication as well as in virus cell-to-cell movement (Gao et al., 2004; Robaglia and Caranta, 2006). As discussed above, BaYMV host range determination is apparently based at both the replication and movement levels, whereas that of WYMV is based primarily at the replication level. Host eIF4E involvement in bymovirus cell-to-cell movement and systemic infection requires further study.

In this study, an association between VPg and eIF4E proteins for replication was clearly shown, but a direct or indirect interaction of the two proteins has not been observed by a yeast two-hybrid assay, as was shown with potyviruses (Léonard et al., 2000; Schaad et al., 2000), or by a co-immunoprecipitation assay (data not shown), as discussed in 2.4.4.

There are 16 amino acid differences within the 215 amino acids throughout the proteins between BaYMV and WYMV VPgs, and 51 amino acid differences within the 187 amino acids throughout the proteins between barley and wheat eIF4Es, as shown in Fig. 3-4A and Fig. 3-3A. Further studies should focus on the precise determination of specific region(s) or amino acid(s) of VPgs and eIF4Es involved in the host tropism of BaYMV and WYMV.

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Chapter 4

General discussion

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Breeding of resistant cultivars is the only ecological and economical approach to control *P. granimis*–transmitted virus disease. Since the first resistance gene against BaYMV was reported in 1973 (Takahashi et al., 1973), total eighteen resistance genes were identified in barley (Kai et al., 2012; Kanyuka et al., 2003; Ordon et al., 2009). However, none of resistance mechanisms was clarified due to the difficulty in working with these monocot–infecting viruses. Until recently, the establishment of a reverse genetics system for BaYMV provided a useful tool for study on bymoviruses. In this study, the virus pathogenicity and resistance mechanisms against bymovirus were studied using *in vitro* transcripts from cDNA clones at the cellular and whole plant levels.

The *rym5* resistance mechanisms against BaYMV were first studied with two BaYMV isolates, Tochigi (JT10, *rym5*–nonbreaking) and Kurashiki (JK05, *rym5*–nonbreaking) isolates. From the virus side, mapping analysis between these two isolates identified VPg as the only virus determinant for breaking *rym5* resistance. Introduction of T-VPg from Tochigi JT10 isolate into Kurashiki JK05 isolate resulted in systemic infection to *rym5* plants. From the host side, the role of eIF4E in breaking *rym5* resistance was examined because the *rym5* resistance gene has been previously mapped to Chromosome 3HL coding for host eIF4E (Stein et al., 2005). Kurashiki isolate could replicate in *rym5* cells but failed to cause systemic infection to *rym5* plants when co-expressed with susceptible eIF4E. Therefore, *rym5* eIF4E confers a resistance against BaYMV at the replication level and there should be some other host resistance factor(s) affecting virus movement in *rym5* plants. This work showed the first experimental evidence that VPg and eIF4E play an important role in bymovirus

infection.

The *rym5* resistance studies provided a useful model for understanding the other resistance mechanisms. The *rym2* resistance mechanisms were also studied here. The Kurashiki JK05 isolate could replicate in *rym2* cells whereas the Tochigi JT10 isolate could not. Mapping analysis between these two isolates showed that the C-terminal region of P3 was identified as a determinant for breaking *rym2* at the cellular level, also suggesting a role of P3 in BaYMV replication. Further study on *rym2* resistance at the movement level could be done with BaYMV-II-2, the only identified BaYMV strain that could cause systemic infection to *rym2* plants. In future, the other resistance mechanisms could be also studied as *rym5* resistance. For instance, the *rym1* resistance mechanisms could be studied with BaYMV-III and the other *rym1*–nonbreaking isolates, the *rym3* resistance mechanisms could be done with BaYMV-IV and the other *rym3*–nonbreaking isolates and the *rym4*/*rym*6 resistance mechanism could be done with Tochigi isolate JT10 and the other *rym*4/*rym*6–nonbreaking isolates.

This study also revealed an association of VPg and eIF4E in the host tropism of BaYMV and WYMV at the cellular level. Replacing the BaYMV VPg gene with that of WYMV abolished BaYMV replication in barley protoplasts, whereas the additional expression of wheat eIF4E from BaYMV genome restored the replication of the BaYMV mutant in barley protoplasts. It seems that VPg and eIF4E required for virus infection is a common feature in the genus *Bymovirus* as shown in the genus *Potyvirus* (Bruun-Rasmussen et al., 2007; Léonard et al., 2000; Ruffel et al., 2002; Schaad et al., 2000). The distinct host ranges of BaYMV and WYMV could also be considered as the result of host resistance and susceptibility. Studies on host range of bymoviruses may be

helpful to identify new resistance genes from different plant species. In future, breeding of resistance cultivars may also be done by distant hybridization between different plant species.

This study was initiated to explain the resistance mechanisms against bymovirus infection. With the current system, more resistance mechanisms could be soon studied in the future. The work here takes a crucial step for understanding the bymoviruses themselves, but it is still far away to interpret the complicated processes during virus infection. Further studies will be required to systemically understand the bymovirus infection, and thereby to better control the bymovirus diseases.

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Chapter 5

Summary

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The genus *Bymovirus* in the family *Potyviridae* consists of viruses with a bipartite plus-sense RNA genome. Two bymoviruses *Barley yellow mosaic virus* (BaYMV) and *Wheat yellow mosaic virus* (WYMV) are agriculturally important pathogens causing yellow mosaic diseases of barley and wheat, respectively, with significant yield losses worldwide. BaYMV occurs in East Asia and Europe and WYMV is found in Japan and China. Breeding of resistant cultivars is the only practical and ecological approach to prevent bymovirus diseases. There have been eighteen resistance genes reported for BaYMV and at least three resistance genes for WYMV but the resistance mechanisms are still poorly understood.

In barley, the *rym5* resistance gene encodes a eukaryotic translation initiation factor 4E (eIF4E) on chromosome 3HL and was utilized for breeding resistant cultivars. However, BaYMV pathogenic strains breaking *rym5* resistance have already emerged in fields.

Bymoviruses have restricted host ranges and each species can infect only one type of host; BaYMV infects only barley and WYMV infects only wheat in fields. So far, the host restriction mechanisms of BaYMV and WYMV are unknown. The distinct host ranges of BaYMV and WYMV could also be due to host resistance and susceptibility.

I studied (1) a BaYMV viral determinant for breaking *rym5* resistance, (2) the mechanism for breaking the eIF4E/*rym5*–mediated resistance, and (3) the association of bymoviral VPg with host eIF4E in the host tropism at the cellular level. All the experiments were carried out using infectious *in vitro* transcripts from cDNA clones with isolated protoplasts or intact plants as the hosts.

1 Analysis of a BaYMV Tochigi isolate JT10 and the host resistance

1.1 Genome characterization

A BaYMV isolate JT10 was obtained from *rym5*-carrying cv. Misato Golden leaves in Tochigi. At first the full-length nucleotide sequences of RNA1 and RNA2 were determined. RNA1 was 7,642 nucleotides and RNA2 was 3,585 nucleotides in length, excluding the poly(A) tail at the 3' end. Sequence comparisons with the previously characterized BaYMV-III isolate, another *rym5*-breaking isolate from a different field in Tochigi, showed that isolate JT10 is distinct from isolate BaYMV-III with 1.7% nucleotide differences in RNA1 and 3.0% in RNA2, although both isolates infected *rym5* cultivars and were isolated in different fields in Tochigi Prefecture in different years.

1.2 Infectivity assay at the cellular and whole plant levels using infectious *in vitro* transcripts

Based on the whole genome sequence information, full-length cDNA clones were constructed and designated as pBY-T1 and pBY-T2 for RNA1 and RNA2 of Tochigi isolate JT10, respectively. Inoculating *rym5*-carrying barley plants (cv. Misato Golden) with the *in vitro* transcripts from pBY-T1 and pBY-T2 resulted in development of yellow mosaic leaf symptoms. Progeny virus genome was examined by PCR/sequencing analysis and it was confirmed that BY-T1 RNA1 + BY-T2 RNA2 were systemically infectious to the *rym5* cultivar.

Next, infectivity of BY-T1 RNA1 + BY-T2 RNA2 to twelve barley varieties was

examined at the cellular and whole plant levels. Ryofu and KoA were used as susceptible controls. Express (*rym4*), Mikamo Golden (*rym5*), Misato Golden (*rym5*), Haruna Nijo (*rym6*), Amagi Nijo (*rym6*) and Miho Golden (*rym6*) were systemically infected with BY-T1 RNA1 + BY-T2 RNA2. Ishuku Shirazu (*rym3*) and Haganemugi (*rym3*) supported RNA replication at the cellular level but were not systemically infected, indicating that the *rym3* varieties were resistant at the movement level. Nittakei 68 (*rym1*) showed limited RNA replication at the cellular level and did not develop systemic infection. Mihori Hadaka 3 (*rym2*) did not support RNA replication at the cellular level at all.

1.3 Viral genome mapping analysis and amino acids identification of VPg for breaking *rym5* resistance

The virus factor involved in breaking *rym5* by the Tochigi isolate JT10 was determined. Previous study showed that Kurashiki isolate JK05 (cDNA clones pBY-K1 for RNA1 and pBY-K2 for RNA2) was non-infectious to *rym5* cells. RNA1 replicates autonomously. Therefore, cDNA fragments were replaced in pBY-K1 with those of pBY-T1 to construct chimeric cDNA clones. Protoplasts prepared from *rym5* Misato Golden leaves were transfected with *in vitro* transcripts from chimeric cDNA clones. The result showed that only chimeric RNA1s containing the JT10 VPg (T-VPg) gene replicated in *rym5* cells. Further, systemic infection in *rym5* plants with BY-K1/T-VPg RNA1 + BY-K2 RNA2 were examined. Progeny viral RNA sequencing results confirmed no additional common spontaneous mutations outside of the T-VPg gene. Thus, T-VPg was identified as a determinant for breaking *rym5* at the whole plant level.

Alignment of amino acid sequences of K-VPg (VPg of Kurashiki isolate JK05) and T-VPg showed that there are six amino acid differences out of 187 amino acids; Tyr in K-VPg and Phe in T-VPg at the position 73; Asp in K-VPg and Glu in T-VPg at the position 85; Thr in K-VPg and Ser in T-VPg at the position 118; Lys in K-VPg and Thr in T-VPg at the position 120; Tyr in K-VPg and His in T-VPg at the position 142; Ala in K-VPg and Val in T-VPg at the position 175.

To precisely identify the key amino acid(s) of VPg for breaking *rym5*, six T-VPg mutants were constructed, in which a single amino acid was substituted with that in K-VPg such as T-VPg_F73Y, T-VPg_E85D, T-VPg_S118T, T-VPg_T120K, T-VPg_H142Y and T-VPg_V175A. Infectivity of each of these VPg mutants was examined in *rym5* cells. The results showed that VPg mutations of F73Y, E85D and V175A negatively affected virus replication (less than 40% was lost) of T-VPg, mutation of T120K sharply reduced virus replication (more than 50% was lost) and mutations of S118T and H142Y were lethal to T-VPg in *rym5* cells, indicating that serine at the position 118 and histidine at the position 142 of VPg were mostly important for virus replication in *rym5* cells. However, converse pBY-K1-derivative mutant of VPg_T118S/Y142H was not able to replicate in *rym5* cells, suggesting that more amino acid mutation(s) may be required for replication of Kurashiki isolate JK05 in *rym5* cells. Later, the pBY-K1-derivative mutant of VPg_T118S/K120T/Y142H containing three amino acid mutations could replicate in *rym5* cells but failed to cause systemic infection to *rym5* plants, indicating that threonine at the position 120 is required for replication and triple VPg mutations at the positions118, 120 and 142 are insufficient for systemic infection to *rym5* plants.

1.4 Expression of susceptible barley eIF4E in *rym5* protoplasts

Previously the *rym5* resistance gene has been reported to code for eIF4E. To testify whether *rym5* resistance is conferred by eIF4E, an eIF4E gene of a susceptible variety KoA (KoA_eIF4E) was introduced into pBY-K2 cDNA clone. Co-expression of the susceptible KoA_eIF4E enabled BY-K1 replication in *rym5* cells, indicating that the resistance by *rym5* is indeed conferred by host eIF4E. However, Kurashiki isolate JK05 could not break *rym5* at the whole plant level even if KoA_eIF4E was co-expressed. Therefore, there must be other resistance factor(s) affecting virus movement in *rym5* plants.

This result shows that host eIF4E functions in bymovirus replication.

1.5 Viral genome mapping analysis to break *rym2* resistance

rym2 cells supported replication of the Kurashiki isolate JK05 but not of the Tochigi isolate JT10. To identify the virus factor responsible for breaking *rym2* at the cellular level, mapping analysis between these two isolates was also done in *rym2* cells. The C-terminal region of P3 of Kurashiki isolate JK05 was identified as a determinant for replication in *rym2* cells, also suggesting a role of P3 in BaYMV replication.

2 Association of VPg and eIF4E in the host tropism of BaYMV and WYMV at the cellular level

The infectious full-length cDNA clones of WYMV RNA1 and RNA2 were constructed and designated as pWY1 and pWY2, respectively. BaYMV and WYMV replication in barley and wheat mesophyll protoplasts was first examined. The results

showed that BaYMV replicated in both barley and wheat cells, but WYMV replicated only in wheat cells not in barley cells. Next, the role of eIF4E in determining the host tropism of BaYMV and WYMV at the cellular level was examined. Two derivative mutant pBY-K2.P1/barley_eIF4E and pWY2.P1/wheat_eIF4E modified from pBY-K2 and pWY2 were constructed, in which viral P2 genes were replaced with barley and wheat eIF4E genes, so that barley and wheat eIF4E genes could be expressed in wheat and barley protoplasts, respectively. Co-expression of wheat eIF4E enabled WYMV replication in barley protoplasts, and co-expression of barley eIF4E increased BaYMV replication in wheat protoplasts, suggesting that eIF4E is an important factor that determines the host tropism of BaYMV and WYMV at the cellular level. The role of VPg was also examined. Replacing the BaYMV VPg gene with that of WYMV abolished BaYMV replication in barley protoplasts, whereas the additional expression of wheat eIF4E from BaYMV genome restored the replication of the BaYMV mutant in barley protoplasts. These results indicate that both VPg and the host eIF4E are involved in the host tropism of BaYMV and WYMV at the replication level.

In this study, I experimentally proved for the first time that (1) BaYMV VPg is the bymovirus factor for breaking eIF4E–mediated resistance in barley, (2) the host eIF4E is functional in bymoviral RNA replication, and (3) VPg/eIF4E association plays an important role in the host tropism at the cellular level. Furthermore, bymoviral P3 was shown to function in RNA replication and involved in *rym2* resistance. This study broadens our understanding in breaking host resistance against bymovirus infection and sets a useful model for understanding other resistance mechanisms.

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Appendixes

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Table S1 Primers used for determining complete sequences of BaYMV Tochigi isolate JT10[a].

ID	Function	Orientation	Position[b]	Sequence (5'→3')
TB87	RNA1 PCR	Forward	ctc/*Xba*I/T7 Promotor/G/1-20 nt	ctc/TCTAGA/**TAATACGACTCACTATA**/G/AA AATAAAACAACCCTAAAC
TB15	RNA1 Seq	Forward	544-560	GGATACACTACTCTTGA
TB39	RNA1 Seq/PCR	Forward	1154-1170	AAGCTAGAAGCACAGCA
TB19	RNA1 Seq	Forward	1769-1785	CTGCCAATCTCGGCAGA
TB21	RNA1 Seq/PCR	Forward	2421-2437	TGATCGACGCAGAGTGA
TB148	RNA1 Seq/PCR	Forward	2911-2937	GTCACAACTTTGAGCAAATTCGATTGG
TB146	RNA1 Seq	Forward	3485-3502	GCAAGTGGCGCGAGCATC
TB9	RNA1 Seq	Forward	4242-4258	TGACATCACGCTCGAAG
TB12	RNA1 Seq/PCR	Forward	4786-4802	ACTCAGGAGGTCGTTGA
TB13	RNA1 Seq	Forward	5502-5518	GTCAACTGGAGTGTGGA
TP210	RNA1 Seq	Forward	6434-6450	CTCAATCCTCAACTGCT
TB57	RNA1 Seq	Forward	7242-7258	CCCACGATCATGGATGA
TB53	RNA1 Seq	Reverse	489-473	GCTGGCATCAGAGGTGA
TB16	RNA1 Seq	Reverse	668-652	AGCAGCGATCCAACACG
TB40	RNA1 Seq	Reverse	1314-1298	GCCTTGATGGTCGTCGA
TB20	RNA1 Seq/PCR	Reverse	1844-1860	CGCAGAACCATACGTCA
TB22	RNA1 Seq	Reverse	2547-2531	AACATCAGGAGAGATGA
TB24	RNA1 Seq/PCR	Reverse	3174-3158	GATGCGAGTAGAAGAGA
TB154	RNA1 Seq	Reverse	3418-3402	TGGTAGCTAGCAGCAGA
TB159	RNA1 Seq	Reverse	4263-4247	AGAGGCTTCGAGCGTGA
TB149	RNA1 Seq/PCR	Reverse	4894-4874	CGAACAAAGCTGTGTGGGATG

TB55	RNA1 Seq	Reverse	5598-5582	TATGGGAGAGGCTGTGA
TB14	RNA1 Seq	Reverse	6187-6171	TGTCGCTAGTGATGTCA
TP211	RNA1 Seq	Reverse	7036-7020	GCATTACTTGATTCTCA
TP213	RNA1 Seq	Reverse	7503-7487	CCGTTCCGAGCCTGATA
TB101R	RNA2 PCR	Forward	aga/*Bam*HI//T7 Promotor/G/1-22 nt	aga/GGATCC/**AATACGACTCACTATA**/G/AAA ATAAAACAACCCTAAACCA
TB28	RNA2 PCR	Forward	370-386	TGGAGCTATGCTACCTA
TB 48	RNA2 Seq/PCR	Forward	953-969	AACTCTGGACATGATCA
TB 161	RNA2 PCR	Forward	1541-1557	CCATCTGCTTGGTGGCA
TB 65	RNA2 PCR	Forward	2059-2075	CGAACTTCAGCTTGAAG
TB 66	RNA2 PCR	Forward	2708-2724	ATGCCAGGAATGCCAGA
TB 93	RNA2 PCR	Forward	3101-3117	CATCAACATCTGCTCGC
TB 60	RNA2 PCR	Reverse	594-578	AGACTGAGGTAACAATA
TB 31	RNA2 Seq/PCR	Reverse	1038-1022	TCAACATCCAGAGCTGA
TB 33	RNA2 PCR	Reverse	1695-1679	GAGTCATCATCACCAGA
TB 63	RNA2 PCR	Reverse	2411-2395	AATCGGTGCTGAAGAGA
TB 160	RNA2 PCR	Reverse	3344-3328	CGGACCTTGAGTGGAGA
TB89	RNA1&2 PCR	Reverse	17-nt random sequence + $(T)_{17}$	GACGACATCGATGGATC$(T)_{17}$[c]

[a] Plain letters, derived from viral genome; underlined letters, restrict enzyme sequences; bold-face letters, T7 promotor sequence; lower case letters, added protective nucleotides for restrict enzyme sites.
[b] Numbers indicate the corresponding position in the genome RNAs of BaYMV Tochigi isolate JT10.
[c] $(T)_{17}$, 17 nucleotides of T.

Table S2 Primers used for constuction of full-length cDNA clones for RNA1 and RNA2 of Tochigi isolate JT10.

Constructs	ID	Orientation	Position[b]	Sequence (5'→3')
pBY-T1	TB87	Forward	ctc/*Xba*I/T7 Promotor/G/1-20 nt	ctc/TCTAGA/**TAATACGACTCACTATA**/G/AAAATAAAACAACCCTAAAC
	TB162	Reverse	aga/*Bam*HI/$(T)_{42}$/7642-7626	aga/GGATCC/$(T)_{42}$[c]/ATTACCTTCTGGTACTC
pBY-T2	TB163	Forward	aga/*Bam*HI/T7 Promotor/G/1-22 nt	aga/GGATCC/**TAATACGACTCACTATA**/G/AAAATAAAACAACCCTACACCA
	TB166	Reverse	cac/*Spe*I/$(T)_{42}$/3585-3568	cac/ACTAGT/$(T)_{42}$/GTCACATTTCCTGTGTAC

[a] Plain letters, derived from viral genome; underlined letters, restrict enzyme sequences; bold-face letters, T7 promotor sequence; lower case letters, added protective nucleotides for restrict enzyme sites.
[b] Numbers indicate the corresponding position in the genome RNAs of BaYMV Tochigi isolate JT10.
[c] $(T)_{42}$, 42 nucleotides of T.

Table S3 Primers used for constuction of GFP-expressed RNA2 of Tochigi isolate JT10[a].

ID	Orientation	Position[b]	Sequence (5'→3')
TB163	Forward	aga/*Bam*HI/T7 Promotor/G/1-22 nt	aga/GGATCC/**TAATACGACTCACTATA**/G/AAAATAAAACAAC CCTACACCA
TB170	Reverse	GFP 5'-12 nt/154-137 /	***TTTGCTAGCCAT***/GATGGAGGGTTTGAAAGC
TB97	Forward	146-154/GFP 5'-17 nt	CCCTCCATC/***ATGGCTAGCAAAGGAGA***
TB169	Reverse	2836-2828/GFP 3'-17 nt	ATGAGAAAT/***TCAGTTGTACAGTTCAT***
TB168	Forward	GFP 3'-9 nt/2828-2844	***TACAACTGA***/ATTTCTCATCACAGCAG
TB166	Reverse	cac/*Spe*I/$(T)_{42}$[c] /3585-3568	cac/ACTAGT/$(T)_{42}$/GTCACATTTCCTGTGTAC

[a] Plain letters, derived from viral genome; underlined letters, restrict enzyme sequences; bold-face letters, T7 promotor sequence; Italic bold-face letters, GFP-coding sequence

[b] Numbers indicate the corresponding position in the genome RNAs of BaYMV Tochigi isolate JT10.

[c] $(T)_{42}$, 42 nucleotides of T.

Table S4 Primers used for constuction of VPg mutants[a].

Constructs	ID	Orientation	Position[b]	Sequence (5'→3')
pBY-K1/VPg	TB146	Forward	3485-3502	GCAAGTGGCGCGAGCATC
	TB204	Reverse	3708-3679	GTTTCCCTTACCTTCAAAGACCAATTTTGTC
	TB203	Forward	3685-3714	ATTGGTCTTGAAGGTAAGGGAAACAAGTAC
	TB147	Reverse	4313-4296	CCAACTCGGGTTGCGACG
pBY-T1/VPg_F73Y	TB207	Reverse	3913-3886	TCAGTGGTGtAGAAAATAGCTTCCAGAA
	TB206	Forward	3895-3922	CTATTTTCTaCACCACTGAAGGCGATGA
pBY-T1/VPg_E85D	TB209	Reverse	3960-3933	CTTATTGGGaTCTGCTGTTCGGAAAAAC
	TB208	Forward	3942-3969	AACAGCAGAtCCCAATAAGGACATGAAC
pBY-T1/VPg_S118T	TB211	Reverse	4057-4030	CCGTGGCTGtTTCTTCTAGCATCTGCCG
	TB210	Forward	4039-4066	CTAGAAGAAaCAGCCACGGTTATTATCA
pBY-T1/VPg_T120K	TB151	Reverse	4042-4068	TTTGATAATAACCtTGGCTGTTTCTTC
	TB150	Forward	4042-4068	GAAGAATCAGCCAaGGTTATTATCAAA
pBY-T1/VPg_H142Y	TB213	Reverse	4129-4102	GCTTTAGATaGTCTGGATCATGCTGTGA
	TB212	Forward	4111-4138	GATCCAGACtATCTAAAGCAGAATGGGT
pBY-T1/VPg_V175A	TB215	Reverse	4229-4202	CCAAACTCAgCTCCAAGATCGTAATCCG
	TB214	Forward	4211-4238	ATCTTGGAGcTGAGTTTGGCACCGACAC
pBY-K1/VPg_T118S /H142Y	TB133	Reverse	4052-4036	AATAACCTTGGCTGaTTCTTCTAGCAT
	TB132	Forward	4036-4052	ATGCTAGAAGAAtCAGCCAAGGTTATT
	TB137	Reverse	4134-4108	ATTCTGCTTTAGATgGTCTGGATCATG
	TB136	Forward	4108-4138	CATGATCCAGACcATCTAAAGCAGAAT
pBY-K1/VPg_T118S/K120T/H142Y	TB202	Reverse	4064-4033	ATAATAACCgTGGCTGaTTCTTCTAGCATCTG
	TB201	Forward	4039-4070	CTAGAAGAAtCAGCCAcGGTTATTATCAAAGA

[a] Underlined letters, codons for substitution; low case letters, substituted nucleotides.

Table S5 Primers used for constuction of eIF4E-expressed mutant RNA2s of Kurashiki isolate JK05 [a].

Constructs	ID	Orientation	Position	Sequence (5'→3')
pBY-K2.P12/KoA_eIF4E(NIb/CP)	TB65	Forward	2059-2075	CGAACTTCAGCTTGAAG
	TB270	Reverse	NIb/CP cleavage site /2824-2804	AGCTTGCAGCCAAATTTCATC/GACTCGAGGGCGCCGGAAGAG
	TB269	Forward	NIb/CP cleavage site/KoA_eIF4E 5'-21 nt	GATGAAATTTGGCTGCAAGCT/ATGGCGGAGGACACGGAGACG
	TB129	Reverse	3558-3542	GTTCGTCAGACTACAAC
pBY-K2.P12/KoA_eIF4E(P1/P2)	TB182	Reverse	KoA_eIF4E 5'-6 nt /(P1/P2) cleavage site /2824-2807	CGCCAT/TGAACCAACAATTCCATTAAG/GACTCGAGGGCGCCGGAA
	TB181	Forward	2819-2824/(P1/P2) cleavage site/KoA_eIF4E 5'-18 nt	CGAGTC/CTTAATGGAATTGTTGGTTCA/ATGGCGGAGGACACGGAG
pBY-K2.P12/*rym5*_eIF4E(P1/P2)	TB241	Forward	*rym5*_eIF4E 599-640 nt	AGGATGCTAAGAGGTCCGACAAAGGCGCCAAGAACCGCTACA
	TB242	Reverse	*rym5*_eIF4E 616-575 nt	CGGACCTCTTAGCATCCTCATGAACGACGAATCCAATGGAGT
	TB243	Forward	*rym5*_eIF4E 350-377 nt	GTAAATGGAGCATCAGTTGTGGCAAAGG
	TB244	Reverse	*rym5*_eIF4E 368-341 nt	CAACTGATGCTCCATTTACCGCCATTGG
	TB245	Forward	*rym5*_eIF4E 469-499 nt	GTGCGTAAGGACAAGGAAAGAGTAGCTATCT
	TB246	Reverse	*rym5*_eIF4E 460-490 nt	CTCTTTCCTTGTCCTTACGCACGCTGACGAC

[a] Plain letters, derived from viral genome; underlined letters, (NIb/CP) or (P1/P2) cleavage site sequences.

Table S6 Primers used for constuction of RNA1 mutants of Tochigi isolate JT10.

Constructs	ID	Orientation	Position[b]	Sequence (5'→3')
pBY-T1/K-CI	TB235	Forward	797-814	TAAAACGTTATATTTATG
	TB232	Reverse	1362-1336	GTCGCCAGCCTGAAGCCCGTAGCTTGC
	TB231	Forward	1345-1371	GGGCTTCAGGCTGGCGACAGTGCTGAG
	TB234	Reverse	3339-3313	GTCGCTAGCTTGGAGCCCAATAATATC
	TB233	Forward	3322-3348	GGGCTCCAAGCTAGCGACACACTTACG
	TB236	Reverse	3732-3251	ATGAGACGAGCGTCTTCG
pBY-T1/K-Kp/6K1	TB15	Forward	544-560	GGATACACTACTCTTGA
	TB252	Reverse	1362-1336	GTCGCCAGCTTGAAGCCCGTAGCTTGC
	TB251	Forward	1345-1371	GGGCTTCAAGCTGGCGACAGTGCTGAG
	TB154	Reverse	3418-3402	TGGTAGCTAGCAGCAGA
pBY-T1/K-6K1	TB15			
	TB254	Reverse	1160-1137	CTAGCCTGAAGCACTATTTTTGGT
	TB253	Forward	1137-1160	ACCAAAATAGTGCTTCAGGCTAG
	TB252			
	TB251			
	TB154			
pBY-T1.K-Kp/P3	TB15			
	TB254			
	TB253			
	TB154			

Table S7 Primers used for constuction of mutants for host range study [a].

Constructs	ID	Orientation	Position[b]	Sequence (5'→3')
pBY-K2.P1/*Hv* eIF4E	TB94	Forward	263-279	TCCAGCACATGGAATGC
	TB172	Reverse	*Hv* eIF4E 5'-9 nt/922-906	**CTCCGCCAT**/TGAACCAACAATTCCAT
	TB173	Forward	*Hv* eIF4E 3'-9 nt /2828-2844	**ACGGTTTGA**/AATTCTCATCACAGCAG
	TB76	Reverse	cac/SpeI/$(T)_{42}$/VN[c]	CACACTAGT /$(T)_{42}$/VN
	TB171	Forward	914-922/*Hv* eIF4E 5'-18 nt	GTTGGTTCA/**ATGGCGGAGGACACGGAG**
	TB174	Reverse	2828-2836/*Hv* eIF4E 3'-18 nt	ATGAGAATT/**TCAAACCGTGTAGCGGT**
pBY-K2.P1/*Ta* eIF4E	TB225	Forward	454-471	GTCCATCTTATGCGCTGA
	TB228	Reverse	*Ta* eIF4E 5'-9 nt/922-905	**CTCGGCCAT**/TGAACCAACAATTCCATT
	TB229	Forward	*Ta* eIF4E 3'-9 nt /2828-2845	**ACCGTTTGA**/AATTCTCATCACAGCAGC
	TB226	Reverse	3178-3161	TCAATTTCTTACCATTTG
	TB227	Forward	914-922/*Ta* eIF4E 5'-18 nt	GTTGGTTCA/**ATGGCCGAGGACACGGAG**
	TB230	Reverse	2828-2836/*Ta* eIF4E 3'-18 nt	ATGAGAATT/**TCAAACGGTGTAGCGGTT**
pBY-K1.WY-VPg	TB152	Forward	2854-2870	GGTGATGACAAGGATGA
	TB217	Reverse	WY-VPg 5'-9nt/3696-3679	***TCCCTTTCC***/TTCAAGACCAATTTTGTC
	TB216	Forward	3688-3696/WY-VPg 5'-18nt/	GGTCTTGAA/***GGAAAGGGAAACAAGTAT***
	TB218	Reverse	4309-4258/WY-VPg 3'-18nt	CTCGGGTTGCGACGTCGACCCCAACTTGT GACAATAATATCCCAGTAGAGGC/***TTCAA GCATGACTTCATC***
	TB148	Forward	2911-2937	GTCACAACTTTGAGCAAATTCGATTGG
	TB147	Reverse	4313-4296	CCAACTCGGGTTGCGACG
pWY2.P1-*Ta* eIF4E	TW68	Reverse	2922-2917/*Ta* eIF4E 3'-24 nt	GGCGGC/**TCAAACGGTGTAGCGGTTCTT GGG**

	TW71	Forward	*Ta* eIF4E 136-152 nt	**TGGACCTTCTGGTTCGA**
	TW67	Forward	931-936/*Ta* eIF4E 5'-24 nt	GGCTCA/**ATGGCCGAGGACACGGAGAC GAGG**
	TW72	Reverse	*Ta* eIF4E 579-563 nt	**GGAGTCCTTGTAGTCCA**
	TW18	Forward	140-156	TGTGCTTCGTTCTCCTA
	TW69	Reverse	*Ta* eIF4E 5'-12 nt /936-916	**GTCCTCGGCCAT**/TGAGCCGACAATGCC GTTGAG
	TW70	Forward	*Ta* eIF4E 3'-12 nt/2917-2934	**TACACCGTTTGA**/GCCGCCCTACTTTCAA CG
	TW37	Reverse	cac/*Spe*I/(T) $_{42}$ [d]/3650-3639	cac/ACTAGT /$(T)_{42}$/GTCACATTTCCT

[a] Plain letters, derived from viral genome; underlined letters, restrict enzyme sequences; bold-face letters, eIF4E sequences derived from barley or wheat; Italic bold-face letters, WY-VPg sequences; lower case letters, added protective nucleotides for restrict enzyme sites.
[b] Numbers indicate the corresponding position in the genome RNAs of BaYMV or WYMV.
[c] VN, mixed bases, V = A or G or C, N = A or G or C or T.
[d] $(T)_{42}$, 42 nucleotides of T.

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Acknowledgements

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Sincerely,

Huangai Li