Studies on the co-infection, pathogenicity, and host resistance mechanisms of soil-borne viruses in barley and wheat

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ムギ類における土壌感染性ウイルスの共感染、病原性、抵抗性遺伝子機能に関する研究

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Abbreviation

SBWMV - soil-borne wheat mosaic virus

JSBWMV - Japanese soil-borne mosaic virus

SBCMV - soil-borne cereal mosaic virus

CWMV - Chinese wheat mosaic virus

BaYMV - barley yellow mosaic virus

BaMMV - barley mild mosaic virus

WYMV - wheat yellow mosaic virus

WSSMV - wheat spindle streak mosaic virus

CP - capsid protein or coat protein

P1 - first protein of the polyprotein precursor

HC-Pro - helper component proteinase

P3 - third protein of the potyviral polyprotein precursor

PIPO - pretty interesting potyviral ORF

6K - membrane-associated protein

VPg - viral genome-linked protein

CI - cylindrical inclusion protein

3' UTR - 3' untranslated regions

5' UTR - 5' untranslated regions

aa - amino acids

nt - nucleotides

Abstract

Barley (*Hordeum vulgare*) and wheat (*Triticum* spp.) are among the most important cereal crops for global human consumption and animal feed. However, they face severe threats from viral pathogens such as *Furovirus* and *Bymovirus*, which are major concerns for winter barley, wheat, and other Triticeae species worldwide. These viruses are transmitted by the widely distributed protozoan *Polymyxa graminis*. As environmental conditions change, the prevalence of soil-borne disease epidemics is increasing, posing a threat to food security, including in regions like Japan. To address these challenges and establish reliable agricultural management practices, it is crucial to uncover the genetic basis of virus susceptibility and resistance. However, there is limited research on the characteristics of coinfection virus multiplication and the resistance mechanisms in plants grown in virus-contaminated soil.

This thesis provided a holistic understanding of the coinfection dynamics, pathogenicity, and host resistance mechanisms against soil-borne viruses in barley and wheat.

Under wild conditions, most infections occur as coinfections involving a *Bymovirus* and/or a *Furovirus* in several Triticeae species. This study explored the differences in how Japanese soil-borne wheat mosaic virus (JSBWMV) and barley yellow mosaic virus (BaYMV) multiply when they co-infect barley. A time series analysis of virus multiplication revealed that JSBWMV infects the roots two weeks prior to BaYMV. Despite this difference in initial infection timing, the systemic movement of both viruses from the roots to the leaves occurred almost concurrently. The subsequent histological analysis detected both viruses in barley root, leaf sheath, and leaf blade sections, though their precise locations varied slightly. Additionally, I examined the response of various barley pan-genome accessions to these viruses. This revealed previously unnoticed genetic variations, providing valuable insights for genetic research and breeding. Notably, my findings indicate that among the wheat pan-genome accessions studied, only the Norin 61 variety was susceptible to JSBWMV, while none showed susceptibility to BaYMV. This suggests an inherent defense mechanism in wheat against BaYMV, highlighting potential avenues for future research and breeding efforts. Co-infection with JSBWMV can influence the pathogenicity of BaYMV in barley, potentially altering BaYMV's symptom severity and impacting disease management strategies (Chapter I).

Next, I explored the specific pathogenic characteristics of single BaYMV infections in barley. In 2018, severe mosaic disease symptoms were observed in the barley cultivar New Sachiho Golden, which carries the *rym3* gene, during early spring. This study aimed to identify key viral genome factors influencing the resistance mechanism associated with the *rym3* gene. The whole genome sequence of BaYMV was determined through RNA sequencing (RNA-seq), revealing a new isolate named BaYMV-Takanezawa. Through a comparative analysis of viral genome sequences, I identified critical mutation sites in the virus-encoded proteins CI and CP, which are associated with overcoming *rym3*-mediated resistance (Chapter II).

Whether in co-infection or single-infection scenarios, the optimal method for controlling soil-borne diseases is resistance breeding, which requires durable and broad-spectrum resistance genes. The third study focuses on elucidating the mechanisms of the resistance gene *Ym2* against wheat yellow mosaic virus (WYMV). This gene functions within the roots by either preventing the initial infection of WYMV or inhibiting viral replication within the roots. Following a previous mapping-based cloning of *Ym2*, PCR amplification, and Sanger resequencing were performed to identify sequence variants of *Ym2* in 91 wheat accessions. These accessions were classified into four groups: 16 with Madsen-type alleles, 12 with missense mutations, 44 with truncated proteins, and 20 with null alleles. Further analysis in a WYMV-contaminated field assessed the correlation between the genetic variants and the host response to natural WYMV infection. The results revealed a clear relationship between specific *Ym2* (*CDS618*) sequence variants and the host's resistance to WYMV (Chapter III).

This is the first report of a natural co-infection of JSBWMV and BaYMV in plants. A newly identified BaYMV isolate has overcome the resistance in widely used Japanese cultivars carrying the *rym3* gene. By analyzing the responses and variations in the *Ym2* gene, this study revealed correlations between host responses and variants in resistance genes against WYMV, highlighting the importance of these findings for resistance breeding.

General introduction

Global importance of Triticeae crops

Since the mid-20th century, the "Green Revolution" has instigated a profound metamorphosis in agricultural methodologies, transitioning from age-old "traditional" towards "industrialized" practices across the developing world. This transition has been instrumental in achieving approximately a 250% enhancement in cereal production (Evenson and Gollin, 2003). Triticeae crops, including wheat (*Triticum* spp.), barley (*Hordeum* spp.), and rye (*Secale* spp.), along with lesser-known wild relatives, are of immense global importance due to their widespread cultivation and consumption (Kilian *et al.*, 2009, Ritchie *et al.*, 2023). These cereals are staple foods, providing a significant portion of the world's caloric intake and essential nutrients (Tester and Langridge, 2010). Wheat alone contributes roughly 20% of the total caloric intake for humans, making it one of the most critical crops for food security (Erenstein *et al.*, 2022). Barley, on the other hand, serves multiple roles, including being a key ingredient in the brewing industry and animal feed, while also being a dietary staple in some regions (Ullrich, 2010). These crops are cultivated across diverse climates, showcasing adaptability to a wide range of environmental conditions (Tilman *et al.*, 2011, Abbo *et al.*, 2014). With a history spanning millennia, Triticeae crops have become integral to diets, economies, and cultural identities globally (Sullivan *et al.*, 2013, Erenstein *et al.*, 2022).

Triticeae crops contribute to various industries, including brewing, animal feed, and biofuel production, underscoring their versatility and importance (Day *et al.*, 2006, Schwarz and Li, 2011, Cornell and Hoveling, 2020). The genetic diversity within these species provides a valuable resource for breeding programs aimed at improving yield, disease resistance, and tolerance to environmental stress (Schreiber *et al.*, 2024). This genetic pool is crucial as global challenges, such as climate change and increasing population pressure, necessitate the development of more resilient and productive crop varieties (Jayakodi *et al.*, 2020, Walkowiak *et al.*, 2020). Numerous studies analyzing global production trends involve examining factors influencing cultivation, yield, and distribution. These factors encompass agronomic practices, breeding advancements, environmental conditions, and socio-economic aspects (Munck and Jespersen, 2011, Linquist *et al.*, 2012). Understanding and optimizing these factors is crucial for ensuring the sustainability and productivity of Triticeae crops in meeting the growing global food demands.

Soil-borne diseases reduce Triticeae crop production

Winter barley and wheat typically yield more than spring cultivars due to their longer maturation periods. However, autumn-sown barley and wheat require vernalization, a process where prolonged exposure to cold temperatures during winter can increase the risk of soil-borne diseases (Panth *et al.*, 2020). These diseases are caused by a variety of pathogens, including fungi, nematodes, bacteria, and viruses, which infect roots or below-ground parts of plants. The resulting symptoms, such as root rot, damping-off, and wilting, can be devastating (Moura *et al.*, 2022). The persistence of these pathogens in the soil exacerbates crop losses, leading to decreased grain quality, increased production costs, and soil degradation.

Effective management of soil-borne diseases necessitates an integrated approach, incorporating cultural, genetic, biological, and chemical control strategies (Panth *et al.*, 2020). Sustainable disease management can be achieved through cultural practices like crop rotation and sanitation, alongside breeding for disease resistance (Panth *et al.*, 2020).

Soil-borne virus-induced mosaic diseases in Triticeae

Among soil-borne pathogens, the genus *Furoviruses* includes soil-borne wheat mosaic virus (SBWMV), which was first reported in the United States, and subsequent pathotypes were identified in Japan, France, Germany, Turkey, China, South Korea, and New Zealand (Kuhne, 2009). Chinese wheat mosaic virus (CWMV) has been extensively investigated in China (Zheng *et al.*, 1999). In the *Bymovirus* genus, barley yellow mosaic virus (BaYMV) was initially reported in Japan (Ikata and Kawai, 1940). Analysis of 23 isolates from various locations in Japan led to the classification of Japanese BaYMV strains into six distinct pathotypes (Kashiwazaki *et al.*, 1989b). Since the 1950s, BaYMV has been identified across East Asia (Jiang *et al.*, 2020). In Europe, both BaYMV and barley mild mosaic virus (BaMMV) were initially documented in Germany (Götz and Friedt, 1993), after which they swiftly spread to other countries, including France (Lapierre, 1980), the United Kingdom (Hill and Walpole, 1989), Italy (Faccini *et al.*, 2001), and Spain (Kuhne, 2009). Wheat spindle streak mosaic virus (WSSMV) has been identified in Canada (Lu *et al.*, 1998), the United States (Bays *et al.*, 1985), and Europe (Signoret *et al.*, 1977), whereas wheat yellow mosaic virus (WYMV) is predominantly found in East Asia (Jiang *et al.*, 2020).

The genetics of resistance against *Furovirus* and *Bymovirus*

Several genes providing resistance to *Furoviruses* in wheat, durum wheat, and barley have been documented. In bread wheat, the resistance genes *Sbm1* and *Sbm2* confer resistance to both SBWMV and soil-borne cereal mosaic virus (SBCMV) and are located on chromosomes 5DL and 2BS, respectively (Okada *et al.*, 2023). In durum wheat, the quantitative trait locus *QSbm.ubo-2BS* exhibits resistance akin to that conferred by *Sbm2* against SBCMV (Russo *et al.*, 2012).

Among the resistance genes against BaYMV/BaMMV, the *rym3* gene has been favored by breeders in East Asia (Oozeki *et al.*, 2017). However, the widely utilized *rym4* gene has been overcome by BaYMV-1 and BaYMV-2 strains, especially in European countries. In contrast to several recessive resistance genes identified in barley breeding, dominant resistance genes or quantitative trait loci (QTLs) against WYMV have been identified in wheat cultivars (Jiang *et al.*, 2020, Chen *et al.*, 2023b). Recently, the *TaRD21A* gene has been shown to enhance resistance against WYMV through an amino acid substitution that reduces the interaction between WYMV-encoded nuclear inclusion protease-a (NIa) and TaRD21A (Liu *et al.*, 2023).

Despite these advances, the mechanisms of virus susceptibility and resistance in barley and wheat against *Furoviruses* and *Bymoviruses* remain poorly understood. This thesis aims to uncover the molecular and genetic basis of virus coinfection, pathogenicity, and host resistance in barley and wheat. This understanding provides novel insights into developing more effective and sustainable strategies for managing viral diseases and ensuring global food security.

Chapter 1. The prominent multiplication of Japanese soil-borne wheat mosaic virus co-infected with barley yellow mosaic virus in barley

Introduction

Viruses belonging to viral genera *Furovirus* and *Bymovirus* are recognized to represent a significant threat to the global production of barley (*Hordeum vulgare*), wheat (*Triticum spp*.), and other Triticeae species (Kuhne, 2009, Jiang *et al.*, 2020). Infected plants exhibit leaf chlorosis, mosaic, and stunted growth during early Spring (Serfling *et al.*, 2017, Mishina *et al.*, 2024). In fields severely infected by these viruses, particularly those in which wheat or barley is cultivated, crop yield losses can reach as high as 80% (Hunger *et al.*, 1989, Myers *et al.*, 1993, Lapierre and Hariri, 2008, Suzuki *et al.*, 2022, Gauthier *et al.*, 2023). Furoviruses are characterized by having a bipartite genome with non-poly(A) viral RNAs 1 & 2, and bymoviruses are characterized by having a bipartite genome with poly(A) viral RNAs 1 & 2. Both *Furovirus* and *Bymovirus* viruses are transmitted by the widespread protozoan *Polymyxa graminis*, which produces long-lasting resting spores (Adams and Swaby, 1988, Shirako *et al.*, 2000, Kanyuka *et al.*, 2003, Kuhne, 2009). The consensus is that resistance breeding represents the most feasible strategy for mitigating the long-term and persistent threat posed by these pathogens (Bayles *et al.*, 2007, Miedaner and Korzun, 2012).

The three Furoviruses of the genus *Furovirus* of the family *Virgaviridae*, soil-borne wheat mosaic virus (SBWMV), Soil-borne cereal mosaic virus (SBCMV), and Chinese wheat mosaic virus (CWMV) can all infect wheat, barley, rye, and triticale (Kuhne, 2009), while wheat cultivars carrying both *Sbm1* and *Sbm2* have been shown to accumulate a notably lower SBWMV and SBCMV titer than those carrying just one of these genes (Narasimhamoorthy *et al.*, 2006, Bayles *et al.*, 2007, Hao *et al.*, 2012, Liu *et al.*, 2014, Liu *et al.*, 2020). Resistance genes protecting barley against the *Furovirus* species Japanese soil-borne wheat mosaic virus (JSBWMV) - a species *Furovirus japonicum* first documented in Japan (Shirako and Brakke, 1984) - have been mapped to sites on both *Jmv1* in chromosome 2H and *Jmv2* in 3H (Okada *et al.*, 2020, Okada *et al.*, 2022). However, as yet, none of these genes have been isolated, and their mode of action remains obscure. Of the genus *Bymovirus* of the family *Potyviridae*, seven viruses are registered on the International Committee on Taxonomy of Viruses (ICTV: https://ictv.global/). Barley yellow mosaic virus

(BaYMV) and barley mild mosaic virus (BaMMV) exclusively infect barley, while wheat yellow mosaic virus (WYMV) solely infects wheat plants. Wheat spindle streak mosaic virus (WSSMV) can infect wheat, rye, and triticale (Kuhne, 2009, Jiang *et al.*, 2020). At least 22 *rym*-mediated genes (resistant to yellow mosaic virus) against BaYMV/BaMMV -a species *Bymovirus hordeiluteum*/*Bymovirus hordei* had been identified in barley (Kanyuka *et al.*, 2005, Stein *et al.*, 2005, Yang *et al.*, 2014, Jiang *et al.*, 2020). The cultivation over a wide area of susceptible barley varieties, specifically those carrying the no longer effective genes *rym4* and/or *rym5*, presents a risk of a major outbreak of disease (Stein *et al.*, 2005, Kuhne, 2009). The Japanese cultivar Sukai Golden (cv. SG), which harbors *rym3* and *rym5*, exhibits resistance to all known Japanese strains of BaYMV (Taniguchi *et al.*, 2001, Arai *et al.*, 2018). The wheat cv. Madsen is protected from infection by WYMV by the presence of the genes *Ym1* and *Ym2*, the latter of which has recently been isolated (Mishina *et al.*, 2023). However, this is not the case for the resistance loci *QYm.njau-3B.1 and QYm.njau-5A.1* harbored by cv. Xifeng (Zhu *et al.*, 2012, Jiang *et al.*, 2020).

While JSBWMV multiplies itself within a host barley plant during the winter, colonizing its roots by early December and its leaves by late January (Okada *et al.*, 2023), BaYMV reaches the roots during January and the leaves by March (Mishina *et al.*, 2024). Barley and wheat plants commonly experience the co-infection of several *Furovirus* and *Bymovirus* pathogens (Kuhne, 2009). European barleys frequently harbor both BaYMV and BaMMV (Pellio *et al.*, 2005, Perovic *et al.*, 2014), while some wheat cultivars are infected by a combination of *Furovirus* and *Bymovirus* species. How these viruses interact with one another *in planta* remains unclear. The rapid development of genomic resources in barley and wheat, along with the availability of a set of pan-genome accessions, now facilitates the identification of target genomic regions and natural variants of resistance genes (Jayakodi *et al.*, 2020, Walkowiak *et al.*, 2020, Mascher, 2021, Wang *et al.*, 2022, Xie *et al.*, 2022). The aim of the present research was to investigate the effect of co-infecting both barley and wheat plants with JSBWMV and BaYMV.

Materials and Methods

Plant materials and growth conditions

The germplasm panel comprised the barley cvs. SG and Kashimamugi, along with a selection of 15 barley pan-genome accessions (Table 1-1) (Jayakodi *et al.*, 2020) and 15 wheat pan-genome accessions (Table 1-2) (Walkowiak *et al.*, 2020). The plants' response to BaYMV and JSBWMV infection was assessed at two locations: the first in a field known to be infested with both viruses, located at Yawara (Tsukubamirai, Ibaraki, Japan) (Ogawa *et al.*, 1995), and the second in a non-infested field at Kannondai (Tsukuba, Ibaraki, Japan) (Mishina *et al.*, 2024). The chosen negative control was cv. SG, validated as resistant to both viruses (Taniguchi *et al.*, 2001, Okada *et al.*, 2023, Taketa *et al.*, 2023), while cv. Kashimamugi (Tonooka *et al.*, 2014), which is susceptible to both viruses, was used as the spreader variety at the infested site. Grain of cvs. SG and Kashimamugi were provided by the Tochigi Prefectural Agricultural Experiment Station. The pan-genome barley accessions were ordered to each institute, while that of the pan-genome wheat accessions was obtained via SeedStor (https://www.seedstor.ac.uk/). The full set of germplasm was sown at Yawara on October 31, 2022, while cv. Kashimamugi was sown at Kannondai on November 27, 2022. Root and leaf tissue was collected every two weeks between the end of November 2022 and the middle of April 2023 from plants whose growth stage was recorded using the Zadok's scale (Zadoks *et al.*, 1974). Root and leaf samples used to measure the titer of JSBWMV and BaYMV were taken from three plants per all 32 accessions. The barley and wheat pan-genome accessions' root and leaf samples used for the TaqMan assay were collected on February 22, 2023 (16 WAS). Data regarding air and soil temperature and relative humidity were obtained from the Institute for Agro-Environmental Sciences, NARO (https://www.naro.affrc.go.jp/archive/niaes/topics/g7/clmres_e.html).

Quantification of viral titre

The methods used to extract total RNA and to perform the TaqMan assay of JSBWMV and BaYMV have been described elsewhere (Okada *et al.*, 2023, Mishina *et al.*, 2024). The number of virus copies present in a 20-ng aliquot of RNA (x) was transformed for the purpose of statistical analysis by applying the function $\log_{10}(x+1)$. The relevant PCR primer sequences are given in

Table 1-5. JSBWMV and BaYMV titers were $\log_{10}$ transformed so that a zero value denotes a titer below the detection threshold.

Detection of *Polymyxa graminis*

The presence of *P. graminis* in the roots of cvs. SG and Kashimamugi, sampled at Yawara on February 28, 2023, were detected using cotton blue staining, as described elsewhere (Liu *et al.*, 2016).

Foliar symptoms of the disease

The mosaic affecting leaves was recorded using an Axio Zoom V16 microscope attached to an Axiocam CCD camera (Carl Zeiss AG, Oberkochen, Germany), using the same parameter settings described by (Mishina *et al.*, 2023).

RNA *in situ* hybridization

The RNA *in situ* hybridization procedure followed the method described by (Mishina *et al.*, 2023). Fresh root, leaf sheath, and leaf blade tissues were collected on February 28, 2023 (17 WAS) from plants raised at Yawara. cDNA from root tissue was used as the template for PCRs primed by sequences in Table 1-5. The resulting amplicons were purified using a QIAquick PCR purification kit (QIAGEN LLC, Germantown, MD, USA). Antisense and sense RNA probes were generated in a Roche digoxigenin-nucleic acid detection kit according to the manufacturer's manual (Roche, Basel, Switzerland).

Viral coat protein immunostaining

Plant samples collected from the Yawara field in 17 WAS were processed for viral coat protein immunostaining described by (Mishina *et al.*, 2023), previously. The required JSBWMV antibody was kindly provided by Dr. Yasuo Ohto (Institute for Plant Protection, NARO), while that recognizing BaYMV antibody was purchased from the Japan Plant Protection Association (https://www.jcpa.or.jp/).

Statistical analysis

Pearson correlation coefficients between BaYMV, JSBWMV titers, and nine environmental factors were analyzed using Origin 2021 software (OriginLab, Northampton, MA, USA).

Identification of resistance genes/loci in the pan-genome accessions

Sequence variation among 22 barley pan-genome accessions within *HvEIF4E* (LOC123445661) and *HvPDIL5*-1 (HG793095.1), genes that have been shown to confer resistance to BaYMV, was identified using the NCBI BLAST + blastn megablast algorithm integrated into the Galaxy tool (Cock *et al.*, 2015).

Results

The accumulation of JSBWMV and BaYMV in the roots and leaves of barley

By four weeks after sowing (4 WAS), the roots of cv. Kashimamugi plants grown at the infected site had already accumulated a JSBWMV titer of 3.81 at a time when the BaYMV titer was zero (Figure 1-1A, Table 1-3). The latter rose to 4.38 by 8 WAS, thereafter gradually increasing to 6.09 by 18 WAS (Figure 1-1A). In the leaf blades, the JSBWMV titer reached 4.90 by 10 WAS (Figure 1-1B), while that of BaYMV remained below the level of detection at 8 WAS, reaching 5.14 at 10 WAS (Figure 1-1B). The JSBWMV titer peaked in the leaves (7.09) at 16 WAS, thereafter remaining relatively constant; during this period, the BaYMV titer gradually declined (Figure 1-1B). Temporal trends in the daily mean and minimum air temperature and soil temperatures, shown in Figure 1-1C, demonstrate that the multiplication of JSBWMV in the roots was favored by temperatures of around 10ºC, while that of BaYMV was more rapid when the air and soil temperature dropped below 5ºC. The translocation of both viruses from the roots to the leaves occurred when the temperature remained below 5ºC for two weeks. In the non-infested field, the titer of both JSBWMV and BaYMV remained at zero (Figure 1-2). The conclusion was that the optimal time for sampling viral titers of both JSBWMV and BaYMV in the roots and the leaves was the period from February to March.

Histological localization of BaYMV and JSBWMV

By 17 WAS, numerous viruliferous resting spores of *P. graminis* were observed in the root cortex cells of both cv. SG and cv. Kashimamugi (Figure 1-3A, B), consistent with some recent observations made by both (Okada *et al.*, 2023) and (Mishina *et al.*, 2024). Unexpectedly, some cv. SG root hair cells were seen to harbor zoospores (Figure 1-3A). While cv. SG leaves exhibited no recognizable disease symptoms (Figure 1-3C), those of cv. Kashimamugi were marked by distinct yellow mosaic(Figure 1-3D), an indication of the translocation of the virus from the roots to the leaves. A histological analysis implied that while throughout the cv. SG plant, the titer of both viral species lay below the detection limit; this was not the case for cv. Kashimamugi plants (Figure 1-3E-X). An *in situ* hybridization assay showed that the latter cultivar's roots harbored an abundance of JSBWMV in broad locations, including in the pith surrounded by the endodermis (Figure 1-3G), while the BaYMV signal was diffused (Figure 1-3H). Confirmatory data was

provided by an immunostaining assay, which was able to identify the presence of JSBWMV coat protein in the roots. The signal of JSBWMV was present in the pith, protoxylem, protophloem, and pericycle, and to a lesser extent in the endodermis (Figure 1-3K), which was more plentiful than that of BaYMV (Figure 1-3L).

Inspection of cross-sections of cv. Kashimamugi leaf sheathes/blades confirmed the presence of both viruses in the vascular bundle and in young leaves but not in the outer leaf sheath (Figures 1-3O and P and 1-4). JSBWMV typically accumulated in young leaves, while BaYMV was concentrated in the vascular cells within the same leaf sheath/blade. Infection by JSBWMV was prominent in some leaf veins, where BaYMV was not detectable (Figures 1-3O, P, and 1-5). RNA diagnostic of both viruses was detected in cv. Kashimamugi bundle sheaths, but while JSBWMV RNA was present in the protoxylem, that of BaYMV was not (Figure 1-3S, T). The RNA *in situ* hybridization assay suggested that root and leaf sheath cells harbored a greater abundance of JSBWMV than of BaYMV. Hybridization experiments using a sense RNA probe constructed for each of the two viruses separately failed to generate any signal throughout the Kashimamugi plant, implying that both viruses present as a positive-sense, single-stranded RNA molecule (Figure 1-4). The conclusion was that the more rapid ability of JSBWMV to colonize the host limits the resources for the colonization of BaYMV (Figure 1-3G, H, K, L).

A reverse transcription quantitative PCR (RT-qPCR) assay was used to quantify the abundance of each virus in plants of the susceptible cv. Kashimamugi. The $\log_{10}$ copy number of JSBWMV was 6.97 in the roots, 7.53 in the young leaf blades/leaf sheaths, and 6.75 in the expanded leaf blades, while the corresponding abundances of BaYMV were 5.93, 5.95, and 5.95 (Figure 1-3Y). The levels of JSBWMV detected in cv. SG tissue was orders of magnitude lower: 2.67 in the roots, 0.00 in the leaf blades, and 0.47 in the leaf sheaths, while those of BaYMV lay below the level of detection (Figure 1-3Y). These outcomes suggested the failure of both the RNA in situ hybridization and the immunostaining assays to detect the presence of JSBWMV in the roots of cv. SG truly reflected a low abundance of JSBWMV, the $\log_{10}$ titer of which - according to the RT-qPCR assay - was <3 (Figure 1-3E, I). In contrast, the $\log_{10}$ titer of JSBWMV in the roots of cv. Kashimamugi was >7, a sufficiently high level to enable the virus' detection using a histological assay. Similarly, it was possible to use these assays to detect the presence of BaYMV in this cultivar, in which the $\log_{10}$ titer was around 6. JSBWMV evidently accumulated to a level

of more than one order of magnitude greater than BaYMV (Figure 1-3Y), consistent with the observed higher abundance of JSBWMV (Figure 1-1A, B). JSBWMV signal was more plentiful than that of BaYMV in regards to the quatitative analysis (Figure 1-3G-X). The overall conclusion is that these two viruses differ markedly in their rate of planta multiplication.

The response of barley pan-genome accessions to exposure to BaYMV and JSBWMV

The pan-genome barley accessions' response to exposure to the two viruses varied both quantitatively and in terms of the effectiveness of the virus translocation *in planta*; there was only limited variation displayed between individuals of a given accession. The observations with respect to JSBWMV are summarized in Figures 1-6A, 1-7, and Table 1-1. The root response of cv. Morex and the two breeding lines HOR7552 and HOR21599 were similar to that of cv. Kashimamugi, with the virus' movement from the roots into the leaves appearing unimpeded. In contrast, the response of the remaining accessions resembled that of cv. SG, scoring 2 and 4 in the roots and zero in the leaves. The corresponding data with respect to BaYMV are summarized in Figures 1-6B, 1-7, and Table 1-1: cv. Akashinriki, like cv. SG, tested negative for the virus in both its roots and leaves, while one or two plants of the two breeding lines HOR10350 and HOR13942, along with cv. Morex tested negatively in their leaves but positively in their roots (scores ranging from 3-5), implying the existence of a partial or stochastic infection. All of the remaining accessions bar one (breeding line HOR3365) tested positively for the virus in their roots (scores ranging from 4-6), a response resembling that of cv. Kashimamugi; the viral score in the leaves of these accessions lay between 3 and 6, again similar to that of the fully susceptible cv. Kashimamugi. The leaves of breeding line HOR3365 featured a lower virus score (0-3), even though its roots tested positive: the inference is that this accession may harbor a gene(s) able to either suppress the translocation of BaYMV from the roots into the leaves and/or to inhibit the virus' multiplication within the leaf. A number of barleys, including cvs. Morex and Golden Promise, and breeding line HOR9043, are known to harbor the resistance gene *rym6* allelic variants of the *HvEIF4E* (Kanyuka *et al.*, 2005, Stein *et al.*, 2005), although in a loss-of-function state (Table 1-4), so the partial BaYMV resistance expressed by cv. Morex is likely conferred by a gene(s) other than *rym6*. All accessions carry the wild-type allele of *HvPDIL5-1* at the *rym1/rym11* locus (Yang *et al.*, 2014) (Table 1-4), so they are not responsible for the BaYMV resistance.

The Response of wheat pan-genome accessions when exposed to JSBWMV

The response to JSBWMV exposure of a sample of the wheat pan-genome accessions was investigated using a qRT-PCR assay (Figures 1-7, 1-9, and Table 1-2). For ten of the accessions, the $\log_{10}$ score was either zero or close to zero in both the roots and the leaves, except for a score of 3 detected in the leaf of one plant of cv. Arina*LrFor*. The roots of one or two plants of cvs. Lancer, CDC Landmark, and Kronos tested positive (scores >3). Plants of cv. Mace inhibited the translocation of JSBWMV from the roots to the leaves, even though its viral titer was quite high (3.86) in the roots (Figure 1-8A). Full susceptibility was displayed by cv. Norin 61, scoring a $\log_{10}$ of 6.12 in the roots and 6.14 in the leaves. BaYMV was undetectable in either the roots or leaves of any of the accessions (Figure 1-8B).

Histological localization of JSBWMV in wheat plants

Two contrasting wheat accessions were used to localize JSBWMV: one was cv. Cadenza was documented as resistant to SBCMV (Kanyuka *et al.*, 2004, Bass *et al.*, 2006); the other was cv. Norin 61, a cultivar shown here to be fully JSBWMV susceptible. In the roots of cv. Norin 61, but not of cv. Cadenza, the presence of viral RNA and coat protein could be established in the pith, protoxylem, protophloem, pericycle, endodermis, and cortex (Figure 1-10C, G); in the leaves, JSBWMV was detectable in the bundle sheath and mesophyll (Figure 1-10K, Q, S). The JSBWMV copy number was lower in the roots of the cv. Cadenza ($\log_{10}$ of 2.40) than in those of cv. Norin 61 (6.39) (Figure 1-10U). The JSBWMV titer was also high in the cv. Norin 61 leaf sheath (7.38) and leaf blade (6.93). No BaYMV was detectable in any tissues of either cultivar.

Discussion

How barley is infected with JSBWMV and BaYMV

Cases are known where a viral infection of a plant based on more than one either related or unrelated pathogen can result in a level of disease severity greater than when only a single viral species is involved (Tatineni *et al.*, 2010, Syller, 2012, Hull, 2014a, Hull, 2014b, Abdullah *et al.*, 2017, Ranabhat *et al.*, 2023). Such mixed infections can inform our understanding of the complexity of plant disease (Kappagantu *et al.*, 2020). The present report addresses the significant differences in JSBWMV and BaYMV accumulation in barley (Figure 1-1A, B) (Gómez-Aix *et al.*, 2019, Okada *et al.*, 2023) and characterizes differences in their infection mechanism (Figure 1-11). The multiplication of JSBWMV in the roots began at least four weeks earlier than that of BaYMY (Figure 1-1A); however, their movement from the roots to the leaves coincided temporally (Figure 1-1B). The *Bymovirus* WYMV accumulates in the stele of the wheat root, while the *barley yellow dwarf virus* is thought to be phloem-limited (Paulmann *et al.*, 2018, Mishina *et al.*, 2023). Both JSBWMV and BaYMV RNA were distributed throughout the roots and leaves of the susceptible barley cv. Kashimamugi (Figure 1-3G, H), an unexpected result given that the coat protein of JSBWMV was largely restricted to the phloem in the root.

A relationship was noted between virus multiplication and the weekly mean air temperature (Figures 1-1C and 1-9). The BaYMV titer in the roots was strongly and negatively correlated with changes in temperature and relative humidity up to 14 WAS (Pearson's correlation coefficients of -0.67 and -0.88, respectively). On the other hand, a strong positive correlation was obtained between the JSBWMV and BaYMV titer in both the roots and leaves over time (Pearson's correlation coefficients ranging from +0.77 to +0.99, see Figures 1-1A, B, and 1-9A). Starting from 16 WAS, the BaYMV titer in both the roots and leaves was suppressed by temperature changes, whereas that of JSBWMV in the roots was promoted by both temperature and rainfall (Pearson's correlation coefficients in the range +0.46 to +0.59. (Figure 1-2B). The reason(s) for these different behaviors remains unclear, but one possibility is that the ability of the phloem to upload, spread systemically, and translocate molecules such as sugars and amino acids is subject to various environmental factors.

JSBWMV is able to maintain a presence in the roots of a resistant barley host

The presence of JSBWMV in a susceptible host can be readily detected via RT-qPCR. In the leaf, a susceptible host recorded a $\log_{10}$ score of 6-7, while in the leaf of a resistant plant, the score was essentially zero (Figure 1-6A); the difference was less stark in the roots, where the score varied between 6 and 7 in a susceptible host and between 2 and 4 (never zero) in a resistant one. The response to JSBWMV infection implies the ability of the host to limit the multiplication of the virus in its leaves to a level below the detection limit of the histological assays, even though the titer of the virus in the roots is as much as a thousand times that of the transcript of actin. The capacity of the virus to maintain itself in the roots of a resistant host has also been noticed by (Okada *et al.*, 2023) those who studied the response of the two JSBWMV-resistant cultivars (cv. Haruna Nijo and cv. SG). Here, more than ten of the pan-genome barley accessions expressed a comparable level of resistance.

The means by which JSBWMV is retained in the host's roots is clearly different from how both BaYMV (in the barley root) and WYMV (in the wheat root) are retained. The BaYMV titer of the resistant barleys (such as cvs. Akashinriki and SG) lay below the detection limit, as is similarly the case for wheat hosts harboring genes determining resistance against WYMV (Mishina *et al.*, 2023). The increase in the copy number of JSBWMV was likely not driven by any growth in the population of *P. graminis*, but rather by either a rise in the viral titer in the vector and/or by a more effective means of multiplying JSBWMV in planta.

With respect to BaYMV titer, and unlike the case for JSBWMV, the viral titer in the root of both resistant hosts (cvs. SG and Akashinriki) was zero (Figure 1-6), mirroring the finding of an earlier study (Mishina *et al.*, 2024). Thus, the multiplication of BaYMV was fully suppressed in these cultivars' root systems. A potentially important difference between these two viruses is that while the BaYMV genome is protected by polyadenylation at its 3' terminus, this is not the case for JSBWMV (Shirako and Wilson, 1993, Shirako *et al.*, 2000, Miyanishi *et al.*, 2002). The translation of a non-poly(A) viral RNA is enhanced by the binding of a viral coat protein or by its polyadenylation (Neeleman *et al.*, 2001), along with the presence of a poly(A)-binding protein (Iwakawa *et al.*, 2012). The presence of JSBWMV in both cvs. SG and Akashinriki suggest that their resistance reflects some restriction of cell-to-cell movement of the virus after its replication. As some of the pan-genome accessions show a level of resistance comparable to that of cv. SG

(for which the $\log_{10}$ score was ~3 in its roots and effectively zero in its leaves), these scores can be assumed to represent threshold values to the JSBWMV in resistance cultivars. The line HOR10350 accumulated a $\log_{10}$ titer of ~4 in its roots and zero in its leaves, indicating that in this host, the virus moves freely between cells within the root system but is unable to be translocated to the leaves (Figure 1-6) (Okada *et al.*, 2023). As yet, the biological basis underlying how the copy number of JSBWMV can be maintained at such a high level in a resistant host's root system remains obscure.

The effect of co-infecting wheat with JSBWMV and BaYMV

Genotypic differences have been demonstrated within both wheat and other cereal crop species with respect to the plants' reaction to exposure to WYMV, CWMV, and SBWMV (Cadle-Davidson *et al.*, 2006, Fukuta *et al.*, 2013, Xu *et al.*, 2018, Chen *et al.*, 2023b, Mishina *et al.*, 2023). The response to infection by *Furovirus* and/or *Bymovirus* pathogens has been widely studied in barley (Lyons *et al.*, 2008, You and Shirako, 2013, Okada *et al.*, 2023, Mishina *et al.*, 2024). Here, given that wheat's response to exposure to either JSBWMV or BaYMV has yet to be investigated (although it is known from the report of (Li and Shirako, 2015) that the latter virus can replicate in isolated wheat cells), it was decided to test members of the wheat pan-genome in order both to reveal any evidence for host specificity and to characterize the consequences of co-infection (Figure 1-8). Most of the accessions did not support the multiplication of JSBWMV in either their roots or leaves, but full susceptibility was displayed by cv. Norin 61, while in cv. Mace, the virus accumulated in the roots but not in the leaves. All of the accessions were immune to BaYMV infection (Walkowiak *et al.*, 2020). Putative candidate genes for resistance against the *Furovirus* SBWMV have been identified by (Liu *et al.*, 2020). It is possible that, with the exception of cv. Norin 61, all of the pan-genome wheat accessions may harbor such genes. Meanwhile cv. Mace appears to carry a gene(s), the product of which limits the translocation of the virus from the root to the shoot – such genes, i.e., encoding products that are active against the translocation of BaYMV in barley, have recently been described by (Mishina *et al.*, 2024).

Wheat cultivars, including cv. Norin 61, which proved to be susceptible to JSBWMV, appears to be immune to BaYMV infection (Figures 1-8, 1-10). In barley, the sites where JSBWMV and BaYMV accumulate differ (Figure 1-5), which suggests that the range of these two viruses may not be identical. If so, neither is likely to provide cross-protection against the other, as was shown

experimentally to be the case (Figures 1-1, 1-3). The ability of a plant virus to infect its host is determined by interactions between various viral proteins (suppressors, translocation proteins, and coat proteins) and host factors (Sato and Yuichiro, 2006). The association of *Bymovirus* VPg and host eIF4E in the host tropism at the cellular level was reported (Li and Shirako, 2015). BaYMV can replicate in isolated wheat cells (Li and Shirako, 2015), so BaYMV's host tropism may reflect its inability to either gain entry to the root, replicate within the root, move from cell to cell, and/or translocate from the root to leaf (Narasimhamoorthy *et al.*, 2006). The mechanisms by which plant viruses establish infection, how the host resists infection, and what determines the host range are all largely unknown (McLeish *et al.*, 2019). The present experiments have established that JSBWMV can infect both barley and wheat and that no cross-protection is provided between JSBWMV and BaYMV.

Figures and tables

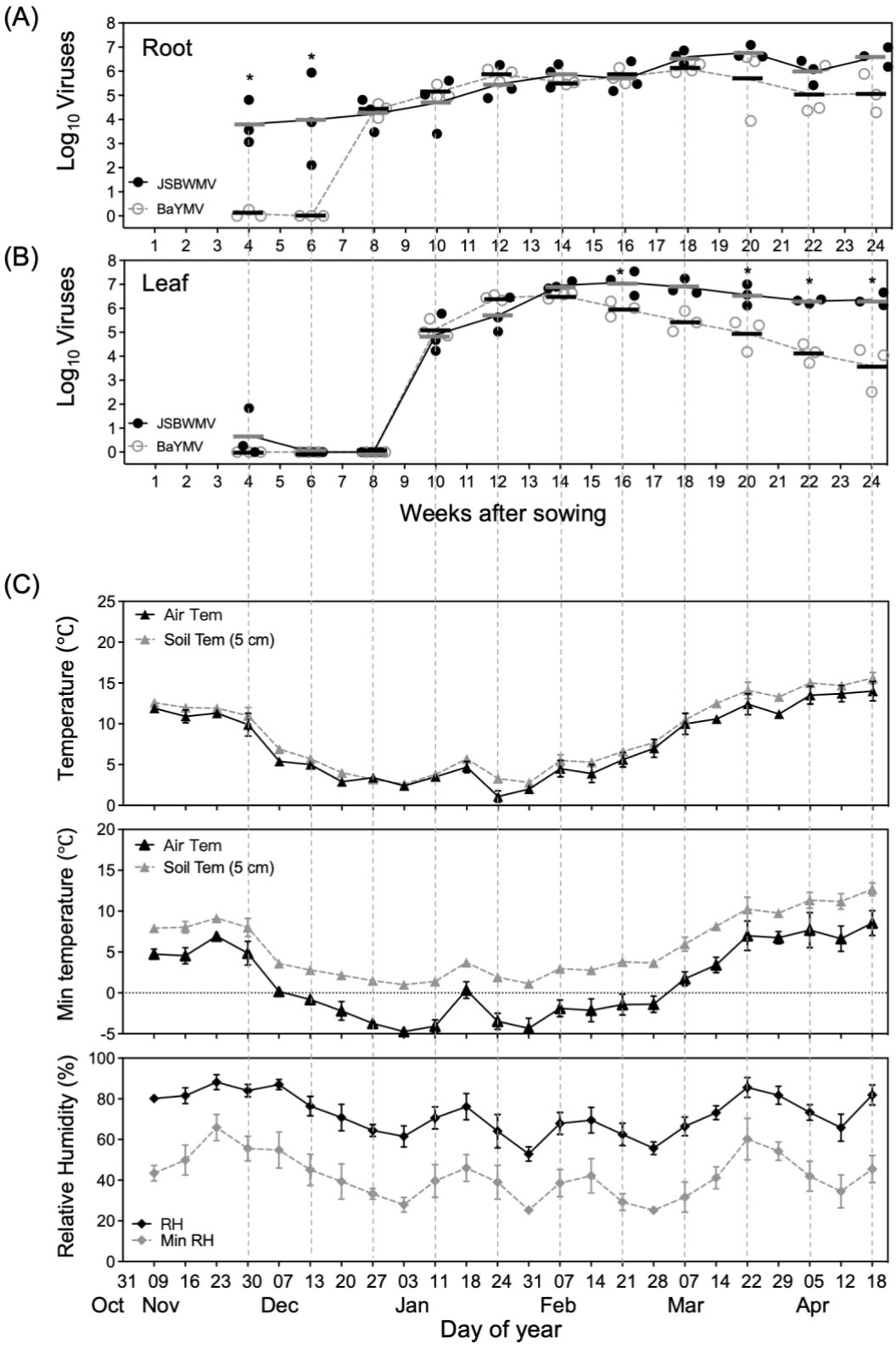


Figure 1-1. Time series analysis of JSBWMV and BaYMV in barley. The $\log_{10}$ transformed copy number of JSBWMV and BaYMV in roots (A) and leaves (B) of individual plant samples of cv. Kashimamugi were detected every two weeks from late November to middle April (n=3). Plants were sown in a JSBWMV/BaYMV contaminated field Yawara 10-4 (Ibaraki, Japan) on October 31st, 2022. * Indicate two-tailed Student's t-test $p < 0.05$. (C) Weekly changes in temperature and minimum temperature during plant growth in Yawara 10-4. Climate data (air and soil at 5 cm depth) were obtained from the Weather Data Acquisition System of the Institute for Agro-Environmental Sciences, NARO. Error bars represent the SD. The dotted line delineates 0 ℃ and the same sampling dates.

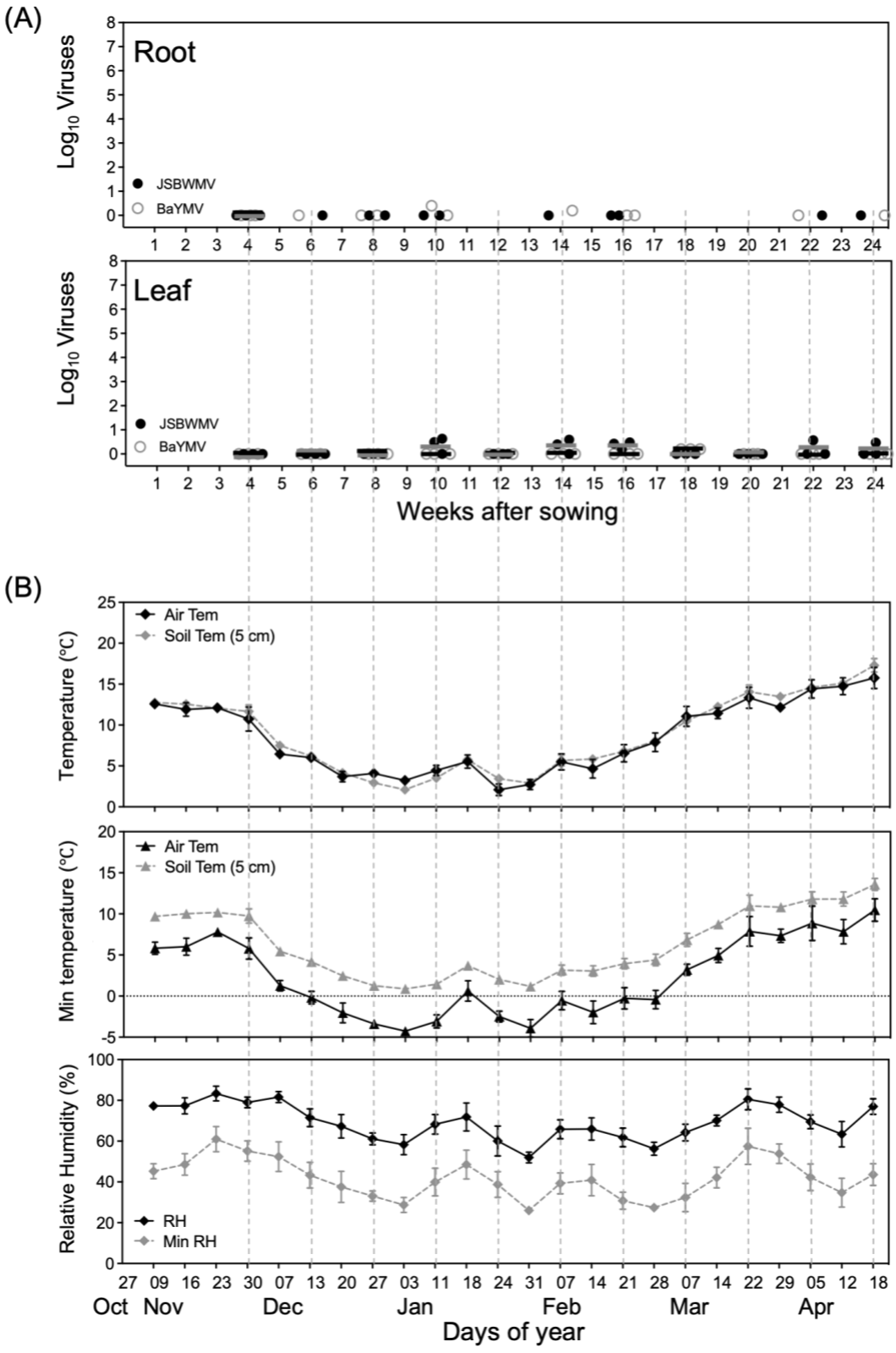

(A)
Root
Leaf
Log10 Viruses
JSBWMV
BaYMV
Weeks after sowing
(B)
Air Tem
Soil Tem (5 cm)
Temperature (°C)
Min temperature (°C)
Relative Humidity (%)
RH
Min RH
Oct
Nov
Dec
Jan
Feb
Mar
Apr
Days of year

Figure 1-2. Investigation of BaYMV and JSBWMV infection in Kannondai B-C1 (JSBWMV and BaYMV uninfested field). (A) JSBWMV and BaYMV titer plot (log_{10} transformed copy number) of cv. Kashimamugi from November to April. (B) Weekly changes in temperature, minimum temperature, and relative humidity during plant growth in Kannondai B-C1. Climate data (air and soil at 5 cm depth) were obtained from the Weather Data Acquisition System of the Institute for Agro-Environmental Sciences, NARO.

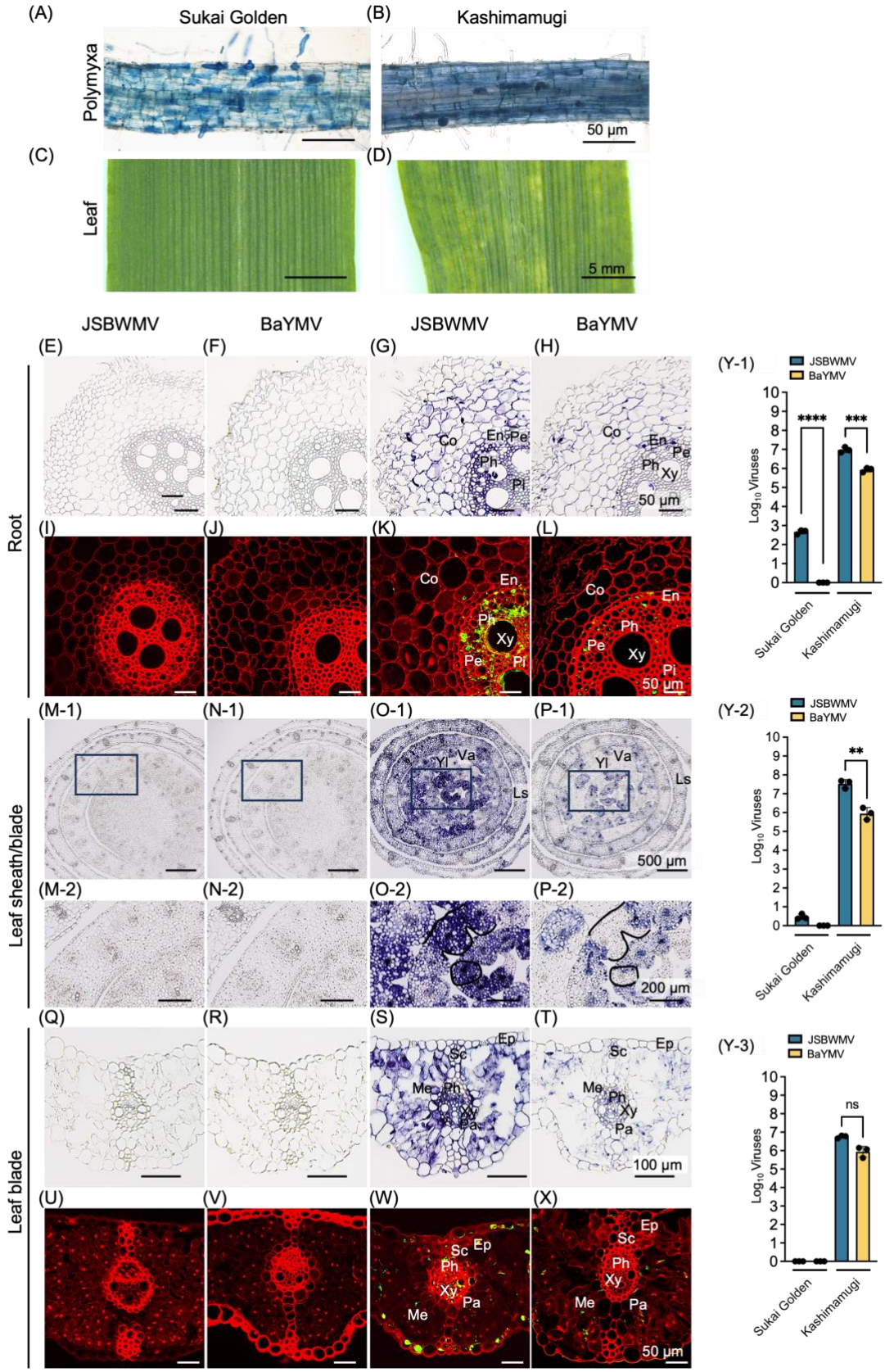

(A)
Sukai Golden
(B)
Kashimamugi
Polymyxa
50 μm
(C)
(D)
Leaf
5 mm
JSBWMV
BaYMV
JSBWMV
BaYMV
(E)
(F)
(G)
(H)
Co
En
Pe
Ph
Xy
Pi
50 μm
Root
(I)
(J)
(K)
(L)
(M-1)
(N-1)
(O-1)
(P-1)
Yl
Va
Ls
500 μm
Leaf sheath/blade
(M-2)
(N-2)
(O-2)
(P-2)
200 μm
(Q)
(R)
(S)
(T)
Ep
Sc
Me
Pa
100 μm
Leaf blade
(U)
(V)
(W)
(X)
(Y-1)
JSBWMV
BaYMV
Log10 Viruses
****
***
Sukai Golden
Kashimamugi
(Y-2)
**
(Y-3)
ns

Figure 1-3. The appearance of yellow mosaic disease and histological localization of JSBWMV and BaYMV in barley. (A) The *P. graminis* colonization of barley seminal roots. (B) The phenotypes of leaves in JSBWMV/BaYMV -resistant Sukai Golden and -susceptible cv. Kashimamugi. The root (E-F), leaf sheath/leaf blade (M-P), and leaf blade (Q-T) of barley-resistant cv. Sukai Golden and susceptible cv. Kashimamugi were hybridized with JSBWMV and BaYMV antisense probes. The coat proteins of JSBWMV and BaYMV were detected by an immunohistological staining assay in root (I-L) and leaf (U-X). The details of the plant tissues were labeled as Co for cortex, En for endodermis, Pe for pericycle, Xy for xylem, Pi for pith, Pr for pericycle, Ph for phloem. The roots, leaf sheaths, and leaf blades were collected on Feb 28th, 2023 (17 weeks after sowing). (Y) JSBWMV and BaYMV titre determined by RT-qPCR. The quantification of JSBWMV and BaYMV in the same individual plants, bars indicate the mean value of three technical replicates with standard deviation (SD). The statistical significance was calculated using a two-tailed Student's t-test, with * indicating $p < 0.05$, *** indicating $p < 0.001$, ns indicating no significance.

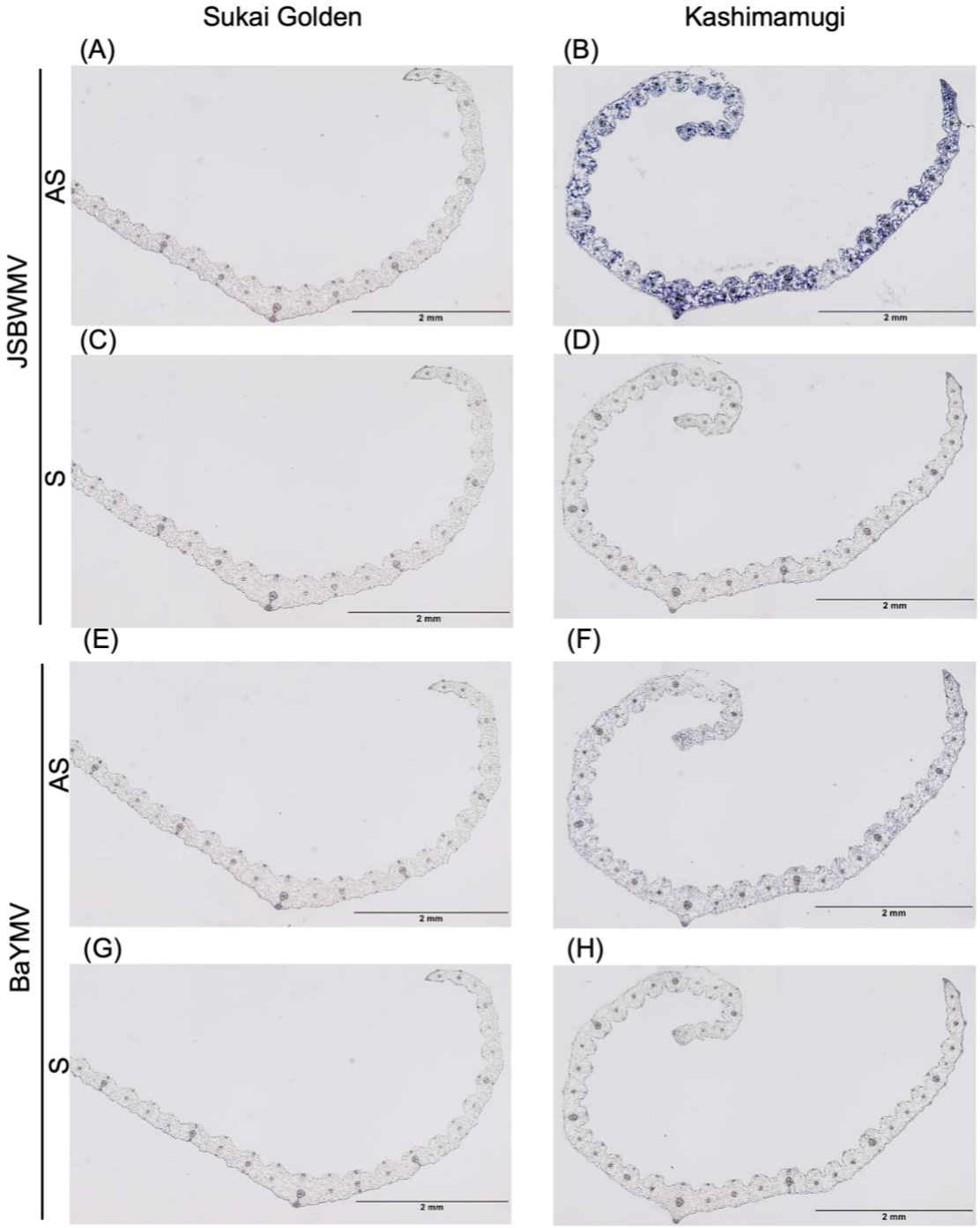


Figure 1-4. Localization of JSBWMV and BaYMV in barley leaf blade by RNA *in-situ* hybridization, AS, antisense probes; S, sense probes. (A, C, E, G) cv. Sukai Golden, (B, D, F, H) cv. Kashimamugi.

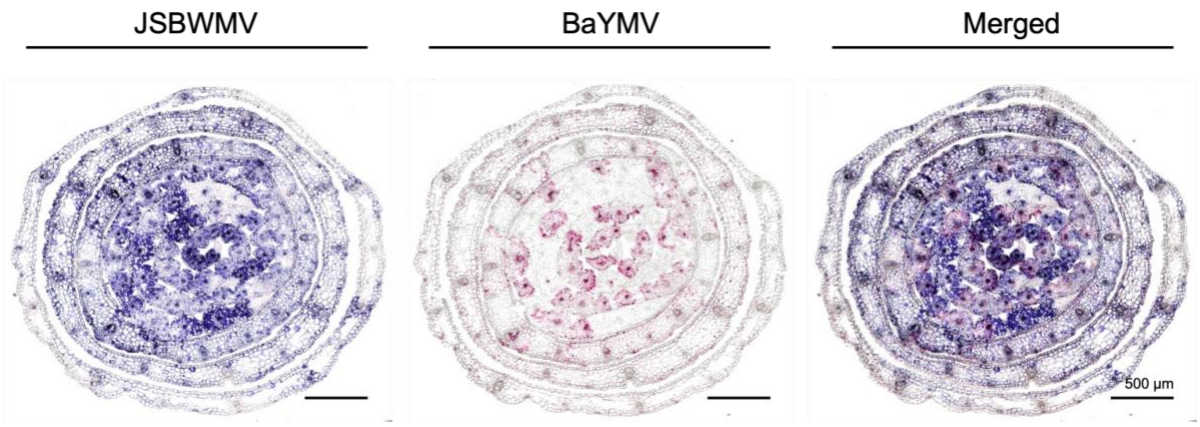


Figure 1-5. Localization of JSBWMV (left) and BaYMV (middle) in barley leaf sheath by RNA *in-situ* hybridization using antisense probes. The merged of their data (right) was indicated.

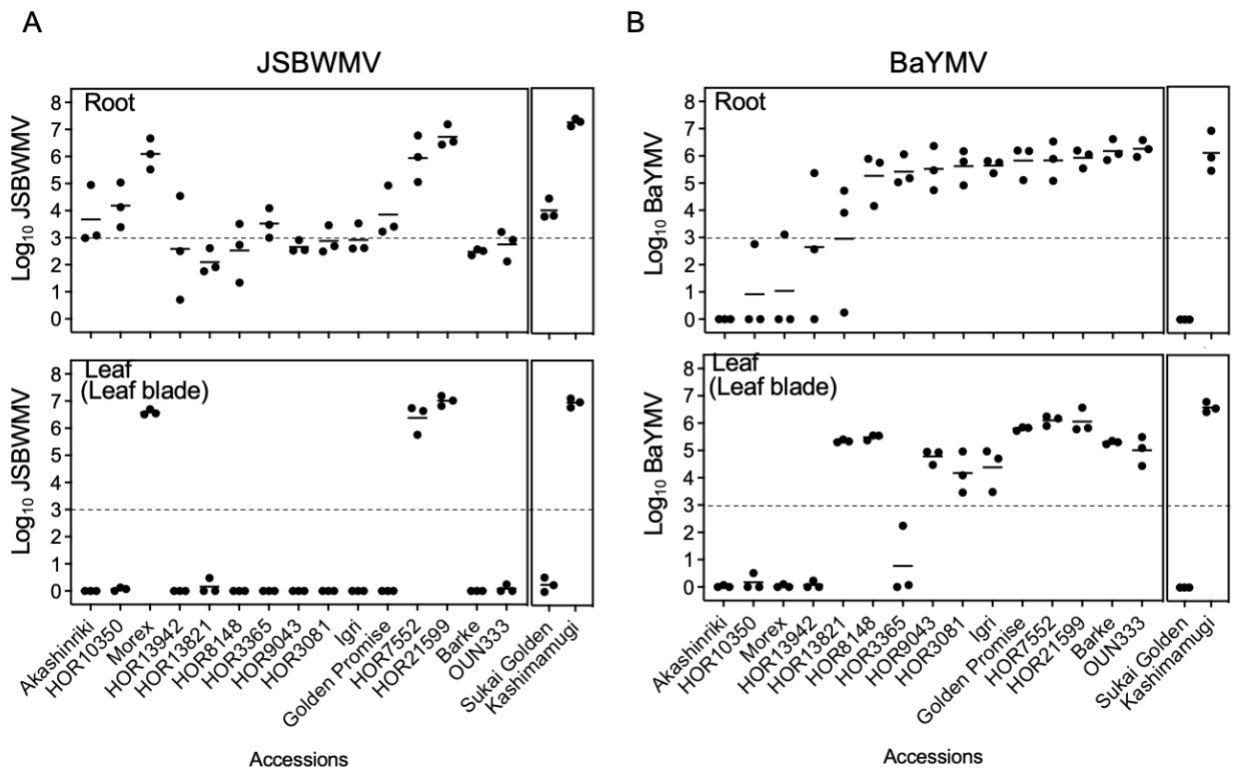


Figure 1-6. Quantification of JSBWMV and BaYMV in the roots (A) and leaves (B) of 15 barley pangenome accessions. The samples were collected on Feb 22nd, 2023. The threshold according to the frequency distribution of the JSBWMV and BaYMV titre is shown as dotted lines indicating a $\log_{10}$ transformed copy number of 3.

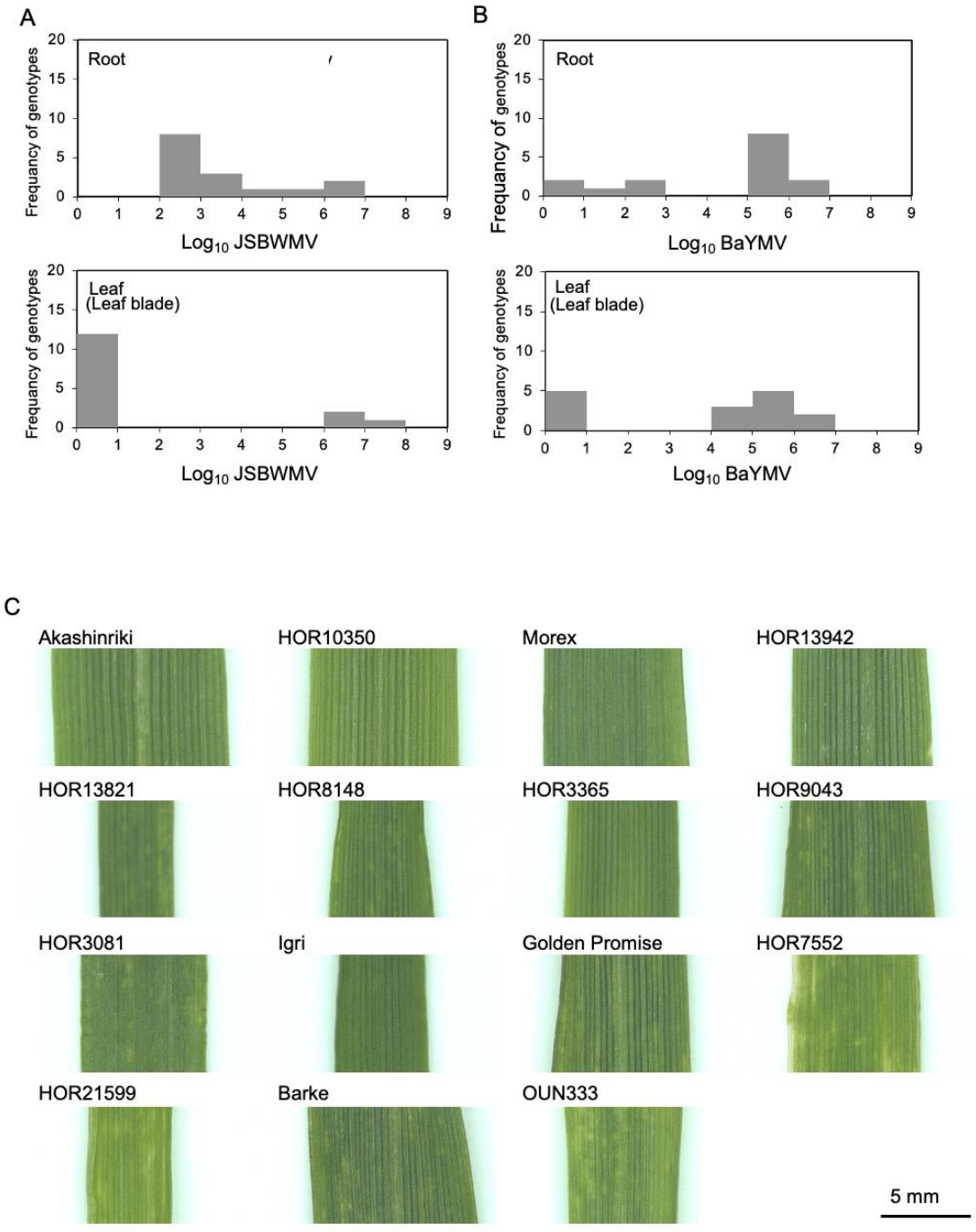


Figure 1-7. Frequency histogram of the BaYMV and JSBWMV titre measured by RT-qPCR in the root and leaf of pan-genome barley accessions (A). Disease symptoms of leaves in 15 barley pangenome accessions (B). All plants were collected on February 22nd 2023, approximately 4 months after sowing.

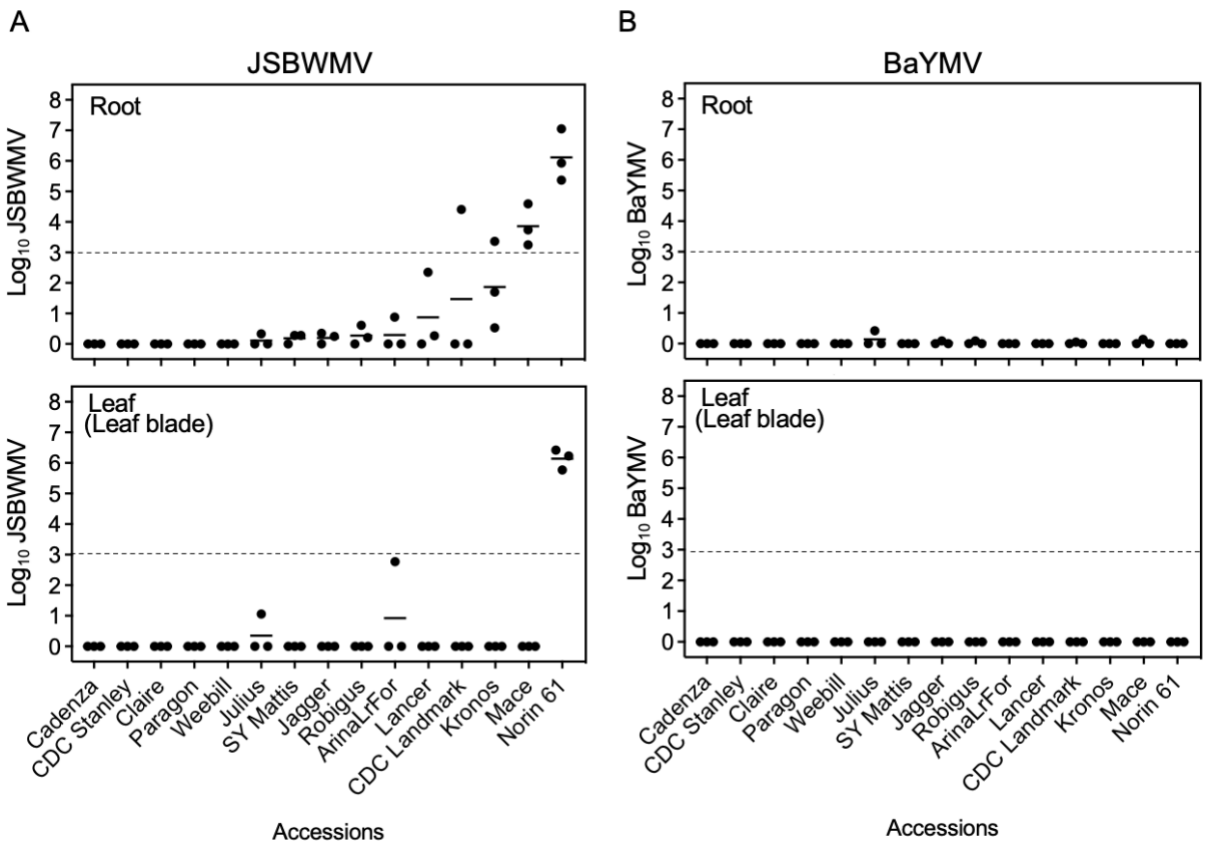


Figure 1-8. Quantification of JSBWMV and BaYMV in the roots (A) and leaves (B) of 15 wheat pangenome accessions. The samples were collected on Feb 22nd, 2023. The threshold according to the frequency distribution of the JSBWMV and BaYMV titre is shown as dotted lines indicating a $\log_{10}$ transformed copy number of 3.

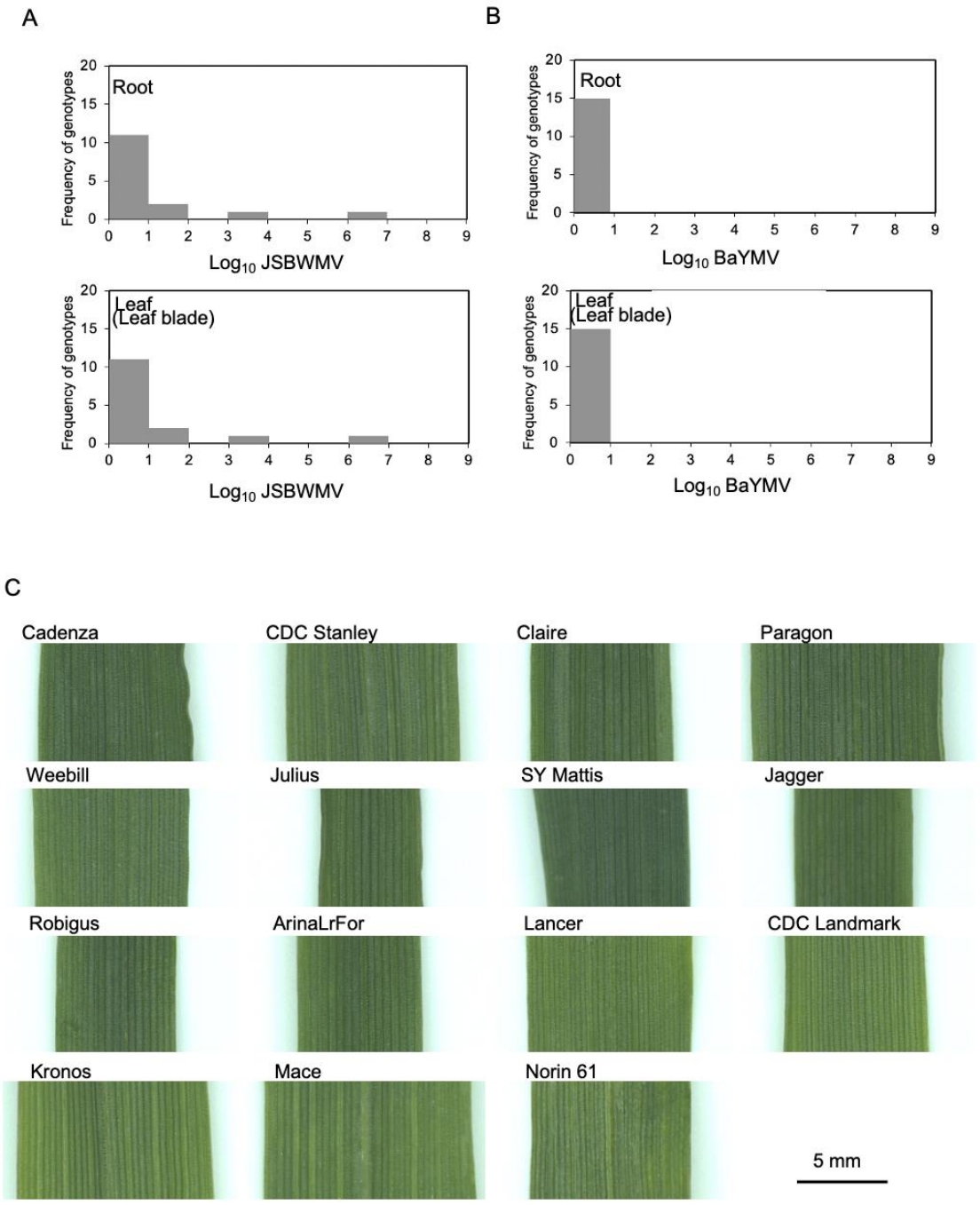


Figure 1-9. Frequency histogram of the JSBWMV and BaYMV titre measured by RT-qPCR in the root and leaf of wheat pan-genome accessions (A). Disease symptoms of leaves in 15 wheat pan-genome accessions (B). All plants were collected on February 22nd 2023, approximately four months after sowing.

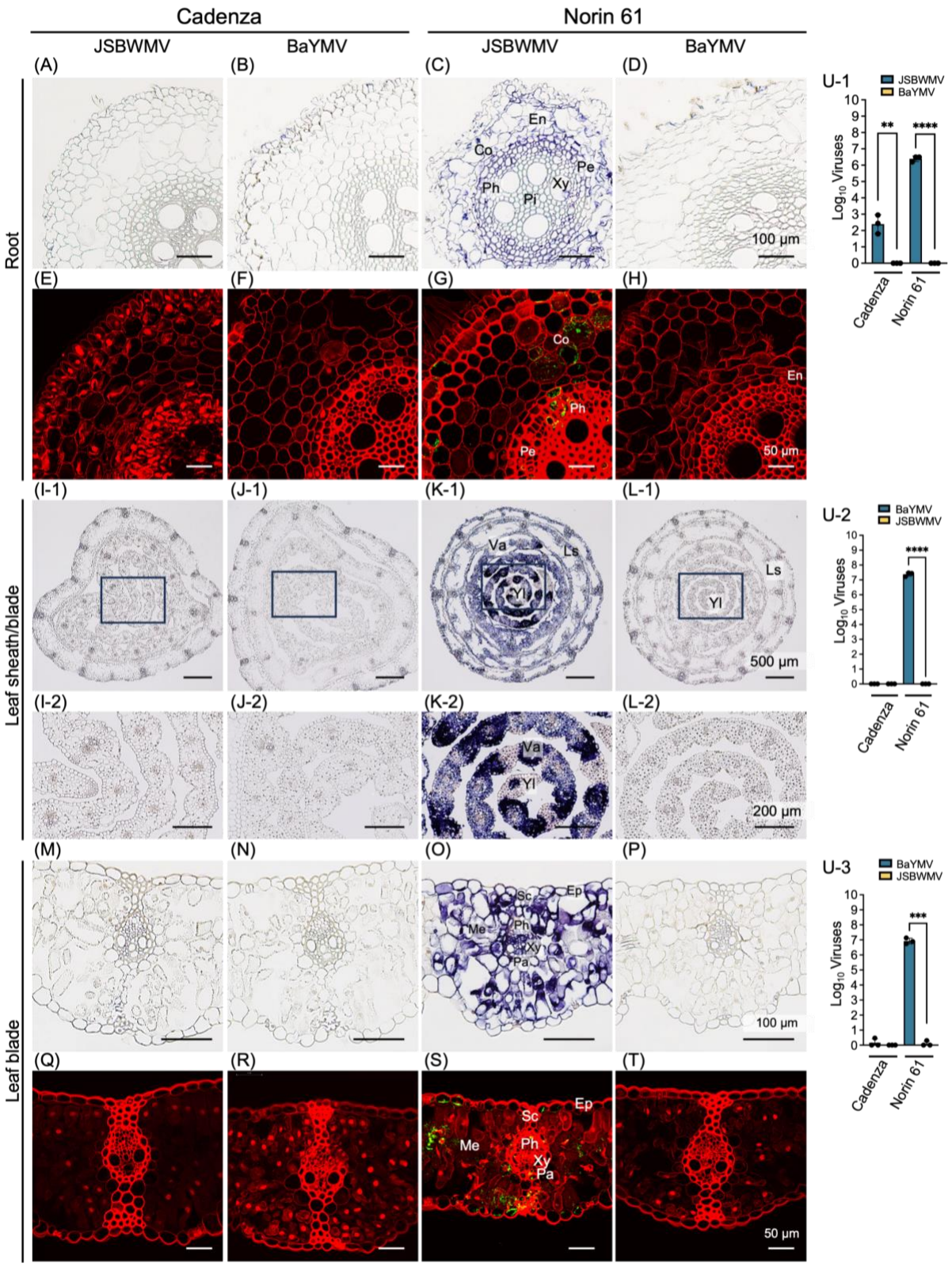

Cadenza
Norin 61
JSBWMV
BaYMV
JSBWMV
BaYMV
(A)
(B)
(C)
(D)
Root
En
Co
Pe
Ph
Pi
Xy
100 μm
(E)
(F)
(G)
(H)
Co
Ph
Pe
En
50 μm
(I-1)
(J-1)
(K-1)
(L-1)
Va
Ls
Yl
Ls
Yl
500 μm
Leaf sheath/blade
(I-2)
(J-2)
(K-2)
(L-2)
Va
Yl
200 μm
(M)
(N)
(O)
(P)
Sc
Ep
Me
Ph
Xy
Pa
100 μm
Leaf blade
(Q)
(R)
(S)
(T)
Ep
Sc
Me
Ph
Xy
Pa
50 μm
U-1
JSBWMV
BaYMV
Log10 Viruses
**
****
Cadenza
Norin 61
U-2
BaYMV
JSBWMV
Log10 Viruses
****
Cadenza
Norin 61
U-3
BaYMV
JSBWMV
Log10 Viruses
***
Cadenza
Norin 61

Figure 1-10. The histological localization of JSBWMV in wheat. The root (A-D), leaf sheath/leaf blade (I-L) and leaf blade (M-P) of wheat cv. Cadenza and cv. Norin 61 were hybridized with JSBWMV and BaYMV antisense probes. The coat proteins of JSBWMV and BaYMV were detected by an immunostaining assay in root (E-H) and leaf (Q-T). The details of the plant tissues were labeled as Co for cortex, En for endodermis, Pe for pericycle, Xy for xylem, Pi for pith, Pr for pericycle, Ph for phloem. The roots, leaf sheaths, and leaf blades were collected on Feb 28th, 2023 (17 weeks after sowing). (Y) BaYMV and JSBWMV titre determined by RT-qPCR. The quantification of JSBWMV and BaYMV in the same individual plants, bars indicate mean value of three technical replicates with standard deviation (SD). The statistical significance was calculated using two-tailed Student's t-test, with * indicating $p < 0.05$, *** indicating $p < 0.001$, **** indicating $p < 0.0001$.

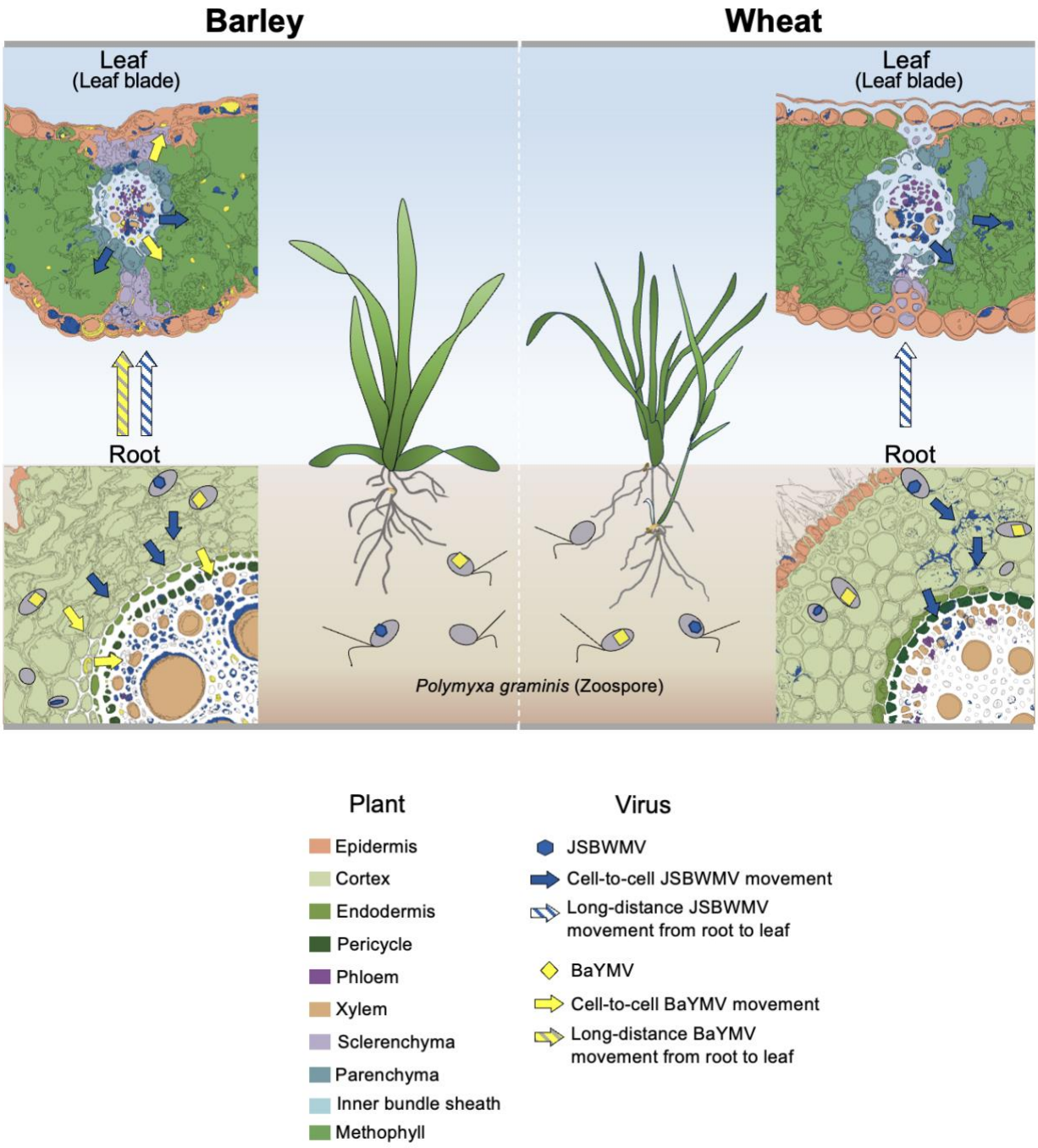


Figure 1-11. Schematic diagram of the soil-borne viral infection in Barley and Wheat in JSBWMV and BaYMV infested field. JSBWMV replicates in both Barley and Wheat. BaYMV replicates in the roots of the host plant barley but not in the non-host plant Wheat.

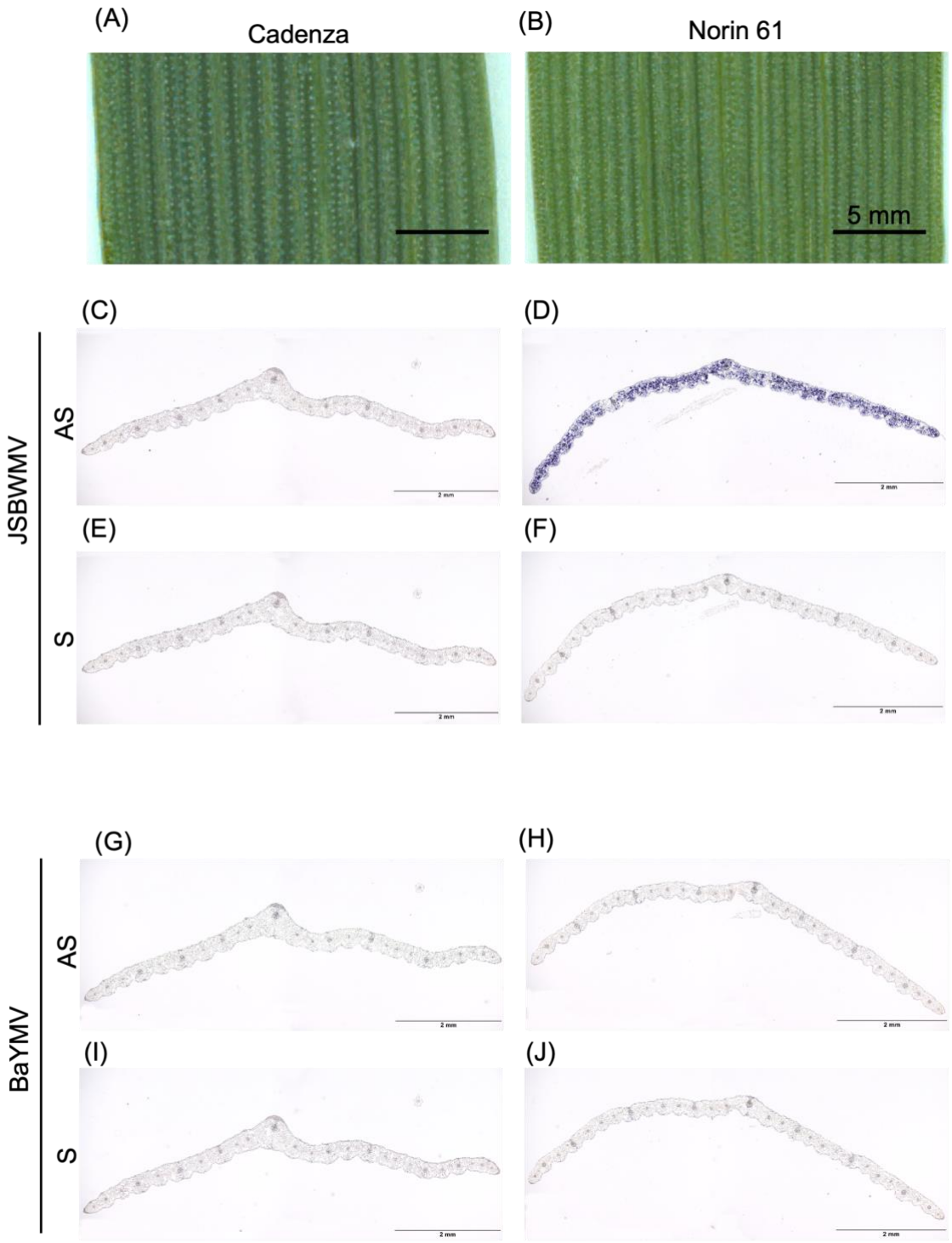
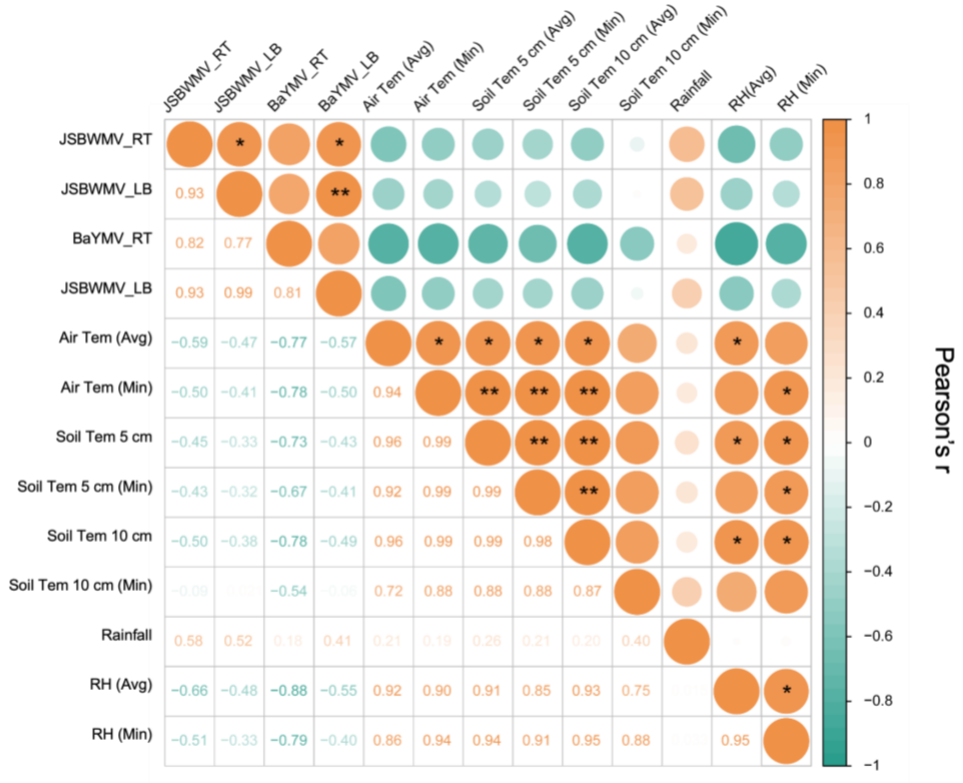


Figure 1-12. Phenotypes and histological localization of JSBWMV in wheat leaf blade by RNA *in-situ* hybridization, AS, antisense probes; S, sense probes. (A, C, E, G, I) cv. Cadenza, (B, D, F, H, J) cv. Norin 61.

(A)


JSBWMV_RT
JSBWMV_LB
BaYMV_RT
BaYMV_LB
Air Tem (Avg)
Air Tem (Min)
Soil Tem 5 cm (Avg)
Soil Tem 5 cm (Min)
Soil Tem 10 cm (Avg)
Soil Tem 10 cm (Min)
Rainfall
RH(Avg)
RH (Min)
Pearson's r
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

(B)

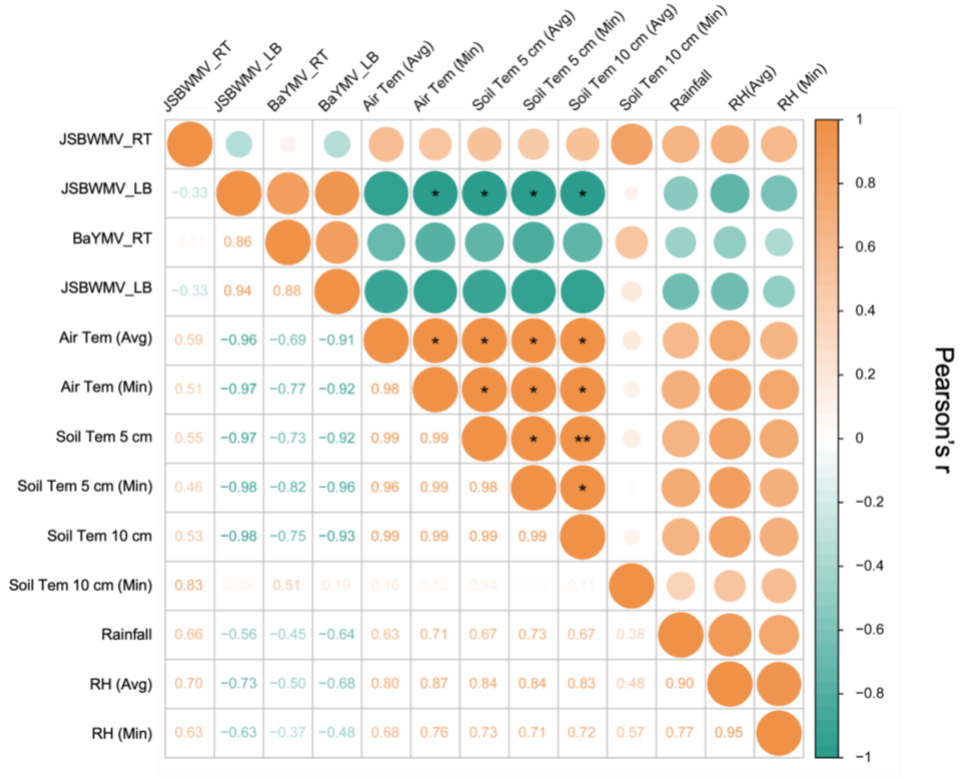

JSBWMV_RT
JSBWMV_LB
BaYMV_RT
BaYMV_LB
Air Tem (Avg)
Air Tem (Min)
Soil Tem 5 cm (Avg)
Soil Tem 5 cm (Min)
Soil Tem 10 cm (Avg)
Soil Tem 10 cm (Min)
Rainfall
RH(Avg)
RH (Min)
Pearson's r
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

Figure 1-13. Pearson's correlation coefficient (two-sided test) of JSBWMV and BaYMV titres and nine environmental variables. A, Correalation coefficient of JSBWMV and BaYMV tirtres in roots (RT) and leaves (LB) of barley cv. Kashimamugi from 4 weeks to 14 weeks after sowing and the related environmental factors. B, Correlation coefficient of JSBWMV and BaYMV tirtres in roots (RT) and leaves (LB) of barley cv. Kashimamugi from 16 weeks to 24 weeks after sowing and the related environmental factors. The environmental factors were weekly air temperatures (Air Tem) and minimum temperature (Air Tem (Min)), average and minimum soil temperature at 5 cm (Soil Tem_5 cm, Soil Tem_5 cm (Min)) and 10 cm (Soil Tem_10 cm, Soil Tem_10 cm (Min)), rainfall, realative humidity (RH), minimum relative humidity (RH (Min)) during plant growth in Yawara 10-4. All climate data were obtained from the Weather Data Acquisition System of the Institute for Agro-Environmental Sciences, NARO. Error bars represent the SEM. Dotted line delineates 0 ℃ and the same sampling dates. Related to Figure 1-1.

Table 1-1. Responses of the a collection of barley pan-genome accessions to exposure to JSBWMV and BaYMV.

Barley cultivars	Species	JSBWMV[a]		BaYMV[a]	
		Root	Leaf (Leaf blade)	Root	Leaf (Leaf blade)
Akashinriki	*Hordeum vulgare*	+	-	-	-
HOR10350	*Hordeum vulgare*	+	-	-	-
Morex	*Hordeum vulgare*	+	+	-	-
HOR13942	*Hordeum vulgare*	+	-	+	-
HOR13821	*Hordeum vulgare*	+	-	+	+
HOR8148	*Hordeum vulgare*	+	-	+	+
HOR3365	*Hordeum vulgare*	+	-	+	+
HOR9043	*Hordeum vulgare*	+	-	+	+
HOR3081	*Hordeum vulgare*	+	-	+	+
Igri	*Hordeum vulgare*	+	-	+	+
Golden Promise	*Hordeum vulgare*	+	-	+	+
HOR7552	*Hordeum vulgare*	+	+	+	+
HOR21599	*Hordeum vulgare*	+	+	+	+
Barke	*Hordeum vulgare*	+	-	+	+
OUN333	*Hordeum vulgare*	+	-	+	+
Sukai Golden	*Hordeum vulgare*	-	-	-	-
Kashimamugi	*Hordeum vulgare*	+	+	+	+

[a]Log10 transformed virus titres < 3 served as -, otherwise served as +.

Table 1-2. Responses of the a collection of wheat pan-genome accessions to exposure to JSBWMV.

Wheat cultivars	Species	JSBWMV[a]		BaYMV[a]	
		Root	Leaf (Leaf blade)	Root	Leaf (Leaf blade)
Cadenza	*Triticum aestivum*	-	-	0	0
CDC Stanley	*Triticum aestivum*	-	-	0	0
Claire	*Triticum aestivum*	-	-	0	0
Paragon	*Triticum aestivum*	-	-	0	0
Weebill	*Triticum aestivum*	-	-	0	0
Julius	*Triticum aestivum*	-	-	0	0
SY Mattis	*Triticum aestivum*	-	-	0	0
Jagger	*Triticum aestivum*	-	-	0	0
Robigus	*Triticum aestivum*	-	-	0	0
Arina*LrFor*	*Triticum aestivum*	-	-	0	0
Lancer	*Triticum aestivum*	-	-	0	0
CDC Landmark	*Triticum aestivum*	-	-	0	0
Kronos	*Triticum aestivum*	-	-	0	0
Mace	*Triticum aestivum*	+	-	0	0
Norin 61	*Triticum aestivum*	+	+	0	0

[a]Log10 transformed virus titres < 3 served as -, otherwise served as +. 0, BaYMV was undetectable in Wheat.

Table 1-3. Sampling dates and their equivalent Zadok's scale growth stages.

Sampling time	Sampling date	Weeks after sowing	Growth Stage	Zadok's Code[a]
1st	2022/11/30	4	Main shoot only	GS20
2nd	2022/12/13	6	Main shoot and 1 tiller	GS21
3rd	2022/12/27	8	Main shoot and 3 tillers	GS23
4th	2023/01/11	10	Main shoot and 5 tillers	GS25
5th	2023/01/25	12	Main shoot and 9 or more tillers	GS29
6th	2023/02/08	14	First node detectable	GS31
7th	2023/02/22	16	Second node detectable	GS32
8th	2023/03/08	18	Third node detectable	GS33
9th	2023/03/22	20	First spikelet of ear just visible above flag leaf ligue	GS51
10th	2023/04/05	22	Gtain watery ripe	GS71
11th	2023/04/18	24	Medium milk	GS75

[a]Zadoks, J., Chang, T., & Konzak, C. 1974, A decimal code for the growth stages of cereals. Weed research, 14(6), 415-421

Table 1-4. Presence/absence variation analysis of known resistance genes to BaYMV among the 15 barley pan-genome accessions.

Genotype	*HvEIF4E* haplotypes	*HvPDIL5-1* haplotypes
Tochinoibuki	n.d.	n.d.
Sukai Golden	*rym5*	WT
HOR21599	n.d.	WT
HOR7552	WT	WT
Morex	*rym6*	WT
Akashinriki	WT	WT
Barke	WT	WT
Golden Promise	*rym6*	WT
HOR10350	WT	WT
HOR13821	WT	WT
HOR13942	WT	WT
HOR3081	WT	WT
HOR3365	WT	WT
HOR8148	WT	WT
HOR9043	*rym6*	WT
Igri	WT	WT
OUN333	WT	WT
B1K_04	WT	WT
Hockett	n.d.	WT
ZDM 01467	n.d.	WT
ZDM 02064	*rym6*	WT
RGT Planet	*rym6*	WT

n.d.- not determined.

Table 1-5. Primers and probes used in this study.

Primers	Sequences 5'-3'
JSBWMV coat protein for in-situ hybridization	
JSBWMVfwG	GCTGTGAAAAATGGTTACACG
T7pro-JSBWMVfwG	CTAATACGACTCACTATAGGGAGAGCTGTGAAAAATGGTTACACG
JSBWMVrvG	TTGGAGTGCTACCGTGAGTCT
T7pro-JSBWMVrvG	CTAATACGACTCACTATAGGGAGATTGGAGTGCTACCGTGAGTCT
BaYMV coat protein for in-situ hybridization	
BaYMVfwG	GCAAGTTAACGCTGGTTTGA
T7pro-BaYMVfwG	CGCGCGTAATACGACTCACTATAGGGGCAAGTTAACGCTGGTTTGA
BaYMVrvG	CATCACTGTAGTTGCGCATGA
T7pro-BaYMVrvG	CGCGCGTAATACGACTCACTATAGGGCATCACTGTAGTTGCGCATGA
JSBWMV coat protein for RT-qPCR[a]	
SBWMV_CP_Fw	GGTGGTGAAGCAGTTATG
SBWMV_CP_Rv	CAACGTCTGATCTGTCTG
SBWMV_CP_Probe	(FAM)ACTCACGGTAGCACTCCAATCC(BHQ1)
BaYMV coat protein for RT-qPCR[b]	
BaYMVFow	GCTGAGAATCAAGTAATG
BaYMVRevG	CACTGTAGTTGCGCATGA
BaYMVProbe	FAM-CCTTCGGAGTCCACCATTCATTCG-BHQ1
Barley actin for RT-qPCR[c]	
HvActinFow	GTACCTTCCAACAGATGTG
HvActinRev	CAGACAACTCGCAACTTA
HvActinProbe	Cy5-TCGCTGGACCTGACTCATCGTA-BHQ1

[a]Okada, K., Xu, W., Mishina, K., et al., 2023 Genetic resistance in barley against Japanese soil-borne wheat mosaic virus functions in the roots, Frontiers in Plant Science, 14, 1149752.

[b,c]Mishina, K., Kai, H., Hamada, M. et al., 2024 Series of resistance genes in barley (Hordeum vulgare L.) that control Barley yellow mosaic virus multiplication and the root-to-leaf systemic movement, Plant Disease 2024 Jan 3. doi: 10.1094/PDIS-07-23-1451-RE.

Chapter 2. Identification of barley yellow mosaic virus isolates breaking *rym3* resistance in Japan

Introduction

Barley yellow mosaic virus (BaYMV) is transmitted through soil-borne protists of the plasmodiophorid *Polymyxa graminis* (Kanyuka et al., 2003). BaYMV is a major pathogen causing up to 50% yield losses in susceptible barley fields worldwide; the disease is particularly severe in East Asia and Europe (Friedt *et al.*, 1987, Kobayashi *et al.*, 1987, Seko, 1987, Yamaguchi *et al.*, 2002, Kihara, 2011, Friedt and Ordon, 2013). Various strains of BaYMV have been isolated globally, indicating its widespread distribution and geographical genetic diversity. Barley breeding programs have incorporated different resistance genes to combat BaYMV infection, driven by the varying pathogenicity of virus strains and the presence of other infectious viruses (Jiang *et al.*, 2020).

BaYMV is a member of the *Bymovirus* genus within the *Potyviridae* family. It exhibits distinctive filamentous particles with a diameter of about 13 nm and modal lengths of 275 and 550 nm, observed in leaf dips and partially purified virus preparations (Inouye, 1964, Inouye, 1968). Its genome consists of two single-stranded, positive-sense RNA molecules: RNA1 (~7.6 kb) and RNA2 (~3.5 kb), which encode essential viral proteins crucial for replication, transcription, and pathogenicity (Adams *et al.*, 2012). *Potyviridae* polyproteins show a common core led by diversified leaders that are enriched in non-core modules, which expand the proteome structural and functional heterogeneity. The layout of BaYMV is leader-less RNA1 encoding the potyvirid polyprotein core and additional RNA2 (Pasin *et al.*, 2022). RNA1, devoid of a leader sequence, encodes eight mature proteins, including P3, 6K1, CI, 6K2, NIa-VPg, NIa-pro, NIb, and CP (Inoue-Nagata *et al.*, 2022). The potyviral cylindrical inclusion (CI) protein with helicase activity for virus replication interacts directly with plasmodesmata, capsid protein-containing ribonucleoprotein complexes (Carrington *et al.*, 1998, Urcuqui-Inchima *et al.*, 2001, Sochor *et al.*, 2012), and potyviral 6K1 protein (Hong *et al.*, 2007) is suggested to facilitate cell-to-cell movement. The Pipo gene embedded within the P3 gene is also predicted to be in the genus *Bymovirus* and translated as a fusion protein P3N-PIPO by +2 frameshifting, facilitating cell-to-cell movement (Chung *et al.*, 2008, Wei *et al.*, 2010, Wen and Hajimorad, 2010,

Vijayapalani *et al.*, 2012). Potyviral VPg enhances viral RNA translation and inhibits reporter mRNA translation in planta (Eskelin *et al.*, 2011). *Bymovirus* VPg is also known to interact with the *eukaryotic translation initiation factor 4E* (*eIF4E*) through the VPg central domain during virus infection in plants (Kanyuka *et al.*, 2005, Stein *et al.*, 2005, Robaglia and Caranta, 2006, Roudet-Tavert *et al.*, 2007). Potyvirus CP also plays a role in cell-to-cell movement, long-distance movement, and aphid transmission (Urcuqui-Inchima *et al.*, 2001, Sochor *et al.*, 2012). RNA2 encodes P1 and P2 proteins, with P1 closely related to HC-pro and P2 unique among potyvirid proteins. These intricate interactions underscore the multifaceted mechanisms of BaYMV's pathogenesis and adaptation to host plants (Adams *et al.*, 2005, You and Shirako, 2010).

BaYMV strains in Japan have evolved into distinct pathotypes, categorized as isolates I to V based on the susceptibility of barley varieties (Kashiwazaki *et al.*, 1989a, Nishigawa *et al.*, 2008, Sotome *et al.*, 2010). The most commonly found isolate is BaYMV-I (pathotype I) (RNA1: AB430765, RNA2: AB430766), while isolates BaYMV-II-1 (pathotype II) (RNA1: D01091), BaYMV-III (pathotype III) (RNA1: AB430767, RNA2: AB430768), BaYMV-IV (pathotype IV) (RNA1: AB430769, RNA2: AB430770), and BaYMV-V (pathotype V) (RNA1: AB450476, RNA2: AB450477) were well classified mostly in Tochigi prefecture, Japan (Kashiwazaki *et al.*, 1989a, Iida *et al.*, 1992, Nishigawa *et al.*, 2008, Tanokami *et al.*, 2021). Tochigi prefecture is a prominent region for two-rowed barley production in Japan, and BaYMV causes particularly serious damage to it. The virus can remain infectious for decades within the thick-walled spores produced by *P. graminis*, making it difficult to eliminate them from infected fields (Vaïanopoulos *et al.*, 2007, Kuhne, 2009, Jiang *et al.*, 2020). Therefore, planting BaYMV-resistant varieties is the only reliable control of this disease (Ordon *et al.*, 2009). For BaYMV-resistant barley varieties, initial efforts were focused on utilizing the varieties carrying the *rym1* and *rym5* genes from cv. Mokusekko 3; after that, many resistant varieties carrying *rym5* and/or other resistance genes were bred (Konishi *et al.*, 1997). However, pathotype III damaged resistant barley varieties with *rym5* (Sotome *et al.*, 2010). A new cultivar, New Sachiho Golden, is developed from the high-yield and high-quality barley cv. "Sachiho Golden", carrying *rym3* (Oozeki *et al.*, 2017, Taketa *et al.*, 2023). Increasing evidence has demonstrated that barley yellow mosaic disease is prevalent in the field at Ohtawara, Tochigi prefecture, Japan, due to the susceptibility of pathotype IV to *rym3*, while it is absent in other regions (Sotome *et al.*, 2010). Subsequently, barley plants showing symptoms similar to barley yellow mosaic disease were also observed in a farmer's field in Takanezawa, Tochigi

prefecture, Japan. Meanwhile, the presence of BaYMV was confirmed by Western blotting analysis of collected barley plants (Sotome *et al.*, 2010). However, to date, the virus genome in Takanezawa and the amino acid substitutions compared with the other types have not yet been studied.

To effectively manage BaYMV disease, it is crucial to conduct research on both the virus's pathogenicity and the host's resistance. Current breeding efforts to develop BaYMV resistance only evaluate the plant's resistance against each BaYMV strain. This is without taking into consideration the research on virus pathogenicity, such as how BaYMV triggers infection. This study aimed to investigate the genetic factors contributing to BaYMV isolates responsible for *rym3* resistance breaking.

Materials and Methods

Plant materials and growth conditions

The malting barley cv. New Sachiho Golden (*Hordeum vulgare* L.), which carries *rym3* (originally inherited from cv. Haganemugi), was developed at the Tochigi Prefecture Agricultural Experiment Station (Oozeki *et al.*, 2017). The Chinese landrace barley cv. Mokusekko 3, which carries *rym1* and *rym5*, is completely resistant to all BaYMV pathotypes (Konishi *et al.*, 1997). All plants were sown in a field infected with BaYMV in Takanezawa, Tochigi Prefecture, Japan, at the end of October 2019. In mid-February 2020, five individual plants were dug up at approximately 9:00 a.m., frozen in liquid nitrogen, and stored at −80 °C until subsequent analyses.

Phenotypic observation

Photos of the field plant were taken using a Canon X7 camera (Canon, Tokyo, Japan). The BaYMV-induced mosaic symptom was observed using an Axio Zoom V16 microscope (Zeiss, Oberkochen, Germany) (Mishina *et al.*, 2023).

RNA extraction and sequencing

Total RNA was extracted from infected plant root tissues using the RNeasy Mini kit (QIAGEN, Hilden, Germany) and treated with the RNase-Free DNase Set (QIAGEN, Hilden, Germany). The concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Five RNA-seq libraries (T01-T05) were constructed from individual plants using the TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA). The libraries were sequenced on the Illumina HiSeq X sequencing platform (Illumina, San Diego, CA, USA), generating 150 bp paired-end reads (Chen *et al.*, 2023a).

Analysis of RNA-seq data

Trimmomatic was used to remove adapters and low-quality bases (Li *et al.*, 2016). The clean reads were aligned to the Morex genome assembly v3 (Mascher *et al.*, 2021) using HISAT (Kim *et al.*, 2015). To obtain unmapped reads, the samtools view ("–F4" option) and the samtools bam2fq

program were used. Unmapped reads were aligned to BaYMV reference sequences, which are BaYMV of Japanese pathotype I RNA1 (AB430765) and RNA2 (AB430766) using bowtie2 (Langmead and Salzberg, 2012). Fragments aligned to those references were counted using the Linux command pipeline command, followed by the samtools view. Variant sites were identified using BCFtools (Danecek *et al.*, 2021). SNP sites were applied if the allele frequency was ≥0.6, and the allele frequencies between >0.4 and <0.6 were called degenerate bases. The abundance of barley mild mosaic virus, wheat yellow mosaic virus, and wheat spindle streak mosaic virus were searched by BLAST of the assembled data in the five root samples in cvs. New Sachiho Golden and Mokusekko 3. The generated RNA-seq data were deposited in the DDBJ BioProject database (accession ID: PRJDB18040).

Phylogenetic analysis of BaYMV strains

The BaYMV-I RNA1 (AB430765) and RNA2 (AB430766) nucleotide sequences were used as queries for a BlastN search against previously documented coding sequences (RNA1: Tochigi II-1 D01091, Tochigi III AB430767, Ohtawara IV AB430769, Dazhong CN DZ MW295871, Nanyang CN NY MW295868, Yancheng AJ132268, Yamaguchi V AB450476, Aschersleben ASL1 AJ515484, Germany G X69757, RNA2: Tochigi III AB430768, Ohtawara IV AB430770, Yancheng AJ132269, Yamaguchi V AB450477, Dazhong CN DZ MW295876, Nanyang CN NY MW295873, Germany D01099, Germany HYT-38 MN107380). The BaYMV-I RNA1 (BAG70349) and RNA2 (BAG70350) amino acid sequences were used as queries for a BlastX search against previously documented coding sequences (RNA1: Tochigi II-1 BAA00875, Tochigi III BAG70351, Ohtawara IV BAG70353, Yamaguchi V BBE49537, Dazhong CN DZ UWL85807, Nanyang CN NY UWL85804, Yancheng CAA10637, Germany G CAA49412, Aschersleben ASL1 CAD56476, RNA2: Tochigi III BAG70352, Ohtawara IV BAG70354, Yamaguchi V BBE49538, Yancheng CAA10638, Dazhong CN DZ UWL85812, NanYang CN NY UWL85809, Germany BAA00884, Germany HYT-38 QHA94748). The database codes of all nucleotide sequences and all amino acid sequences used in this study were deposited in GeneBank. The e-value threshold applied was 1×10^{-50}, and the identity threshold was 90%. Query coverage of more than 90% was utilized to remove partial hits. Nucleotide sequences were aligned using the Clustal Omega algorithm (Sievers and Higgins, 2021).

Correlation analysis between BaYMV encoded protein residue and the virus infection to *rym3*-carrying barley cv. New Sachiho Golden

To analyze the responses of *rym3*-carrying barley plants against BaYMV, they were scored "−1" for resistant and "1" for susceptible (You and Shirako, 2013). The difference in the amino acid residue was scored "−1" for identical and "1" for different from the consensus sequences of BaYMV-Takanezawa-T01 DRR552862. The coefficient of determination (R^2) is calculated between the resistance response score and the amino acid residue score. The *p*-value threshold of 0.01 is calculated from the t-score, t = r√(n − 2)/√(1 − r2), and Excel function "tdist". For the correlation analysis, the scores (0/1 correspondence with BaYMV-Takanezawa-T01 DRR552862) of all amino acids (inferred from the RNA sequences) of different BaYMV isolates (Ohtawara IV BAG70353, Yamaguchi V BBE49537, Tochigi II-1 BAA00875, Tochigi III BAG70351, Tochigi I BAG70349, Germany G CAA49412) were compared with their capability to cause visible symptoms on the plants of barley cv. New Sachiho Golden in the Takanezawa field trial.

Genome-wide association analysis

The R package rrBLUP (Endelman, 2011) is used to verify correlation analysis results. The Q (population structure) + K (relative kinship) model was used to calculate the GRM (genetic relationship matrix). Minor allele frequency is set at 0.05. The FDR threshold of 0.05 was used to determine the significant peaks.

Results

Identification of barley yellow mosaic virus isolates on breaking *rym3* varieties

In the autumn of 2019, cv. New Sachiho Golden was sown in the primary malting barley production fields located in Takanezawa, Tochigi prefecture, Japan (the field is hereinafter called Takanezawa). Barley yellow mosaic virus (BaYMV) isolates were identified on the *rym3*-carrying variety barley plant, cv. New Sachiho Golden. The leaves exhibited severe yellow mosaic symptoms at the end of February (Figure 2-1).

Root samples randomly selected from five individual plants grown at various parts of the same field were collected and subjected to RNA sequencing. Raw reads from 2.4 to 3.4 Gbases were obtained. From the clean reads, 81.8–87.3% were mapped to the Morex V3 genome, and the BaYMV consensus sequences were matched to Tochigi I (RNA1, AB430765; RNA2, AB430766) (Mascher, 2021). Quantities of BaYMV ranged from 3.8 to 6.6% in total. Subsequently, complete genome consensus sequences were obtained for RNA1 and RNA2 of the five BaYMV isolates, BaYMV-Takanezawa-T01 to BaYMV-Takanezawa-T05 (Table 2-1). The viral polyprotein coding sequences are consistent across all isolates and consist of the following: 7642 bp of RNA1 nucleotide sequences, which translate to 2412 aa protein; 3585 bp of RNA2 nucleotide sequences, which translate to 890 aa protein. BaYMV remained at almost zero in cv. Mokusekko 3, as a negative control. In the test of cvs. New Sachiho Golden and Mokusekko 3, several soil-borne viruses belonging to the genus *Bymovirus* (*Potyviridae*)—barley mild mosaic virus, wheat yellow mosaic virus, and wheat spindle streak mosaic virus—were not present in these samples (Table 2-1).

The existence of two distinct BaYMV isolates in Takanezawa

The nucleotide identity among the various sequences of East Asian and European isolates ranged from 0 to 514 mismatches (93.27%) for RNA1 and up to 344 mismatches (90.40%) for RNA2 (Table 2-2). Amino acid identities varied from 95.69% (104 aa) to 100% for RNA1 and 96.85% (28 aa) to 100% for RNA2 (Table 2-3).

The RNA1 of BaYMV isolates detected in Takanezawa were genetically distinct from isolates reported from China and Europe. All BaYMV-Takanezawa-T01 RNA1 to BaYMV-Takanezawa-T05 RNA1 were nearly identical. There were 40 out of 7642 sites of nucleotide variation (99.48%

identity) for nucleotide sequences and 3 out of 2412 sites of variation (99.88% identity) for amino acid sequences compared with closely Japanese isolate Ohtawara IV RNA1 (AB430769) (Figure 2-2, Table 2-2 and 2-3).

All five nucleotide sequences of RNA2 were highly similar to Tochigi I (BAG70350) with over 99% identity. Regarding the amino acid sequences, BaYMV-Takanezawa-T02 (DRR552863) and BaYMV-Takanezawa-T03 (DRR552864) were closely related to Ohtawara IV with 99.78% identity. In comparison, BaYMV-Takanezawa-T01 (DRR552862) and BaYMV-Takanezawa-T04 (DRR552865) were more similar to Tochigi III (BAG70352) with 99.44% identity, suggesting that two isolates of BaYMV RNA2 were identified (Figure 2-2, Table 2-3). Between BaYMV-Takanezawa-T01 (DRR552862) and BaYMV-Takanezawa-T02 (DRR552863), 22 out of 3585 sites of nucleotide variation (99.39% identity) (Table 2-2) and 5 out of 890 sites of amino acid variation (99.44% identity) were identified (Table 2-3).

Variable sites of virus sequence in amino acid

Variable sites within the NIa-VPg, NIa-pro, and CP proteins exhibited diversification between the consensus sequence of BaYMV-Takanezawa-T01 DRR552862 and Ohtawara IV (BAG70353) (Figure 2-3 and Figure 2-4, Table 2-4). The most variable sites were identified in the NIa-VPg (amino acid variation of 19.79%, 37 of 187 aa) and N-terminal region of CP (amino acid variation of 7.69%, 23 of 299 aa) proteins among the East Asian and European isolates (Figure 2-3). These sites were related to the co-evaluation between resistance genes and the virus genome. Otherwise, permissive sites may not affect the virus's pathogenicity.

Amino acid changes in NIa-VPg correlated with pathogenicity

To determine whether the amino acid changes in the newly discovered BaYMV genome are associated with breaking *rym3* resistance, the correlation coefficient between the amino acid variations was computed in RNA1 (representative BaYMV-Takanezawa-T01 DRR552862 and BaYMV-Takanezawa-T02 DRR552863) of BaYMV and the responses observed in the Takanezawa field (Figure 2-5 and Table 2-4). The VPg and the CP proteins were the most variable regions, with correlation coefficients (R^2) greater than 0.5 and *p*-values smaller than 0.01. The changes in the CI (RNA1 amino acid residue 459) and CP (RNA1 amino acid residue 2138) proteins correlated with pathogenicity (Figure 2-5 and Table 2-5). Furthermore, one significant amino acid sequence difference was observed between polyproteins encoded by RNA2. This

difference was located at amino acid residue 359, within the P2 protein. The isolates BaYMV-Takanezawa-T01 (DRR552862), Ohtawara IV (BAG70354), and Yamaguchi V (BBE49538) encoded Threonine at amino acid 359, while BaYMV-Takanezawa-T02 (DRR552863), Tochigi I (BAG70350), Tochigi III (BAG70352), and the other three Chinese (BaYMV-CN DZ UWL85812, CN NY UWL85809, BaYMV-Yancheng CAA10638) and two German (BaYMV-Germany, BAA00884, Germany HYT-38 QHA94748) isolates encoded Alanine at this position (Figure 2-5 and Table 2-4).

Discussion

rym3 resistance breaking in BaYMV-infected field

The resistance gene of *rym3* is commonly used in Japanese cultivars (Konishi *et al.*, 1997). However, cultivating genotypes carrying a single resistance gene is in danger of breaking resistance under selection pressure. The *rym3* resistance has been broken in the Ohtawara and Yamaguchi fields (Sotome *et al.*, 2010). This study provides a comprehensive understanding of barley plants' responses to BaYMV through the RNA-Seq technique. The RNA1 sequence of the Takanezawa field is similar to that of the Ohtawara isolate. These two locations are 33 km apart but share the same RNA1 origin and are likely to be prevalent in the northern part of Tochigi prefecture. The RNA1 sequence of Yamaguchi is in a different clade than the BaYMV-Takanezawa isolates despite the two locations being 850 km apart. It is possible that the two breaking events of *rym3* resistance in Ohtawara and Yamaguchi are independent and do not share the same mutation sites in the virus genomes. It suggests that our approach was deemed useful because it was assumed that all *rym3*-breaking events corresponded to the same mutation. The *rym4* resistance-breaking strain was identified among the German isolates, and its VPg mutation was determined, but Japanese strains that break *rym4* resistance have not been identified (Li *et al.*, 2016). An association analysis of virus variants was proposed to identify the corresponding mutations responsible for pathogenicity changes. Our preliminary small-scale genome-wide association study analysis indicates the same site of amino acid variation in the polyprotein, suggesting that our proposal was supported (Figure 2-5 and Figure 2-6). To date, many sequences have been registered, but their pathotype response is still largely unknown. Additionally, responses of resistant genotypes in the BaYMV-infected field need further study.

Co-existence isolates of BaYMV identified in Takanezawa

Our data revealed that at least two viruses with different genetic backgrounds of breaking resistance exist in the BaYMV-Takanezawa isolates. In virus sequence assembly, single colony isolation or RNA transcript assembly methods are typically utilized, particularly for viruses with unique sequences. Here shows that, in the Takanezawa field, the analysis of RNA2 revealed the presence of a mixture of two distinct BaYMV isolates across the five biological replicates (Tables 2-2 and 2-3, and Figure 2-4), making it challenging to use RNA transcript assembly without producing chimeric outputs. To overcome this challenge, a consensus call was used along a

reference genome. However, T05 (DRR552866) failed to reach a consensus call due to the presence of many SNPs with even allele frequencies (40–60%), resulting in degenerate bases (Arai *et al.*, 2018). It is worth noting that different isolates can intermix and result in the misdirection of pathotypes if treated as a single strain in the multiple pathotype field.

The determinant protein responsible for breaking *rym3* resistance

In this study, the correlation between amino acid substitutions and disease responses was examined. Out of 2412 amino acid residues in RNA1, 25 aa residues were candidates responsible for breaking *rym3* resistance (Table 2-4). Out of 890 amino acid residues in RNA2, only 1 was a candidate (Table 2-5). If multiple mutation events of breaking *rym3* happen, an R^2 not equal to 1 can be assumed. Our analysis listed two amino acid residues in RNA1 with $R^2 = 1$, residue 459 in CI protein, and residue 2138 in CP (Table 2-4). CI is involved in resistance to *Wheat yellow mosaic virus*, genus *Bymovirus*, in the family *Potyviridae* (Ohki et al., 2019). CI protein of *Soybean mosaic virus*, genus *Potyvirus*, in the family *Potyviridae*, is a pathogenic determinant provoking a lethal systemic hypersensitive response, which the function varied with its single amino acid substitution (Seo *et al.*, 2009, Zhang *et al.*, 2009). VPg often plays a crucial role in interacting with recessive resistance genes, while CP interacts with dominant resistance genes (Hull, 2014b). Previous studies have suggested that VPg is identified as a pivotal protein in overcoming *eIF4E*-mediated recessive resistance genes like *rym4/5/6* (Charron et al., 2008, Jiang and Laliberte, 2011, Li and Shirako, 2015, Li et al., 2016).

Our analysis listed one amino acid residue in RNA2 with an $R^2 = 0.56$, residue 359 in the TMV-like coat protein (TMV-like CP) domain of the P2 protein, which is conserved in all full-length *Bymovirus* accessions except *Oat mosaic virus* (Pasin et al., 2022) (Table 2-5). TMV-like CP sequences of bymoviruses cluster within a monophyletic clade, suggesting their common origin. The CP of family *Virgaviridae* and *Benyviridae* members shows homology with *Bymovirus* P2 (Arai *et al.*, 2018, Pasin *et al.*, 2022). A recent study reveals that a single amino acid substitution mutation of WYMV P2 affects the function of its viral suppressor of RNA silencing activity and its own protein stability (Chen *et al.*, 2023b); however, P2 protein biochemical properties and function are still largely unknown. More sequences and *rym3* responses were required to perform a correlation analysis. Although many isolates are currently registered, the response to different pathotypes remains to be further studied. It would also be useful to test resistant genotypes in fields

infected by BaYMV. Any potential mutations should be verified through artificial inoculation (Tanokami *et al.*, 2021).

Figures and tables

Figure 2-1. The disease symptoms of *Barley yellow mosaic virus* (BaYMV) on the cv. New Sachiho Golden in the Takanezawa field in late March 2020. (**A**) The overall appearance of the affected plants in the field. (**B**) Leaf mosaic symptoms of plants. (**C**) Observation of the second leaf from the upper new leaf.

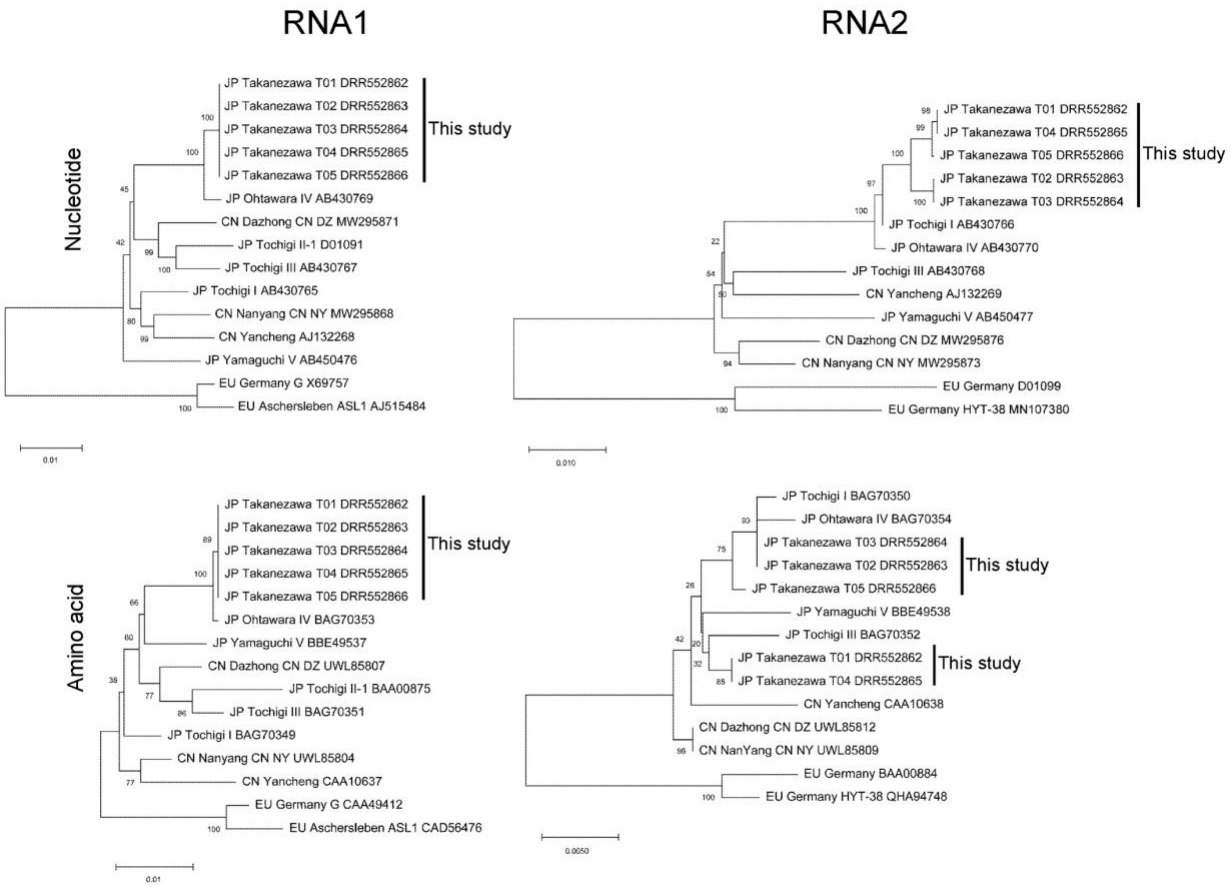


Figure 2-2. Phylogenetic tree using nucleotide and amino acid sequences of RNA1 and RNA2. A neighbor-joining phylogenetic tree was produced by the MEGA 11 program based on a ClustalW alignment of the nucleotide and amino acid sequences. The robustness of internal branches was tested using bootstrap analyses (1000 replicates).

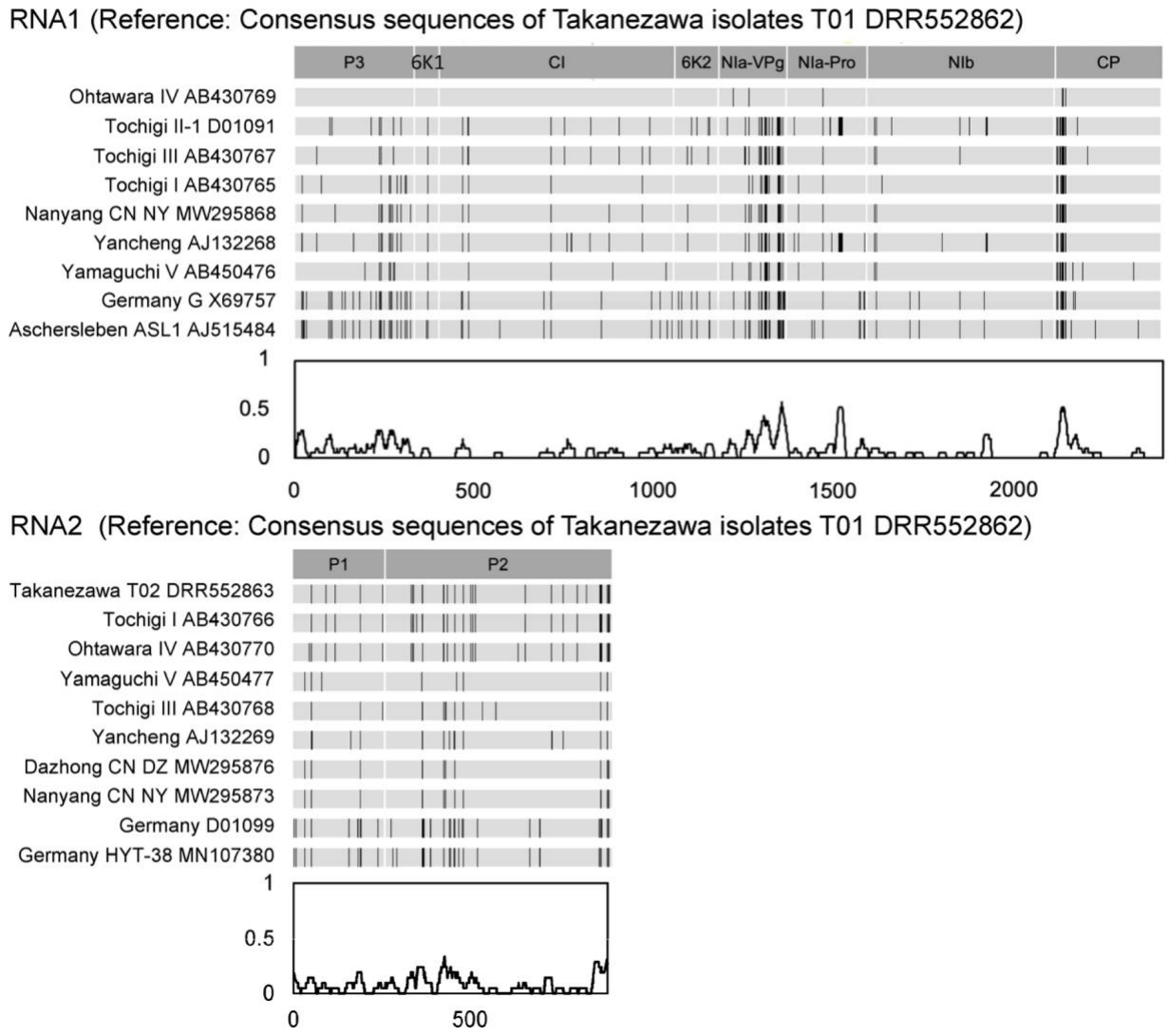


Figure 2-3. The sites of amino acid variation in the polyproteins from Barley yellow mosaic virus (BaYMV) isolates. The consensus sequences of BaYMV-Takanezawa-T01 DRR552862, RNA1, and RNA2 were used as references. Each black-colored vertical bar represents amino acid substitutions compared to the consensus sequence. Line plots at the bottom show sliding window frequency summaries in 20 aa on average.

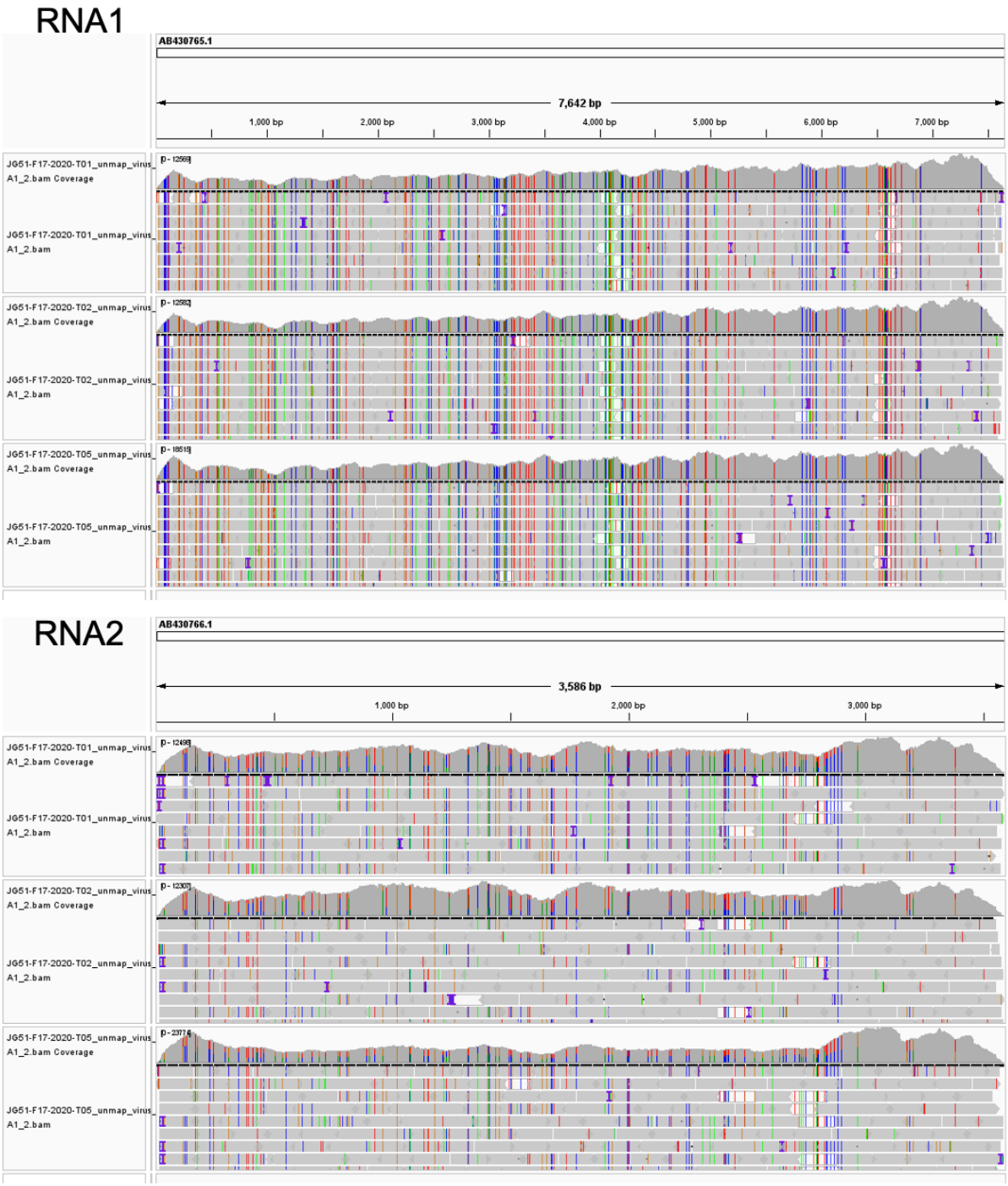


Figure 2-4. Visualization with Integrative Genomics Viewer (IGV) of the reads aligned with the barley yellow mosaic virus (BaYMV) strain BaYMV-I reference genome (AB430765.1)

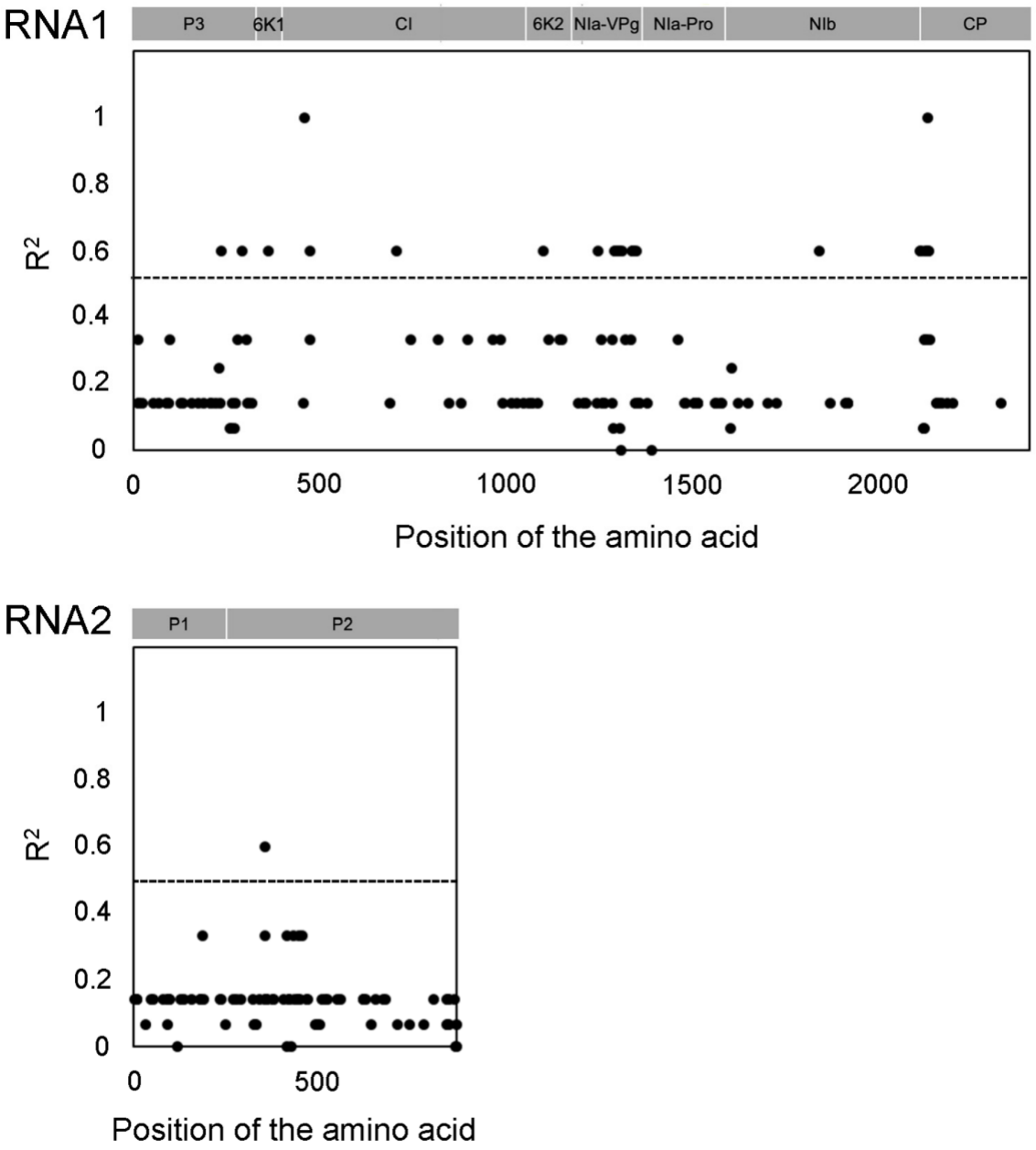


Figure 2-5. The correlation coefficient plot between the phenotypes and the sites of amino acid variations in the polyproteins. The sequences used for this analysis are summarized in Table 2-4. For each position of the amino acid within the protein (horizontal axis), the positions without any dots are excluded due to no correlations. The *y*-axis represents the coefficient of determination (R^2) between amino acid variations and phenotypes for every site. The horizontal gray lines represent the significance thresholds for amino acid variations ($p = 0.01$ and $R^2 = 0.5$).

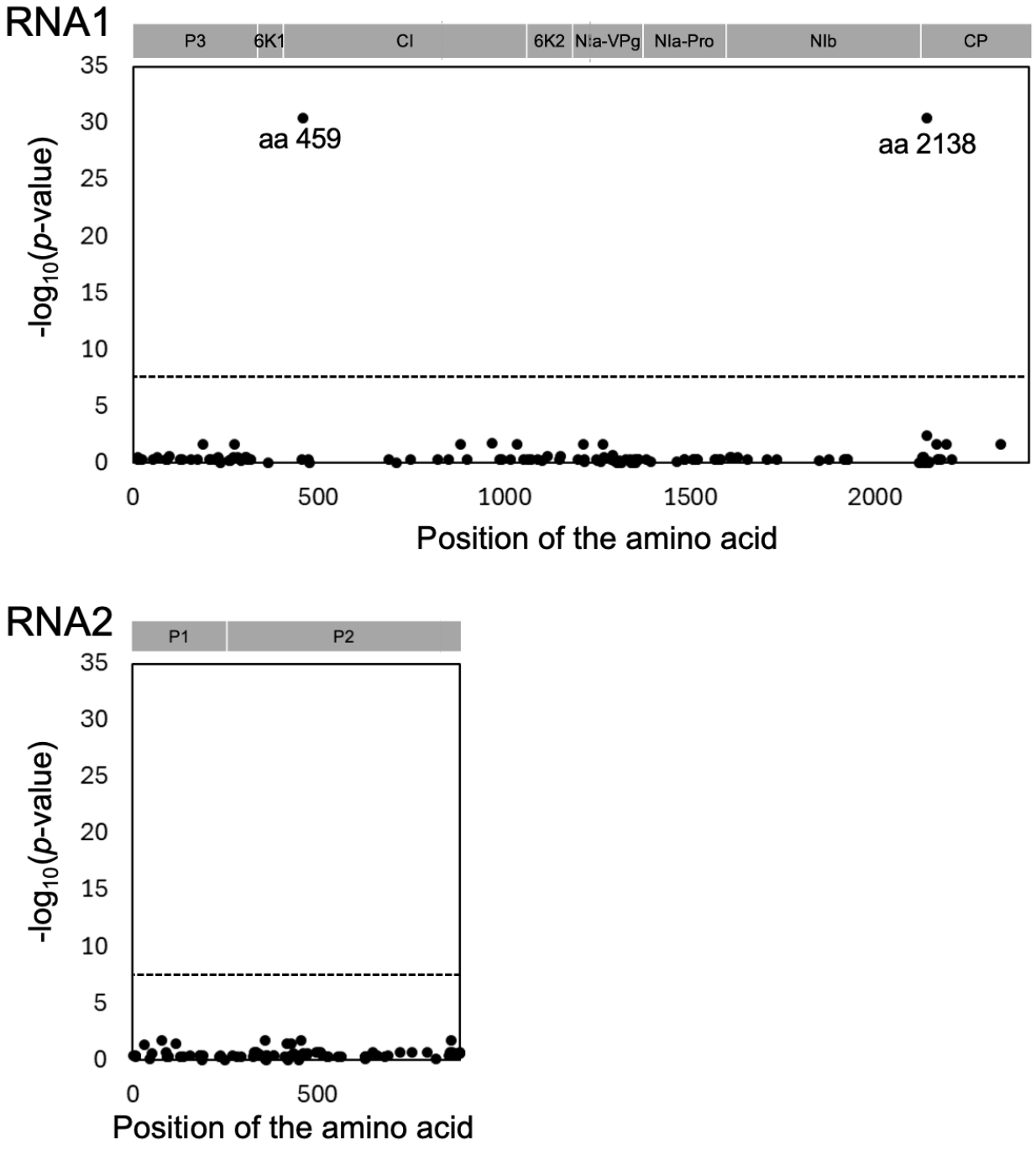


Figure 2-6. Small-scale genome-wide association studies analysis reveals the sites of amino acid variations in the polyproteins.

Table 2-1. Summary of the RNA-seq data and mapping results in Takanezawa.

												Barley (GCA_904849725)		BaYMV RNA1 (AB430765)		
Id	Barley cultivar	Biological Replicate	Run	Raw R1 reads	Raw R1 bases	Raw R2 reads	Raw R2 bases	Clean R1 reads	Clean R1 bases	Clean R2 reads	Clean R2 bases	Mapped fragments to Morex V3*	Mapping rates to Morex V3	Mapped fragments to BaYMV_RNA	Mapping rates to BaYMV_RNA1	BaYMV_RNA 1_FPKM
T01	New Sachiho Golden	1	DRR552862	8,514,039	1,285,619,889	8,514,039	1,285,619,889	5,148,243	712,036,892	5,148,243	655,083,000	4,442,595	86.3%	166,038	3.2%	4220.3
T02	New Sachiho Golden	2	DRR552863	11,023,709	1,664,580,059	11,023,709	1,664,580,059	6,163,266	851,088,163	6,163,266	790,590,692	5,351,688	86.8%	170,519	2.8%	3620.4
T03	New Sachiho Golden	3	DRR552864	11,201,100	1,691,366,100	11,201,100	1,691,366,100	6,402,638	885,537,789	6,402,638	815,586,283	5,396,085	84.3%	184,611	2.9%	3773.0
T04	New Sachiho Golden	4	DRR552865	7,798,270	1,177,538,770	7,798,270	1,177,538,770	4,312,084	593,390,960	4,312,084	548,363,940	3,763,370	87.3%	125,525	2.9%	3809.2
T05	New Sachiho Golden	5	DRR552866	9,456,614	1,427,948,714	9,456,614	1,427,948,714	5,395,952	757,089,190	5,395,952	699,499,935	4,414,921	81.8%	269,932	5.0%	6546.0
M01	Mokusekko 3	1	DRR552867	3,456,738	521,967,438	3,456,738	521,967,438	2,453,680	335,574,843	2,453,680	308,887,046	2,337,805	93.9%	0	0.0%	0.0
M02	Mokusekko 3	2	DRR552868	1,855,612	280,197,412	1,855,612	280,197,412	1,328,266	182,059,854	1,328,266	168,652,593	1,247,994	95.8%	1	0.0%	0.1
M03	Mokusekko 3	3	DRR552869	4,151,350	626,853,850	4,151,350	626,853,850	2,949,475	402,377,197	2,949,475	372,615,931	2,811,595	87.6%	0	0.0%	0.0

*Mascher, M.; Wicker, T.; Jenkins, J.; Plott, C.; Lux, T.; Koh, C.S.; Ens, J.; Gundlach, H.; Boston, L.B.; Tulpova, Z.; et al. Long-read sequence assembly: a technical evaluation in barley. Plant Cell 2021, 33, 1888-1906, doi:10.1093/plcell/koab077.
Nishigawa, H., Hagiwara, T., Yumoto, M., Sotome, T., Kato, T., & Natsuaki, T. (2008). Molecular phylogenetic analysis of Barley yellow mosaic virus. Archives of virology, 153, 1783-1786.
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Hodge,B.A.; Stewart,L.R.; Paul,P.A. (Plant Pathology, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, USA). Prevalence and Sequence Diversity of Viruses Infecting Wheat in Ohio, 2019. Unpublished work.

Table 2-1. Summary of the RNA-seq data and mapping results in Takanezawa (continued).

BaYMV RNA2 (AB430766)			BaMMV RNA1 (D83408)			BaMMV RNA2 (D83409)			WYMV RNA1 (AB627808)		
Mapped fragments to BaYMV_RNA	Mapping rates to BaYMV_RNA	BaYMV_RNA 2_FPKM	Mapped clean fragments against BaMMV RNA1	Rates against BaMMV RNA1	FPKM of BaMMV RNA1	Mapped clean fragments against BaMMV RNA2	Rates against BaMMV RNA2	FPKM of BaMMV RNA2	Mapped clean fragments against WYMV RNA1	Rates against WYMV RNA1	FPKM of WYMV RNA1
106,513	1.3%	2707.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
111,213	1.0%	2361.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
103,257	0.9%	2110.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
83,733	1.1%	2541.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
148,588	1.6%	3603.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0	0.0%	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0	0.0%	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0	0.0%	0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected

Table 2-1. Summary of the RNA-seq data and mapping results in Takanezawa (continued).

WYMV RNA2 (AB627823)			WSSMV RNA1 (MN046367)			WSSMV RNA2 (MN046369)		
Mapped clean fragments against WYMV RNA2	Rates against WYMV RNA2	FPKM of WYMV RNA2	Mapped clean fragments against WSSMV RNA1	Rates against WSSMV RNA1	FPKM of WSSMV RNA1	Mapped clean fragments against WSSMV RNA2	Rates against WSSMV RNA2	FPKM of WSSMV RNA2
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2-2. Percentage identity/distance matrix of RNA1 and RNA2. Estimation of the divergence between nucleotide sequences of BaYMV is illustrated by distance matrix (upper block) percentage identity (lower block).

Nucleotide of RNA1[1]	Takanezawa T01 DRR552862	Takanezawa T02 DRR552863	Takanezawa T03 DRR552864	Takanezawa T04 DRR552865	Takanezawa T05 DRR552866	Ohtawara IV AB430769	Dazhong CN DZ MW295871	Tochigi II-1 D01091	Tochigi III AB430767	Tochigi I AB430765	Nanyang CN NY MW295868	Yancheng AJ132268	Yamaguchi V AB450476	Germany G X69757	Aschersleben ASL1 AJ515484
Takanezawa T01 DRR552862		0	0	0	0	40	203	219	200	174	205	208	204	493	514
Takanezawa T02 DRR552863	100.00%		0	0	0	40	203	219	200	174	205	208	204	493	514
Takanezawa T03 DRR552864	100.00%	100.00%		0	0	40	203	219	200	174	205	208	204	493	514
Takanezawa T04 DRR552865	100.00%	100.00%	100.00%		0	40	203	219	200	174	205	208	204	493	514
Takanezawa T05 DRR552866	100.00%	100.00%	100.00%	100.00%		40	203	219	200	174	205	208	204	493	514
Ohtawara IV AB430769	99.48%	99.48%	99.48%	99.48%	99.48%		205	223	207	178	205	215	204	485	506
Dazhong CN DZ MW295871	97.34%	97.34%	97.34%	97.34%	97.34%	97.32%		163	140	183	190	203	207	466	488
Tochigi II-1 D01091	97.13%	97.13%	97.13%	97.13%	97.13%	97.08%	97.87%		122	204	221	233	231	475	495
Tochigi III AB430767	97.38%	97.38%	97.38%	97.38%	97.38%	97.29%	98.17%	98.40%		184	202	214	216	489	510
Tochigi I AB430765	97.72%	97.72%	97.72%	97.72%	97.72%	97.67%	97.61%	97.33%	97.59%		137	148	182	447	470
Nanyang CN NY MW295868	97.32%	97.32%	97.32%	97.32%	97.32%	97.32%	97.51%	97.11%	97.36%	98.21%		140	209	463	484
Yancheng AJ132268	97.28%	97.28%	97.28%	97.28%	97.28%	97.19%	97.34%	96.95%	97.20%	98.06%	98.17%		216	457	478
Yamaguchi V AB450476	97.33%	97.33%	97.33%	97.33%	97.33%	97.33%	97.29%	96.98%	97.17%	97.62%	97.27%	97.17%		466	487
Germany G X69757	93.55%	93.55%	93.55%	93.55%	93.55%	93.65%	93.90%	93.78%	93.60%	94.15%	93.94%	94.02%	93.90%		64
Aschersleben ASL1 AJ515484	93.27%	93.27%	93.27%	93.27%	93.27%	93.38%	93.61%	93.52%	93.33%	93.85%	93.67%	93.75%	93.63%	99.16%	

Nucleotide of RNA2[1]	Takanezawa T01 DRR552862	Takanezawa T04 DRR552865	Takanezawa T05 DRR552866	Takanezawa T02 DRR552863	Takanezawa T03 DRR552864	Tochigi I AB430766	Ohtawara IV AB430770	Tochigi III AB430768	Yancheng AJ132269	Yamaguchi V AB450477	Dazhong CN DZ MW295876	Nanyang CN NY MW295873	Germany D01099	Germany HYT-38 MN107380
Takanezawa T01 DRR552862		0	3	22	22	23	31	143	147	155	138	130	344	323
Takanezawa T04 DRR552865	100.00%		3	22	22	23	31	143	147	155	138	130	344	323
Takanezawa T05 DRR552866	99.92%	99.92%		19	19	23	31	143	147	155	138	130	344	323
Takanezawa T02 DRR552863	99.39%	99.39%	99.47%		0	22	30	142	146	156	137	129	342	322
Takanezawa T03 DRR552864	99.39%	99.39%	99.47%	100.00%		22	30	142	146	156	137	129	342	322
Tochigi I AB430766	99.36%	99.36%	99.36%	99.39%	99.39%		8	120	124	134	115	107	323	301
Ohtawara IV AB430770	99.14%	99.14%	99.14%	99.16%	99.16%	99.78%		124	126	134	117	109	326	304
Tochigi III AB430768	96.01%	96.01%	96.01%	96.04%	96.04%	96.65%	96.54%		103	114	101	93	303	291
Yancheng AJ132269	95.90%	95.90%	95.90%	95.93%	95.93%	96.54%	96.48%	97.13%		128	114	104	312	300
Yamaguchi V AB450477	95.68%	95.68%	95.68%	95.65%	95.65%	96.26%	96.26%	96.82%	96.43%		119	104	322	293
Dazhong CN DZ MW295876	96.15%	96.15%	96.15%	96.18%	96.18%	96.79%	96.74%	97.18%	96.82%	96.68%		60	302	287
Nanyang CN NY MW295873	96.37%	96.37%	96.37%	96.40%	96.40%	97.01%	96.96%	97.41%	97.10%	97.10%	98.33%		288	262
Germany D01099	90.40%	90.40%	90.40%	90.46%	90.46%	90.99%	90.90%	91.55%	91.29%	91.02%	91.57%	91.96%		149
Germany HYT-38 MN107380	90.99%	90.99%	90.99%	91.02%	91.02%	91.60%	91.52%	91.88%	91.63%	91.82%	91.99%	92.69%	95.84%	

[1]Nishigawa, H., Hagiwara, T., Yumoto, M., Sotome, T., Kato, T., & Natsuaki, T. (2008). Molecular phylogenetic analysis of Barley yellow mosaic virus. Archives of virology, 153, 1783-1786.

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Table 2-3. Percentage identity/distance matrix of RNA1 and RNA2. Estimation of the divergence between amino acid sequences of BaYMV is illustrated by distance matrix (upper block) percentage identity.

Amino acid of RNA1[1]	Takanezawa T01 DRR552862	Takanezawa T02 DRR552863	Takanezawa T03 DRR552864	Takanezawa T04 DRR552865	Takanezawa T05 DRR552866	Ohtawara IV BAG70353	Dazhong CN DZ UWL85807	Tochigi II-1 BAA00875	Tochigi III BAG70351	Tochigi I BAG70349	Nanyang CN NY UWL85804	Yancheng CAA10637	Yamaguchi V BBE49537	Germany G CAA49412	Aschersleben ASL1 CAD56476
Takanezawa T01 DRR552862		0	0	0	0	3	41	43	69	48	40	47	67	80	90
Takanezawa T02 DRR552863	100.00%		0	0	0	3	41	43	69	48	40	47	67	80	90
Takanezawa T03 DRR552864	100.00%	100.00%		0	0	3	41	43	69	48	40	47	67	80	90
Takanezawa T04 DRR552865	100.00%	100.00%	100.00%		0	3	41	43	69	48	40	47	67	80	90
Takanezawa T05 DRR552866	100.00%	100.00%	100.00%	100.00%		3	41	43	69	48	40	47	67	80	90
Ohtawara IV BAG70353	99.88%	99.88%	99.88%	99.88%	99.88%		42	42	68	48	39	46	66	79	89
Dazhong CN DZ UWL85807	98.30%	98.30%	98.30%	98.30%	98.30%	98.26%		40	66	44	35	39	63	79	89
Tochigi II-1 BAA00875	98.22%	98.22%	98.22%	98.22%	98.22%	98.26%	98.34%		50	32	38	32	57	74	84
Tochigi III BAG70351	97.14%	97.14%	97.14%	97.14%	97.14%	97.18%	97.26%	97.93%		37	66	63	61	94	104
Tochigi I BAG70349	98.01%	98.01%	98.01%	98.01%	98.01%	98.01%	98.18%	98.67%	98.47%		46	43	67	83	93
Nanyang CN NY UWL85804	98.34%	98.34%	98.34%	98.34%	98.34%	98.38%	98.55%	98.42%	97.26%	98.09%		28	47	64	74
Yancheng CAA10637	98.05%	98.05%	98.05%	98.05%	98.05%	98.09%	98.38%	98.67%	97.39%	98.22%	98.84%		38	68	78
Yamaguchi V BBE49537	97.22%	97.22%	97.22%	97.22%	97.22%	97.26%	97.39%	97.64%	97.47%	97.22%	98.05%	98.42%		83	93
Germany G CAA49412	96.68%	96.68%	96.68%	96.68%	96.68%	96.72%	96.72%	96.93%	96.10%	96.56%	97.35%	97.18%	96.56%		24
Aschersleben ASL1 CAD56476	96.27%	96.27%	96.27%	96.27%	96.27%	96.31%	96.31%	96.52%	95.69%	96.14%	96.93%	96.77%	96.14%	99.00%	

Amino acid of RNA2[1]	Tochigi I BAG70350	Ohtawara IV BAG70354	Takanezawa T03 DRR552864	Takanezawa T02 DRR552863	Takanezawa T05 DRR552866	Yamaguchi V BBE49538.1	Tochigi III BAG70352.1	Takanezawa T01 DRR552862	Takanezawa T04 DRR552865	Yancheng CAA10638	Dazhong CN DZ UWL85812	NanYang CN NY UWL85809	Germany BAA00884	Germany HYT-38 QHA94748
Tochigi I BAG70350		3	1	1	3	9	8	6	6	10	6	6	27	25
Ohtawara IV BAG70354	99.66%		2	2	4	10	9	7	7	11	7	7	28	26
Takanezawa T03 DRR552864	99.89%	99.78%		0	2	8	7	5	5	9	5	5	26	24
Takanezawa T02 DRR552863	99.89%	99.78%	100.00%		2	8	7	5	5	9	5	5	26	24
Takanezawa T05 DRR552866	99.66%	99.55%	99.78%	99.78%		7	7	3	3	9	5	5	26	24
Yamaguchi V BBE49538	98.99%	98.88%	99.10%	99.10%	99.21%		9	6	6	11	7	7	28	26
Tochigi III BAG70352	99.10%	98.99%	99.21%	99.21%	99.21%	98.99%		5	5	10	5	5	28	26
Takanezawa T01 DRR552862	99.33%	99.21%	99.44%	99.44%	99.66%	99.33%	99.44%		0	8	5	5	26	24
Takanezawa T04 DRR552865	99.33%	99.21%	99.44%	99.44%	99.66%	99.33%	99.44%	100.00%		8	5	5	26	24
Yancheng CAA10638	98.88%	98.76%	98.99%	98.99%	98.99%	98.76%	98.88%	99.10%	99.10%		9	9	27	25
Dazhong CN DZ UWL85812	99.33%	99.21%	99.44%	99.44%	99.44%	99.21%	99.44%	99.44%	99.44%	98.99%		0	23	21
NanYang CN NY UWL85809	99.33%	99.21%	99.44%	99.44%	99.44%	99.21%	99.44%	99.44%	99.44%	98.99%	100.00%		23	21
Germany BAA00884	96.97%	96.85%	97.08%	97.08%	97.08%	96.85%	96.85%	97.08%	97.08%	96.97%	97.42%	97.42%		6
Germany HYT-38 QHA94748	97.19%	97.08%	97.30%	97.30%	97.30%	97.08%	97.08%	97.30%	97.30%	97.19%	97.64%	97.64%	99.33%	

1Nishigawa, H., Hagiwara, T., Yumoto, M., Sotome, T., Kato, T., & Natsuaki, T. (2008). Molecular phylogenetic analysis of Barley yellow mosaic virus. Archives of virology, 153, 1783-1786.

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Table 2-4. Correlated variations of RNA1 amino acids. Fields breaking *rym3* shaded in gray.

Genome	aa position	BaYMV[1]							R^2	p-value
		Takanezawa T01 DRR552862	Ohtawara IV BAG70353	Yamaguchi V BBE49537	Tochigi II-1 BAA00875	Tochigi III BAG70351	Tochigi I BAG70349	Germany G CAA49412		
RNA1	236	T	T	A	A	A	A	K	0.53	0.003018
RNA1	292	V	V	V	L	V	L	L	0.56	0.002006
RNA1	364	S	S	C	C	C	C	C	0.53	0.003018
RNA1	459	H	H	H	E	E	Q	K	1	-
RNA1	476	V	V	A	A	A	A	A	0.53	0.003018
RNA1	709	Q	Q	H	H	H	H	H	0.53	0.003018
RNA1	1102	I	I	I	L	L	I	T	0.56	0.002006
RNA1	1250	I	I	I	T	T	I	T	0.56	0.002006
RNA1	1295	E	E	E	K	T	E	N	0.56	0.002006
RNA1	1297	I	I	I	V	I	V	V	0.56	0.002006
RNA1	1305	T	T	A	A	A	A	A	0.53	0.003018
RNA1	1307	Y	Y	K	H	H	K	K	0.53	0.003018
RNA1	1310	V	V	M	I	I	I	I	0.53	0.003018
RNA1	1317	Y	Y	F	H	H	F	F	0.53	0.003018
RNA1	1342	R	R	T	K	K	V	V	0.53	0.003018
RNA1	1344	L	L	S	A	A	S	G	0.53	0.003018
RNA1	1346	T	T	Y	Y	Y	Y	Y	0.53	0.003018
RNA1	1349	A	A	G	G	G	G	E	0.53	0.003018
RNA1	1355	E	E	D	D	D	D	D	0.53	0.003018
RNA1	1848	R	R	R	K	K	R	K	0.56	0.002006
RNA1	2119	S	S	P	P	P	P	P	0.53	0.003018
RNA1	2122	E	E	D	D	D	D	D	0.53	0.003018
RNA1	2133	V	V	A	A	A	A	A	0.53	0.003018
RNA1	2135	K	K	Q	G	R	R	R	0.53	0.003018
RNA1	2138	A	A	A	F	F	L	L	1	-
RNA1	2139	D	D	D	E	E	E	D	0.56	0.002006
RNA1	2143	V	V	A	A	A	A	A	0.53	0.003018

[1]Nishigawa, H., et al. "Molecular phylogenetic analysis of Barley yellow mosaic virus." Archives of virology 153 (2008): 1783-1786.
Tanokami, Mai, et al. "Utility of a GFP-expressing Barley yellow mosaic virus for analyzing disease resistance genes." Breeding science 71.4 (2021): 484-490.
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Table 2-5. Correlated variations of RNA2 amino acids. Fields breaking *rym3* shaded in gray.

Genome	aa position	BaYMV[1]							R^2	p-value
		Takanezawa T01 DRR552862	Takanezawa T02 DRR552863	Ohtawara IV BAG70354	Yamaguchi V BBE49538	Tochigi I BAG70350	Tochigi III BAG70352	Germany BAA00884		
RNA2	359	T	A	T	T	A	A	A	0.5625	0.0020062

[1]Nishigawa, H., et al. "Molecular phylogenetic analysis of Barley yellow mosaic virus." Archives of virology 153 (2008): 1783-1786.
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Chapter 3. Re-sequencing of the *Ym2* gene reveals the relationship between its natural variations in wheat and host responses to wheat yellow mosaic virus

Introduction

Wheat yellow mosaic virus (WYMV) is a damaging soil-borne pathogen of wheat. The vegetative growth during the spring of infected plants is stunted, and their leaves develop chlorotic stripes and become discolored (Liu *et al.*, 2005, Takeuchi *et al.*, 2010). Heavily infected crops can suffer losses in grain yield of up to 80% (Nishimura *et al.*, 2010, Zhang *et al.*, 2011, Suzuki *et al.*, 2022). Similar disease symptoms are displayed by rye, triticale, and wheat plants harboring wheat spindle streak mosaic virus (WSSMV), and by barley plants harboring either barley yellow mosaic virus (BaYMV) or barley mild mosaic virus (BaMMV); all of the above viruses are members of *Bymovirus*, *Potyviridae* (Jiang *et al.*, 2020). WYMV was first documented in Japan (Sawada, 1927), but there was no report of a symptomatically similar disease until WSSMV was identified in North America (Slykhuis, 1960, Wiese *et al.*, 1970). More recently, WYMV has been identified in both China (Han *et al.*, 2000) and Europe (Kuhne, 2009). The level of severity of the disease is influenced by several factors, including the pathogenicity of the viral strain, the extent of the host plants' susceptibility, and various climatic factors (particularly temperature) (Clover and Henry, 1999). The WYMV genome comprises two linear, single-stranded, positive sense RNA molecules, one of length 7.7 kb and the other 3.7 kb (Namba *et al.*, 1998). The entry of WYMV into a wheat host requires the prior colonization of its roots by the plasmodiophorid vector *Polymyxa graminis* (Inouye, 1969), a process which is initiated by the encystment of a vector zoospore on the surface of a root cell. Thereafter, a tubular structure develops, enabling the contents of the zoospore to be injected into the root cells (Tamada and Kondo, 2013). The coat protein of WSSMV is retained within the resting spore of the vector, allowing the pathogen to be transmitted in particle form (Driskel *et al.*, 2004); however, it is not yet known whether this is also the case for WYMV. It has been established that under favorable environmental conditions, WYMV is protected within the resting spores of *P. graminis* (Kanyuka *et al.*, 2003) and can survive for at least 10 y without the host (Ohto and Naito, 1997, Chen, 2005). As fumigation of soils to control the virus is neither cost-

effective nor ecologically sustainable, the only currently viable control method is to breed for genetically determined resistance.

Strategies combating viral infection in host plants include blocking the entry of the vector, silencing the viral RNA, suppressing viral multiplication, and inhibiting viral movement within the host (Soosaar *et al.*, 2005, Hou *et al.*, 2012). The most promising of these are interference with viral multiplication and control over viral movement (Liu *et al.*, 2016, Ohki *et al.*, 2019). Genes measuring resistance against WYMV have been mapped to various genomic locations (Jiang *et al.*, 2020, Suzuki *et al.*, 2022). The US winter wheat cultivar (cv.) Madsen is highly resistant: when grown in the presence of inoculum, plants neither develop disease symptoms nor do they harbor a detectable titer of WYMV coat protein (Takeuchi *et al.*, 2010). Genetic mapping has shown that the resistance exhibited by cv. Madsen relies on a pair of genes, namely *Ym1* on chromosome arm 2DL and *Ym2* on chromosome arm 3BS cultivar (Suzuki *et al.*, 2015, Suzuki *et al.*, 2022). The cv. Madsen *Ym1* allele is incompletely dominant over the allele carried by the susceptible host cv. Hokushin, whereas the *Ym2* allele is completely dominant.

The resistance gene *Ym2* (*CDS618*) encodes a coiled-coil (CC, positions 11 to 129) nucleotide-binding site (NBS, 190 to 458) leucine-rich repeat (LRR, 534 to 896) protein and is expressed in the root, it was recently isolated from wheat cv. Madsen revealed the molecular basis of WYMV resistance (Mishina *et al.*, 2023). A comparison of the cvs. Madsen and Chinese Spring sequences revealed that a 1 bp deletion formed a premature stop codon in cv. Chinese Spring, leading to a truncated (763-residue) translation product. However, the sequence diversity of *Ym2* (*CDS618*) in other wheat accessions was unknown.

The current study aimed to investigate the natural variations of *Ym2* (*CDS618*) and the relationship between sequence variants and host response to WYMV in wheat accessions.

Materials and Methods

Plant materials and field infection with WYMV

WYMV-resistance cv. Madsen is a soft white winter wheat released in 1988 for cultivation in the Pacific Northwest of the United States. The WYMV-susceptible cv. Hokushin was bred by the Hokkaido Agriculture Experimental Station and released in 1994 (Yanagisawa *et al.*, 2000). Grain samples of the other hexaploidy and tetraploid wheat used in this analysis were obtained from HRO (Naganuma, Japan), INRA (Clermont-Ferrand, France), Kyoto University (Kyoto, Japan), the international Maize and Wheat Improvement Center (Texcoco, Mexico), the John Innes Center (Norwich, UK), and NARO (Tsukuba, Japan) (Table 3-1).

Plants were sown at the end of September in a WYMV-infested field at Naka-Mareppu, Date (Hokkaido, Japan), and at the end of October in a WYMV-free field at NICS (NARO, Tsukuba, Japan). The plants were sampled in late November to monitor the presence of *P. graminis* and again in February to assess the disease reaction.

Detection of WYMV using ELISA

WYMV in leaf tissue was detected using a plate-trapped antigen enzyme-linked immuno-sorbent assay (ELISA) as described by (Suzuki *et al.*, 2015) based on an established polyclonal anti-WYMV antibody (Ueda *et al.*, 1998).

TaqMan assay for quantifying WYMV

RNA was extracted from 50 mg samples of roots, leaf sheaths, or leaf blades using an RNAeasy plant mini kit (www.qiagen.com); contaminating genomic DNA was removed by passage through an On-column DNase I treatment set (www.qiagen.com). RNA was quantified using a Nanodrop 2000 device (www.thermofisher.com/). The template for the synthesis of the first cDNA strand was a 4 μL aliquot of 10 to 200 ng/μL RNA provided for a 10 μL reaction containing Invitrogen SuperScript III reverse transcriptase, oligo$(dT)_{12\text{-}18}$ primer and RNaseOUT ribonuclease inhibitor according to the manufacturer's instructions (www.thermofisher.com/). The resulting cDNA was finally massed up to 40 μL. The multiplex TaqMan assay used to quantify WYMV RNA1 was modified from the method given by (Liu *et al.*, 2016). Each 10 μL reaction contained 2× SsoAdvanced Probe Supermix (www.bio-rad.com/), 150 nM WYMV primers, 125 nM WYMV

probe, 150 nM Actin primers, 125 nM Actin probe, and 1 μL of 0.05 to 1 pg/μL cDNA. The reactions were subjected to an initial denaturing (95 °C/30 s), followed by 50 cycles of 95 °C/30 s, 58 °C/30 s in CFX96 real-time PCR system (Bio-Rad, Tokyo). The FAM and Texas red baseline thresholds were set to 60 in order to estimate the Cq value. WYMV was quantified in reference to a standard curve according to the formula, $\log_{10}$ (number of copies) = (39.3 – Cq)/3.4. A Cq value above 39.3 or a "Not Detected" response was interpreted as indicating a WYMV copy number of zero. Similarly for the *Actin* sequence, $\log_{10}$ (number of copies) = (41.8 – Cq)/3.4. The number of WYMV units present in a 20-ng aliquot of RNA (x) was transformed for the purpose of statistical analysis by applying the function $\log_{10}$ (x + 1). Each measurement was supported by three technical replicates.

DNA extraction

Genomic DNA was extracted from young leaf tissue using a standard cetyl trimethyl ammonium bromide (CTAB)-based method (Wilkie *et al.*, 1997): 0.2 g frozen, shredded leaf was homogenized using an Retsch MM300 TissueLyser (www.retsch.com), by including two 5-mm-diameter zirconia-silica beads in each tube, along with 0.8 mL 1.5× CTAB buffer and 0.1% (v/v) 2-mercaptoethanol. After homogenization, 0.8 mL 24:1 chloroform/isoamyl alcohol was added, and following centrifugation, the aqueous layer was mixed with 0.5 mL isopropanol. The DNA pellet recovered after further centrifugation was rinsed twice in 0.5 mL 70% ethanol and dried under vacuum. Finally, the DNA was dissolved in 0.2 mL 1 mM Tris-hydrochloride, 0.1 mM ethylenediaminetetraacetic acid buffer (pH 8.0), and diluted to 80 to 100 ng/μL for use as a PCR template.

PCR amplification of genomic DNA

PCR primer sequences designed using Primer3 software (bioinfo.ut.ee/primer3-0.4.0/) are listed in SI Appendix, Table S12, and the primer orientation for resequencing *CDS618* is shown in SI Appendix, Fig. S12. Each 10 μL PCR contained 0.25 U PrimeSTAR GXL DNA polymerase (www.takarabio.com/), 0.3 μM of each primer, 200 μM dNTP, 1× PrimeSTAR GXL buffer, and 20 ng genomic DNA. The reactions were subjected to an initial denaturation (98 °C/60 s), followed by 35 cycles of 98 °C/10 s, 58 °C or 60 °C/15 s (58 °C for Actin, 60 °C for *CDS618*), 68 °C/60 s per kb target; 68 °C for 7 min. Where necessary, the amplicons were subjected to electrophoresis through 2% SeaPlaque agarose (FMC, Rockland, ME, USA).

Amplicon purification and sequencing and identification of nucleotide variants

Amplicons were purified using a QIAquick PCR Purification kit (www.qiagen.com). A 2 μL aliquot of eluate was electrophoresed through a 1% agarose gel to verify the presence of the target fragment present prior to Sanger sequencing, as described earlier (Pourkheirandish *et al.*, 2015). The dye terminator was removed using Agencourt CleanSEQ (www.beckman.com/). Fragments were assembled using GeneStudio sequence analysis software v2.0.0 (GeneStudio, Suwanee, GA, USA). A BlastN search was conducted against the 10+ genome genomic database (www.10wheatgenomes.com) using coding sequences obtained from cv. Madsen as the query. Matched sequences were aligned to the cv. Madsen coding sequences using ClustalW software, and nucleotide variants called using a customized Perl script. Variant sites were annotated using SNPeff v4 software (Cingolani *et al.*, 2012).

Results

Natural variation of *Ym2* (*CDS618*) in wheat accessions

A number of *CDS618* sequence variants present in a panel of 91 wheat accessions were identified via a combination of PCR amplicon resequencing and a search of public sequence data (Walkowiak *et al.*, 2020) (Figure 3-1A). High-quality sequences were obtained from 16 wheat accessions for 809 bp of exon1 and 2014 bp of exon2 of *CDS618*, respectively. Nonsynonymous polymorphisms were detected at 26 nucleotide sites within bread wheat (*Triticum aestivum*), synthetic hexaploid wheat, spelt wheat (*Triticum spelta*), and tetraploid durum wheat (*Triticum durum*). In addition, seven different premature stop codons were identified among the hexaploid members of the panel and three among the tetraploid ones; the former resulted in predicted truncated proteins of length 68, 100, 178, 213, 253, 278, and 763 residues (Table 3-1). A set of 12 accessions harbor a missense variant of the full 940-residue product (Figure 3-1A and Table 3-2), while four accessions, namely Chogokuwase, Minaminokomugi, and Norin 61 (two accessions), harbor two copies of the gene, of which one encodes a truncated (213 residue) protein and the other a 940-residue chimera between *CDS618* and *TraesCS3B02G035800* (Figure 3-2). A set of 19 accessions, both hexaploid and tetraploid accessions, carry a null allele (Table 3-3).

The relationship between the *Ym2* (*CDS618*) sequence and the host response to WYMV inoculation

To estimate the WYMV titre in wheat, the primers of the WYMV coat protein and TaqMan probe were used to quantify the accumulation of virus following infested-field inoculation in 83 of the 90 accessions (Figure 3-1B). 59 of 83 wheat accessions show a lower level with the $\log_{10}$ titer of WYMV < 2. Among these accessions, all 16 accessions carry the cv. Madsen allele was shown to accumulate a very low titer of the virus RNA1 ($\log_{10}$ transformed copy number <2) indicated were all able to limit WYMV replication, although it remains to be established whether either the missense variant or the chimeric protein is responsible since the accessions may also harbor other genes, such as *Ym1* or *Qym4* (Yamashita *et al.*, 2020). The disease response of the 56 accessions that do not generate a 940-residue protein varied widely, implying the presence of genes for WYMV resistance other than *CDS618*.

Discussion

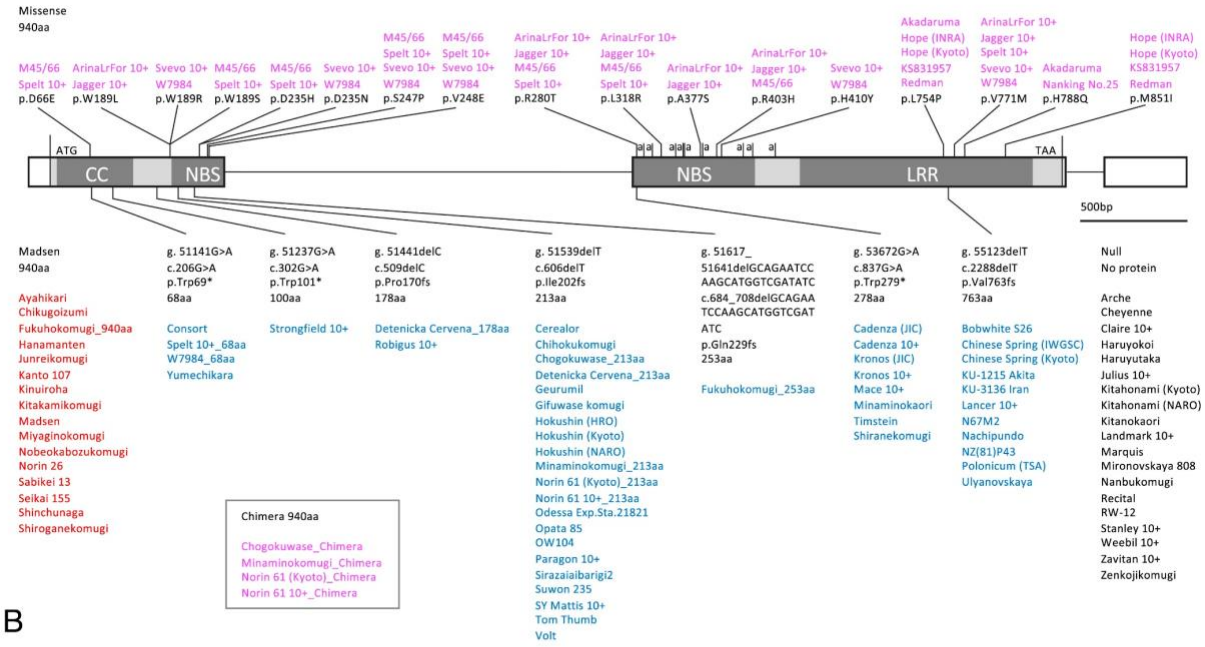
This study combines previously identified *Ym2* (*CDS618*), which encodes an NBS-LRR protein, and its newly generated sequence variations involved in WYMV infection (Mishina *et al.*, 2023). This gene belongs—along with a diverse range of major genes in the cereals encoding resistance against a variety of pathogens, including *Pm21* and *Mla* (resistance to *B. graminis*), *Sr13*, *Sr22* and *Sr35* (*Puccinia graminis*), *Lr10* (*Puccinia triticina*) and *Yr10* (*Puccinia striiformis*)—to the "Intron1 clade." The wheat genome harbors some 3,000 NBS-LRR genes; most of those characterized to date encode resistance against a fungal rather than a viral or bacterial pathogen (Steuernagel *et al.*, 2020). Like *Ym2*, however, many NBS-LRR genes isolated from dicotyledonous species are determinants of resistance against viruses (de Ronde *et al.*, 2014). As previously reported, the presence of *Ym2* reduces the viral titer by six orders of magnitude. Consistently, the *Ym2* alleles present in cv. Chinese Spring (Pearce *et al.*, 2015) and some other cultivars (www.wheat-expression.com) are also transcribed in the root, as is the *Ym2* barley ortholog MLOC_62179 (ics.hutton.ac.uk/morexGenes/view_gene2.cgi?seq_name=MLOC_62179&dataset=assembly3_WGSMorex_rbca.fasta#fpkm_rep). While WYMV can enter the roots of susceptible or resistant cultivars, it is not readily translocated to the latter's leaves, implying that the resistance gene product acts to either inhibit viral movement to the shoot (Ohto and Naito, 1997, Ohki *et al.*, 2019) and/or suppress its multiplication within the root. A similar conclusion has been reached with respect to resistance against the soil-borne cereal mosaic virus and Japanese Soil-borne wheat mosaic virus (Kanyuka *et al.*, 2003, Okada *et al.*, 2023). The previous observations imply that plants carrying *Ym2* either hinder the initial movement of WYMV from *P. graminis* into host root cells or suppress its multiplication within root tissue (Liu *et al.*, 2016, Mishina *et al.*, 2023).

By investigating haplotypes for resistance, this work demonstrated that a number of *Ym2* haplotypes have evolved in bread wheat germplasm. Each of the eight accessions carries a *Ym2* haplotype encoding a 940-residue polypeptide despite varying from the cv. Madsen haplotype, at 15 nucleotide sites, was resistant to WYMV, implying that the key residues required for function were all maintained (unless other resistance genes(s) were present). The protein encoded by the haplotype present in the susceptible cvs. Chinese Spring, Bobwhite, Ulyanovskaya, and Nachipundo lack four of the LRRs represented in the cv. Madsen gene product. Given that the C-

terminal end of the LRR region of the product of potato virus X resistance gene Rx is responsible for the recognition of virus coat protein (Rairdan and Moffett, 2006), it is plausible that these missing C-terminal LRRs are required for the wheat plant to recognize WYMV. The haplotype present in the resistant cv. Yumechikara harbors a premature stop codon within the CC domain, so its resistance must be due to possessing a gene other than *Ym2*. Overall, the differences in gene diversity patterns observed for *Ym2* (*CDS618*) were found in wheat accessions; our data support plants carrying *Ym2* or its missense pattern limited the replication of WYMV in roots.

Figures

A


Missense
940aa
M45/66 Spelt 10+ p.D66E
ArinaLrFor 10+ Jagger 10+ p.W189L
Svevo 10+ W7984 p.W189R
M45/66 Spelt 10+ p.W189S
M45/66 Spelt 10+ p.D235H
Svevo 10+ W7984 p.D235N
M45/66 Spelt 10+ Svevo 10+ W7984 p.S247P
M45/66 Spelt 10+ Svevo 10+ W7984 p.V248E
ArinaLrFor 10+ Jagger 10+ M45/66 Spelt 10+ p.R280T
ArinaLrFor 10+ Jagger 10+ M45/66 Spelt 10+ p.L318R
ArinaLrFor 10+ Jagger 10+ p.A377S
ArinaLrFor 10+ Jagger 10+ M45/66 p.R403H
Svevo 10+ W7984 p.H410Y
Akadaruma Hope (INRA) Hope (Kyoto) KS831957 Redman p.L754P
ArinaLrFor 10+ Jagger 10+ Spelt 10+ Svevo 10+ W7984 p.V771M
Akadaruma Nanking No.25 p.H788Q
Hope (INRA) Hope (Kyoto) KS831957 Redman p.M851I
ATG
CC
NBS
NBS
LRR
TAA
500bp
Madsen
940aa
Ayahikari
Chikugoizumi
Fukuhokomugi_940aa
Hanamanten
Junreikomugi
Kanto 107
Kinuiroha
Kitakamikomugi
Madsen
Miyaginokomugi
Nobeokabozukomugi
Norin 26
Sabikei 13
Seikai 155
Shinchunaga
Shiroganekomugi
g. 51141G>A
c.206G>A
p.Trp69*
68aa
Consort
Spelt 10+_68aa
W7984_68aa
Yumechikara
g. 51237G>A
c.302G>A
p.Trp101*
100aa
Strongfield 10+
g. 51441delC
c.509delC
p.Pro170fs
178aa
Detenicka Cervena_178aa
Robigus 10+
g. 51539delT
c.606delT
p.Ile202fs
213aa
Cerealor
Chihokukomugi
Chogokuwase_213aa
Detenicka Cervena_213aa
Geurumil
Gifuwase komugi
Hokushin (HRO)
Hokushin (Kyoto)
Hokushin (NARO)
Minaminokomugi_213aa
Norin 61 (Kyoto)_213aa
Norin 61 10+_213aa
Odessa Exp.Sta.21821
Opata 85
OW104
Paragon 10+
Sirazaiaibarigi2
Suwon 235
SY Mattis 10+
Tom Thumb
Volt
g. 51617_
51641delGCAGAATCC
AAGCATGGTCGATATC
c.684_708delGCAGAA
TCCAAGCATGGTCGAT
ATC
p.Gln229fs
253aa
Fukuhokomugi_253aa
g. 53672G>A
c.837G>A
p.Trp279*
278aa
Cadenza (JIC)
Cadenza 10+
Kronos (JIC)
Kronos 10+
Mace 10+
Minaminokaori
Timstein
Shiranekomugi
g. 55123delT
c.2288delT
p.Val763fs
763aa
Bobwhite S26
Chinese Spring (IWGSC)
Chinese Spring (Kyoto)
KU-1215 Akita
KU-3136 Iran
Lancer 10+
N67M2
Nachipundo
NZ(81)P43
Polonicum (TSA)
Ulyanovskaya
Null
No protein
Arche
Cheyenne
Claire 10+
Haruyokoi
Haruyutaka
Julius 10+
Kitahonami (Kyoto)
Kitahonami (NARO)
Kitanokaori
Landmark 10+
Marquis
Mironovskaya 808
Nanbukomugi
Recital
RW-12
Stanley 10+
Weebil 10+
Zavitan 10+
Zenkojikomugi
Chimera 940aa
Chogokuwase_Chimera
Minaminokomugi_Chimera
Norin 61 (Kyoto)_Chimera
Norin 61 10+_Chimera

B

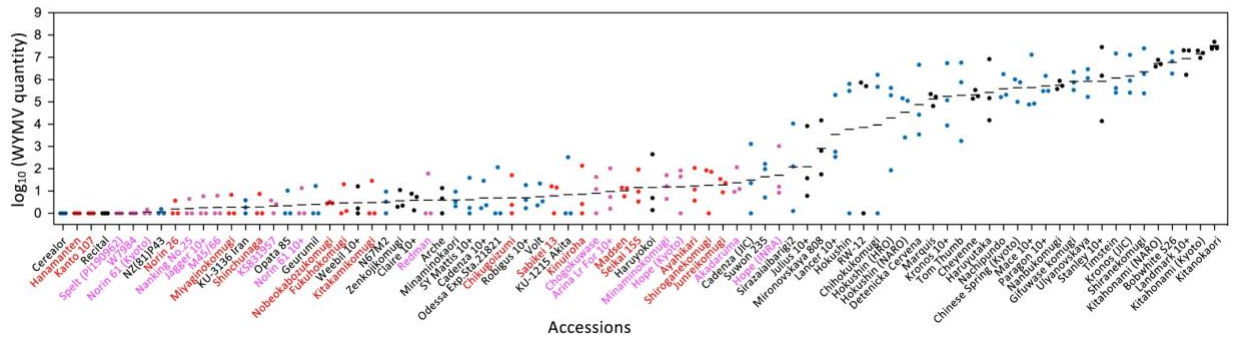

log10 (WYMV quantity)
Accessions

Figure 3-1. Sequence variation at *Ym2* (*CDS618*) and its effect on WYMV resistance. (A) Variable nucleotide sites in the cv. Madsen sequences are represented in red are shown on the Left, missense variants in magenta (shown above the coding sequence), and premature stop codons in blue (shown below the sequence). Accessions lacking any *CDS618* product (null mutations) are shown in black to the Right, and those harboring a chimeric version of the gene are boxed. g: genome, c: coding sequence, p: amino acid position, fs: frameshift, *: stop codon, a: p.F291C, p.I306V, p.G345D, p.N360K, p.K361E, p.E381V, p.K445R, p.S464A and p.T508M specific for cultivar M45/66. Parentheses: duplicated gene products from multiple plant accessions. The cultivars Chogokuwase, Minaminokomugi, and Norin61 (two accessions) harbor two copies of *CDS618,* one encoding a truncated 213-residue protein and the other a chimera between *CDS618* and *TraesCS3B02G035800*, predicted to encode a 940-residue protein. (B) WYMV copy number (log_{10} transformed) in the roots of plants exposed to WYMV.

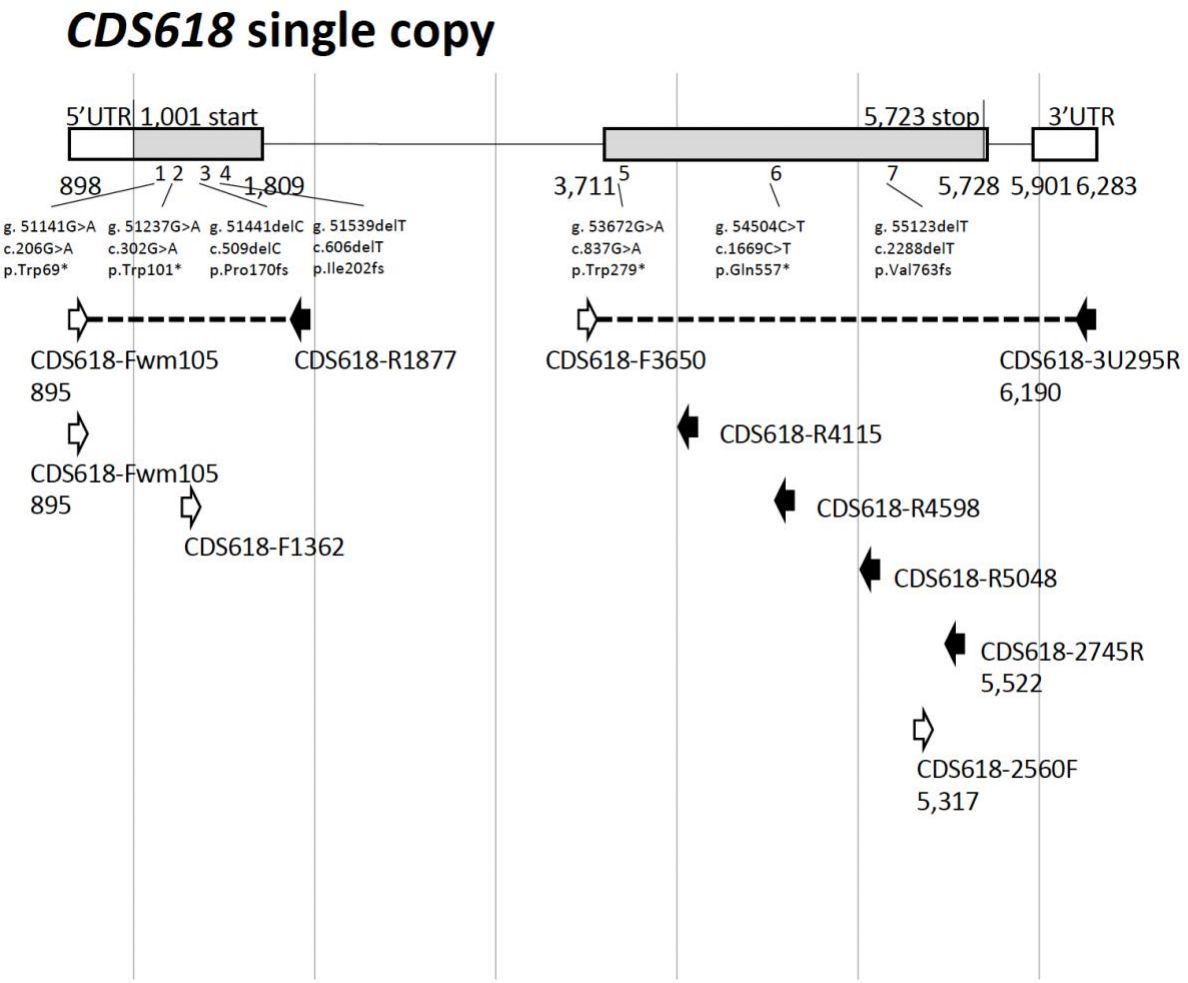


Figure 3-2. PCR amplification and sequencing strategy of *CDS618*. *CDS618* encoding 940aa protein in cv. Madsen.

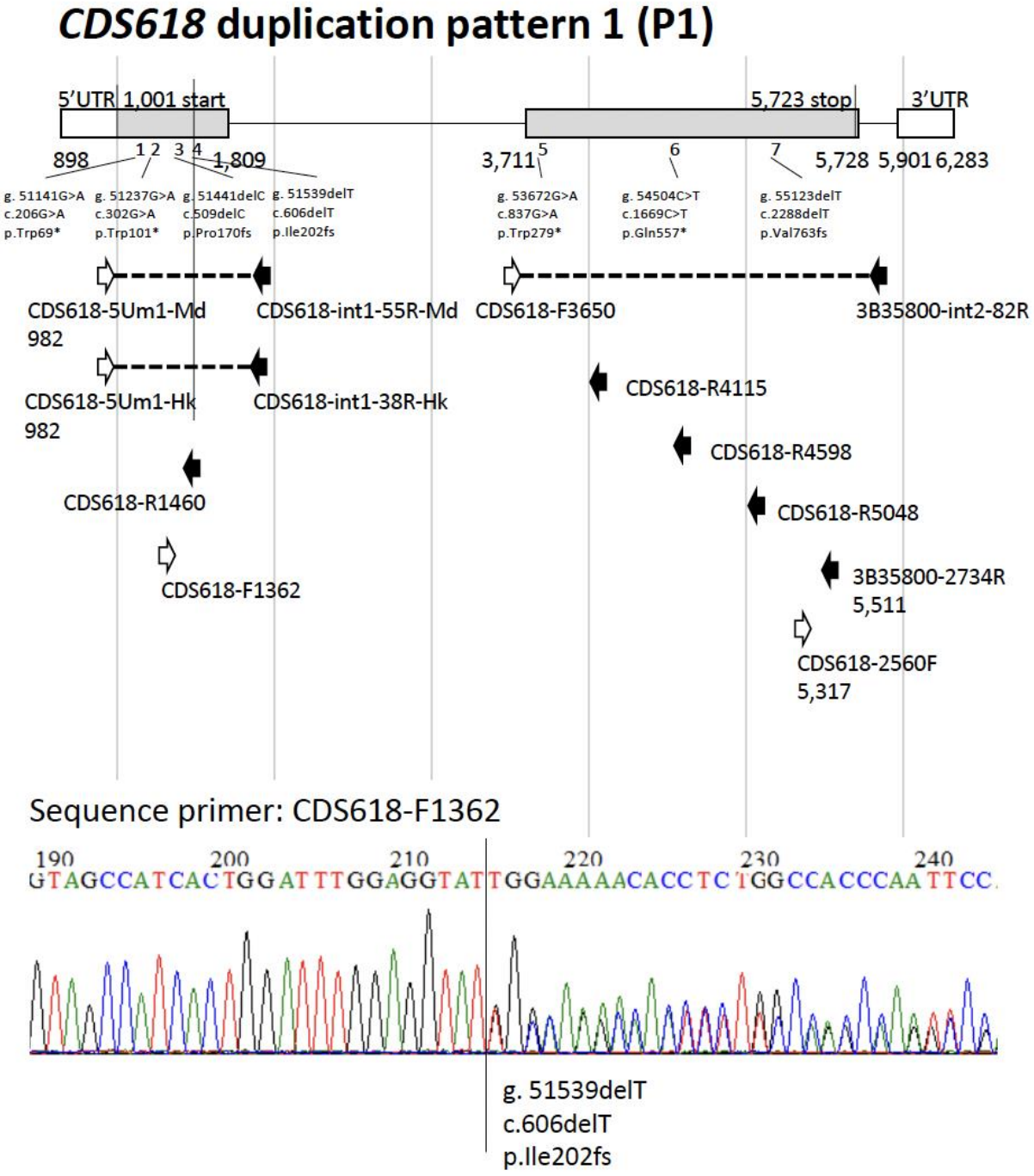


Figure 3-2. (continued) (B) Mixed sequences in Norin 61, Minaminokomugi and Chogokuwase consisted of *CDS618* encoding 213aa protein and chimeric gene encoding 940aa protein.

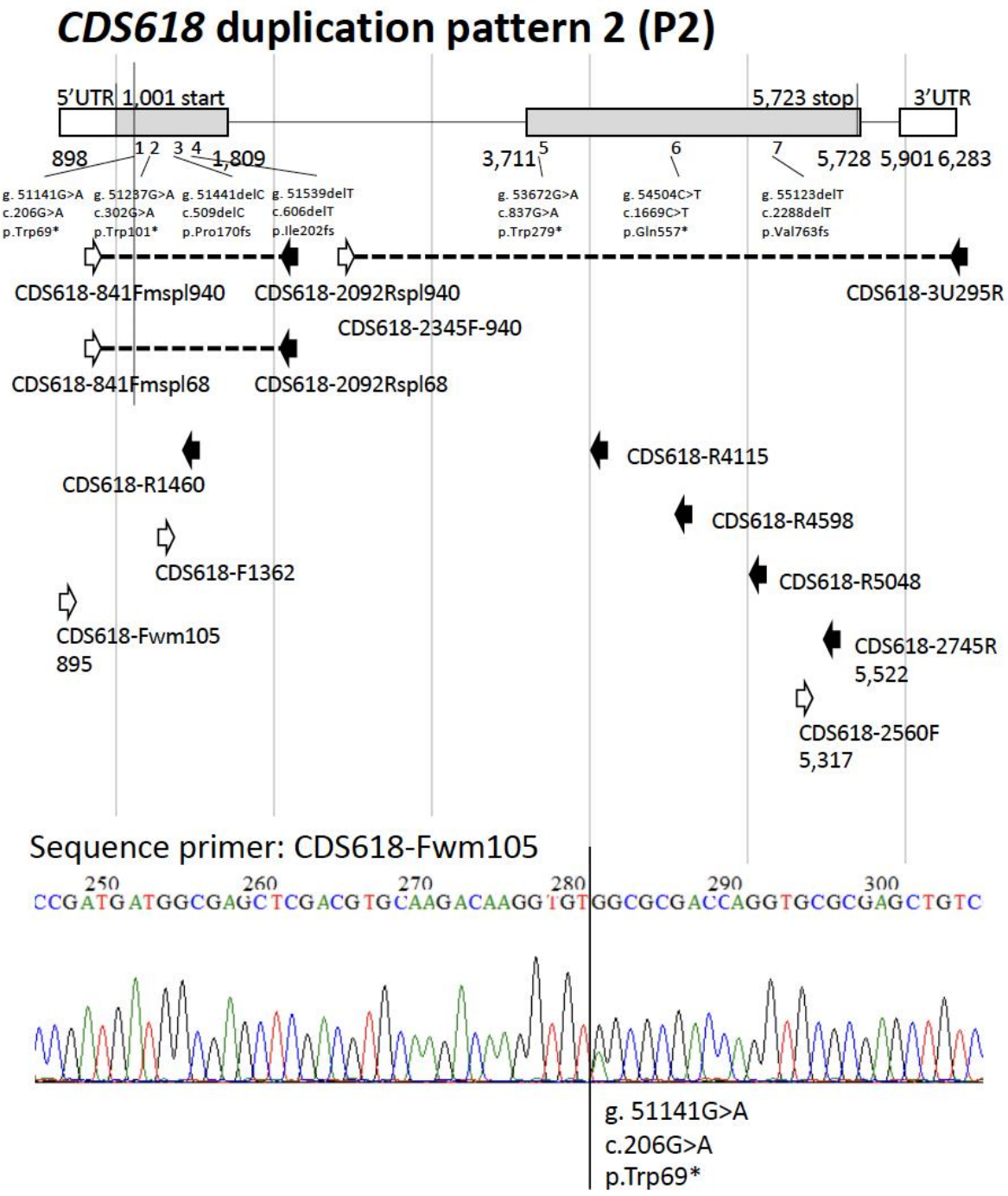


Figure 3-2. (continued) (C) Mixed sequences in cv. W7984 consisted of *CDS618* homologous genes encoding 940aa and 68aa proteins, a pattern similar to Spelt.

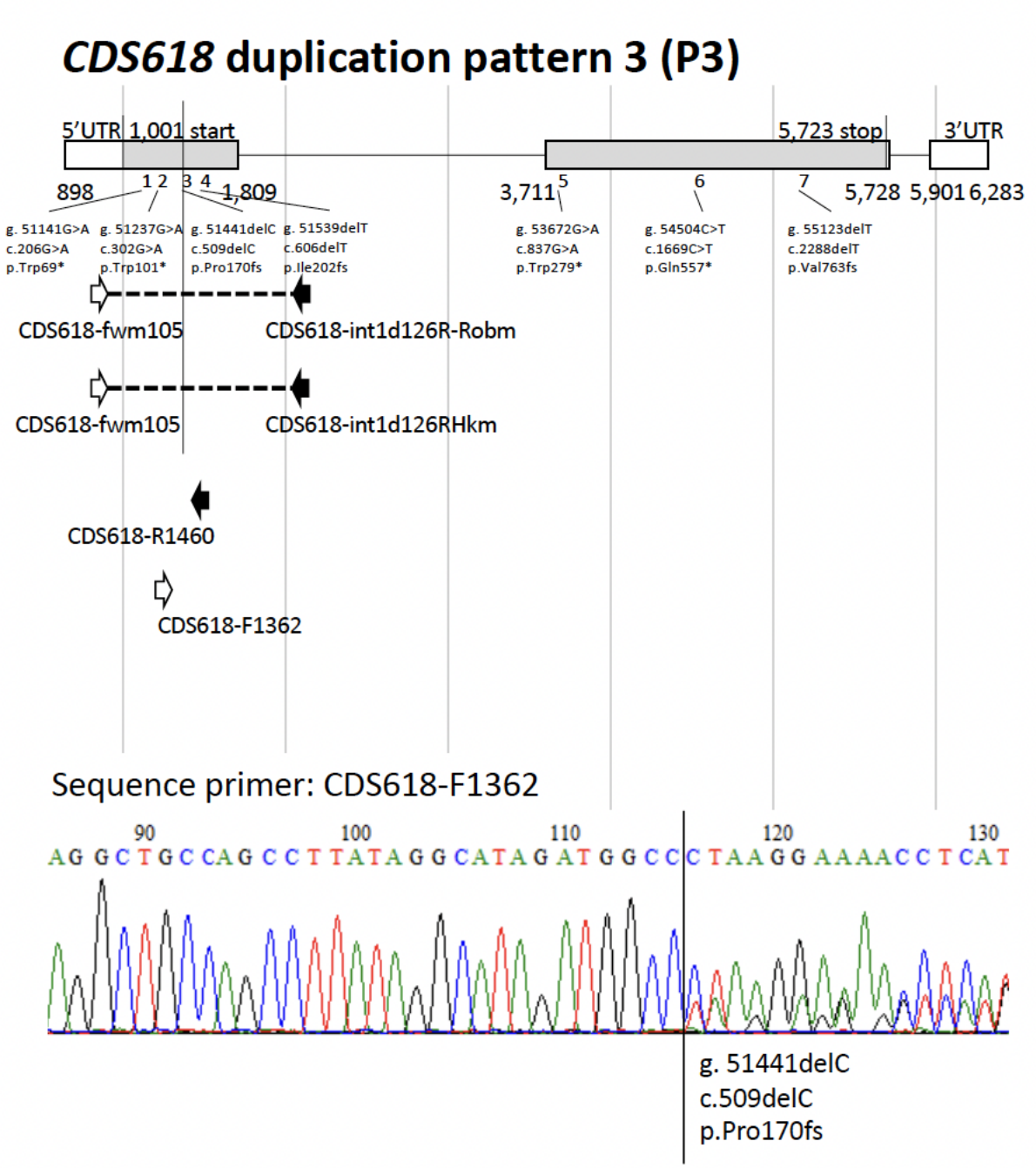


Figure 3-2. (continued) (D) Mixed sequences in cv. Detenicka Cervena consisted of *CDS618* homologous genes encoding 178aa protein (Robigus like) and 213aa protein (Hokushin like).

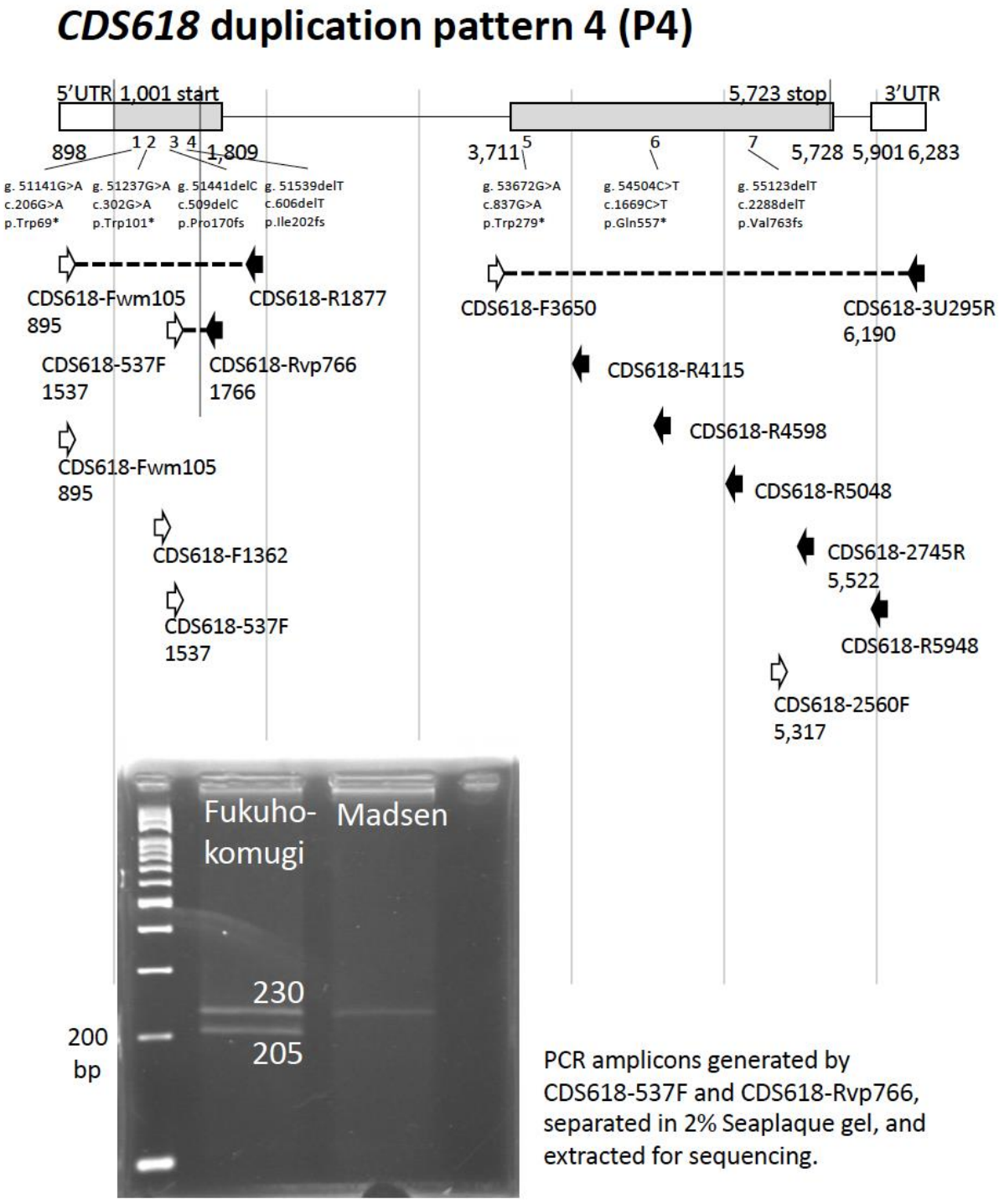


Figure 3-2. (continued) (E) Mixed amplicons in cv. Fukuhokomugi consisted of *CDS618* homologous genes encoding 940aa and 253aa proteins (by 25-bp deletion).

Table 3-1. *CDS618* variants and their effects on WYMV resistance in a panel of 91 cultivars. Related to Figure 3-1A.

#	Cultivar (donor)	Species	Origin	Yrn2 DNA re-sequencing			Exon1 (bp)	Exon1 acc. ID	Exon2 (bp)	Exon2 acc. ID	Protein (aa)	Protein type	TaActin bp	WYMV RT-qPCR (n=3)[4]		WYMV ELISA O.D. (n=6)[5]		Sequence reference
				Sample ID	Seed source	Re-sequencing ID								Mean	S. E.	Mean	S. E.	
1	Madsen	*Triticum aestivum*	USA	I50	HRO	Madsen	809	TBA	2014	TBA	940	Madsen	350	1.015	0.126	0.215	0.079	This study
2	Fukuhokomugi	*Triticum aestivum*	JPN	I54	INRA	Fukuhokomugi_940aa	809	LC651727	2014	LC651742	940	Madsen	350	0.471	0.419	0.247	0.077	This study
						Fukuhokomugi_253aa	762	LC651728	n.t.	-	253	Truncated						
3	Kanto 107	*Triticum aestivum*	JPN	W6	Kyoto U	Kanto 107	809	LC651689	2014	LC651756	940	Madsen	350	0.000	0.000	0.380	0.053	This study
4	Norin 26	*Triticum aestivum*	JPN	W7	Kyoto U	Norin 26	809	LC651690	2014	LC651757	940	Madsen	350	0.192	0.192	0.369	0.026	This study
5	Nobeokabozukomugi	*Triticum aestivum*	JPN	W10	Kyoto U	Nobeokabozukomugi	809	LC651705	2014	LC651758	940	Madsen	350	0.468	0.022	0.318	0.051	This study
6	Ayahikari	*Triticum aestivum*	JPN	W40	Kyoto U	Ayahikari	809	LC651692	2014	LC651761	940	Madsen	350	1.227	0.431	0.381	0.077	This study
7	Seikai 155	*Triticum aestivum*	JPN	W41	Kyoto U	Seikai 155	809	LC651730	2014	LC651762	940	Madsen	350	1.161	0.424	0.337	0.058	This study
8	Shiroganekomugi	*Triticum aestivum*	JPN	W43	Kyoto U	Shiroganekomugi	809	LC651693	2014	LC651763	940	Madsen	350	1.265	0.633	0.329	0.055	This study
9	Junreikomugi	*Triticum aestivum*	JPN	W44	Kyoto U	Junreikomugi	809	LC651694	2014	LC651764	940	Madsen	350	1.280	0.176	0.334	0.050	This study
10	Shinchunaga	*Triticum aestivum*	JPN	W45	Kyoto U	Shinchunaga	809	LC651737	2014	LC651765	940	Madsen	350	0.290	0.290	0.332	0.064	This study
11	Chikugoizumi	*Triticum aestivum*	JPN	W49	Kyoto U	Chikugoizumi	809	LC651695	2014	LC651767	940	Madsen	350	0.699	0.519	0.407	0.087	This study
12	Kinuiroha	*Triticum aestivum*	JPN	W50	Kyoto U	Kinuiroha	809	LC651696	2014	LC651768	940	Madsen	350	0.855	0.855	0.431	0.067	This study
13	Hanamanten	*Triticum aestivum*	JPN	W52	Kyoto U	Hanamanten	809	LC651706	2014	LC651770	940	Madsen	350	0.000	0.000	0.449	0.077	This study
14	Kitakamikomugi	*Triticum aestivum*	JPN	W55	Kyoto U	Kitakamikomugi	809	LC651739	2014	LC651771	940	Madsen	350	0.487	0.487	0.431	0.047	This study
15	Miyaginokomugi	*Triticum aestivum*	JPN	W59	Kyoto U	Miyaginokomugi	809	LC651738	2014	LC651773	940	Madsen	350	0.278	0.278	0.425	0.072	This study
16	Sabikei 13	*Triticum aestivum*	JPN	W61	Kyoto U	Sabikei 13	809	LC651703	2014	LC651774	940	Madsen	350	0.789	0.395	0.435	0.057	This study
17	Hope (INRA)	*Triticum aestivum*	USA	I55	INRA	Hope (INRA)	809	LC651729	2014	LC651743	940	Missense	350	1.716	0.857	0.252	0.075	This study
18	M45/66	*Triticum aestivum*	ARG	I56	INRA	M45/66	809	LC651682	2014	LC651744	940	Missense	350	0.264	0.264	0.214	0.043	This study
19	Nanking No.25	*Triticum aestivum*	CHN	I60	INRA	Nanking No.25	809	LC651707	2014	LC651747	940	Missense	350	0.217	0.217	0.206	0.060	This study
20	Redman	*Triticum aestivum*	CAN	I65	INRA	Redman	809	LC651702	2014	LC651750	940	Missense	350	0.594	0.594	0.312	0.052	This study
21	W7984	*Triticum aestivum*	MEX	I70	INRA	W7984[3]	809	LC651686	2014	LC651751	940	Missense	350	0.000	0.000	0.334	0.093	This study
						W7984_66aa	207	LC651685	n.t.	-	66	Truncated						
22	Hope (Kyoto)	*Triticum aestivum*	USA	W5	Kyoto U	Hope (Kyoto)	809	LC651688	2014	LC651755	940	Missense	350	1.192	0.601	0.392	0.096	This study
23	Akadaruma	*Triticum aestivum*	JPN	W16	Kyoto U	Akadaruma	809	LC651691	2014	LC651759	940	Missense	350	1.375	0.348	0.317	0.047	This study
24	KS831957	*Triticum aestivum*	USA	W58	Kyoto U	KS831957	809	LC651704	2014	LC651772	940	Missense	350	0.331	0.172	0.434	0.069	This study
25	AvinaLrFox	*Triticum aestivum*	CHE	IT12	Kyoto U	AvinaLrFox 10+	809	GCA_903993985	2014	GCA_903993985	940	Missense	350	0.988	0.537	n.t.	n.t.	Walkowiak et al., 2020
26	Jagger	*Triticum aestivum*	USA	IT14	Kyoto U	Jagger 10+	809	GCA_903993795	2014	GCA_903993795	940	Missense	350	0.257	0.257	n.t.	n.t.	Walkowiak et al., 2020
27	Spelt (PI190962)	*Triticum spelta*	EUR	IT17	Kyoto U	Spelt 10+[2]	809	GCA_903994165	2014	GCA_903994165	940	Missense	350	0.000	0.000	n.t.	n.t.	Walkowiak et al., 2020
						Spelt 10+_68aa	207	GCA_903994165	n.t.	-	68	Truncated						
28	Svevo	*Triticum turgidum*	ITA	n.a. (seq)[2]	n.a.	Svevo	809	GCA_900231445	2014	GCA_900231445	940	Missense	350	n.t.	n.t.	n.t.	n.t.	Maccaferri et al., 2019
29	Chinese Spring (Kyoto)	*Triticum aestivum*	CHN	CS	Kyoto U	Chinese Spring (Kyoto)	809	LC651701	1483	LC651752	783	Truncated	350	5.636	0.316	1.118	0.365	This study
30	Hokushin (HRO)	*Triticum aestivum*	JPN	HK	HRO	Hokushin (HRO)	642	LC651708	n.t.	-	213	Truncated	350	4.289	1.181	n.t.	n.t.	This study
31	Bobwhite S26	*Triticum aestivum*	MEX	I44	CIMMYT	Bobwhite S26	809	LC651679	1483	LC651740	783	Truncated	350	6.778	0.278	3.225	0.295	This study
32	Kronos (JIC)	*Triticum turgidum*	USA	I45	JIC	Kronos (JIC)	809	LC651680	28	LC651680	278	Truncated	350	6.163	0.499	2.823	0.440	This study
33	Cadenza (JIC)	*Triticum aestivum*	GBR	I46	JIC	Cadenza (JIC)	809	LC651681	28	LC651741	278	Truncated	350	1.489	0.901	0.221	0.048	This study
34	Hokushin (NARO)	*Triticum aestivum*	JPN	I49	NARO	Hokushin (NARO)	642	TBA	n.t.	TBA	213	Truncated	350	4.543	0.567	1.462	0.427	This study
35	Cereal*x*	*Triticum aestivum*	FRA	I52	INRA	Cereal*x*	642	LC651724	n.t.	-	213	Truncated	350	0.000	0.000	0.224	0.049	This study
36	Betenicka Cervena	*Triticum aestivum*	CZE	I53	INRA	Betenicka Cervavena_178aa	537	LC651725	n.t.	-	178	Truncated	350	4.877	0.930	2.887	0.402	This study
						Betenicka Cervavena_213aa	642	LC651726	n.t.	-	213	Truncated						
37	M67M2	*Triticum aestivum*	ISR	I58	INRA	M67M2	809	LC651683	1483	LC651745	783	Truncated	350	0.500	0.282	0.189	0.039	This study
38	Nachipundo	*Triticum aestivum*	NPL	I59	INRA	Nachipundo	809	LC651684	1483	LC651746	783	Truncated	350	5.595	0.327	3.293	0.282	This study
39	NZ[81]P43	*Triticum aestivum*	NZL	I61	INRA	NZ[81]P43	809	LC651719	1483	LC651748	783	Truncated	350	0.065	0.065	0.338	0.059	This study
40	Odessa Exp.Sta.21821	*Triticum aestivum*	PRT	I62	INRA	Odessa Exp.Sta.21821	642	LC651714	n.t.	-	213	Truncated	350	0.689	0.689	0.323	0.074	This study
41	Ulyanovskaya	*Triticum aestivum*	RUS	I63	INRA	Ulyanovskaya	809	LC651720	1483	LC651749	783	Truncated	350	5.923	0.365	0.518	0.212	This study
42	Siazaisibaoqi2	*Triticum aestivum*	JPN	I66	INRA	Siazaisibaoqi2	642	LC651715	n.t.	-	213	Truncated	350	2.083	1.133	0.308	0.044	This study
43	Tom Thumb	*Triticum aestivum*	CHN	I67	INRA	Tom Thumb	642	LC651716	n.t.	-	213	Truncated	350	5.301	1.056	1.571	0.357	This study
44	Volt	*Triticum aestivum*	HUN	I68	INRA	Volt	642	LC651717	n.t.	-	213	Truncated	350	0.746	0.300	0.305	0.039	This study
45	Opata 85	*Triticum aestivum*	MEX	I69	INRA	Opata 85	642	LC651718	n.t.	-	213	Truncated	350	0.340	0.340	0.302	0.047	This study
46	Gifuwase komugi	*Triticum aestivum*	JPN	W2	Kyoto U	Gifuwase komugi	642	LC651709	n.t.	-	213	Truncated	350	5.919	0.236	1.848	0.378	This study
47	KU-1215 Akita	*Triticum aestivum*	JPN	W3	Kyoto U	KU-1215 Akita	809	LC651687	1483	LC651753	783	Truncated	350	0.841	0.841	0.378	0.048	This study
48	KU-3136 Iran	*Triticum aestivum*	IRN	W4	Kyoto U	KU-3136 Iran	809	LC651723	1483	LC651754	783	Truncated	350	0.283	0.169	0.372	0.065	This study
49	Chihokukomugi	*Triticum aestivum*	JPN	W8	Kyoto U	Chihokukomugi	642	LC651711	n.t.	-	213	Truncated	350	3.965	1.989	0.997	0.345	This study
50	Minaminokomugi	*Triticum aestivum*	JPN	W14	Kyoto U	Minaminokomugi_213aa	642	LC651731	n.t.	-	213	Truncated	350	1.190	0.307	0.328	0.049	This study
						Minaminokomugi_Chimera	809	LC651732	2014	LC651775	940	Chimeric						
51	Chogokuwase	*Triticum aestivum*	JPN	W15	Kyoto U	Chogokuwase_213aa	642	LC651733	n.t.	-	213	Truncated	350	0.907	0.480	0.312	0.038	This study
						Chogokuwase_Chimera	809	LC651734	2014	LC651776	940	Chimeric						
52	Timstein	*Triticum aestivum*	USA	W39	Kyoto U	Timstein	809	LC651735	n.t.	LC651760	278	Truncated	350	6.074	0.554	0.395	0.109	This study
53	Suwon 235	*Triticum aestivum*	KOR	W42	Kyoto U	Suwon 235	642	LC651710	n.t.	-	213	Truncated	350	1.642	0.467	0.314	0.072	This study
54	Shirasekomugi	*Triticum aestivum*	JPN	W47	Kyoto U	Shirasekomugi	809	LC651736	28	LC651766	278	Truncated	350	6.349	0.593	2.330	0.302	This study
55	Minaminokaori	*Triticum aestivum*	JPN	W51	Kyoto U	Minaminokaori	809	LC651700	28	LC651769	278	Truncated	350	0.806	0.195	0.410	0.060	This study
56	Hokushin (Kyoto)	*Triticum aestivum*	JPN	W56	Kyoto U	Hokushin (Kyoto)	642	LC651712	n.t.	-	213	Truncated	350	3.770	1.887	0.471	0.115	This study
57	Geummil	*Triticum aestivum*	KOR	W57	Kyoto U	Geummil	642	LC651713	n.t.	-	213	Truncated	350	0.409	0.409	0.450	0.051	This study
58	Norin 61 (Kyoto)	*Triticum aestivum*	JPN	N61	Kyoto U	Norin 61 (Kyoto)_213aa	642	LC651721	n.t.	-	213	Truncated	350	0.056	0.056	0.372	0.029	This study
						Norin 61 (Kyoto)_Chimera	809	LC651722	2014	LC651777	940	Chimeric						
59	Norin 61 (10+)	*Triticum aestivum*	JPN	IT1	Kyoto U	Norin 61 10+_213aa	642	GCA_904066035	n.t.	-	213	Truncated	350	0.379	0.379	n.t.	n.t.	Walkowiak et al., 2020
						Norin 61 10+_Chimera	809	GCA_904066035	2014	GCA_904066035	940	Chimeric						
60	Cadenza	*Triticum aestivum*	GBR	IT3	Kyoto U	Cadenza 10+	809	GCA_902810645	28	GCA_902810645	278	Truncated	350	0.888	0.389	n.t.	n.t.	Walkowiak et al., 2020
61	Consort	*Triticum aestivum*	GBR	n.a.	HRO	Consort	207	LC651697	n.t.	-	68	Truncated	350	n.t.	n.t.	n.t.	n.t.	This study
62	Chinese Spring (IWGSC)	*Triticum aestivum*	CHN	n.a. (seq)	n.a.	Chinese Spring (IWGSC)	809	GCA_900519105	1483	GCA_900519105	783	Truncated	350	n.t.	n.t.	n.t.	n.t.	Zhu et al., 2021
63	Kronos (10+)	*Triticum turgidum*	USA	IT2	Kyoto U	Kronos 10+	809	GFFU01000000	28	GFFU01000000	278	Truncated	350	5.255	0.810	n.t.	n.t.	URL[6]
64	Lancer	*Triticum aestivum*	AUS	IT6	Kyoto U	Lancer 10+	809	GCA_903993975	1483	GCA_903993975	783	Truncated	350	3.539	0.890	n.t.	n.t.	Walkowiak et al., 2020
65	Mace	*Triticum aestivum*	AUS	IT5	Kyoto U	Mace 10+	809	GCA_903994175	28	GCA_903994175	278	Truncated	350	5.645	0.739	n.t.	n.t.	Walkowiak et al., 2020
66	OW104	*Triticum aestivum*	JPN	n.a.	HRO	OW104	642	LC651699	n.t.	-	213	Truncated	350	n.t.	n.t.	n.t.	n.t.	This study
67	Paragon	*Triticum aestivum*	GBR	IT4	Kyoto U	Paragon 10+	642	GCA_902810665	n.t.	-	213	Truncated	350	5.717	0.225	n.t.	n.t.	Walkowiak et al., 2020
68	Polonicum	*Triticum turgidum*	CHN	n.a. (seq)	n.a.	Polonicum (TSA)	809	TSA_GEBP01315160	1483	TSA_GEBP01315160	783	Truncated	350	n.t.	n.t.	n.t.	n.t.	TSA_GEBP01315160
69	Robigus	*Triticum aestivum*	GBR	IT7	Kyoto U	Robigus 10+	537	GCA_902810665	n.t.	-	178	Truncated	350	0.702	0.305	n.t.	n.t.	Walkowiak et al., 2020
70	Stronghold	*Triticum turgidum*	CAN	n.a. (seq)	n.a.	Stronghold 10+	303	SAMN11375118	n.t.	-	100	Truncated	350	n.t.	n.t.	n.t.	n.t.	URL[7]
71	SY Mattis	*Triticum aestivum*	FRA	IT10	Kyoto U	SY Mattis 10+	642	GCA_903994185	n.t.	-	213	Truncated	350	0.615	0.495	n.t.	n.t.	Walkowiak et al., 2020
72	Yumechikara	*Triticum aestivum*	JPN	n.a.	HRO	Yumechikara	207	LC651698	n.t.	-	68	Truncated	350	n.t.	n.t.	n.t.	n.t.	This study
73	Kitahonami (NARO)	*Triticum aestivum*	JPN	I48	NARO	Kitahonami (NARO)	null	n.a.	null	n.a.	0	no potein	350	6.726	0.086	3.329	0.188	This study
74	Arche	*Triticum aestivum*	FRA	I51	INRA	Arche	null	n.a.	null	n.a.	0	no potein	350	0.600	0.328	0.236	0.198	This study
75	Marquis	*Triticum aestivum*	CAN	I57	INRA	Marquis	null	n.a.	null	n.a.	0	no potein	350	5.135	0.164	0.880	0.341	This study
76	Recital	*Triticum aestivum*	FRA	I64	INRA	Recital	null	n.a.	null	n.a.	0	no potein	350	0.000	0.000	0.312	0.071	This study
77	Zenkojikomugi	*Triticum aestivum*	JPN	W9	Kyoto U	Zenkojikomugi	null	n.a.	null	n.a.	0	no potein	350	0.565	0.241	0.360	0.075	This study
78	Kitanokaori	*Triticum aestivum*	JPN	W11	Kyoto U	Kitanokaori	null	n.a.	null	n.a.	0	no potein	350	7.501	0.099	1.509	0.464	This study
79	Hasuyokoi	*Triticum aestivum*	JPN	W12	Kyoto U	Hasuyokoi	null	n.a.	null	n.a.	0	no potein	350	1.166	0.782	0.332	0.064	This study
80	Kitahonami (Kyoto)	*Triticum aestivum*	JPN	W13	Kyoto U	Kitahonami (Kyoto)	null	n.a.	null	n.a.	0	no potein	350	7.160	0.096	2.615	0.284	This study
81	Mironovskaya 808	*Triticum aestivum*	RUS	W31	Kyoto U	Mironovskaya 808	null	n.a.	null	n.a.	0	no potein	350	2.914	0.701	0.330	0.043	This study
82	Nanbukomugi	*Triticum aestivum*	JPN	W46	Kyoto U	Nanbukomugi	null	n.a.	null	n.a.	0	no potein	350	5.752	0.105	1.278	0.369	This study
83	Hasuyutaka	*Triticum aestivum*	JPN	W48	Kyoto U	Hasuyutaka	null	n.a.	null	n.a.	0	no potein	350	5.426	0.800	0.344	0.048	This study
84	Cheyenne	*Triticum aestivum*	USA	W54	Kyoto U	Cheyenne	null	n.a.	null	n.a.	0	no potein	350	5.315	0.114	0.966	0.324	This study
85	RW-12	*Triticum aestivum*	JPN	W60	Kyoto U	RW-12	null	n.a.	null	n.a.	0	no potein	350	3.962	1.931	0.461	0.152	This study
86	Claire	*Triticum aestivum*	GBR	IT9	Kyoto U	Claire 10+	null	GCA_902810655	null	GCA_902810655	0	no potein	350	0.588	0.227	n.t.	n.t.	Walkowiak et al., 2020
87	Julius	*Triticum aestivum*	BEU	IT13	Kyoto U	Julius 10+	null	GCA_903994195	null	GCA_903994195	0	no potein	350	2.096	0.941	n.t.	n.t.	Walkowiak et al., 2020
88	Landmark	*Triticum aestivum*	CAN	IT11	Kyoto U	Landmark 10+	null	GCA_903995565	null	GCA_903995565	0	no potein	350	6.948	0.364	n.t.	n.t.	Walkowiak et al., 2020
89	Stanley	*Triticum aestivum*	CAN	IT8	Kyoto U	Stanley 10+	null	GCA_903994155	null	GCA_903994155	0	no potein	350	5.928	0.966	n.t.	n.t.	Walkowiak et al., 2020
90	Weebil	*Triticum aestivum*	MEX	IT15	Kyoto U	Weebil 10+	null	GCA_902810675	null	GCA_902810675	0	no potein	350	0.480	0.374	n.t.	n.t.	Walkowiak et al., 2020
91	Zavitan	*Triticum dicoccoides*	ISR	n.a. (seq)	n.a.	Zavitan	null	GCA_002162155	null	GCA_002162155	0	no potein	350	n.t.	n.t.	n.t.	n.t.	Avni et al. 2017

[1]Root samples. Scores log10 (WYMV copies in 20 ng RNA) transformed.

[2]Leaf samples. Negative control scores obtained from cv. Hokushin in un-infested field was 0.355 ± 0.043, buffer control 0.371 ± 0.037.

[3]On-line sequence data

[4]Missense type of 940 aa

[5]https://opendata.earlham.ac.uk/opendata/data/Triticum_turgidum/EI/v1.1/

[6]https://iwgsc.versailles.inra.fr/download/iwgsc/TGAC_WGS_assemblies_of_other_wheat_species/

n.t.: not tested, n.a.: not applicable, null: no CS618 amplicon produced.

Table 3-2. Cultivars with *Ym2* encoding a 940-residue protein that had amino acid changes from that of Madsen.

	Amino acid positions													
Cultivars	66	189	189	235	247	248	280	318	377	403	410	754	771	788	851
Spelta10+	D66E		W189S	D235V	S247P	V248E	R280Y								
M45/66	D66E			D235V	S247P	V248E	R280Y								
Svevo10+, W7984		W189R		D235N	S247P	V248E					H410T		V771M		
ArinaLrFor10+, Jagger10+			W189S					L318R	A377S	R403H			V771M		
Akadaruma, Nanking No. 25														H788Q	
Hope (INRA), Hope (Kyoto), Redman, KS831957												L754P			M851I

Underlined – with phenotype data of response to WYMV shown in Figure 3-1B.

Table 3-3. The presence/absence of *Ym2*-like genes in wheat and related germplasm. Related to Figure 3-1.

Species	Accession	Data sourse	Genome	TraesCS3B02G037500[a]	TraesCS3B02G035800[b]	TraesCS3D02G03290[c]	Homologs in outgroup[d]
Triticum aestivum	Chinese Spring	Ensemble plants (GCA_900519105.1)	A	–	–	–	–
			B	TraesCS3B02G037500_763aa	TraesCS3B02G035800_949aa	–	–
			D	–		TraesCS3D02G032900_943aa	–
Triticum aestivum	Madsen	BAC(NCBI acc.), RNA-seq(SRA acc.)	A	–	–	–	–
			B	BAC_2213I06_g5_940aa	RNAseq_3B35800_949aa	–	–
			D	–		RNAseq_3D32900_943aa	–
Triticum aestivum	Hokushin	BAC(NCBI acc.), RNA-seq(SRA acc.)	A	–	–	–	–
			B	BAC_1412C02_g9_213aa	RNAseq_3D32900_943aa	–	–
			D	–		RNAseq_3D32900_943aa	–
Triticum aestivum	ArinaLrFor	10wheatgenomes	A	–	–	–	–
			B	chr3B_20110263_940aa	chr3B_20775958_947aa	–	–
			D	–		chr3D_9542612_943aa	–
Triticum aestivum	Cadenza	10wheatgenomes	A	–	–	–	–
			B	sca.001377_24430_278aa	sca.003374_235100_962aa	–	–
			D	–		sca.084955_14659_943aa	–
Triticum aestivum	Jagger	10wheatgenomes	A	–	–	–	–
			B	chr3B_23537852_940aa	chr3B_22866793_947aa	–	–
			D	–		chr3D_11226920_943aa	–
Triticum aestivum	Lancer	10wheatgenomes	A	–	–	–	–
			B	chr3B_19302639_763aa	chr3B_18687308_949aa	–	–
			D	–		chr3D_12354461_943aa	–
Triticum aestivum	Mace	10wheatgenomes	A	–	–	–	–
			B	chr3B_26723490_278aa	–	–	–
			D	–		chr3D_8450472_943aa	–
Triticum aestivum	Norin61	10wheatgenomes	A	–	–	–	–
			B	chr3B_21373380_213aa, chr3B_20617482_940aa	chr3B_20654632_949aa	–	–
			D	–		chr3D_12400431_943aa	–
Triticum aestivum	Paragon	10wheatgenomes	A	–	–	–	–
			B	sca.095501_24151_213aa	sca.018950_25913_947aa	–	–
			D	–		sca.087109_16095_943aa	–
Triticum aestivum	Robigus	10wheatgenomes	A	–	–	–	–
			B	sca.021276_94711_178aa	sca.069992_10491_948aa	–	–
			D	–		sca.090996_32997_943aa	–
Triticum aestivum	SY-Mattis	10wheatgenomes	A	–	–	–	–
			B	chr3B_20220435_213aa	chr3B_19431736_947aa	–	–
			D	–		chr3D_14132369_293aa	–
Triticum spelta	Spelt	10wheatgenomes	A	–	–	–	–
			B	chr3B_20943856_68aa, chr3B_20693493_940aa	chr3B_20044714_949aa	–	–
			D	–		chr3D_13367638_943aa	–
Triticum aestivum	Julius	10wheatgenomes	A	–	–	–	–
			B	–	chr3B_19757752_949aa	–	–
			D	–		chr3D_13174930_943aa	–
Triticum aestivum	Landmark	10wheatgenomes	A	–	–	–	–
			B	–	chr3B_22978738_947aa	–	–
			D	–		chr3D_12890089_943aa	–
Triticum aestivum	Stanley	10wheatgenomes	A	–	–	–	–
			B	–	chr3B_24121859_947aa	–	–
			D	–		chr3D_17367265_943aa	–
Triticum aestivum	Claire	10wheatgenomes	A	–	–	–	–
			B	–	sca.014286_23958_947aa	–	–
			D	–		sca.073631_15297_943aa	–
Triticum turgidum	Kronos	10wheatgenomes	A	–	–	–	–
			B	sca.018730_125583_278aa	sca.015583_25599_949aa	–	–
Triticum turgidum	Svevo	Ensembl plants (GCA_900231445.1)	A	–	–	–	–
			B	TRITD3Bv1G009550_940aa	TRITD3Bv1G009200_949aa	–	–
Triticum turgidum	polonicum	NCBI TSA_GEDP01315160	A	–	–	–	–
			B	TSA_GEDP01315160_763aa	TSA_GEDL01011566_949aa	–	–
Triticum turgidum	Strongfield	iwgsc WGS v1	A	–	–	–	–
			B	TGAC_c703851_100aa	TGAC_c5030037_949aa	–	–
Triticum turgidum	Cappelli	iwgsc WGS v1	A	–	–	–	–
			B	TGAC_c175416_278aa	TGAC_c346567_c5400887_949aa	–	–
Triticum dicoococides	Zavitan	Ensembl plants (GCA_002162155.1)	A	–	–	–	–
			B	–	–	–	–
Triticum urartu	G1812	Ensembl plants	A	–	–	–	–
Aegilops speltoides	subsp. *speltoides*	iwgsc WGS v1	S	–	TGAC_remap_WGS_TraesCS3B02G035800_498aa	–	–
Aegilops sharonensis	subsp. *sharonensis*	iwgsc WGS v1	S	TGAC_c23505_940aa	–	–	–
Aegilops tauschii	subsp. *strangulata*	Ensembl plants	D	–	–	AET3Gv20064800_942aa	–
Scale cereale	Lo7	NCBI WGS GCA_900079665.1	R	–	–	–	–
Scale cereale	Muskateer and Prima	NCBI TSA GCJW01009180	R	TSA_GCJW01009180_938aa	–	–	–
Hordeum vulgare	Morex	Ensembl plants (IBSC v2)	H	HORVU3Hr1G004100_940aa	–	–	–
Lolium perenne		NCBI BioProject PRJEB12921	Lp	–	–	–	PRJEB12921_sca.2000_62344_949aa
Brachypodium distachyon	Bd21	Ensembl plants	Bd	–	–	–	BRADI_2g11931v3_938aa
Oryza sativa	Nipponbare	Ensembl plants	Os	–	–	–	–
Oryza brachyantha		Ensembl plants	Ob	–	–	–	OB01G22970_955aa
Zea mays	B73	Ensembl plants	Zm	–	–	–	–
Sorghum bicolor	Moench	Ensembl plants	Sb	–	–	–	EER98470_924aa

[a] *Ym2* ortholog in cv. Chinese Spring. [b,c] *Ym2* paralogs in cv. Chinese Spring B genome and D genome, respectively. [d] RNAseq_3B35800 of Madsen was a query.

Table 3-4. Primers used in this study.

Primers	Sequences (5'-3')	Remarks
TaqMan assay for quantifying WYMV and *Actin*		
WYMV Fw	GTCACYTCCAGAAACAAA	
WYMV Rv	CTGACCACTTCTTAACTTC	
WYMV probe	[6-FAM]-CAACGCAGGACTAAAGC-[IABkFQ]	
WYMV F2	CCGTCAACGCAGGACTAAA	
WYMV R2	TGGTGATGCCAAGACTGGT	
Actin Fw	GCCAGAATAGATTCAGAA	
Actin Rv	GAGAGGAAGTACAGTGTC	
Actin probe	[TEX615]-AGACAACTCGCAACTTAGA-[IAbRQSp]	
Amplicon purification, sequencing and the identification of nucleotide variants		
CDS618Fwm105	CATACCACTTCACCCACCTTC	Single copy detection
CDS618-F1362	AATATCGGGTGGTGGAAGTG	Single copy detection
CDS618-R1877	CTWGAACGGGAGCGCTGAAC	Single copy detection
CDS618-F3650	TTGGTGATGATCCTATTGTGTG	Single copy detection
CDS618-R4115	AGAGCACGGTTACCTTCCAA	Single copy detection
CDS618-R4598	GACCCCCAAAAGTTGAATGA	Single copy detection
CDS618-R5048	CCTTCCCGGACACCTTTATC	Single copy detection
CDS618-2560F	TGGTGCACCTTACACTGCAT	Single copy detection
CDS618-2745R	CTCAAGTAAATCACCGCCTTG	Single copy detection
CDS618-R5948	ATCTCCAGTGCGTGAATGTG	Single copy detection
CDS618-3UTR295R	CGTTGACAAGGGACAAGACA	Single copy detection
CDS618-5Um1-Md	ACGATCGATCCTGCCTTGC	Duplication pattern 1 detection
CDS618-int1-55R-Md	GCGCTGAACACTCCATACAG	Duplication pattern 1 detection
CDS618-5Um1-Hk	ACGATCGATCCTGCCACC	Duplication pattern 1 detection
CDS618-int1-38R-Hk	AGCGCTGAACACTCCATATTA	Duplication pattern 1 detection
CDS618-1460R	GACAAGGGTCAATGGTCACA	Duplication pattern 1 detection
CDS618-2734R	GTGTACCCGCTCTGCTTCTT	Duplication pattern 1 detection
CDS618-141R	CATGCTGCTCATCTCGTCAC	Duplication pattern 1 detection
CDS618-841Fm-spl940	AGTTCTATATAGTGCTGGCGA	Duplication pattern 2 detection
CDS618-841Fm-spl68	AGTTCTATATAGTGCTGGCTA	Duplication pattern 2 detection
CDS618-2092R-spl940	ATGTTTATGAGACATGCTTG	Duplication pattern 2 detection
CDS618-2092R-spl68	ATGTTTATGAGACATGCTCA	Duplication pattern 2 detection
3B35800-e2_2734R	GTGTACCCGCTCTGCTTCTT	Duplication pattern 1 detection
3B35800-int2-82R	AGGGAGCATGCAACAGTAGAA	Duplication pattern 1 detection
CDS618-537F	GAGACAGGATGAATATGCCAAT	Duplication pattern 4 detection
CDS618-Rvp766	GCTCGTGATTCTGCCATGAAG	Duplication pattern 4 detection
Actin Fw	GAGAGGAAGTACAGTGTC	Housekeeping genes (positive control) (Liu et al., 2016)
Actin Rv	AGCCAGAATAGATTCAGAA	Housekeeping genes (positive control) (Liu et al., 2016)
CDS618-F2475	TTTGCGCCTTTATGTTTTCC	Duplication pattern 1 detection
CDS618-2200R	CGGACGGTTAGGGACTCAAG	Duplication pattern 1 detection
CDS618-F3246	TGCAAATATGTCGCATCACC	Duplication pattern 1 detection
CDS618-1621F	GTCAGAAATTCGCAGCACAAT	Duplication pattern 1 detection
CDS618-F663-Robm	GCAGTACATCAACAAACTGG	Duplication pattern 3 detection
CDS618-F662-Hkm	TGCAGTACATCAACAAACTAG	Duplication pattern 3 detection
CDS618-int1d126R-Robm	GGATAATAACTTGTTACCTGC	Duplication pattern 3 detection
CDS618-int1d126R-Hkm	GGATAATAACTTGTTACCTCC	Duplication pattern 3 detection

General discussion and conclusion

This study provided significant insights into the complex interactions between soil-borne viruses and Triticease crops, specifically focusing on barley and wheat. A comprehensive time series analysis was conducted to examine the multiplication of JSBWMV and BaYMV in the barley cultivar Kashimamugi using RT-qPCR from November to April (Okada *et al.*, 2023, Mishina *et al.*, 2024). The results revealed a sequential pattern of infection, with JSBWMV infecting roots prior to BaYMV, although both viruses migrate to leaves nearly simultaneously. This finding highlighted the intricate timing and coordination in virus coinfection scenarios. Severe infection persists until late February or early March, with JSBWMV dominating over BaYMV in co-infection scenarios, emphasizing the importance of early infection monitoring. Additionally, the histological analysis confirmed the presence of both viruses in various barley tissues. Implementing robust monitoring systems, including molecular diagnostics and histological analysis, can aid in the early detection and management of virus outbreaks, potentially reducing the spread and severity of infections. This study further explored resistance mechanisms in barley and wheat pangenome accessions (Jayakodi *et al.*, 2020, Walkowiak *et al.*, 2020), suggesting the involvement of genes beyond known resistance factors. However, this study employed only one susceptible genotype to compare their infection process. The infection dynamics may vary significantly across different genotypes (Mishina *et al.*, 2023, Okada *et al.*, 2023), virus isolates (Li and Shirako, 2015, Arai *et al.*, 2018), and environmental conditions (Mishina *et al.*, 2024). Future studies can incorporate a broader range of susceptible genotypes, including those susceptible to either JSBWMV or BaYMV and those resistant to these viruses. Evaluating infections under single and co-infection scenarios across multiple years and varying environmental conditions, such as temperature and humidity, would provide a more comprehensive understanding of virus interactions and host responses. Investigating the pathogenicity and resistance mechanisms in genotypes resistant to JSBWMV, such as cv. Sukai Golden, would also be an intriguing direction for future research (Okada *et al.*, 2020, Okada *et al.*, 2022, Okada *et al.*, 2023). Understanding the environmental factors that contribute to the prevalence of soil-borne diseases, such as the role of *Polymyxa graminis* as a vector, can inform cultural practices. For instance, crop rotation, proper soil management, and the use of clean seed material can minimize the risk of virus transmission and reduce disease incidence in subsequent planting seasons.

This study also investigated the genetic diversity and pathogenicity of BaYMV isolates. Previous reports have identified more than five BaYMV isolates in Japan (Arai *et al.*, 2018). The present study focuses on newly identified Japanese pathotype isolates, specifically BaYMV-Takanezawa. This investigation reveals rapid viral genome evolution within novel mutations in the CI and CP proteins associated with increased pathogenicity, particularly in the wildly cultivated barley cv. New Sachiho Golden (Oozeki *et al.*, 2017). Beyond the previously reported determinant role of the VPg protein in breaking *eIF4E*-mediated recessive resistance (Li *et al.*, 2016). These findings provided new information on the molecular mechanisms of resistance breakdown and underscored the necessity for continuous monitoring and genetic analysis of BaYMV evolution and the challenges it poses to breeding programs. Further studies, such as the elucidation of the interactions between BaYMV and other *rym*-mediated resistance genes, are needed. To control this new BaYMV isolate, developing varieties with pyramiding different resistance genes to increase resistance durability is a more reliable method. Continuous monitoring of virus populations, coupled with research into new resistance mechanisms, can help anticipate and mitigate the impact of evolving viral threats.

Furthermore, the *Ym2* gene protects wheat by blocking the WYMV's entry or suppressing its multiplication in roots (Liu *et al.*, 2016, Mishina *et al.*, 2023). Using a positional cloning approach, *Ym2* (*CDS618*) was previously isolated from the bread wheat cultivar Madsen, this gene encodes a CC-NBS-LRR protein (Suzuki *et al.*, 2015, Mishina *et al.*, 2023). Combining existing and new re-sequencing data, the study analyzed natural variations in 91 wheat accessions using the full length of *Ym2* (*CDS618*). Four distinct protein patterns were identified: 16 accessions of the Madsen haplotype, 12 with missense mutations, 44 showed truncated proteins, and 19 had null mutations. Correlations between allelic variations and disease responses revealed that the Madsen haplotype and missense mutations effectively limit WYMV accumulation in roots, demonstrating their importance in breeding for virus resistance. These findings revealed that *Ym2* is an important resistance gene against WYMV, which has been used to control this disease in Hokkaido, Japan. This study added another layer of complexity to the understanding of host-pathogen interactions and offered a pathway for selecting and developing resistance varieties. Further analysis of *Ym2* gene variants can be conducted on wheat accessions from the Wheat Resistance gene enrichment sequencing collection (WatRenSeq) (Jupe *et al.*, 2013, Wingen *et al.*, 2014, Arora *et al.*, 2019),

and employ genome-wide association analysis (GWAS) to identify new resistance genes against WYMV in wheat.

This study advances our understanding of the virus-host interactions at a cellular level and the genetic and molecular basis of resistance to soil-borne mosaic viruses in cereal crops. It highlights the need for continuous monitoring and genetic analysis to develop sustainable and effective strategies for managing viral diseases in agriculture, thereby contributing to global food security.

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