Research Paper

Utility of a GFP-expressing *Barley yellow mosaic virus* for analyzing disease resistance genes

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The soil-borne plasmodiophorid *Polymyxa graminis* is a vector for *Barley yellow mosaic virus* (BaYMV), which can severely damage barley plants. Although 22 disease resistance genes have been identified, only a few have been used for breeding virus-resistant cultivars. Recently, BaYMV strains capable of overcoming the effects of some of these genes have been detected. In this study, green fluorescent protein (GFP)-expressing BaYMV was constructed and used to examine viral dynamics in inoculated barley plants. Leaf inoculations resulted in higher infection rates than root or crown inoculations. Additionally, inoculations of some resistant cultivars produced infections that were similar to those observed in a field test. The results of this study indicate that the GFP-expressing virus is a useful tool for visualizing virus replication and dynamics, and for understanding resistance mechanisms.

Key Words: GFP, BaYMV, *rym* genes, barley, resistance gene.

Introduction

Barley yellow mosaic virus (BaYMV) belongs to the genus *Bymovirus* in the family *Potyviridae*. It has a bipartite genome comprising RNA1 and RNA2, and its particles are approximately 275 and 550 nm long (Huth *et al.* 1984). The plasmodiophorid protist *Polymyxa graminis* serves as a vector enabling BaYMV to infect barley, with infected plants exhibiting mosaic symptoms (Kusaba and Toyama 1970, Toyama and Kusaba 1970). The virus remains viable in resting spores for several years (Adams *et al.* 1988, Kanyuka *et al.* 2003). Because the resting spores are stable in soil and root debris, it is almost impossible to eliminate them from the field. The only practical method for controlling BaYMV outbreaks involves breeding disease-resistant cultivars (Kühne 2009). To date, 22 resistance (*rym*) genes have been identified (Jiang *et al.* 2020), but newly emerged virus strains have already managed to overcome the effects of some of these genes (Kashiwazaki *et al.* 1989, Kühne 2009, Sotome *et al.* 2010, You and Shirako 2013). Therefore, breeders should aim to develop new disease-resistant cultivars carrying two or more resistance genes (Werner *et al.* 2005).

In Japan, there are five (I to V) strains (pathotypes) of BaYMV, which differ regarding the susceptibility of host cultivars (Arai *et al.* 2018, Iida *et al.* 2013, Nishigawa *et al.* 2008, Sotome *et al.* 2008, 2010). For example, barley cultivar ‘Sachiho Golden’, which has *rym3*, is resistant to pathotypes I, II, and III, but is susceptible to pathotypes IV and V, whereas ‘Mikamo Golden’, which has *rym5*, is resistant to pathotypes I, II, IV, and V, but is susceptible to pathotype III. ‘Sukai Golden’, which has both *rym3* and *rym5*, is resistant to all pathotypes (Sotome *et al.* 2008, 2010). Although previous studies revealed *rym4/5/6* encode a eukaryotic translation initiation factor (eIF4E) (Kanyuka *et al.* 2005, Stein *et al.* 2005), *rym3* has not been functionally characterized. Both *rym1* and *rym11* encode protein disulfide isomerase-like 5-1 (HvPDIL5-1), which catalyzes the higher-order formation of proteins and ensures viral proteins form properly (Yang *et al.* 2014a, 2014b). The functions of the other *rym* genes remain unknown. Therefore, selecting effective resistance genes to accumulate in new cultivars is challenging. The accumulation of different functional genes may lead to stable viral resistance. In the field, *P. graminis* transmits BaYMV into the roots of susceptible plants. Therefore, root inoculations may mimic natural infections. In this study, we constructed a green fluorescent protein (GFP)-expressing virus, which was then used to inoculate selected cultivars to evaluate the utility of the virus as a tool for understanding resistance mechanisms.

Communicated by Nils Stein

Received February 25, 2021. Accepted July 2, 2021.

First Published Online in J-STAGE on September 2, 2021.

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Materials and Methods

Barley cultivars and cultivation

Barley cultivars ‘New Golden’, which has no *rym* gene and is susceptible to all BaYMV isolates, ‘Nittakei 68’ (*rym1*), ‘Sachiho Golden’ (*rym3*), ‘Miharu Gold’ (*rym5*), and ‘Muju Covered2’ (*rym12*), were cultivated in soil. More specifically, seeds were sown in polyvinyl pots containing soil and incubated at 20°C with a 10-h photoperiod. The resulting seedlings were incubated in a growth chamber with a 10-h light (13°C)/14-h dark (5°C) cycle until the four-leaf stage (about 30 days after sowing). The plants were then inoculated and incubated in a growth chamber with a 10-h light (10°C)/14-h dark (5°C) cycle.

To cultivate plants under hydroponic conditions, ‘New Golden’ barley seeds were placed on a thin sheet that was impenetrable to roots and incubated in a growth chamber at 20°C with a 10-h photoperiod. Individual seedlings were stabilized in sponge at the one-leaf stage and then incubated in a growth chamber with a 10-h light (13°C)/14-h dark (10°C) cycle until the four-leaf stage. Plants were inoculated and then incubated in a growth chamber at 13°C with a 10-h photoperiod.

Construction of infectious clones of BaYMV pathotypes

Total RNA was extracted from plants infected by BaYMV pathotype I (Nishigawa *et al.* 2008) using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) as described by the manufacturer. The RNA served as the template for synthesizing cDNA using the First-Strand cDNA Synthesis Kit Oligo (Cytiva, Tokyo, Japan) and the NotI-(dT)18 primer. A PCR amplification was completed using KOD-Plus-Neo (TOYOBO, Osaka, Japan) or the KOD One® PCR Master Mix (TOYOBO) as described by the manufacturer with some modifications; 35 cycles, 30 s at 51–54°C for annealing, and 10 s–2 min at 68°C for extension (Supplemental Table 1). Four fragments (A to D) and three fragments (A to C) covering the RNA1 and RNA2 sequences, respectively, were amplified and introduced into the p35SIV(19) vector previously used to construct infectious *Turnip mosaic virus* clones (Suchiro *et al.* 2004) to produce a full-length cDNA clone. Diagrams of the construction of pathotype I are presented in Supplemental Fig. 1. The same strategy was used to construct clones of pathotypes II (Kashiwazaki *et al.* 1990, 1991), III, IV (Nishigawa *et al.* 2008), and V (Sotome *et al.* 2010). The GenBank accession numbers of RNA1 and RNA2 of pathotype V are AB450476 and AB450477, respectively.

Construction of GFP-expressing viruses

Using pBaYMV-I-RNA2 as a template, two pathotype I DNA fragments (A and B) were amplified by PCR and sequentially cloned into pUC19 (Thermo Fisher Scientific) and then into pBaYMV-I-RNA2 to generate the empty vector pBaYMV-I-RNA2-P2/MCS (Fig. 1). This empty vector

Fig. 1. Diagram of the construction of the empty vector and the GFP expression vector. The PCR primers are presented at both ends of the amplified DNA fragments. The empty vector (pBaYMV-I-RNA2-P2/MCS) has a multiple cloning site (BamHI, SalI, and StuI). The recognition sequence for cleavage (underlined) was introduced between P2 and the stop codon. The GFP gene was introduced between the BamHI and SalI sites to generate the GFP expression vector (pBaYMV-I-RNA2-P2/GFP). These plasmids include the 35S promoter and the NOS terminator upstream and downstream of the genome, respectively.

encodes the same protease recognition site as P1/P2 and a multiple cloning site (MCS: BamHI, SalI, and StuI) between the end of the P2 gene and the stop codon. To introduce the GFP gene into this plasmid, a PCR amplification was conducted using 35S-sfGFP-nosT (Fujii and Kodama 2015) as the template and GFP-Bam-F and GFP-Sal-R as the primers. The PCR conditions were the same as those used for constructing the infectious clones. The amplified DNA fragment (735 bp) was digested with BamHI and SalI. The resulting fragment was introduced into pBaYMV-I-RNA2-P2/MCS digested with the same enzymes to produce pBaYMV-I-RNA2-P2/GFP (Fig. 1). Details regarding the PCR primers are listed in Supplemental Table 2. The same method was used to generate GFP-expressing viruses for the other pathotypes.

Inoculation

Gold particles were coated with equal amounts of RNA1 and RNA2 plasmids (2 µg/µl each). The Helios® Gene Gun System (Bio-Rad, Hercules, CA, USA) was used for inoculating plants as described by the manufacturer. Plants were inoculated at the four-leaf stage. Specifically, three older

leaves were inoculated. Additionally, the upper, middle, and lower parts of the root were inoculated. Finally, the crown was injected three times, each from a different direction.

Regarding the sap inoculation method, 300 mg GFP-expressing leaves ground in 6 ml of 0.1 M phosphate buffer, pH 7.0 (Muto Pure Chemicals, Tokyo, Japan) were used as the inoculum. Before inoculating plants, their leaves were sprinkled with carborundum. The inoculum was rubbed into four leaves per plant at the four-leaf stage.

Examination of GFP fluorescence

The MZ16F fluorescence stereomicroscope (Leica Microsystems, Wetzlar, Germany) and filters for GFP were used to detect fluorescence. Another stereomicroscope SZX12 (Olympus, Tokyo, Japan) with an adapter for GFP (NIGHTSEA, Lexington, MA, USA) was also used.

Detection of the virus by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), after which cDNA was synthesized using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The cDNA served as the template for a PCR amplification using the BaYMV-1-1-6398F (5'-CTGAGGAGCACGAAGCAGAA-3') and BaYMV-1-1-7381R (5'-CCAGAACCCGTGTGGTTGGTT-3') primers targeting RNA1 of the virus and the SapphireAmp® Fast PCR Master Mix (Takara Bio). Temperature and time parameters were 94°C for 1 min of an initial denaturation, followed by 30 cycles of 98°C for 5 s, 58°C for 5 s, and 72°C for 10 s. The final extension step was carried out at 72°C for 7 min. Another RT-PCR assay was conducted to evaluate the stability of the GFP-expressing viruses. The ISOSPIN Plant RNA kit (Nippon Gene, Tokyo, Japan) was used to extract RNA, which was then used to synthesize cDNA as previously described (Arai *et al.* 2018). The PCR amplification was completed using the RNA2-Sph-F and RNA2-Nhe-KpnI-R primers (Supplemental Table 2) targeting RNA2 of the virus and MyTaq™ DNA Polymerase (Meridian, Cincinnati, OH, USA). Temperature and time parameters were 95°C for 2 min of an initial denaturation, followed by 30 cycles of 95°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min. The final extension step was carried out at 72°C for 7 min.

Results

Construction of infectious clones and GFP-expressing viruses

We constructed infectious clones and GFP-expressing viruses for all five BaYMV pathotypes (Fig. 1). After using a gene gun to inoculate the leaves of the susceptible barley cultivar 'New Golden' (no *rym* genes) with GFP-expressing viruses at the four-leaf stage, GFP fluorescence was detected in the upper leaves that emerged at 3–4 weeks

(pathotypes I, II, IV, and V) and 6 weeks (pathotype III) post-inoculation (Fig. 2A, 2B). Disease symptoms appeared approximately 1 week after the detection of GFP. Additionally, GFP fluorescence was also observed in some roots, including lateral roots (Fig. 2D). Similar results were obtained for 'New Golden' plants infected with the GFP-expressing viruses produced for the other pathotypes (data not shown). To confirm the presence of the virus in plant parts in which GFP fluorescence was detected, GFP-expressing leaves and roots as well as non-GFP-expressing leaves and roots were analyzed by RT-PCR using virus-specific primers. Viruses were detected in both non-GFP-expressing roots as well as GFP-expressing leaves and roots (Fig. 2E). The detection of the virus in non-GFP-expressing roots may indicate the viruses did not replicate

Fig. 2. Analysis of GFP fluorescence in plants inoculated with GFP-expressing viruses. Leaves of the susceptible cultivar 'New Golden' at 6 weeks post-inoculation (pathotype V). Images are of leaves (A, B) and roots (C, D) under white light (A, C) and under UV light using a long-pass filter (B, D). Under UV light, GFP fluorescence was detected in the upper leaves and lateral roots. The MZ16F fluorescence stereomicroscope with magnification of 7.1x was used. Similar results were obtained for the other pathotypes. Detection of BaYMV in different parts of an infected plant by RT-PCR (E). 'New Golden' plants were examined at 3 months post-inoculation (pathotype I) using PCR primers (BaYMV-1-1-6398F and BaYMV-1-1-7381R) specific for the CP gene of the virus. The amplified product was approximately 1 kbp (denoted by an arrowhead). M: 1 kb DNA Ladder (New England Biolabs, Ipswich, MA, USA); 1: root of an uninoculated plant (negative control); 2: leaf of an uninoculated plant (negative control); 3: root with no GFP fluorescence from an inoculated plant; 4: leaf with no GFP fluorescence from an inoculated plant; 5: root with GFP fluorescence from an inoculated plant; 6: leaf with GFP fluorescence from an inoculated plant.

Fig. 3. Images of GFP fluorescence around the inoculation site. Susceptible cultivar ‘Miharu Gold’ (A–C) and resistant cultivar ‘Sachiho Golden’ (D–F) were examined at 1 month post-inoculation (pathotype III). (A, D) Under white light without a filter. (B, E) Under UV light with a band-pass filter. (C, F) Under UV light with a long-pass filter. The MZ16F fluorescence stereomicroscope with magnification of 7.1x was used. Small areas with replicating viruses were detected, even in the resistant cultivar. Similar images were obtained for most of the other pathotypes, but some pathotype–cultivar combinations produced different results (Supplemental Fig. 2). The presence of the virus was confirmed by RT-PCR and sequencing.

or replicated minimally in the root cells, resulting in undetectable GFP fluorescence.

An examination of the leaves inoculated using a gene gun revealed some GFP fluorescence around the inoculation site (Fig. 3). Damaged cells also produced green fluorescence, but it was distinguished from GFP fluorescence using the long-pass filter. Some resistant cultivars also had detectable GFP fluorescence around the inoculation site (Fig. 3F, Supplemental Fig. 2). The size of the GFP fluorescence area did not change, indicative of a low cell-to-cell movement activity.

Stability of GFP-expressing viruses

We subsequently analyzed the stability of the GFP gene via the sap inoculation of the susceptible cultivar ‘New Golden’. The GFP-expressing leaves were used as the inoculum. Gene stability was assessed by RT-PCR at 2 months post-inoculation. Although minor deletions in the GFP gene were detected in some plants, they did not diminish the intensity of GFP fluorescence, implying all pathotypes were stable (Fig. 4). The GFP fluorescence and disease symptom areas were almost identical in size, but GFP fluorescence was detected about 1 week earlier than the disease symptoms. Accordingly, the viral dynamics in inoculated plants can be analyzed by observing GFP fluorescence. Moreover, this experimental system may be useful for the early identification of viral replication sites.

Infection rates of BaYMV pathotypes in the susceptible cultivar ‘New Golden’

The GFP-expressing viruses were used for the sap inoculation of susceptible and resistant cultivars. The results of an RT-PCR analysis indicated the virus was present in the

Fig. 4. Stability of the GFP gene. The leaves of six (1–6) susceptible ‘New Golden’ plants were inoculated with GFP-expressing viruses (pathotype I). The upper leaves with GFP fluorescence were analyzed by RT-PCR to detect the GFP gene. Primers RNA2-Sph-F and RNA2-Nhe-KpnI-R (Supplemental Table 2) anneal to sequences flanking the GFP gene. Therefore, the amplified products were approximately 1.3 kbp (complete GFP gene) and 580 bp (truncated GFP gene). The GFP gene of only one sample had a minor deletion (lane 4). Similar results were obtained for the other pathotypes. Lanes 1 and 2: 9 weeks post-inoculation (wpi); lanes 3–6: 8 wpi; M: 500 bp DNA Ladder (Takara Bio).

upper leaves in which GFP fluorescence was detected, whereas the virus was undetectable in the non-GFP-expressing upper leaves (Fig. 2E, Table 1). The viral infection rates for ‘New Golden’ were approximately 60% for pathotypes I, II, and V, whereas they were 21% and 36% for pathotypes III and IV, respectively. We confirmed the virus was able to infect barley cultivars with and without *rym* genes, and that the infections were almost the same as those under field conditions (Table 1). However, the infection rates were lower for the cultivars with *rym* genes than for ‘New Golden’, which lacks *rym* genes.

Inoculation of crowns and roots with GFP-expressing viruses

The sap inoculation method was inappropriate for infecting roots with GFP-expressing viruses, possibly because of the hard surface and thin structure of the roots. Therefore, although the resulting infection rate was not high, the roots were inoculated using a gene gun. An examination of the inoculated ‘New Golden’ roots revealed GFP fluorescence was detectable only in some roots, including lateral roots that may have emerged after the inoculation. The RT-PCR analysis confirmed the presence of the virus in the roots, but neither the virus nor GFP fluorescence was detected in the upper leaves (Table 2).

In this study, a gene gun was used to inoculate the crown of ‘New Golden’ plants. In plants inoculated with pathotypes I, II, or IV, GFP fluorescence was observed in the newly emerged upper leaves. In plants infected with pathotype III, GFP fluorescence was detected only in the roots. In contrast, plants inoculated with pathotype V appeared to be uninfected. The RT-PCR results indicated the virus was present in the GFP-expressing upper leaves, which exhibited

Table 1. Results of the sap inoculation of leaves with GFP-expressing viruses

Patho- types	Barley cultivars	<i>rym</i> genes	Infectivity in the field ^a	The no. of inoculated plants	The no. of plants that showed GFP fluorescence ^b			The no. of virus- detected plants ^c	Infection rate (%)
					2 wpi	1 mpi	2 mpi	2 mpi	2 mpi
I	New Golden	(no)	S	13	0	4	8	8	62
	Nittakei 68	<i>rym1</i>	R	13	0	0	0	0	0
	Sachiho Golden	<i>rym3</i>	R	13	0	0	0	0	0
	Miharu Gold	<i>rym5</i>	R	13	0	0	0	0	0
	Muju Covered 2	<i>rym12</i>	R	13	0	0	0	0	0
II	New Golden	(no)	S	25	0	11	15	15	60
	Nittakei 68	<i>rym1</i>	R	15	0	0	0	0	0
	Sachiho Golden	<i>rym3</i>	R	15	0	0	0	0	0
	Miharu Gold	<i>rym5</i>	R	15	0	0	0	0	0
	Muju Covered 2	<i>rym12</i>	S	15	0	2	3	3	20
III	New Golden	(no)	S	110	0	20	23	23	21
	Nittakei 68	<i>rym1</i>	S	35	0	0	0	0	0
	Sachiho Golden	<i>rym3</i>	R	35	0	0	0	0	0
	Miharu Gold	<i>rym5</i>	S	35	0	1	3	3	9
	Muju Covered 2	<i>rym12</i>	S	35	0	1	2	2	6
IV	New Golden	(no)	S	25	0	7	9	9	36
	Nittakei 68	<i>rym1</i>	S	15	0	1	4	4	27
	Sachiho Golden	<i>rym3</i>	S	15	0	0	1	1	7
	Miharu Gold	<i>rym5</i>	R	15	0	0	0	0	0
	Muju Covered 2	<i>rym12</i>	R-S	15	0	0	0	0	0
V	New Golden	(no)	S	23	0	7	13	13	57
	Nittakei 68	<i>rym1</i>	S	18	0	0	1	1	6
	Sachiho Golden	<i>rym3</i>	S	18	0	2	3	3	17
	Miharu Gold	<i>rym5</i>	R	18	0	0	0	0	0
	Muju Covered 2	<i>rym12</i>	R	18	0	0	0	0	0

^a Results of studies by Arai *et al.* (2018) and Iida *et al.* (2013); ^b GFP fluorescence was observed in the upper leaves that emerged after inoculations; ^c RT-PCR detection of viruses in the upper leaves; R-S: infections in the field only in some years; wpi: weeks post-inoculation; mpi: months post-inoculation.

Table 2. Results of the gene gun inoculation of roots with GFP-expressing viruses

Pathotypes	The no. of inoc- ulated plants	The no. of plants that showed GFP fluorescence ^a				The no. of virus-infected plants ^b (leaf, root)	Infection rate (%) (leaf, root)
		2 wpi	1 mpi	1.5 mpi	2 mpi	2 mpi	
I	6	0	0	1	0	0, 0	0, 0
II	6	0	4	2	2	0, 2	0, 33
III	6	0	0	0	0	0, 0	0, 0
IV	6	0	0	0	2	0, 2	0, 33
V	6	0	0	0	0	0, 0	0, 0

The susceptible cultivar ‘New Golden’ was used for inoculations. ^a GFP fluorescence was observed in some young roots, including lateral roots;

^b RT-PCR detection of viruses in the leaves and roots at 2 mpi; leaf: viruses were detected in the upper leaves with GFP fluorescence; root: viruses were detected in the roots with GFP fluorescence; wpi: weeks post-inoculation; mpi: months post-inoculation.

disease symptoms. The infection rates of pathotypes I, II, III, IV, and V were 50%, 50%, 0%, 25%, and 0%, respectively (Table 3). These rates were similar to those resulting from the root inoculation.

Discussion

In the present study, we constructed GFP-expressing viruses for all five BaYMV pathotypes in Japan. These

GFP-expressing viruses were able to infect susceptible cultivars and induce disease symptoms, confirming they maintained their biological features in infected plants. Because GFP was produced during viral gene expression, we were able to examine the plant cells in which the viruses were replicating. The viruses were detected in the GFP-expressing areas by RT-PCR, which confirmed they were highly stable. Therefore, GFP-expressing viruses can be used for analyzing viral dynamics. In this study, the GFP

Table 3. Results of the gene gun inoculation of crowns with GFP-expressing viruses

Pathotypes	The no. of inoculated plants	The no. of plants that showed GFP fluorescence ^a (leaf, root)				The no. of virus-infected plants ^b (leaf, root)	Infection rate (%) (leaf, root)
		2 wpi	1 mpi	1.5 mpi	2 mpi	2 mpi	
I	4	0, 0	1, 1	1, 1	2, 2	2, 4	50, 100
II	4	0, 0	0, 2	2, 2	2, 2	2, 2	50, 50
III	4	0, 0	0, 0	0, 1	0, 2	0, 2	0, 50
IV	4	0, 0	0, 1	1, 1	1, 1	1, 1	25, 25
V	4	0, 0	0, 0	0, 0	0, 0	0, 0	0, 0

The susceptible cultivar ‘New Golden’ was used for inoculations. ^a GFP fluorescence was observed in the upper leaves that emerged after inoculations; ^b RT-PCR detection of viruses in the roots at 2 mpi; leaf: viruses were detected in the upper leaves with GFP fluorescence; root: viruses were detected in the roots with GFP fluorescence; wpi: weeks post-inoculation; mpi: months post-inoculation. If GFP fluorescence was undetectable in a plant, the roots were used for detecting viruses.

gene was inserted downstream of P2, but other positions were also tested. For example, the GFP gene positioned between NIb (replicase) and CP was expressed, but the detection of its fluorescence was delayed by about 2 weeks (data not shown).

After leaves were inoculated, GFP fluorescence was detected around the inoculation site in the susceptible and resistant cultivars (Fig. 3), but GFP fluorescence was detected in the upper leaves only in the susceptible cultivars. This observation is consistent with the results of an earlier study involving furovirus inoculations of resistant barley cultivars (Lyons *et al.* 2008). Therefore, the resistance genes (*rym* genes) analyzed in this study may encode proteins that prevent long-distance viral movement, but it is also possible that the viral replication rate was lower in the resistant plants than in the susceptible plants. When susceptible cultivars were inoculated, GFP fluorescence was detected in some roots, including the lateral roots, but some plants had weak or no fluorescence. However, the RT-PCR results indicated the virus was detectable in the roots lacking GFP fluorescence. These findings imply that GFP analyses are less sensitive than RT-PCR analyses. Additionally, the virus may not have been replicating in these root cells. These observations may be associated with differences in cellular activities, including cell division and metabolism. The infection rates resulting from root inoculations were very low, even for the susceptible cultivars. Moreover, root inoculations of the resistant cultivars did not lead to successful root infections (data not shown). These results suggest the virus was not actively replicating in the inoculated root cells, the hardness of the root surface inhibited inoculations using the gene gun, and *rym* genes were expressed and the encoded proteins were functional in the roots. Hence, we used the gene gun to inoculate the crown, in which cells are actively dividing. Although the infection rate increased slightly, for some pathotypes, it was still lower than the infection rate following the sap inoculation of leaves. Moreover, pathotypes III and V were unable to infect the susceptible cultivar ‘New Golden’. The reason for the lack of infection was not determined, but crown inoculations are likely unsuitable for testing BaYMV infections.

Leaf inoculations produced results that were almost the same as field test results. Although infections of susceptible cultivars carrying a *rym* gene were confirmed, the infection rates were lower than the infection rate for the susceptible cultivar ‘New Golden’, which lacks *rym* genes (Table 1). Thus, the expression of these *rym* genes seems to inhibit BaYMV infections. The sap inoculation of the *rym*-possessing susceptible cultivar ‘Nittakei 68’ with pathotype III did not result in an observable infection, reflecting the low infectivity of this pathotype, which also weakly infected ‘New Golden’. Under natural conditions, the virus is continuously introduced into plants via the invasion of virus-infested *P. graminis*, which may explain the high infection rates in the field.

We aimed to clarify the diversity in the effects of the *rym* genes by inoculating barley cultivars with differing *rym* genes with GFP-expressing viruses. We assumed that root inoculations would mimic natural viral infections because *P. graminis* invades plant roots. In contrast to the low infection rates following root inoculations, the infections resulting from leaf inoculations were almost identical to those observed in a field test. Accordingly, the *rym* genes examined in this study (*rym1*, 3, 5, and 12) appear to be expressed in the leaves. Viruses often break resistance mechanisms via mutations. Therefore, accumulating (pyramiding) two or more resistance genes may be necessary when breeding new cultivars. More specifically, the accumulated *rym* genes should be functionally diverse. If not, it is possible that the resistance mediated by the introduced *rym* genes will be broken simultaneously. On the basis of the data presented herein, analyses involving GFP-expressing viruses may be useful for screening resistance genes. Future studies should elucidate the effects of the *rym* genes on viral replication and dynamics. Furthermore, efficient root inoculation methods may need to be established. The findings of the current study may have implications for the breeding of new barley cultivars resistant to BaYMV and related viruses.

Author Contribution Statement

YN, TN, and HN developed the methods and designed the experiments. WW, MYumoto, TH, SM, and AT constructed the infectious clones and GFP-expressing viruses. YT and SK optimized the hydroponic cultivation conditions. MT, WW, and MYamamoto inoculated the plants. MT, WW, YNakazawa, and MYamamoto conducted microscopic analyses. TK and HN wrote the manuscript.

Acknowledgments

We thank Dr. Koichi Ishikawa for his valuable advice. This research was supported by the research program on development of innovative technology grants from the Project of the Bio-oriented Technology Research Advancement Institution (BRAIN) (30011A). We thank Yuka Egwuatu and Edanz (<https://jp.edanz.com/ac>) for proofreading a draft of this manuscript.

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