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K. Saeki · C. Miyazaki · N. Hirota · A. Saito · K. Ito
T. Konishi

RFLP mapping of BaYMV resistance gene *rym3* in barley (*Hordeum vulgare*)

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Abstract The *rym3* (formerly designated *ym3*) gene conferring resistance to barley yellow mosaic virus (BaYMV) is effective against all strains of the virus but up to now has not been mapped to any chromosome. We performed a linkage analysis, using DNA extracted from individually harvested mature leaves of 153 F_2 plants derived from a cross between BaYMV-resistant cv 'Ishuku Shirazu' carrying *rym3* and susceptible cv 'Ko A'. Additionally, the F_3 lines derived from F_2 plants were grown in the BaYMV-infested field and examined for their reaction to BaYMV. Our results indicated that *rym3* is located on the short arm of chromosome 5H and flanked by RFLP markers MWG28 and ABG705A at distances of 7.2 and 11.7 cM, respectively. The chromosomal configuration estimated by DNA markers around *rym3* and the utilization of these molecular markers for pyramiding with the BaYMV resistance genes in barley breeding programs are discussed.

Key words *Hordeum vulgare* · BaYMV · Resistance gene · RFLP · Mapping

Introduction

One of the most serious diseases of barley is caused by barley yellow mosaic virus (BaYMV) (Ikata and Kawai 1940; Huth and Lesemann 1978) which is transmitted by a soil-borne fungus, *Polymyxa graminis* Led (Toyama and Kusaba 1970). Barley seedlings infected with BaYMV show the symptoms of yellow streaks and brown necrotic patches on young leaves. Similar symptoms also appear in barley seedlings infected with barley mild mosaic virus (BaMMV), which is of the same bymovirus group as BaYMV. In Europe, BaYMV naturally attacks winter barley as a mixed infection with BaMMV (Huth et al. 1984; Huth and Adams 1990), while in Japan it is BaYMV that is predominantly widespread and recognized as the most economically damaging viral pathogen of two-rowed malting barley. BaMMV has been detected only at two limited sites, and its mixed infection with BaYMV has hardly been observed at all (Kashiwazaki et al. 1990).

BaMMV is closely related to BaYMV with respect to particle morphology of the virus and is transmitted by the same fungal vector (Huth et al. 1984). However, these two viruses clearly differ in serological properties (Huth et al. 1984; Kashiwazaki et al. 1989), nucleotide sequence of the capsid proteins (Kashiwazaki et al. 1992; Schlichter et al. 1993) and reaction to barley genotypes (Kashiwazaki et al. 1989; Götz and Friedt 1993; Ordon et al. 1997). The viruses are differentiated into strains that are distinguishable by the spectrum of pathogenicity to barley genotypes. In Japan, six strains of BaYMV have been classified, namely, BaYMV I-1, I-2, I-3, II-1, II-2 and III (Kashiwazaki et al. 1989); in Europe, two strains have been isolated, BaYMV-1 and BaYMV-2 (Huth 1989). In Japan, two strains of BaMMV, BaMMV-Ka1 and BaMMV-Na1, have been isolated (Nomura et al. 1996).

The most effective approach to prevent BaYMV and BaMMV infection is the introgression of the resistance gene(s) into modern barley cultivars. In Japan, the *rym5* gene conferring BaYMV resistance [revised from *ym5* by the new system of three-letter locus symbols in barley (Franckowiak et al. 1996)] was introgressed from a Chinese barley landrace, Mokusekko 3, into two-rowed malting cultivars, and several BaYMV resistant cultivars were released to the farmers. About 10 years later, how-

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K. Saeki · N. Hirota · K. Ito (✉)
Plant Bioengineering Research Laboratories,
Sapporo Breweries Ltd, Nitta, Gunma 370-0393, Japan

C. Miyazaki
Nagasaki Laboratory, Japan Science and Technology Corporation,
Ikeda, Ohmura 856-0026, Japan

A. Saito
Kyushu National Agricultural Experiment Station,
Nishigoshi, Kumamoto 861-1192, Japan

T. Konishi
294 Okada, Mabi-cho, Kibi-gun, Okayama 710-1311, Japan

ever, the *rym5* cultivars were suddenly damaged by BaYMV, the result of a new strain of the virus, BaYMV III, that appeared in the growing areas of the *rym5* cultivars (Ogawa et al. 1987). A similar incident also occurred in Europe. BaYMV-resistant cultivars containing *rym4* derived from a Dalmatian landrace of spring barley, Ragusa, were attacked by a new strain of the virus, BaYMV-2, in Germany and England (Huth 1989), and also in France and Belgium (Hariri et al. 1990). Consequently, the pyramiding of multiple resistance genes into a cultivar appears to be the best way to delay the breakdown of the resistance.

The *rym3* gene has the major advantage of facilitating the pyramiding for BaYMV resistance because the resistant spectrum of *rym3* is very broad against all strains of BaYMV in Japan (Kashiwazaki et al. 1989), China (Chen et al. 1992) and Europe (Ordon et al. 1992; Götz and Friedt 1993). The *rym3* gene was first detected in a BaYMV-resistant mutant, Ea 52, that was induced from susceptible cv 'Chikurin Ibaraki 1' by gamma-ray irradiation (Ukai and Yamashita 1980). This *rym3* gene is allelic to the BaYMV resistance gene of cv 'Ishuku Shirazu' (Kawada and Tsuru 1987). Though *rym3* is not effective against BaMMV (Ordon et al. 1992, 1997; Götz and Friedt 1993), BaMMV has been found only at small limited sites, and the disease symptoms of BaMMV are very mild compared with the BaYMV infection in Japan (Nomura et al. 1996).

Up to now, most of the resistance genes to BaYMV and BaMMV have been located on the respective barley chromosomes: *Ym* (renamed *rym1*) on chromosome 4H (Takahashi et al. 1973; Konishi et al. 1997), *Ym2* on chromosome 7H (Takahashi et al. 1973), *ym4*, *rym5* and *rym6* on chromosome 3H (Kaiser and Friedt 1989; Konishi et al. 1997; Iida and Konishi 1994, respectively), *ym7* on chromosome 1H (Graner et al. 1995) and *ym8*, *ym9* and *ym11* on chromosome 4H (Graner et al. 1995; Bauer et al. 1997). However, *rym3* has not been mapped to any of the chromosomes. The objectives of the investigation presented here were to locate the *rym3* resistance gene to a chromosome and to identify any restriction fragment length polymorphism (RFLP) markers linked to *rym3* that can be used for marker-assisted selection in barley breeding programs for BaYMV resistance.

Materials and methods

Plant materials and their disease assessment

A total of 153 F_2 plants derived from a cross between BaYMV-resistant cv 'Ishuku Shirazu' carrying *rym3* and susceptible cv 'Ko A' were cultivated in the BaYMV-free field and individually harvested. Since the BaYMV infestation was not uniform in the field, 10 seedlings per F_3 line were grown in three different randomized hill plots of the field infested with only the BaYMV I-1 strain (a total of 30 seedlings per line) and investigated for their reaction to BaYMV. With respect to the reaction of the F_3 lines, the F_2 individuals were classified into susceptible homozygotes, susceptible/resistant heterozygotes and resistant homozygotes.

A set of wheat-barley disomic addition lines (Islam et al. 1981) and their parents, wheat cv 'Chinese Spring' and barley cv 'Betzes', were used for the chromosome assignment of the RFLP markers by the Southern hybridization method.

RFLP probes

A total of 323 RFLP probes were kindly provided by Dr. A. Graner of the Munich Mapping Program (Graner et al. 1991), Dr. A. Kleinhofs of the North American Barley Genome Mapping Project (Kleinhofs et al. 1993b) and Dr. M. Sorrells of Cornell University (Heun et al. 1991). Most of their RFLP probes had been assembled in a consensus map (Qi et al. 1996), while some MWG probes were shown in the map constructed by Graner and Mechelen (1992). Probe labeling was performed with DIG-High Prime (Boehringer Mannheim) or by the polymerase chain reaction (PCR) direct labeling method. PCR direct labeling was conducted as described below. A reaction mixture containing 1 μ*M* M13 forward primer or SP6 promoter primer, 1 μ*M* M13 reverse primer or T7 promoter primer, 0.2 m*M* dNTP (except dTTP), 0.13 m*M* dTTP, 0.07 m*M* DIG-11-dUTP alkali-labile (Boehringer Mannheim), 2.5 U of Ampli Taq DNA polymerase (Perkin Elmer), 1×concentration of PCR reaction buffer (appended to Ampli *Taq*) and an appropriate amount of template DNA (plasmid DNA containing the insert fragment utilizable as probe) was amplified by 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min.

DNA extraction and RFLP screening by Southern hybridization

DNA was extracted from the mature leaves of the F_2 plants and their parents as described in the standard protocol of the CTAB method (Murray and Thompson 1980). Three to five micrograms of each DNA sample was individually digested with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RV, *Hin*dIII and *Xba*I. These digested DNAs were electrophoresed on a 0.8% agarose gel (LO3, Takara Shuzo) and transferred to positively charged nylon membranes (Boehringer Mannheim) by the capillary method (Sambrook et al. 1989). The prehybridization, hybridization and detection procedures were performed essentially according to the DIG-system (Boehringer Mannheim) manual. We employed the 50% formamide-high SDS buffer for the hybridization. Following the hybridization, membranes were washed for 5 min at room temperature in 2×SSC, 1% SDS; for 15 min at 56°C in 2×SSC, 1% SDS and then twice for 30 min at 56°C in 0.1×SSC, 0.1% SDS.

Linkage analysis

Segregation of the genotypes at each locus was checked by a chi-square test against the expected 1:2:1, 3:1 or 1:3 ratio in the F_2 generation. Recombination values between the markers and *rym3* were estimated by the maximum likelihood method (Allard 1956). Map positions of the *rym3* locus and the RFLP markers were calculated using MAPMAKER version 3.0 (Lincoln et al. 1993) with a minimum LOD of 3.00 using the Kosambi function (Kosambi 1944).

Results

The infection of barley seedlings grown in the field by BaYMV was so effective that resistant plants were easily distinguished from susceptible ones in the F_3 lines. The seedlings of the susceptible homozygous lines were completely infected, as was the susceptible parent, 'Ko A', whereas the resistant homozygous lines and the resistant parent, 'Ishuku Shirazu', did not show any disease symptoms. The number of susceptible plants within the susceptible/resistant heterozygous lines varied depending

Table 1 Linkage relationships between *rym3* and RFLP markers reported on chromosome 2H and the position of their markers on the chromosome

Marker	χ^2 for linkage	Recombination value (%)	Position (cM)
Group A			
ABG703	0.58	(Independent)	0.0
cMWG682	1.64	(Independent)	5.1
MWG878	2.33	(Independent)	14.2
cMWG655	3.45	(Independent)	21.3
ABG358	0.63	(Independent)	35.6
CDO64	0.49	(Independent)	48.0
ABC454	0.84	(Independent)	57.3
MWG2054	1.12	(Independent)	73.0
Group B			
ksuF15	12.81*	38.8±3.6	0.0
cMWG720	0.64	(Independent)	32.8
BCD410	13.11*	45.2±3.8	35.5
WG645	14.14**	46.5±3.8	36.5
MWG2076	14.54**	45.0±3.7	38.8
MWG989	10.86*	40.6±4.5	40.8
MWG2225	12.80*	46.0±3.7	40.9
Group C			
Bmy	6.19	(Independent)	
Group D			
CDO57A	3.01	(Independent)	
CDO57B	32.41**	28.4±3.3	
ABG316	3.28	(Independent)	
ABG316B	13.39**	13.4±3.1	
BG123B	202.37**	7.5±1.6	

*, ** Significant at the 5% and 1% levels, respectively

on genetic background and environment. Based on the reaction of each F_3 line to BaYMV, we deduced that the 153 F_2 plants consisted of 40 resistant homozygotes, 76 heterozygotes and 37 susceptible homozygotes. The segregation fit a 1:2:1 ratio well (χ^2=0.124, P=0.90–0.95), clearly indicating that the BaYMV resistance of 'Ishuku Shirazu' is governed by a single gene symbolized as *rym3*.

A preliminary analysis using F_3 lines derived from a cross between 'Ishuku Shirazu' and a multiple marker stock for chromosome 2H, *e-v-li* (Konishi et al. unpublished) gave a loose linkage between *rym3* and the *li* (*lig*) locus encoding liguleless on chromosome 2H. A similar result has also been reported from other linkage analysis of the F_2 and F_3 populations of 'Ishuku Shirazu'×several multiple marker stocks (Kawada et al. 1997).

As an initial step to mapping the *rym3* gene on barley chromosome, we focused our attention on screening for polymorphisms between the parents, 'Ishuku Shirazu' and 'Ko A', using 56 RFLP markers previously mapped on chromosome 2H (Qi et al. 1996; Graner and Mechelen 1992). Of these, 19 RFLP markers (33.9%) showed polymorphic DNA fragments; their linkage relationships with *rym3* are indicated in Table 1. Nineteen RFLP markers were classified into 4 groups by the linkage analysis. Group A consisted of 8 markers that were completely independent of *rym3* and linked in the order from ABG703 to MWG2054 on a 73-cM chromosomal segment. Referral to a consensus map (Qi et al. 1996) shows that this order is from the distal end to the proximal end on the short arm of chromosome 2H. Group B contained 7 markers that were linked and arranged from ksuF15 to MWG2225 along 40.9 cM. A chi-square test for linkage revealed that every marker of group B was loosely linked with *rym3*, except cMWG720. Judging from the magnitude of each recombination value and its standard error, however, we deduced that all the markers of group B were independent of *rym3*. These markers were located on the long arm of chromosome 2H of the consensus map. The Bmy marker of group C was completely independent of *rym3* and of the markers of groups A and B, suggesting that Bmy may be distant from groups A and B on chromosome 2H or located on a chromosome other than chromosome 2H. Linkages between *rym3* and the 3 markers CDO57, ABG316 and BG123 of group D were found. In this investigation, CDO57 and ABG316 gave two polymorphic DNA fragments between the parents. One of the CDO57 markers was completely independent of *rym3*, but the other was linked to *rym3*. Referral to the consensus map indicates that CDO57A is located on

Table 2 Segregation of RFLP markers and their linkages with *rym3* on chromosome 5H. Genotypes of F_2 plants are P_1/P_1 of 'Ishuku Shirazu' type, P_1/P_2 of heterozygous type and P_2/P_2 of 'Ko A' type

Marker	Segregation in F_2 population				Linkage with *rym3*
	P_1/P_1 :	P_1/P_2 :	P_2/P_2	Total	χ^2	Recombination value (%)
ABG316B	37	: 83	: 33	153	1.31	13.4±2.94
ABG705A	40	: 71	: 41	152	0.67	11.5±1.96
rym3	40	: 76	: 37	153	0.12	
MWG 28	36	: 77	: 39	152	0.14	7.2±1.54
CDO749	38	: 73	: 39	150	0.12	5.5±1.35
MWG2040B		112	: 40	152	0.14	8.7±2.37
MWG596	41	: 72	: 39	152	0.47	5.4±1.33
cMWG770	41	: 73	: 39	153	0.37	5.7±1.36
MWG526	40	:	113	153	0.11	4.5±1.71
BG123B	43	: 71	: 38	152	0.99	7.5±1.57
ABC302	38	: 77	: 31	146	1.11	18.3±2.55
CDO57B	37	: 83	: 33	153	1.31	28.4±3.17
MWG635C	32	: 82	: 36	150	1.52	32.4±3.46

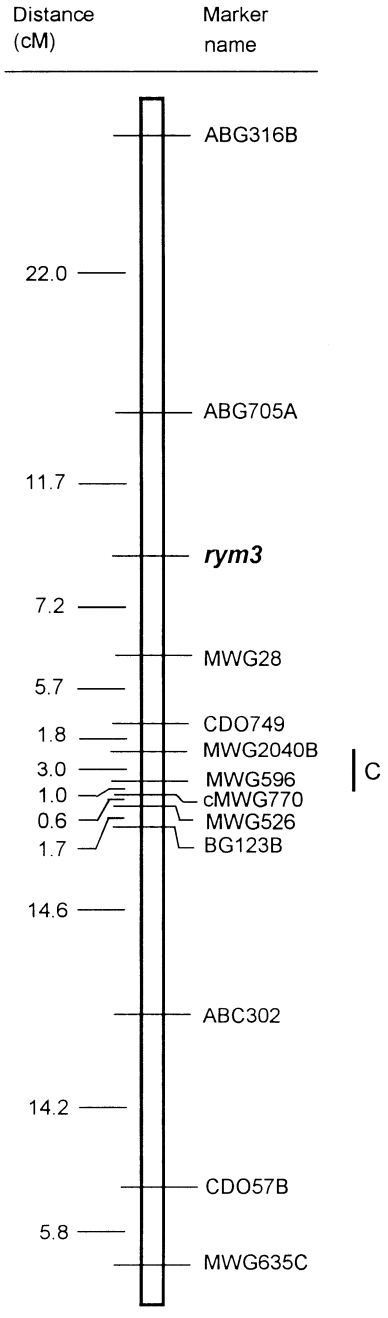

Fig. 1 Partial RFLP linkage map of chromosome 5H harboring the BaYMV resistance gene *rym3*. The positions of the RFLP markers used for linkage analysis are indicated to the *right* of the *vertical bar*, and the approximate position of the centromere is indicated by the *short vertical bar* with *C* (Kleinhofs et al. 1993). We designate the new locus mapped by the MWG635 marker as MWG635C because on the same chromosome, MWG635C is about 60 cM from the previously mapped MWG635A

chromosome 2H, whereas CDO57B is on chromosome 5H. ABG316 markers have been localized on several chromosomes in the consensus map; ABG316A on chromosome 3H, ABG316B on chromosome 5H, ABG316C, D and E on chromosome 4H and ABG316F on chromosome 1H. In addition, BG123 examined here gave one polymorphic DNA fragment, which was tightly linked with *rym3*. The consensus map indicates that BG123A is located on chromosome 2H, while BG123B is on chromosome 5H. These results suggested that *rym3* might be located on chromosome 5H.

Based on the information on the 3 RFLP markers of group D, we further examined the polymorphism against the parents using additional 16 RFLP markers mapped on chromosome 5H. As a result, 12 out of 19 RFLP markers (63.2%) gave polymorphic DNA fragments. Linkage relationships between *rym3* and these 12 markers were examined. As shown in Table 2, all markers were linked to *rym3* and their segregation patterns fit the monogenic ratio well (1:2:1, 3:1 or 1:3). The 12 markers were arranged in the order illustrated in Fig. 1, and *rym3* is flanked with ABG705A and MWG28 at map distances of 11.7 cM and 7.2 cM, respectively. To ensure that *rym3* is located on chromosome 5H, we examined the chromosomal location of 3 markers (CDO749, MWG596 and BG123B) using the wheat-barley disomic addition lines (Islam et al. 1981). In this study, their markers were recognized as being single-copy, and the polymorphic RFLP fragments identified in barley were also detected in the chromosome 5H disomic addition line (data not shown). This confirmed that the RFLP linkage group including *rym3* was mapped on chromosome 5H. Referral to the map of chromosome 5H constructed from 'Steptoe'×'Morex' data (Kleinhofs et al. 1993a) indicated that *rym3* is located on the short arm of chromosome 5H since the centromere is between *MWG2040B* and *ksuA3A*.

Discussion

One of the parents, 'Ishuku Shirazu', is characterized by resistance to all strains of BaYMV and stiff stem against lodging. The initial objective of the barley breeding program for 'Ishuku Shirazu' was to introgress an agriculturally important trait, stiff stem, from a six-rowed barley cultivar, 'Haganemugi', into two-rowed barley cultivars. During the breeding process, 'Ishuku Shirazu' was found to possess resistance to BaYMV and was released as a cultivar for feed barley at Kyushu National Agricultural Experiment Station in 1981. The pedigree of the parents, 'Ishuku Shirazu' and 'Ko A', is illustrated in Fig. 2. Kawada and Tsuru (1987) demonstrated that 'Aizu 6' and 'Haganemugi' carry the same *rym3* gene, indicating that the BaYMV resistance gene was introduced from 'Aizu 6' through 'Haganemugi' and 'Hakei J-7' into 'Ishuku Shirazu'. 'Aizu 6' was bred from a cross between 'Ken-yoshi 1' and 'Iwate Omugi 1', but it is uncertain which parent contains the BaYMV resistance gene, *rym3*. The other parent, 'Ko A', is a tworowed malting cultivar and seriously damaged by BaYMV.

The coefficient of relationship between 'Ishuku Shirazu' and 'Ko A' is estimated to be 0.1211 ($=1/2^4+1/2^7+1/2^8+1/2^6+1/2^7+1/2^8+1/2^7$) through the common ancestors 'Ebisu' and 'Golden Melon'. This coefficient shows that the parents, 'Ishuku Shirazu' and 'Ko A', are genetically not very different. It has been

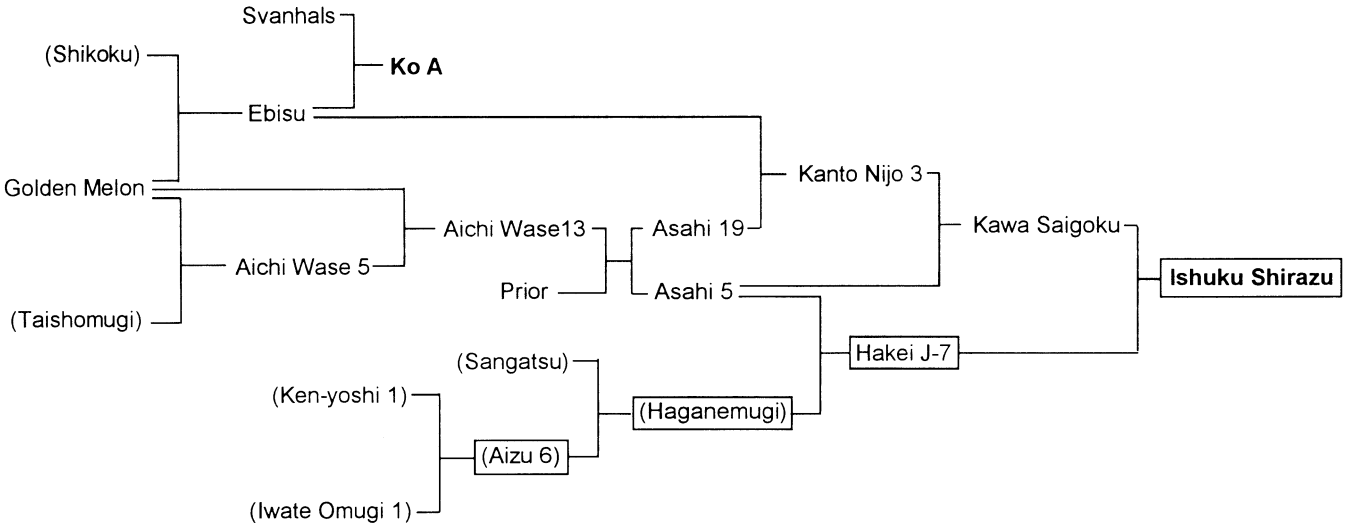

Fig. 2 Pedigree of the parents, 'Ishuku Shirazu' and 'Ko A'. The parents are shown in *bold letters*, and BaYMV-resistant varieties are indicated in *boxes*. Six-row varieties are in *parentheses*

pointed out that six-rowed varieties of the Oriental regions are highly diverged from two-rowed ones with respect to genetic constitution (Takahashi 1955; Ordon et al. 1997). Two-rowed varieties were introduced from the Occidental to the Oriental regions about 100 years ago, and cross-breeding between two-rowed and six-rowed varieties has rarely been conducted in Japan. The percentage of markers showing polymorphism on this segment of chromosome 5H (63.2%) was distinctly higher than that of chromosome 2H (33.9%), suggesting that the segment containing *rym3* derived from the BaYMV-resistant, six-rowed cv 'Haganemugi' might have been conserved through multiple crosses with susceptible two-rowed varieties.

'Ishuku Shirazu' is the first cultivar resistant to BaYMV released in Japanese two-rowed barley breeding programs, but only a few genetic studies on its BaYMV resistance have been conducted. This is largely due to the lack of suitable morphological and physiological markers for linkage analysis on the short arm of chromosome 5H around *rym3* compared with other regions of barley chromosomes. Our RFLP analysis revealed that *rym3* is mapped between ABG705A and MWG28 on the short arm of chromosome 5H. However, as indicated by Künzel and Korzun (1996), recombination on the short arm of chromosome 5H was severely suppressed. Therefore, it is necessary to determine the more tightly linked molecular markers to *rym3* for the marker-assisted selection.

As mentioned above, many "resistant" varieties have been infected by new strains of BaYMV. Among the BaYMV resistance genes, *rym3* has a broad resistant spectrum to all strains of the virus. In Japan, barley breeding for BaYMV resistance has been principally conducted using a Chinese barley landrace, Mokusekko 3, as the totally resistant parent. However, most of the cultivars and lines possessing the resistance introduced from Mokusekko 3 are infected with a new BaYMV III strain, whereas Mokusekko 3 is still resistant. The reason why Mokusekko 3 is completely resistant to all strains of the virus, including BaYMV III, is that Mokusekko 3 simultaneously contains two different *rym1* and *rym5* resistance genes, while BaYMV "resistant" cultivars and lines carry only the *rym5* gene (Konishi et al. 1997). This suggests that it is important to accumulate at least more than two resistance genes into a cultivar in barley breeding programs for complete and sustainable resistance to BaYMV, since the breakdown of the *rym3* resistant cultivars may occur by a new strain of BaYMV appearing in future.

The pyramiding of the BaYMV resistance genes *rym3* and *rym5* has been practiced at barley breeding stations in Japan. The *rym5* homozygous resistant plants are easily selected from hybrid populations between the *rym3* and *rym5* genotypes at the seedling stage in the laboratory using an esterase isozyme marker that is tightly linked to *rym5* (Konishi et al. 1989; Konishi and Kaiser 1991). The seedlings selected by marker assistance are transplanted into the BaYMV-infested field to again prove their disease resistance. Further examination for introgression of *rym3* into the *rym5* homozygotes is made by a test cross with the *rym3* tester or selection of the resistant plants grown in the field infested by BaYMV-III. When *rym3* has not been introgressed into the *rym5* homozygotes, they are susceptible to the BaYMV strain (Furusho and Fukuoka 1997; Sotome et al. 1995). These steps are laborious and time-consuming in barley breeding. Therefore, the development of suitable molecular markers closely linked to *rym3* would facilitate the preselection of the homozygous *rym3* plants at the seedling stage. In addition to *rym3*, we are attempting to tag other BaYMV resistance genes with molecular markers for the pyramiding of the BaYMV resistance in barley.

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