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Towards isolation of the BaMMV resistance gene *rym15* derived from the Japanese cultivar Chikurin Ibaraki 1

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Towards isolation of the BaMMV resistance gene *rym15* derived from the Japanese cultivar Chikurin Ibaraki 1

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Contents

List of abbreviations

AFLP	amplified fragment length polymorphism
BAC	bacterial artificial chromosome
BaMMV	*barley mild mosaic virus*
BaYMV	*barley yellow mosaic virus*
CAPS	cleaved amplified polymorphic sequences
C×U	Chikurin Ibaraki 1 × Uschi
CCCH	Cysteine3Histidine
CRISPR- Cas9	clustered regularly interspaced short palindromic repeats-associated protein 9
DArT	Diversity Arrays Technology
eIF4E	eukaryotic translation initiation factor 4E
GAB	genomics-assisted breeding
GBS	genotyping-by-sequencing
HC	high confidence
IBSC	International Barley Genome Sequencing Consortium
InDels	insertions/deletions
I×C	Igri × Chikurin Ibaraki 1
KH	K homology
KASP	competitive allele specific PCR
LC	low confidence
MAGIC	multi-parent advanced generation inter-cross
MAS	marker-assisted selection
NGS	next generation sequencing
NSE4	non-structural maintenance of chromosome element 4
PDIL5-1	protein disulfide isomerase like 5-1
QTL	quantitative trait locus
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
STS	sequence tagged site
TGS	third-generation sequencing
WGS	whole genome sequencing

List of publications

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1. Summary

Barley mild mosaic virus (BaMMV) and *Barley yellow mosaic virus* (BaYMV), members of the genus *Bymovirus* in the family *Potyviridae*, are the causal agents for barley yellow mosaic disease in winter barley in Europe and Asia. Due to transmission of BaMMV and BaYMV via the soil-borne plasmodiophorid *Polymyxa graminis*, which can survive in the soil for many years, can reinfect the roots of barley plants given the suitable environmental conditions, thus breeding of resistant cultivars is the only efficient and environmentally friendly way to prevent high yield losses caused by this disease.

In 2004, it was shown that the BaMMV resistance of Chikurin Ibaraki 1 is imparted by a single recessive gene named *rym15* that is located on chromosome 6HS. This resistance gene was previously localized in a genetic map of Chikurin Ibaraki 1 × Plaisant, however the order of flanking markers EBmac0874 and Bmag0173 was found to be inverted compared to the previous genetic map of *Hordeum vulgare* Lina × *Hordeum spontaneum* Canada Park. Therefore, in the present study, the first step towards identifying the causal gene was to construct a medium-resolution map of the chromosome segment containing *rym15*. This was achieved using a set of 522 F_2 plants derived from the two F_2 populations Igri × Chikurin Ibaraki 1 (I×C, 180 plants) and Chikurin Ibaraki 1 × Uschi (C×U, 342 plants), respectively, derived from crosses of different susceptible parents with the resistance donor. The phenotypic results revealed segregation ratios of 250s:92r (I×C, χ^2=0.659) and 140s:40r (C×U, χ^2=0.741), suggesting the presence of a single recessive resistance gene against BaMMV in Chikurin Ibaraki 1. The order of all markers was the same in both F_2 populations and in accordance with the physical map (Morex v2 genome assembly). Two single nucleotide polymorphisms (SNPs)-based competitive allele specific PCR (KASP) markers designated *rym15*_1 and *rym15*_8 were selected as new flanking markers for the target locus *rym15*. Using these two flanking markers, two sets of 139 (I×C) and

284 (C×U) segmental recombinant inbred lines (RILs) were selected from 2174 (I×C) and 5728 (C×U) F_2-plants, respectively. Subsequently, a total of 32 KASP markers were used for marker saturation of the target locus *rym15* in these RILs. High-resolution maps were constructed and the target interval was downsized to 0.161 cM and 0.036 cM in the two respective crosses, corresponding to a physical interval of 11.3 Mbp in the I×C RILs and 0.281 Mbp in the CxU RILs according to the Morex v3 genome sequence.

In the target region of 0.281 Mbp, a set of six high confidence (HC) and two low confidence (LC) genes was identified. Blast analysis revealed functional SNPs in two HC genes. This work lays the foundation for gene identification of the target locus *rym15*.

1. Zusammenfassung

Die Gelbmosaikviren BaMMV (*Barley mild mosaic virus*) und BaYMV (*Barley yellow mosaic virus*) gehören zur Gattung *Bymovirus* in der Familie der *Potyviridae* und sind die Erreger der Gelbmosaikvirose der Gerste, die insbesondere in Europa und Asien auftritt. Da BaMMV und BaYMV über den bodenbürtigen Protisten *Polymyxa graminis* übertragen werden, dessen Dauersporen über viele Jahre im Boden überdauern und bei entsprechenden Umweltbedingungen die Wurzeln der Gerstenpflanzen infizieren, ist die Züchtung resistenter Sorten der effizienteste und umweltfreundlichste Weg, um hohe Ertragsverluste durch diese Krankheit zu vermeiden.

Im Jahr 2004 wurde gezeigt, dass die BaMMV-Resistenz der japanischen Herkunft Chikurin Ibaraki 1 durch ein rezessives Resistenzgen (*rym15*) auf Chromosom 6HS bedingt ist, welches in der Population Chikurin Ibaraki 1 × Plaisant lokalisiert wurde. Es zeigte sich jedoch, dass die flankierenden Marker EBmac0874 und Bmag0173 im Vergleich zu der früheren genetischen Karte der Population *Hordeum vulgare* Lina × *Hordeum spontaneum* Canada Park invertiert vorlagen. In der vorliegenden Studie bestand daher der erste Schritt zur Isolation von *rym15* in der Erstellung einer Karte mit mittlerer Auflösung zur exakten Lokalisierung von *rym15*. Dazu wurden 522 F_2-Pflanzen derPopulationen Igri (s) × Chikurin Ibaraki 1 (r, I×C, 180 Pflanzen) bzw. Chikurin Ibaraki 1 (r) × Uschi (s, C×U, 342 Pflanzen) verwendet. Die Phänotypisierungen ergaben Spaltungsverhältnisse von 250s:92r (I×C, χ^2=0,659) bzw. 140s:40r (C×U, χ^2 =0,741), was auf das Vorhandensein eines einzigen rezessiven Resistenzgens gegen BaMMV in Chikurin Ibaraki 1 hindeutet. Die Reihenfolge aller Marker war in beiden F_2-Populationen gleich und in Übereinstimmung mit der physikalischen Karte (Morex v2 Genom-Assembly). Zwei auf SNPs (Single nucleotide polymorphisms) basierende KASP- (kompetitive allelspecific PCR) Marker (*rym15*_1, *rym15*_8) wurden als neue flankierende Marker für den Ziellocus *rym15* ausgewählt. Unter Verwendung dieser beiden flankierenden Marker wurden

aus 2174 (I×C) bzw. 5.728 (C×U) F_2-Pflanzen zwei Sätze von 139 (I×C) und 284 (C×U) segmentalen RILs (rekombinante Inzuchtlinien) erstellt. Anschließend wurden insgesamt 32 KASP-Marker für die Markerabsättigung des Ziellocus *rym15* in diesen segmentalen RILs verwendet. Basierend auf dieser hochauflösenden Kartierung, wurde das Zielintervall in den beiden Kreuzungen auf 0,161 cM bzw. 0,036 cM verkleinert. Dies entspricht gemäß der Morex v3-Genomsequenz einem physischen Intervall von 11,3 Mbp in der I×C-Population und von 0,281 Mbp in der CxU-Population.

In der Zielregion von 0,281 Mbp wurden sechs Gene mit hoher Signifikanz (HC) und zwei mit niedrigerer Signifikanz (LC) identifiziert. Die Blast-Analyse ergab funktionelle SNPs in zwei HC-Genen. Diese Arbeit bildet die Grundlage für die Isolation des Resistenzgens *rym15*.

2. Introduction

2.1. Barley

Barley (*Hordeum vulgare* L.) belongs to the genus *Hordeum* in the tribe *Triticeae* of the grass family *Poaceae* (Gaut, 2002). It is the fourth most important cereal crop with respect to production area after wheat, maize, and rice. To date, barley is produced in more than 100 countries (FAOSTAT, 2022). In 2020, barley was cultivated on 51.6 million hectares with a production of 157.03 million tons worldwide, of which 61%, 14.9%, 13.1%, 4.5% and 6.6% were produced in Europe, Asia, Americas, Africa and Oceania, respectively (FAOSTAT, 2022). In the last decade (2011-2020), as the largest producer of barley, the average production in each year was around 18.1 million tons in the Russian Federation, followed by France and Germany with an average production of 11.3 and 10.6 million tons, respectively (FAOSTAT, 2022).

As one of the founder crops of Old World agriculture, wild barley (*Hordeum spontaneum*) is the progenitor of cultivated barley (*Hordeum vulgare* L.) (Zohary et al., 2012). Evidence from ancient barley grains discovered in the Near East Fertile Crescent indicate that barley was domesticated in that region about 8,000 B.C (Nesbitt and Samuel, 1996). This hypothesis was supported by Badr et al. (2000), who used data from 400 amplified fragment length polymorphism (AFLP) markers in 317 wild and 57 cultivated barley lines to determine that the Israel-Jordan region is the domestication area of barley. Additional studies confirmed that the Fertile Crescent is the domestication region of barley (Lev-Yadun et al., 2000; Zohary et al., 2012). Furthermore, analysis of the *Bkn-3* allele indicated that the Himalayas may be considered a region of domesticated barley diversification (Badr et al., 2000). Using 1,309 DArT (Diversity Arrays Technology) markers, Dai et al. (2012) analyzed the genetic differentiation between wild barleys from the Near East and Tibet. The results indicated that the Tibetan Plateau and its vicinity is another domestication center of cultivated barley.

Barley is mainly used for animal feed, malting and human food. Between 2011 and 2020, around 65%, 20%, 5% and 7% of the total global barley consumption were respectively used for animal feed, industrial, human food and other purposes (Badea and Wijekoon, 2021). In most countries, barley is mainly used as livestock feed and only a small portion is used for human nutrition (Giraldo et al., 2019). However, in some areas of North Africa and the Near East, which are characterized by harsh living conditions, barley is still a major food (Badea and Wijekoon, 2021). Moreover, more than 69% of farmland is used for barley cultivation in Tibet. Naked (hulless) barley is still a major stable food in this region, accounting for more than 65% of the total food production (Al-Menaie et al., 2013).

Thanks to its specific chemical composition and health benefits, the use of barley in processed foods has recently increased. Barley grains contain significant quantities of medicinally beneficial functional compounds, such as β-glucan, vitamin E and resistant starch. Those compounds can reduce serum cholesterol and blood glucose levels, and promote intestinal function (La et al., 2022). Additionally, barley grass is rich in functional ingredients, such as calcium, iron, magnesium, β-carotene, chlorophyll, gamma-aminobutyric acid, tryptophan, vitamins (A, B1, B2, B6, C and K), alkaloid and polyphenols (Lahouar et al., 2015; Zeng et al., 2018).

2.2. Barley yellow mosaic disease

Plant diseases are major factors causing severe yield losses and quality reductions. Barley yellow mosaic disease was first reported in Japan (Ikata and Kawai, 1940). Subsequently, during the 1950s, the disease was found in China (Kühne, 2009; Ruan et al., 1984; Zhou and Cao, 1985) and South Korea (Lee et al., 1996, 2006; Park et al., 2005). Around 1980s, due to the barley yellow mosaic disease, serious yield losses occurred in Northern Europe, especially in Germany (Huth and Lesemann, 1978; Huth, 1984; Proeseler et al., 1984). Later on, the disease was detected in many other countries such as the United Kingdom (Hill and Evans, 1980), the Netherlands (Langenberg and Van Der Wal, 1986), Ukraine (Fantakhun et al., 1987), France

(Signoret and Huth, 1993), Spain (Achon et al., 2005) and Poland (Jezewska and Trzmiel, 2009). Moreover, a BaYMV isolate was identified in Iran, which is the first occurrence of such viruses in this region (Hosseini et al., 2014). Barley yellow mosaic disease can cause yield losses up to 50% when susceptible barley varieties are grown on infested soils (Plumb et al., 1986; Huth, 1989a; Adams and Hill, 1992). In case of extended periods of frost, infected susceptible barley varieties are more sensitive to frost damage, which can lead to complete yield losses (Huth, 1988). In China, the occurrences of this disease caused nearly 100% yield losses of barley in the mid-1970s (Chen, 2005; Kühne, 2009).

Barley yellow mosaic disease is caused by two different viruses, *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) (Huth and Adams, 1990). Both viruses have similar particle morphology (Huth et al., 1984) and belong to the genus *Bymovirus* in the family *Potyviridae*, transmitted by the root-inhabiting fungal-like plasmodiophorid *Polymyxa graminis* (Adams et al., 1988). The differences between two viruses are mainly reflected in the 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 the reactions of barley cultivars to virus inoculation (Huth and Adams, 1990; Götz and Friedt, 1993; Ordon et al., 1997). The virus can be differentiated into strains according to the spectrum of pathogenicity to barley genotypes. In Europe, two strains of BaYMV (BaYMV and BaYMV-2) and three strains of BaMMV (BaMMV, BaMMV-SIL and BaMMV-Teik) have been identified (Huth, 1989b; Huth and Adams, 1990; Hariri et al., 2003; Kühne et al., 2003; Kanyuka et al., 2004; Habekuss et al., 2008). In Japan, eight strains in five pathological groups (I to V) of BaYMV, i.e. I-1, I-2, I-3, II-1, II-2, III, IV, and V (Kashiwazaki et al., 1989; Okada et al., 2004; Sotome et al., 2010) as well as two Japanese BaMMV strains (BaMMV-Kal and BaMMV-Nal) have been classified (Nomura et al., 1996). In Korea, six BaYMV isolates (Yeonggwang, Gunsan, Goseong, Jeonju, Gangin, and Daegu) and two BaMMV isolates (Yeonggwang and Gunsan) are known (Jo et al., 2018). In China, more than 10 strains of both viruses were identified so far (Chen et al., 1992; Chen et al., 1999; Zheng et al., 1999).

BaMMV and BaYMV are positive-sense single stranded RNA-viruses with a similar genomic organization comprising two RNA molecules designated RNA1 and RNA2 (Kashiwazaki et al., 1990; Kashiwazaki et al., 1991; Davidson et al., 1991; Peerenboom et al., 1996). RNA1 is approximately 7.7 kbp and encodes for the coat protein (CP), the cytoplasmatic inclusion protein (C1), the protease (NIa), the genome-linked viral protein (VPg), the RNA-dependent RNA polymerase (NIb) as well as the 6K1, 6K2 and P3 proteins (Kashiwazaki et al., 1990). RNA2 is approx. 3.6 kbp and only encodes for the two proteins P1 (Cysteine proteinase activity) and P2 (unknown function), which might not be absolutely required for viral replication in infected cells (Davidson et al., 1991; You and Shirako, 2010). The comparison of the complete nucleotide sequence of RNA1 and RNA2 of BaMMV and BaYMV revealed a low level of sequence identity (Kashiwazaki, 1996; Meyer and Dessens, 1996).

In natural infection, the virus moves from the roots to the leaves, which usually occur in autumn at temperatures below 20 °C. Symptoms of the virus infection will be visible on the leaves in winter or early spring. During spring, when the average temperature increases and exceeds 20 °C, disease symptoms disappear (Jiang et al., 2020). In general, infected plants show a mosaic of pale green and yellow discolorations on young leaves, stunted growth, reduced number of tillers and a delayed maturation (Jiang et al., 2020). The virus may remain virulent inside the resting spores of the vector *P. graminis* for longer than a decade (Huth, 1991). When environmental conditions are suitable, the virus infection will be reinitiated on the susceptible host plants, thus the use of virus-resistant barley cultivars is the only practical and environmentally friendly way to control soil-borne barley yellow mosaic disease (Kanyuka et al., 2003).

2.3. BaMMV/BaYMV resistance genes in barley

Up to now, twenty-two resistance genes have been reported, of which 19 are recessively inherited resistance genes derived from the primary (*H. vulgare*/*spontaneum*) gene pool of barley, two are dominant resistance loci (*Rym14*

and *Rym16*) that originate from the secondary gene pool of *H. bulbosum* and a third dominant locus *Rym17* is derived from a Pakistani barley accession (see review of Jiang et al., 2020). The first isolated BaYMV/BaMMV resistance gene *rym4*, which encodes the eukaryotic translation initiation factor 4E (*eIF4E*), was initially mapped on chromosome 3HL in the early 1990s (Graner and Bauer, 1993; Kanyuka et al., 2005; Stein et al., 2005). Subsequently, the amino acid substitutions and/or insertions/deletions (InDels) of *eIF4E* were reported to be the causal function of several allelic genes including *rym5*, *rym6*, *rym10*, $rym_{HOR4224}$ and $rym_{HOR3298}$ (see review of Jiang et al., 2020). Another cloned BaMMV/BaYMV resistance gene, *rym1*/*11*, encodes a protein disulfide isomerase like 5-1 (*PDIL5-1*), which causes resistance due to non-functional alleles or amino acid substitutions of the host factor gene *HvPDIL5-1* (Yang et al., 2014a, b).

In Japan, initial barley resistance breeding programs used only a single resistance gene *rym5* (Ogawa et al., 1987). However, the resistance of this gene was rapidly overcome by the new isolate BaYMV III (Kashiwazaki et al., 1989). Similarly, the majority of resistant barley cultivars in Europe carried *rym4* as the only resistance source (Stein et al., 2005). However, in the late 1980s, *rym4* was overcome by the new virus isolate BaYMV-2 in Germany and the United Kingdom, and later in other European countries (Huth, 1989b; Hariri et al., 1990; Adams, 1991; Steyer et al., 1995; Kühne et al., 2003). In addition, the *rym4* allelic resistance gene *rym5*, which is also efficient against BaYMV-2, was overcome by the European isolates BaMMV-Teik and BaMMV-SIL (Habekuss et al., 2008; Kanyuka et al., 2004). In East Asia, BaYMV resistance conferred by *rym1*/*11* has also been overcome (Shi et al., 2019; Jiang et al., 2022). In China, the resistance gene *rym4* is not effective against BaYMV isolates BaYMV-CN_NY and BaYMV-CN_YZ, and isolates of both BaMMV (BaMMV-CN_NY and BaMMV-CN_YZ) and BaYMV (BaYMV-CN_DZ and BaYMV-CN_NY) are virulent to *rym5* (Jiang et al., 2022). Thus, the identification of new BaMMV/BaYMV resistance resources is critical in future barley breeding programs. Another option is the creation of new alleles of resistance genes by targeted mutagenesis of the cloned recessive resistance genes *rym4*/*5* and *rym1*/*11* by Cas9

endonuclease in BaMMV/BaYMV-susceptible barley cutivals (Hoffie et al., 2021).

2.4. Molecular markers

The development of DNA marker technologies in the 1980s had a major impact on plant breeding (Nadeem et al., 2018), and molecular markers are now a powerful tool for marker-assisted selection (MAS), quantitative trait locus (QTL) analysis and genetic association studies. DNA markers include (1) hybridization-based restriction fragment length polymorphism (RFLP), (2) PCR-based markers like AFLPs, cleaved amplified polymorphic sequences (CAPS), sequence tagged sites (STS) or simple sequence repeat (SSR) markers, and (3) single nucleotide polymorphism (SNP) markers identified by DNA sequencing (Jones et al., 2009).

The RFLP technique was developed in the beginning of 1980s. In this marker technology, restriction enzymes cut DNA at restriction sites resulting in fragments with varying length that can be sorted by gel electrophoresis. Next, Southern blotting is conducted and the membranes are exposed to fluorescence- or radioactive-labelled DNA probes, which hybridize with complementary DNA sequences (Beckmann and Soller, 1983). RFLPs are co-dominant, reliable, highly reproducible and can be used for gene mapping, QTL analysis and genetic fingerprinting. However, RFLPs have three disadvantages: (1) the number of detected independent loci is low, (2) a high quality and large amount of DNA is needed, and (3) it is a laborious and expensive approach (Beckmann and Soller, 1983). For these reasons, RFLP has now been superseded by PCR- or sequencing-based markers.

The PCR-based CAPS technique was originally named PCR-RFLP markers due to a combination of RFLP and PCR (Maeda et al., 1990). First the target DNA is amplified by PCR, then the products are cleaved by restriction enzymes on specific sites, and an agarose gel or acrylamide gel is used to detect the presence/absence of restriction sites (Konieczny and Ausubel, 1993). CAPS markers are co-dominant, have no requirement for radioactivity or blotting and have been applied in MAS and map-based cloning studies (Weiland and Yu, 2003; Spaniolas et al., 2006). As for

CAPS, AFLP markers also combine the RFLP and PCR technology to detect variations in the restriction sites, i.e. DNA is digested followed by PCR (Vos et al., 1995). First, two restriction enzymes are used for the digestion of DNA, next each end of the resulting fragments is ligated with adapters. Then, using primers developed according to the known sequences of adapters plus additional bases, a subset of fragments is amplified using PCR and visualized by gel electrophoresis (Becker et al., 1995). AFLPs are dominant, detect a large number of loci and reveal a high number of polymorphisms (Thomas et al., 1995; Keim et al., 1997; Qi et al., 1998). Co-dominant STS markers are a class of molecular markers for specific amplification with a specific primer sequence, which is identified by a known and unique DNA sequence of 200 to 500 base pairs (Jones et al., 2009). SSR markers, also known as microsatellites (Litt and Luty, 1989), are short (one to six nucleotides), tandemly repeated DNA sequences. They are amplified by PCR to generate DNA fragments which can be distinguished by high-resolution gel or capillary electrophoresis based on the varying number of repeated sequences in microsatellite regions between individuals (Kalia et al., 2011). The development of SSR markers is expensive and time consuming. However, SSR analysis requires only a small amount of DNA. Due to the properties of co-dominance and high reproducibility, SSR markers have been used for mapping, population genetic analyses, determination of hybridity, protection of germplasm, cultivar identification, gene pool variation analysis and as diagnostic markers of economically valuable traits (Powell et al., 1996; King et al., 2008).

In the late 1990s, as increasing quantities of DNA sequence became available, focus changed to SNP markers. SNPs are the most common DNA variants in eukaryotic genomes, occurring in the range of one SNP every 100–300 bp (Xu, 2010). SNPs derive from single-nucleotides substitutions which can be transversions [purine-pyrimidine exchanges (C/G, T/A, C/A or G/T)] or transitions [purine-purine or pyrimidine-pyrimidine exchanges (C/T or G/A)], and are distinguished from InDels (Vignal et al., 2002; Hayward et al., 2012). They are co-dominant markers with high reproducibility and are usually bi-allelic (Casci, 2010). SNPs have arisen by mutations during evolution. Thus, the distribution patterns are different among

individuals. However, those individuals that have a common ancestor are more likely to share identical SNP alleles (Jones et al., 2009).

Due to the above mentioned characteristics, SNPs are the basis for SNP microarrays such as the Illumina InfiniumTM assay (Imelfort et al., 2009). In case of barley, the 9k Illumina SNP chip (Comadran et al., 2012) was constructed using SNP data from 10 different cultivated barley genotypes. This SNP chip includies roughly 7,900 SNPs, of which 36% (2,832) are derived from the former Barley Oligonucleotide Pooled Assay (BOPA) markers (Close et al., 2009) and 64% (5,010) are new. Five years later, the 50k Illumina SNP chip (Bayer et al., 2017) was developed from exome capture data of 170 cultivated accessions and the published barley pseudomolecule assembly (Beier et al., 2017; Mascher et al., 2017). The 50k Illumina SNP chip contains 44,040 working assays, of which 14% (6,251) are from the 9k iSelect platform, and the rest (86%) are new SNPs (Bayer et al., 2017). Today, SNP markers are the most commonly used markers for the detection of genetic diversity in various crops (Baloch et al., 2017) and for construction of linkage maps in several plant species (Semagn et al., 2006; Majeed et al., 2019).

Another widely used SNP genotyping platform is Competitive Allele Specific PCR (KASP) designed by LGC Genomics (https://www.lgcgroup.com/). Three components are needed for this assay: (1) template DNA of samples, (2) a common primer and two competitive allele-specific primers having one fluorophore (FAM or HEX) attached as a tail, and (3) Taq polymerase and fluorescence resonance energy transfer (FRET) cassettes (KASP Master Mix) in the buffer solution. The KASP assay can detect both alleles in a single reaction. It has many advantages, for example being cost effective, simple, tolerant to variability in DNA quality and able to assay a large number of individuals in a very short time (He et al., 2014a). KASP markers are frequently applied in marker-assisted recurrent selection, marker-assisted backcrossing and fine mapping of genes of interest (Semagn et al., 2014).

In addition, genotyping-by-sequencing (GBS) is an important tool for generating new markers (He et al., 2014b). Nowadays, GBS has become a low-cost, powerful and simple platform to detect SNPs or InDels in almost all species (Elshire et al., 2011).

First, the sample DNA is digested with one or two specific restriction enzymes and a barcode adapter is ligated to the end of DNA fragments. Then, the sample DNA is amplified by PCR and the products are pooled. The pooled samples are then processed with next generation sequencing (NGS) technologies (He et al., 2014b). GBS is rapid, cost-effective and highly reproducible. It can simultaneously perform SNP discovery and genotyping across individual lines within a population (Sonah et al., 2013). It has been applied in the characterization of germplasm, population studies and breeding of diverse plant species (Poland and Rife, 2012). GBS-derived SNPs are also used in genotyping and genetic analysis (Beissinger et al., 2013). In addition, compared to array-derived SNPs, the SNPs from GBS have more power to detect rare alleles in diverse germplasm collections (Darrier et al., 2019).

2.5. Barley genomic resources

As a self-pollinating diploid plant species with a genome size of around 5.1 Gbp (Doležel et al., 1998), barley has become a model plant for genetic studies in *Triticeae* (Schulte et al., 2009). For a long time, due to the large size and repeat-rich composition of the *Triticeae* genomes, the genome sequence assembly of barley was an intractable problem. Over the last decades, gene isolation by map-based cloning was limited due to the lack of barley genomic resources. During this period the published genetic linkage/consensus maps have been an important source of genetic markers for mapping of target genes in barley. These resources included different marker systems from RFLP (Graner et al., 1991; Kleinhofs et al., 1993) to SSR markers (Ramsay et al., 2000; Varshney et al., 2007), as well as SNP markers (Close et al., 2009; Comadran et al., 2012; Muñoz-Amatriaín et al., 2011). In addition, different genetic maps have been published based on RFLP, SSR and SNP markers, with different marker densities (Rostoks et al., 2005; Stein et al., 2007).

Moreover, by using an approach which incorporated chromosome sorting, NGS, array hybridization, and systematic exploitation of conserved synteny with model grasses, an ordered, information-rich scaffold of the barley genome (“genome zipper”)

containing an estimated 86% of the barley genes was developed (Mayer et al., 2011). This resource was applied in genetic mapping of several genes in different cereal species, for example the BaMMV/BaYMV resistance gene *rym11* (Lüpken et al., 2013), the BaMMV resistance gene *rym7* (Yang et al., 2013), the *Rrs1* gene against scald in barley (Hofmann et al., 2013), the restorer gene *Rfp3* in rye (Hackauf et al., 2017) and the Russian wheat aphid resistance gene *Dn2401* in wheat (Staňková et al., 2015).

Nowadays, as NGS and third-generation sequencing (TGS) became achievable and affordable, the significant improvement of the barley reference genome has facilitated marker saturation of target genes of interest. The initial barley reference genome assembly from cultivar Morex meanwhile has three different pseudomolecule versions: Morex v1 (hierarchical short-read assembly based on bacterial artificial chromosomes (BACs); Mascher et al., 2017); Morex v2 (whole genome sequencing (WGS) short-read; Monat et al., 2019); and Morex v3 (whole-genome long-read; Mascher et al., 2021). In order to generate a high-quality barley genome sequence, the International Barley Genome Sequencing Consortium (IBSC) was established in 2006 (Schulte et al., 2009). A physical map of 4.98 Gbp was developed comprising 9,265 BAC contigs with an estimated N50 contig size of 904 kilobases. More than 3.90 Gbp were anchored to a high-resolution genetic map and a set of 26,159 high confidence (HC) genes with homology support from other plant genomes was identified (The International Barley Genome Sequencing Consortium, 2012). In 2017, the chromosome-conformation capture sequencing (Hi-C) was used to derive the linear order of sequences. The updated assembly Morex v1 represents 4.79 Gbp of the genome with an N50 value of 1.9 Mbp. A set of 39,734 HC loci and 41,949 low confidence (LC) loci was identified (Mascher et al., 2017). Two years later, TRITEX was used to generate an improved annotated reference genome assembly with a physical size of 4.65 Gbp and a scaffold N50 value of 43.7 Mbp. In total, 32,787 HC and 30,871 LC gene models were annotated on the v2 pseudomolecules (Monat et al., 2019). Recently, the new version of barley reference genome Morex v3 has been published, with a scaffold N50 value of 118.9 Mbp. The updated assembly was

obtained by using accurate circular consensus long-read sequencing on the PacBio platform, which captures a much higher amount of highly repetitive sequence than short-read assemblies. This generated an updated reference genome assembly for barley with near-complete representation of the repeat-rich intergenic space (Mascher et al., 2021). The Morex v3 pseudomolecules were annotated using the same transcriptomic resources as used for Morex v2, but with an improved version of the PGSB annotation pipeline. Finally a set of 35,827 HC and 45,860 LC genes was identified (Mascher et al., 2021). Barley genome sequence datasets are available via several online databases, such as *EnsemblPlants*, *Nord-Gen*, *BARLEX*, *MorexGenes*, *GrainGenes*, *HvGDB*, *Bex-DB*, *BarleyDB* and *BarleyVarDB*, which contain different information and offer different tools for simple analysis such as BLAST, genome-specific primer design and creation of graphical figures of a specified genomic region based on the genomic resources (for a review see Riaz et al., 2021).

Meanwhile, several consensus maps in barley were released with different densities based on a different number of examined populations (Muñoz-Amatriaín et al., 2014; Silvar et al., 2015; Abed et al., 2021). In addition, a first barley pan-genome was established, comprising one wild barley and 19 cultivated accessions (Jayakodi et al., 2020) and based on a comprehensive survey of GBS data from more than 22,000 accessions from the German National gene bank (Milner et al., 2019). Subsequently, a short-read assembly of the wild barley accession ‘OUH602’ has been released, with comparable quality to the recently published pan-genome assemblies (Sato et al., 2021). Moreover, a chromosome-scale assembly of the Japanese elite malting barley cultivar ‘Haruna Nijo’ has been published recently that is of similar quality to the Morex v2 reference (Sakkour et al., 2022). In conclusion, the updated barley cultivar Morex reference assemblies, the pan-genome assemblies with 20 diversity accessions and the additional assembly resources for wild and elite barley not only make previously hidden genetic variation accessible to genetic studies and breeding, but also promote the use of wild barley alleles in breeding programs.

2.6. Map-based gene isolation

Currently, there are two basic approaches for studying gene functions: reverse genetics from gene to phenotype and forward genetics from phenotype to gene (Takahashi et al., 1994). Reverse genetics strategies disrupt or modify a specific gene and then the phenotype is scored, while forward genetics requires the cloning of the causative gene underlying a particular phenotype for the trait of interest. As a forward genetics strategy, the efficient strategy for gene isolation called map-based cloning has been widely applied and well advanced in crops (Stein and Graner, 2005). This approach is based on the phenotype analysis of the mutant individual, the initial genetic mapping and fine mapping of the target gene, the physical map construction of the corresponding causative molecular locus and the functional analysis of the candidate genes (Peters et al., 2003). Many factors, e.g. the population size (Dinka et al., 2007), the distribution of crossovers along chromosomes (The International Barley Genome Sequencing Consortium, 2012), the genetic/physical ratio across the genome (Kunzel et al. 2000), phenotyping (Cobb et al., 2013) and the number of markers, mainly determine the success of map-based cloning.

Recombination is a main driver of genetic variation, however recombination rates are highly variable between different species (Nachman, 2002; Auton et al., 2012; Stapley et al., 2017), between populations of the same species (Kong et al., 2010; Salomé et al., 2012; Spence and Song, 2019) and among individuals of the same population (Wang et al. 2012). Recombination rates vary even along chromosomes. On Triticeae chromosomes, gene density and recombination rate are higher in telomeric regions than centromeric regions (Dvorák, 2009). This general trend has been observed in sequence data from barley (The International Barley Genome Sequencing Consortium, 2012; Zeng et al., 2015; Muñoz-Amatriaín et al., 2015; Dreissig et al., 2019), wheat (Raats et al., 2013; Choulet et al., 2014) and *Ae. tauschii* (Luo et al., 2013). Variation in recombination rate is influenced by epigenetic information, such as DNA methylations (Melamed-Bessudo and Levy, 2012; Mirouze et al., 2012; Yelina et al., 2012; Habu et al., 2015), histone modifications and nucleosome positions (Choi et al.,

2013). In addition, environmental conditions are reported to effect recombination rates. However, there is no clear consensus due to the differences between species and experimental systems. For example, the relationship between temperature and recombination was found to be positive or negative (Bomblies et al., 2015; Jackson et al., 2015; Phillips et al., 2015). Other studies found that the relationship between temperature and recombination resembles an U-shaped curve, with elevated recombination rates found at low and high temperatures (Plough, 1917; Plough, 1921; Lloyd et al., 2018; Modliszewski et al., 2018), or a reverse U-shaped curve (Wilson, 1959). Moreover, recombination rates were shown to vary drastically depending on variations in meiotic genes (Brand et al., 2018; Dreissig et al., 2020; Barakate et al., 2021).

Recombination events may be increased by using multi-parent advanced generation inter-crosses (MAGIC) to generate fully inbred recombinant populations by crossing multiple founders through two-way, four-way and eight-way crossing (Cavanagh et al., 2008). Substantial changes in recombination patterns in plants may be achieved by clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9), thus recombination rates could also be increased by using the latest tools in genome editing (Schmidt et al., 2020). In addition, it is reported that a shift of just 10°C in growth temperature is sufficient to increase overall recombination frequency (Phillips et al., 2015). Moreover, in the post-NGS era, the advances in molecular biology, biotechnology and genomics, and the dramatic reduction in sequencing cost have reduced half the time span taken for fine mapping and gene cloning using non-NGS-based markers (Jaganathan et al., 2020).

3. The goals of this work

As mentioned above, barley yellow mosaic disease causes yield loss of barley up to 50% and the widely used resistance genes *rym4*/*5* and *rym1*/*11* have been overcome, stressing the importance of the identification of the new BaMMV/BaYMV resistance resources.

The present study is based on the work of Le Gouis et al. (2004), in which the BaMMV resistance locus *rym15* derived from Chikurin Ibaraki 1 was initially mapped on the short arm of barley chromosome 6H between the flanking markers EBmac0874 and Bmag0173. However the order of these two markers in that study is inverted compared to the genetic map of *Hordeum vulgare* Lina × *Hordeum spontaneum* Canada park (Ramsay et al. 2000). As this gene is effective only against BaMMV, it was hypothesized that this gene may be different from the previously isolated BaMMV resistance genes mentioned above. Therefore, the main objectives of the present PhD thesis were (i) remap the target locus *rym15* and identify SNP-based flanking markers by medium-resolution mapping, (ii) develop a high-resolution mapping population for *rym15*, (iii) saturate the locus using data from the 50K Infinium SNP chip, GBS and PacBio SMRT sequencing, the barley reference genome and the barley pan-genome resources, and (iv) predict potential candidate genes for the BaMMV resistance locus *rym15*.

4. Delineating the elusive BaMMV resistance gene *rym15* in barley by medium-resolution mapping

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Delineating the elusive BaMMV resistance gene *rym15* in barley by medium-resolution mapping

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Abstract *Barley mild mosaic virus* (BaMMV), transmitted by the soil-borne protist *Polymyxa graminis*, has a serious impact on winter barley production. Previously, the BaMMV resistance gene *rym15* was mapped on chromosome 6HS, but the order of flanking markers was non-collinear between different maps. To resolve the position of the flanking markers and to enable map-based cloning of *rym15*, two medium-resolution mapping populations Igri (susceptible)×Chikurin Ibaraki 1 (resistant) (I×C) and Chikurin Ibaraki 1×Uschi (susceptible) (C×U), consisting of 342 and 180 F_2 plants, respectively, were developed. Efficiency of the mechanical inoculation of susceptible standards varied from 87.5 to 100% and in F_2 populations from 90.56 to 93.23%. Phenotyping of F_2 plants and corresponding F_3 families revealed segregation ratios of 250 s:92r (I×C, χ^2=0.659) and 140 s:40r (C×U, χ^2=0.741), suggesting the presence of a single recessive resistance gene. After screening the parents with the 50 K Infinium chip and anchoring corresponding SNPs to the barley reference genome, 8 KASP assays were developed and used to remap the gene. Newly constructed maps revealed a collinear order of markers, thereby allowing the identification of high throughput flanking markers. This study demonstrates how construction of medium-resolution mapping populations in combination with robust phenotyping can efficiently resolve conflicting marker ordering and reduce the size of the target interval. In the reference genome era and genome-wide genotyping era, medium-resolution mapping will help accelerate candidate gene identification for traits where phenotyping is difficult.

Keywords Barley · BaMMV mechanical inoculation · *rym15* · Medium-resolution mapping · SSR · KASP

Introduction

Barley yellow mosaic disease is caused by two related viruses, *barley yellow mosaic virus* (BaYMV) and *barley mild mosaic virus* (BaMMV). The disease can heavily impact winter barley cropping, with 40–80% yield loss in 2-rowed barley in Japan (Usugi 1988; Ohto 2000), and 50% losses in Europe (Plumb et al. 1986; Adams et al. 1992; Overthrow et al. 1999) up to complete yield loss, e.g., in some counties of the Yangtze River Valley (Chen 1993, 2005; Chen and

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Ruan 1992). Both viruses belong to the genus *Bymovirus* in the family *Potyviridae* and are transmitted by the root-infecting plasmodiophorid *Polymyxa graminis* L. However, the two causal viruses differ in their temperature optima, serological properties, and transcriptomes and their ability to infect different barley genotypes (Huth and Adams 1990; Habekuß et al. 2008). Use of resistant cultivars is the most economical and environmentally friendly way to control these soil-borne viruses (Kanyuka et al. 2003). So far, 22 resistance genes against barley yellow mosaic disease have been reported, of which most are recessive genes (see review of Jiang et al. 2020). However, many of these resistance genes are no longer effective. For example, the resistance gene *rym4* is ineffective against BaYMV-2, which appeared in the late 1980s; the resistance gene *rym5* was overcome by the strain BaMMV-Sil in France and BaMMV-Teik in Germany (Hariri et al. 2003; Vaianopoulos et al. 2007; Habekuß et al. 2008). It may therefore be expected that this trend will continue in the future; based on this, it is essential to identify and further characterize new sources of resistance and to develop diagnostic markers for marker-assisted selection (MAS) in barley.

About half of the known virus resistance genes in crops are recessive (Kang et al. 2005; Robaglia and Caranta 2006; Wang and Krishnaswamy 2012). Plant viruses need to recruit the host cells' machinery to complete the infectious life cycle; thus, mutation in the host factors genes may result in virus resistance (Garcia-Ruiz, 2018). Several of these recessive resistance genes are isoforms of *eukaryotic translation initiation factor 4E* (*eIF4E*), and *eIF4G* (Moffett 2009; Hashimoto et al. 2016). Up to now, two recessive resistance genes against BaMMV/BaYMV in barley have been isolated. The resistance to BaMMV/BaYMV impacted by the *rym4/5* locus is due to the host factor gene *HvEIF4E* (Kanyuka et al. 2005; Stein et al. 2005), while *rym1/11* resistance is caused by sequence variations of the host factor gene *Protein Disulfide Isomerase Like 5–1* (*HvPDIL5-1*) (Yang et al. 2014a). Out of twenty-two reported BaMMV/BaYMV resistance genes, six are allelic forms of *HvEIF4E*, i.e., *rym4*, *rym5*, *rym6*, *rym10*, $eIF4E_{HOR4224}$, and $eIF4E_{HOR3298}$, while two (*rym1* and *rym11*) are allelic forms of *HvPDIL51* (Perovic et al. 2014; Yang et al. 2014a; Shi et al. 2019).

The Japanese barley landrace Chikurin Ibaraki 1 is susceptible to BaYMV in Japan (Ukai and Yamashita 1980). In contrast to this, Chikurin Ibaraki 1 was found to be resistant in response to three European strains, i.e., BaMMV, BaYMV-1, and BaYMV-2 (Götz and Friedt 1993; Lapierre and Signoret 2004). Werner et al. (2003) demonstrated that an uncharacterized recessive resistance locus on chromosome 5HS effective against BaYMV and BaYMV-2 originates from Chikurin Ibaraki 1 and segregates independently from the Carola-derived *rym4* resistance that is effective against BaYMV and BaMMV. Further analysis of a doubled haploid (DH) mapping population derived from the cross of the Chikurin Ibaraki 1 and the susceptible winter barley cv. Plaisant located the recessive resistance gene effective against BaMMV on the short arm of chromosome 6H that was subsequently named *rym15* (Le Gouis et al. 2004). However, the study showed that the order of flanking markers EBmac0874 and Bmag0173 is inverted compared to the genetic map of Lina×*Hordeum spontaneum* Canada Park (Ramsay et al. 2000). To date, this discrepancy in the marker order spanning the resistance locus has hindered further map-based cloning efforts for *rym15*.

During BaMMV/BaYMV testing in fields, there are many obstacles, e.g., an uneven distribution of the virus, simultaneous occurrence of two viruses (BaMMV and BaYMV), and similarity of the symptoms (Huth et al. 1984). In addition, only 1 cycle of winter barley resistance testing per year highlights the demand for a reliable and efficient testing method of soil-borne viruses of barley. Consequently, the mechanical inoculation method could overcome the variation in year-to-year scoring of the resistance reaction from the same genotype in the same field that is due to the abovementioned variable environmental factors (Friedt 1983; Pandey 2006). Up to now, several mechanical inoculation methods for BaMMV were developed, e.g., based on soaked sponge rubbing (Friedt 1983), airbrush (Adams et al. 1986), finger rubbing (Kashiwazaki et al. 1989; Habekuß et al. 2008), spray gun (Ordon and Friedt 1993), or stick with gauze (SWG) methods (Jonson et al. 2006). Those studies suggested that the additives, the inoculation stages, the temperatures, and the inoculation techniques of the virus might influence the inoculation efficiency. While BaMMV is readily transmissible, the efficiency of BaYMV is much lower and is usually below 50% (So et al. 1997). Therefore, the knowledge of various degrees of mechanical inoculation efficiency should be taken in account for optimization of map-based cloning projects.

In the past 25 years, molecular markers have been increasingly used in the genetic analysis of various traits and nowadays have become the basic tool

for effective mapping of resistance genes in all crop plant species (Garrido-Cardenas et al. 2018; Perovic et al. 2019). Various codominant marker platforms have been used effectively to map resistance genes in crop plants. Simple sequence repeat (SSR) markers or microsatellites are highly polymorphic and reproducible; however, they are not amenable for high throughput even in the case of modified capillary systems (Perovic et al. 2013a) nor as abundant as single-nucleotide polymorphism (SNP). Due to the property of abundance and high throughput, SNP markers have become the most amenable for gene mapping and breeding (Silvar et al 2011; Rasheed et al. 2017; Lu et al. 2020).

In case of barley, SNP arrays (Comadran et al. 2012; Bayer et al. 2017) provide the accurate physical marker position based on the most recent reference genome assembly data (Mascher et al. 2017; Monat et al. 2019). This feature greatly enhances the efficiency of breeding and genetic studies in barley (Perovic et al. 2020). Based on the published barley reference sequence (Mascher et al. 2017) and exome capture data (Russell et al. 2016), the 50 K Illumina Infinium genotyping array was developed, featuring 49,267 SNP markers that were converted into 44,040 working assays (Bayer et al. 2017). Compared with the 9 K Infinium iSelect array, which contained 7842 markers (Comadran et al. 2012), the 50 K Illumina Infinium array possesses around six times more markers, resulting in cheaper genotyping costs per sample.

The main objectives of the present study were to construct two medium-resolution maps for the BaMMV resistance gene *rym15*, resolve the discrepancy in the order of flanking markers, and develop robust high-throughput amenable flanking markers as a prerequisite for map-based cloning of the resistance gene *rym15*.

Material and methods

Plant material

The resistant Japanese cultivar Chikurin Ibaraki 1 was crossed with the susceptible cultivars Igri and Uschi. A set of 342 and 180 F_2 plants derived from the crosses Igri×Chikurin Ibaraki 1 (I×C) and Chikurin Ibaraki 1×Uschi (C×U) was used to construct the medium-resolution maps for BaMMV resistance gene *rym15* (Supplementary Table 1). In addition, F_3 progeny was used for the validation of phenotypic data (Supplementary Table 2).

Resistance test

A set of 522 F_2 plants and corresponding F_3 families (Supplementary Table 2) was mechanically inoculated by the isolate BaMMV-ASL, and the crossing parents (Chikurin Ibaraki 1, Igri, and Uschi) and Maris Otter (positive control) were included in all 15 batches of the phenotypic analysis. After sowing, the plants were cultivated in the greenhouse for 7 days followed by cultivation in a growth chamber at 12 °C, 70% relative humidity, and 16-h (14:00–6:00) photoperiod (illuminance 20 klux). The mechanical inoculation was conducted according Perovic et al. (2014) with minor changes. All plant samples were inoculated at the 2- to 3-leaf stage two times at an interval of 5–7 days using sap extracted from the leaves of infected Maris Otter by homogenization in 0.1 M K_2HPO_4 buffer, pH 9.8. Approximately, 0.2 mL of buffer was used for each 1 g of infected leaf material. To aid mechanical inoculation, 0.1 g of carborundum (mesh 400) was added per 1 mL sap. Six weeks after the first inoculation, the leaves of tested plants were sampled and the double antibody sandwich ELISA (DAS-ELISA) was carried out according to Clark and Adams (1977) using polyclonal antibodies prepared at JKI (Quedlinburg, Germany). Virus particles were estimated via extinction at 405 nm using a Dynatech MR 5000 microtiter-plate reader at 30 min and 60 min after addition of p-nitrophenyl phosphate (PNPP) substrate buffer. All F_2 and F_3 plants with an extinction $E405 > 0.1$ were qualitatively scored as susceptible.

Based on the phenotypic data of susceptible parental lines Igri, Uschi, and positive control Maris Otter, the success rate of the mechanical inoculation method firstly was calculated by dividing the number of ELISA-positive susceptible plants with the total number of inoculated ones of these three genotypes. To evaluate the inoculation efficiency in the populations, the genotypic data of susceptible F_2 (homozygous/heterozygous) and F_3 (homozygous) plants were compared with the phenotypic data; the efficiency was calculated using the following equation:

$$\text{BaMMV inoculation efficiency} = \frac{\text{number of susceptible plants (based on ELISA scores)}}{\text{total number of plants analysed for BaMMV (based on marker analysis)}} \times 100\%$$

DNA extraction and SSR marker analysis

In order to make the genetic analysis by SSR markers for all F_2 plants and parental lines Chikurin Ibaraki 1, Igri, and Uschi, DNA was extracted from barley seedlings of 14 days old using CTAB (cetyltrimethylammonium bromide) method according to Stein et al. (2001). The concentration and quality of DNA were estimated using the NanoDrop ND-1000 spectrophotometer (PeQLab, Erlangen, Germany). A set of six SSR markers linked to *rym15* (Bmac0127, Bmac0018, Bmag0867, Bmag0870, EBmac0874, and Bmag0173; Le Gouis et al. 2004) was chosen for genotyping the parental lines and 522 F_2 plants. PCR reaction consisting of 1 µL of template DNA (25–30 ng/µL), 1 µL of 10×buffer, 1 µL of 25 mM $MgCl_2$, 0.2 µL of 10 mM dNTP-Mix, 0.25 µL of each forward primer (10.0 pmol/µL) and reverse primer (10.0 pmol/µL), and 0.08 µL of 5 U HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia). M13-tails were added to the forward primers, for SSR amplification, so that 0.1 µL of M13 primer (10.0 pmol/µL) (5′-CAC GAC GTT GTA AAA CGA C-3′) labeled with 5′ fluorescent dyes was added to the reaction mix in a final volume of 10 µL (Macdonald et al. 2006; Perovic et al. 2013b). DNA was amplified in a GeneAmp PCR System 9700 (Applied Biosystems) for all SSR markers under the following conditions: 94 °C for 5 min; followed by touchdown PCR with 12 cycles of 30 s at 94 °C, 30 s at 62 °C, 30 s at 72 °C, and then 35 cycles with 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C, and a final extension at 72 °C for 10 min. Amplified products (1 µL) were checked on an agarose gel (1.5%). For the capillary-based scoring, 1 µL of the PCR product was mixed with Hi-Di™ formamide (Applied Biosystems) and GeneScan™-500 ROX™ size standard (Applied Biosystems) (0.03 µL ROX: 14 µL HiDi™ formamide). The mixture was then denatured for 5 min at 94 °C and subjected to capillary electrophoresis in an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Data was collected using 3130xl data collection software v3.0 (Applied Biosystems). The size of the detected alleles was determined using the GeneMapper v4.0 (Applied Biosystems).

The physical position of the SSR markers was determined by blasting forward and reverse primers against the barley reference genome sequence (http://webblast.ipk-gatersleben.de/barley_ibsc/) using default parameters of blastN.

50 K Illumina Infinium genotyping array and KASP marker development

In order to identify polymorphisms between parental lines (Chikurin Ibaraki 1, Igri, and Uschi) and develop markers for genetic analysis for both populations, the DNA of three parental lines (Chikurin Ibaraki 1, Igri, and Uschi) was analyzed by using the 50 K Illumina Infinium genotyping array at the company TraitGenetics (Gatersleben, Germany). The additional information (locus name, position, and sequence) on 50 K array SNPs was downloaded from iSelect (http://bioinf.hutton.ac.uk/iselect/app/). The SNP dataset was filtered using Excel software; on each chromosome, the homozygous SNPs between Chikurin Ibaraki 1 and Igri were identified and the same analysis was conducted for Chikurin Ibaraki 1 and Uschi. Based on the Infinium 50 K data, a set of eight SNPs was selected for the design of competitive allele-specific PCR (KASP) assays (*rym15*_1, *rym15*_4, *rym15*_6, *rym15*_8, *rym15*_11, *rym15*_13, *rym15*_15, *rym15*_17, Supplementary Table 3) by using the website BatchPrimer3 (You et al. 2008); the parameter of product size is 70–150 base pair. All eight KASPs were used to genotype the three parental lines and 522 F_2 plants. The PCR reaction consisted of 2.2 µL of template DNA (25–30 ng/µL), 0.2 µL of common primer (10.0 pmol/µL), 0.08 µL of each allele-specific primer 1 and allele-specific primer 2 (10.0 pmol/µL), and 2.5 µL of 2×KASP Master Mix. DNA was amplified in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with the following conditions: 94 °C for 15 min; followed by PCR with 9 cycles (−0.6 °C/cycle) of 20 s at 94 °C, 1 min at 61 °C, and then 25 cycles with 20 s at 94 °C, 1 min at 55 °C, and a final cool down at 30 °C for 1 min. If necessary, recycling with the following conditions was performed: 94 °C for 3 min, followed by PCR with 9 cycles of 20 s at 94 °C, 1 min at 57 °C, and a final cool down at 30 °C for 1 min. The fluorescence signals from HEX and FAM for the specific alleles were detected using the same Detection System (Bio-Rad) at 37 °C after thermal cycling was complete. At the end of

the run, the results were displayed in the data analysis software under "Allelic Discrimination" (LGC, Guide to running KASP genotyping on the Bio-Rad CFX-series instruments).

Linkage analysis

The observed segregation ratios of $F_{1:2}$ (1:3) and $F_{2:3}$ (1:2:1) for the inheritance of a single recessive gene were tested using chi-squared (χ^2). Based on the genotypic and verified phenotypic data of all F_2 plants, the genetic maps were constructed using the software JoinMap v.4 (Van Ooijen 2006) applying the Kosambi function (Kosambi 1944) and a LOD score of 3.

Results

Phenotypic analysis

A set of 522 F_2 plants was mechanically inoculated using BaMMV-ASL isolate. In order to test integrity of individual F_2 plants, the phenotypic analysis of corresponding F_3 families was conducted (Supplementary Table 4). Based on phenotyping of the F_2 and $F_{2:3}$ generations, 342 (I×C) and 180 (C×U) F_2 plants showed the segregation of 250 s:92r (χ^2=0.659) and 140 s:40r (χ^2=0.741), respectively. Chi-square test indicated that these ratios fit to a 3 s:1r segregation ratio (Supplementary Table 4). In the F_3 generation, the ratio of non-segregating susceptible to segregating susceptible to resistant $F_{2:3}$ plants from I×C and C×U was 74:176:92 (χ^2=2.187) and 53:87:40 (χ^2=2.078), respectively. Chi-square test indicated that these ratios fit to a 1:2:1 segregation ratio (Supplementary Table 4). Overall, the resistance data of F_2 populations I×C and C×U suggest a single recessive gene causing resistance against BaMMV in Chikurin Ibaraki 1.

The entire phenotypic analysis of all F_2 plants and corresponding F_3 families was accomplished in 15 batches due to the space and time constraints in the growth chamber. Regarding analysis of susceptible control genotype, out of 204 DAS-ELISA-analyzed Maris Otter plants, seven escaped from the virus inoculation. At the same time, for the parental line Igri, five out of 40 inoculated ones escaped, while all of 36 Uschi plants were successfully inoculated. Based on these data, the inoculation rates in the susceptible control Maris Otter as well as the susceptible parental lines Igri and Uschi are 96.35%, 87.5%, and 100%, respectively. In the populations I×C, 16 false positives and 13 escapes were identified among the F_2 plants, while in the population C×U, nine plants were false positive and 13 escaped (Supplementary Table 5). Accordingly, 29 (8.47%, I×C) and 22 (12.22%, C×U) F_2 plants with the deduced F_2 phenotypic data based on $F_{2:3}$ phenotyping analysis were used for further linkage analysis. Based on all phenotypic data of the susceptible F_2 (homozygous/heterozygous/recombinant) and F_3 (homozygous) plants in the populations I×C and C×U, the efficiency of inoculation method varied from 90.56 to 93.23% (Table 1).

Molecular marker genotyping

The genotyping of three parental lines using the 50 K array identified 14,863 (Chikurin Ibaraki 1 and Igri) and 13,678 (Chikurin Ibaraki 1 and Uschi) polymorphic SNPs (Fig. 1; Supplementary Table 6). In total, 9310 SNPs (68.06%) were identical among parental combinations. On the target chromosome 6H, 1679 (Chikurin Ibaraki 1 and Igri) and 1565 (Chikurin Ibaraki 1 and Uschi) SNPs were identified, of which 1076 SNPs (68.75%) were in common.

Table 1 Efficiency of the mechanical inoculation method of BaMMV

F_2 population	Heterozygous susceptible F_2	Homozygous susceptible F_2	Recombinant susceptible F_2	Homozygous susceptible F_3	Total	Efficiency
Igri×Chikurin Ibaraki 1	161[a]	74[a]	7[a]	150[a]	329[a]	90.56%
	151[b]	72[b]	6[b]	126[b]	355[b]	
Chikurin Ibaraki 1×Uschi	79[a]	50[a]	7[a]	130[a]	266[a]	93.23%
	70[b]	47b	6[b]	125[b]	248[b]	

[a]The number of different types of susceptible plants based on the genotypic analysis

[b]The number of susceptible plants based on phenotypic analysis

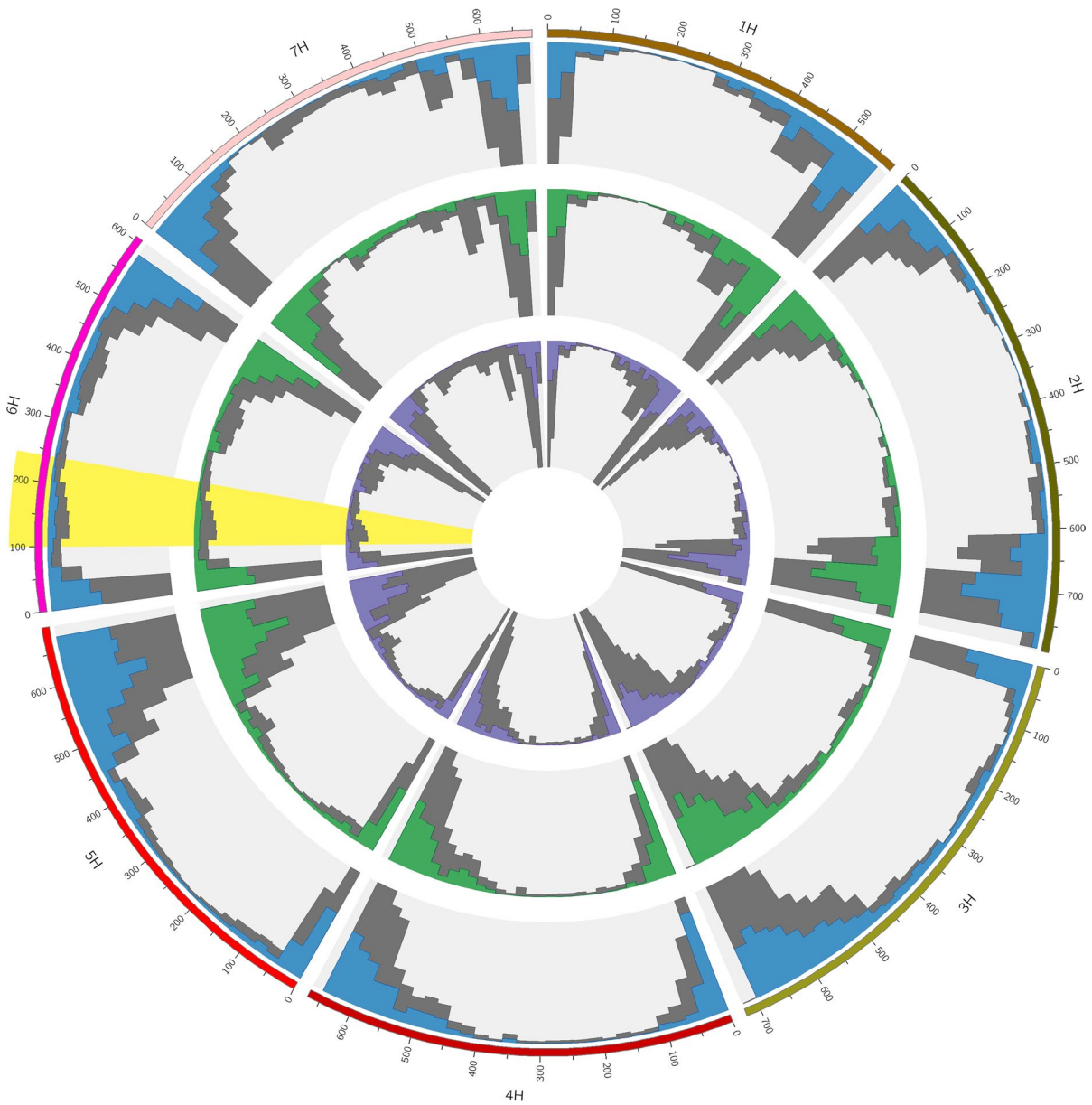

Fig. 1 Landscape of the 50 K SNP array marker distribution on seven barley chromosomes. All SNPs from the 50 K Illumina Infinium iSelect genotyping array are presented in grey. SNPs between Chikurin Ibaraki 1 and Igri are presented in blue. SNPs between Chikurin Ibaraki 1 and Uschi are presented in green. Consensus SNPs from the comparison are presented in purple (Chikurin Ibaraki 1 and Igri; Chikurin Ibaraki 1 and Uschi). The interval between flanking markers *rym15*_1 and *rym15*_8 is presented in yellow

Three parental lines were genotyped using six SSR and eight KASP markers (Table 2; Fig. 2). The genotypic ratios of all markers in F_2 families from both the F_2 populations fitted to a 1:2:1 segregation ratio (Supplementary Table 7). The physical position of all used markers was determined using the blastN alignment algorithm against both publicly available Morex assemblies (Table 2). The two distal, telomeric SSR markers Bmag0173 and Bmag0870 span around 299.78 Mb on chromosome 6H according to the Morex v2 assembly. Controversially, for the SSR marker Bmag0173, no hits on chromosome 6H in Morex v1 could be found,

Table 2 Physical position and allele size/types of SSR and KASP markers

Marker		Bmag0176	EBmac0874	Bmag0867	Bmac0127	Bmac0018	Bmag0870	*rym15*_1	*rym15*_4	*rym15*_6	*rym15*_8	*rym15*_11	*rym15*_13	*rym15*_15	*rym15*_17
Physical position_Morex v1	Start	-	150,284,733	261,541,306	271,882,721	293,925,242	397,650,916	100,092,059	175,284,342	200,044,740	240,373,116	287,521,970	319,250,935	338,666,322	348,226,696
	End	-	150,284,844	261,541,434	271,182,699	293,925,223	397,651,036	-	-	-	-	-	-	-	-
Physical position_Morex v2	Start	-	148,343,963	258,379,492	272,699,163/272699616	295,207,402	395,517,134	99,216,348	174,152,954	198,364,820	235,707,335	289,171,679	321,032,499	339,011,251	347,643,455
	End	95,736,832	148,344,153	258,379,622	272,699,278/272699733	-	395,517,254	-	-	-	-	-	-	-	-
Allele size/type	Igri	142	214	149	135	156	135	A	A	A	T	A	A	C	T
	Uschi	142	209	144	144	156	122	A	A	A	T	A	A	C	T
	Chikurinibaraki 1	149	191	137	137	150	148	G	G	G	C	C	G	G	C

while the blastN alignment of the reverse primer against Morex v2 revealed a hit on chromosome 6H (Supplementary Table 8).

Medium-resolution map construction

The resistance gene *rym15* was mapped between the two flanking markers *rym15*_1 and *rym15*_8 (Supplementary Fig. 1) within a genetic window of 3.5 cm and 3.7 cm in the F_2 populations I×C and C×U, respectively (Fig. 3). At the same time, the physical size of the interval according to Morex v2 assembly was estimated to be 137 Mb. Between the two flanking markers, 141 and 109 SNPs were identified at the I×C and C×U populations, respectively, of which a set of 85 SNPs (77.98%) was in common.

Discussion

Following initial genetic mapping of the gene of interest, the next step towards positional isolation of candidate genes is an immediate screening of a large population with dense markers segregating at the locus of interest, commonly referred to as high-resolution mapping. In the present study, due to the non-collinear order of previously published flanking markers (Le Gouis et al. 2004; Ramsay et al. 2000) and the high rate of resistant genotypes that were identified during infection of the first batch of F_2 plants, we decided to remap the gene at medium resolution in a smaller population to resolve the previous discrepancies. Instead of developing the high-resolution mapping populations by using the previous flanking markers, analysis of 342 (I×C) and 180 (C×U) F_2 plants was conducted. The main aim of this step was to take into account an actual ratio of hampered phenotyping and to optimize map base cloning by mapping the gene to a smaller interval. The use of KASP markers with a precise position in contrast to the previously flanking SSR markers (Le Gouis et al. 2004), together with the construction of a medium size mapping population, might help optimizing costs and time constrains during map-based cloning.

Since the BaMMV resistance gene *rym15* originates from a non-adapted landrace and is currently not used in breeding programs in Germany, a detailed evaluation of the mechanical inoculation was performed. The ELISA score revealed that 96.35%, 87.5%, and 100% of susceptible control Maris Otter and susceptible

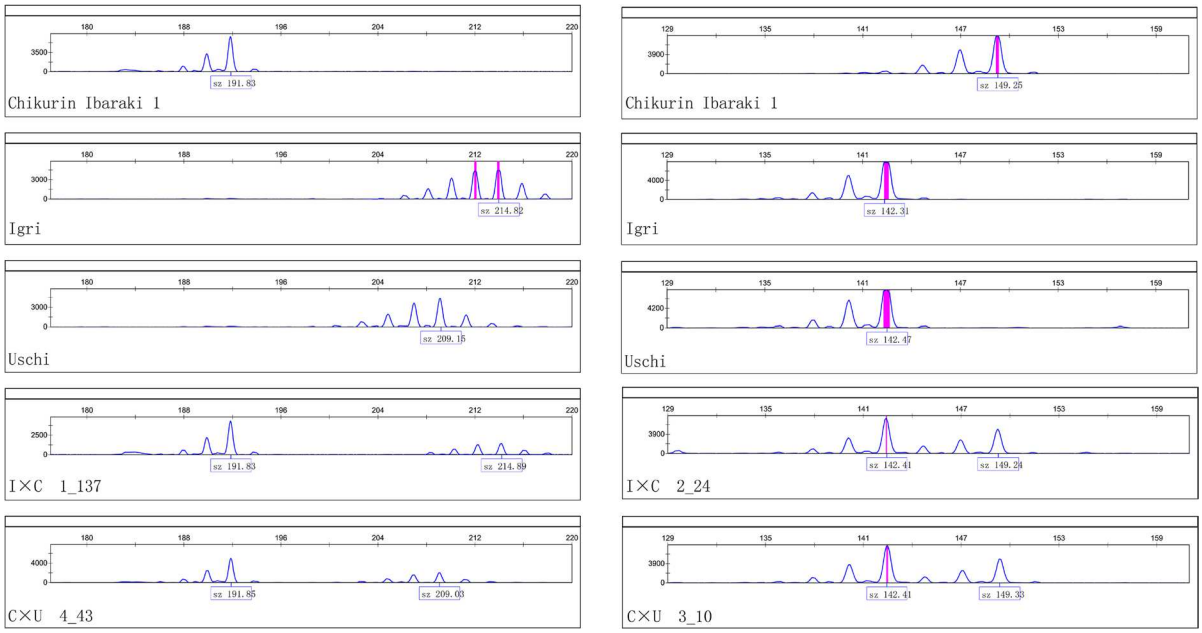

Fig. 2 Chromatograms of the SSR markers EBmac0874 (left) and Bmag0173 (right) used for mapping of the resistance gene *rym15*. The order of genotypes for both markers are resistant parent Chikurin Ibaraki 1, susceptible parent Igri, second susceptible parent Uschi, one F_2-plants from cross Igri×Chikurin Ibaraki 1, and one F_2-plants from cross Chikurin Ibaraki 1×Uschi

parental lines Igri and Uschi, respectively, were infected. The susceptible control cultivar Maris Otter showed a higher rate of infectivity than parental cultivar Igri, corresponding to previous studies (Yang et al. 2014b; Shi et al. 2019). At the same time, parental cultivar Uschi revealed a higher infection rate than Maris Otter, albeit with a lower number of tested plants. In the case of F_2 and F_3 plants, the efficiency of the mechanical inoculation varied from 90.56 to 93.23% in the I×C and C×U populations, respectively. In the F_2 populations, we analyzed a currently used method based on finger rubbing for mechanical inoculation which revealed about 10% escapes. However, the efficiency in the present study was much higher than in similar studies (Shi et al. 2019; Pidon et al. 2020), although a similar inoculation method (Habekuß et al. 2008) was used.

The combined F_2 and $F_{2:3}$ analysis revealed that a single recessive resistance gene on chromosome 6H named *rym15* conferred the resistance against BaMMV in the Japanese cultivar Chikurin Ibaraki 1. The order of markers in two constructed medium-resolution maps turned out to be collinear, and all mapped markers showed the same order in the genetic map and physical map according to the reference position at the Morex v1 and v2 assemblies (Mascher et al. 2017; Monat et al. 2019). In both constructed maps, the markers EBmac0874 and Bmag0173 are inverted compared to the previous map (Le Gouis et al. 2004); in addition, in the present study, the interval fixed by these two markers is out the frame of the target region containing *rym15* locus. According to the physical position of the reverse primer at the Morex v2 genome assembly, the Bmag0173 revealed to be distal to EBmac0874, which corresponds to the order in the present study. These two markers have been used in several studies, and some maps show the same order as the present study (Ramsay et al. 2000; Varshney et al. 2007; Friesen et al. 2006; Gupta et al. 2011), and some show the discrepancy in the order in comparison with the present study (Cakir et al. 2003a, b; Le Gouis et al. 2004). In addition, the distances of these two markers are very different between the maps, which could be explained by the use of a different type of population, the size of the population, and the differences in the genetic background of the genotypes used. In conclusion, the discrepancy of the SSR markers EBmac0874 and Bmag0173 is commonly known and not unique. A hypothetical explanation of discrepant mapping could be co-migration of fragments from two or more loci in

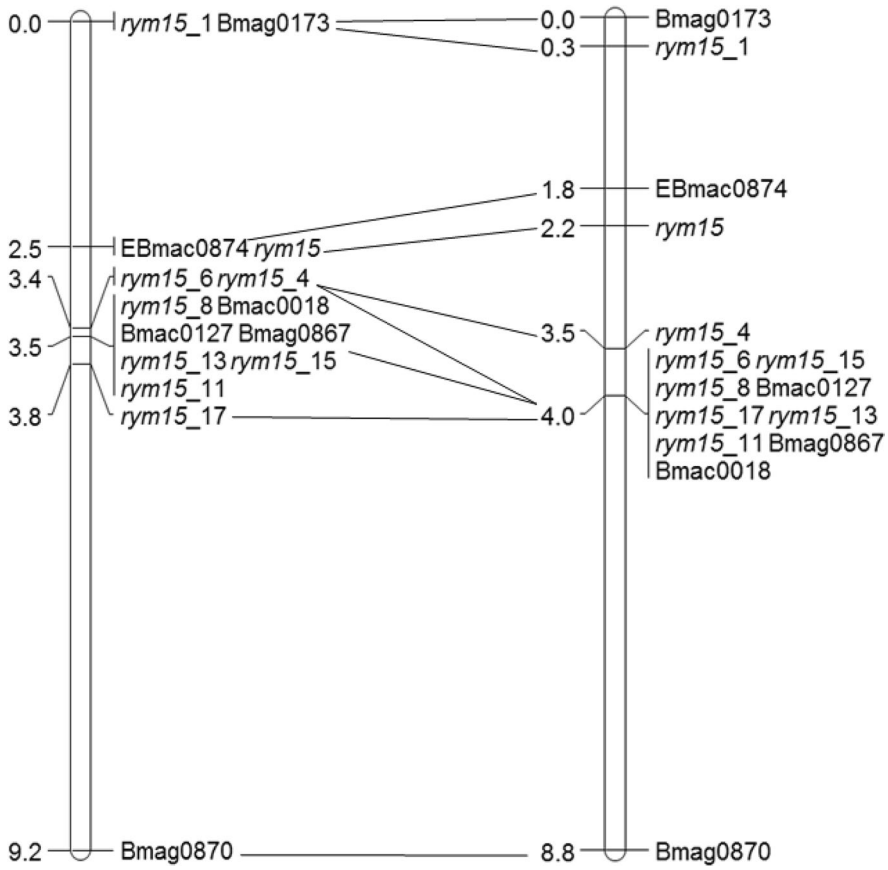

Fig. 3 Genetic maps of BaMMV resistance gene *rym15*. Maps were constructed based on analysis of 342 and 180 F_2 plants derived from the crosses Igri × Chikurin Ibaraki 1 (left) and Chikurin Ibaraki 1 × Uschi (right). Collinearity between the two genetic maps is shown with black lines

certain genotypes versus presence of single bands in other genotypes.

The high-quality barley reference sequences Morex v1 and Morex v2 (Mascher et al. 2017; Monat et al. 2019) provide more precise information than the draft barley genome sequence (The International Barley Genome Sequencing Consortium, 2012). The study of leaf rust resistance gene $Rph_{MBR1012}$ (Fazlikhani et al. 2019) has shown the efficient use of the barley reference sequence (Morex v1), especially in marker saturation. In the present study, the SNPs derived from the 50 K Illumina Infinium genotyping array were positioned on the physical map based on the published barley reference sequence. For the previous flanking SSR marker Bmag0173, no information about physical position on chromosome 6H could be found in Morex v1, while based on the Morex v2 assembly, the physical position of the reverse primer provide more precise information; thus, it could be used as reference information when comparing the order of these two previous flanking markers, reflecting the improvement of Morex v2 compared to the Morex v1.

A major constraint in map-based cloning projects is the interplay between the size of the target region defined by flanking markers and the number of F_2 plants needed for delineation of a single candidate gene. However, barley and other Triticeae are rich in repetitive DNA which hampers gene isolation (Krattinger et al. 2009). Nevertheless, nowadays, based on the reference sequence of Morex, high-throughput genotyping (e.g., via genotyping-by-sequencing or high-density SNP arrays) can considerably improve the efficiency of marker development in barley. In the present study, the KASP markers were developed in a short time based on the screening of parental lines by using 50 K Illumina Infinium genotyping array. The medium-resolution maps we constructed provide more reliable results for delineating the target gene. In case of incorrectly scored phenotypes, this step greatly reduces the risk that a gene of interest may lie outside of putative flanking markers which span a very short interval.

The next step for isolating the resistance gene *rym15* is the construction of a high-resolution map. For this, high-resolution mapping populations will be constructed by screening the newly developed, robust flanking markers in around 8000 F_2 plants from both F_2 populations. For marker saturation, a set of 85 informative SNP markers was identified between the flanking markers *rym15*_1 and *rym15*_8 based on the 50 K SNP array screen. Based on information on corresponding candidate genes (high confidence and low confidence) in the genome interval covered by these SNPs, promising genes will be selected for marker development for further saturation of the *rym15* locus. Meanwhile, the KASP markers developed in the present study can already be efficiently used in breeding programs attempting to transfer *rym15* to elite barley cultivars.

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Author contribution FO and DP conceived and designed the study; AH provided the material and performed phenotypic analysis; YP performed the experiments; YP and DP analyzed the data and drafted the manuscript; all authors read and approved the final manuscript.

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Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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5. High-resolution mapping of *Barley mild mosaic virus* resistance gene *rym15*

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High-Resolution Mapping of *Barley mild mosaic virus* Resistance Gene *rym15*

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Barley yellow mosaic virus (BaYMV) and *Barley mild mosaic virus* (BaMMV), which are transmitted by the soil-borne plasmodiophorid *Polymyxa graminis*, cause high yield losses in barley. In previous studies, the recessive BaMMV resistance gene *rym15*, derived from the Japanese landrace Chikurin Ibaraki 1, was mapped on chromosome 6HS of *Hordeum vulgare*. In this study, 423 F_4 segmental recombinant inbred lines (RILs) were developed from crosses of Chikurin Ibaraki 1 with two BaMMV-susceptible cultivars, Igri (139 RILs) and Uschi (284 RILs). A set of 32 competitive allele-specific PCR (KASP) assays, designed using single nucleotide polymorphisms (SNPs) from the barley 50 K Illumina Infinium iSelect SNP chip, genotyping by sequencing (GBS) and whole-genome sequencing (WGS), was used as a backbone for construction of two high-resolution maps. Using this approach, the target locus was narrowed down to 0.161 cM and 0.036 cM in the Igri × Chikurin Ibaraki 1 (I × C) and Chikurin Ibaraki 1 × Uschi (C × U) populations, respectively. Corresponding physical intervals of 11.3 Mbp and 0.281 Mbp were calculated for I × C and C × U, respectively, according to the Morex v3 genome sequence. In the 0.281 Mbp target region, six high confidence (HC) and two low confidence (LC) genes were identified. Genome assemblies of BaMMV-susceptible cultivars Igri and Golden Promise from the barley pan-genome, and a HiFi assembly of Chikurin Ibaraki 1 together with re-sequencing data for the six HC and two LC genes in susceptible parental cultivar Uschi revealed functional SNPs between resistant and susceptible genotypes only in two of the HC genes. These SNPs are the most promising candidates for the development of functional markers and the two genes represent promising candidates for functional analysis.

Keywords: barley, BaMMV resistance, high-resolution mapping, *rym15*, candidate gene

INTRODUCTION

Barley (*Hordeum vulgare* ssp. *vulgare*), the fourth most cultivated cereal in the world (FAOSTAT, 2022), is mainly used for animal feed and malting. The soil-borne barley yellow mosaic disease, caused by *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV), significantly affects the yield of winter barley in large parts of Europe and East Asia (Kühne, 2009).

Due to transmission of BaMMV and BaYMV *via* the soil-borne plasmodiophorid *Polymyxa graminis* (Adams et al., 1988; Kanyuka et al., 2003), it is of prime importance to improve the genetic resistance in modern cultivars to ensure winter barley cultivation despite the increasing frequency of infested fields.

A total of 22 resistance genes against BaYMV and/or BaMMV were reported up to now, of which the two recessive genes *rym1/11* and *rym4/5* have been the predominant sources of breeding for commercial BaMMV/BaYMV resistant barley cultivars (Jiang et al., 2020). However, a predominant European isolate BaYMV-2 became virulent on *rym4*-carrying barley varieties (Kühne et al., 2003; Rolland et al., 2017). Another widespread BaYMV-2 resistance gene *rym5* is overcome by the European isolates BaMMV-Teik and BaMMV-SIL (Kanyuka et al., 2004; Habekuß et al., 2007), the Japanese isolate BaYMV-III (Nishigawa et al., 2008) and isolates of BaMMV in France (Rolland et al., 2017). In addition, in China, BaYMV isolates BaYMV-CN_NY and BaYMV-CN_YZ were virulent to *rym4*, and *rym5* was overcome by BaYMV isolates BaYMV-CN_DZ and BaYMV-CN_NY, as well as BaMMV isolates BaMMV-CN_NY and BaMMV-CN_YZ. Remarkably, the isolate BaYMV-CN_NY was also virulent to accessions, which carried *rym1/11* and *rym5* (Jiang et al., 2022). Thus, it is critical to search for alternative BaMMV/BaYMV resistance resources and identify diagnostic markers for marker-assisted selection.

During recent decades, in addition to SNP arrays (Bayer et al., 2017), next-generation sequencing (NGS) technologies have been widely applied in plant breeding. For instance, using NGS technology, cost-effective genotyping-by-sequencing (GBS) approaches have been developed and widely used in barley genetic studies (Poland et al., 2012). SNPs assayed with high-density SNP arrays and GBS enable navigation between genetic maps and physical genome positions. Using both kinds of markers in tandem can be advantageous because polymorphisms of GBS-derived SNPs and SNPs included in arrays tend to target complementary haplotypes or genome regions (Darrier et al., 2019; Negro et al., 2019). Furthermore, GBS-derived SNPs have more power to detect rare alleles in diverse germplasm collections, while SNP arrays are prone to ascertainment bias. On the other hand, array-derived SNPs have the advantage of highly robust calling of alleles at the same SNPs across multiple populations (Darrier et al., 2019).

Third-generation sequencing technologies, such as Pacific Biosciences (PacBio) and Oxford Nanopore Technologies, operate on different principles (Eid et al., 2009; Jain et al., 2015). Compared to the short-read approaches, the assembly data obtained by using long-read sequencing methods can provide more information regarding variants residing in the repeat-rich intergenic space or copy-number variants at complex loci (Mascher et al., 2021). However, until very recently, error rates of both sequencing platforms were significantly higher than short-read NGS methods (Hu et al., 2021). Depending on the DNA fragment length and quality, Oxford Nanopore Technologies MinION/GridION can provide reads longer than 1 Mb, with read accuracy of 87–98% and reads for an N50 of 10–60 kb, and the newest PacBio sequencing improvement Sequel 2 can generate high-fidelity (HiFi) reads up to 20 kb with more than 99% accuracy with N50 of 10–20 kb using the single-molecule circular consensus sequence technology (Wenger et al., 2019; Logsdon et al., 2020; Miga et al., 2020).

Recently, a barley pan-genome was assembled comprising 19 cultivated accessions and one wild barley (Jayakodi et al., 2020). Furthermore, the updated barley reference genome Morex v3 was released by the use of accurate circular consensus long-read sequencing, and a set of 35,827 high confidence (HC) and 45,860 low confidence (LC) genes was identified (Mascher, 2020; Mascher et al., 2021). The availability of those online resources facilitates the study of the genome and its relationship with target traits in barley. For the present study, the assembly of our susceptible parental line Igri is of particular relevance as a sequence resource for narrowing down and annotating the *rym15* target region.

In the past 20 years, map-based cloning turned out to be efficient for the isolation of candidate genes for important traits (Jaganathan et al., 2020). Up to now, two BaMMV/BaYMV resistance loci were cloned through map-based cloning: *rym4*, *rym5*, and $rym_{HOR3298}$, as allelic variants of the eukaryotic translation initiation factor 4E (*eIF4E*; Kanyuka et al., 2005; Stein et al., 2005; Shi et al., 2019), and *rym1/11* encoding a protein disulfide isomerase like 5–1 (*PDIL5-1*; Yang et al., 2014). The updated and improved genomic resources for barley have simplified marker saturation and accelerated gene isolation (Perovic et al., 2018). The availability of public reference genome assemblies and low-cost, high throughput sequencing platforms, which can generate millions of polymorphisms for genetic mapping, provide a great opportunity for genetic mapping studies (Jaganathan et al., 2020).

Chikurin Ibaraki 1 is susceptible to BaYMV in Japan (Ukai and Yamashita, 1980). Interestingly, this Japanese cultivar was found to be resistant to three European strains, that is, BaMMV, BaYMV-1, and BaYMV-2 (Götz and Friedt, 1993; Lapierre and Signoret, 2004). The first genetic mapping of the Chikurin Ibaraki 1 derived BaMMV resistance locus *rym15* revealed that it is inherited recessively and located on chromosome 6HS (Le Gouis et al., 2004). In a previous publication (Wang et al., 2021), two medium-resolution maps were constructed by using a set of 180 (I × C) and 342 (C × U) F_2 plants. In this publication mapping was done by the use of six SSR markers and eight KASP markers (*rym15*_1 to *rym15*_17) that were developed based on a 50 K Illumina Infinium iSelect screen of three parental lines and phenotyping of corresponding F_2-F_3 families, the gene was fixed between KASP markers *rym15*_1 and *rym15*_8 in an interval around 137 Mb according to the barley reference assembly Morex v2 (Wang et al., 2021). Based on this information, in a current study, two high-resolution mapping populations comprising 2,218 (I × C) and 5,870 (C × U) F_2 plants were developed and corresponding F_4 segmental RILs were phenotyped using the BaMMV-ASL isolate, the present study aimed to (1) construct a high-resolution mapping population of *rym15*, (2) narrow down the target region, and (3) predict potential candidate genes for BaMMV resistance gene *rym15*.

MATERIALS AND METHODS

Plant Material and Construction of the High-Resolution Mapping Populations

To construct high-resolution mapping populations for *rym15*, two segregating F_2 populations comprising 2,218 and 5,870 F_2 plants were produced based on the crosses between the resistant cultivar Chikurin Ibaraki 1 and the susceptible cultivars Igri and Uschi, respectively. DNA of F_2 plants was extracted at the two-leaf stage using the efficient 96-sample multiplex DNA extraction protocol described by Milner et al. (2019). All F_2 plants were analyzed using the co-dominant flanking markers *rym15*_1 and *rym15*_8 which we identified in a previous study (Wang et al., 2021). Those F_2 plants carrying a recombination event within the target interval were self-pollinated and selfed seeds were harvested. For each recombinant F_2 plant, a set of 12 seeds was sown in 96 Quick pot trays (8 × 12). DNA of F_3 plants was extracted as described above and subsequently analyzed with the same markers, that is, *rym15*_1 and *rym15*_8, in order to identify segmental homozygous recombinants. Homozygous recombinant F_3 plants were selfed and corresponding F_4 plants were subsequently used for the construction of a high-resolution mapping population. By this approach, two high-resolution mapping populations of 139 (I × C) and 284 (C × U) F_4 segmental RILs were developed and subsequently used for resistance testing (**Table 1**).

Resistance Test

All 423 RILs were mechanically inoculated with a predominant isolate BaMMV-ASL (derived from Aschersleben, Germany) under controlled growth chamber conditions according to Perovic et al. (2014). A set of 6 plants per segmental RIL were sown randomly in 60 Quick pot trays (6 × 10). In each tray, a set of 6 plants of cultivar Maris Otter was used as positive control, and three plants of the resistant parent Chikurin Ibaraki 1 and three plants of the susceptible parent Igri or Uschi were sown. Five to six weeks after the first inoculation, the mosaic symptoms on the plants were estimated visually and the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was carried out according to Clark and Adams (1977), using polyclonal antibodies and conjugate IgG (Loewe Biochemica, Sauerlach, Cat. No.07006S). The virus titer was estimated *via* extinction at 405 nm using a Dynatech MR 5000 microtiter-plate reader at 45 min and 90 min after addition of p-Nitrophenyl Phosphate (PNPP). Plants with an extinction E405 > 0.10 were qualitatively scored as susceptible. Segregation of resistant and susceptible F_4 RILs was analyzed using the chi-square tests for goodness of fit to the expected segregation ratios (1r:1 s).

GBS Library Construction, Sequencing, and Data Analysis

Genomic DNA of the parental lines (Chikurin Ibaraki 1, Igri, and Uschi) was extracted using the CTAB (cetyl trimethylammonium bromide) method according to Stein et al. (2001) and digested with *PstI* and *MspI* (New England Biolabs) according to Wendler et al. (2014). GBS libraries were loaded on 2% Ultra PureTM Agarose Gel from Invitrogen stained with SYBRGold. Size selection from 250 bp to 600 bp was performed visually and gel extraction of cut gel pieces was performed using MinElute Gel Extraction Kit from Qiagen. The GBS libraries were sequenced in loading concentration of 10pM on Illumina® MiSeq™ (Illumina, San Diego, United States) with 150 cycles, single-end reads, using a custom sequencing primer. Sequence data were analyzed using a Galaxy web server (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). The sequencing reads were trimmed by using the tool Trim Galore (version 0.4.0) with a quality threshold of 30 to remove the low-quality reads and also the reads shorter than 50 bp. Alignment was performed against the genome assembly Morex v3 (Mascher, 2020) by using the trimmed sequencing reads of three parental lines. This step was conducted using BWA-MEM (version 0.7.17; Li, 2013) with default parameters. SNP calling was performed using MPileup version 1.8 (Li and Durbin, 2009) and the polymorphisms between resistant (Chikurin Ibaraki 1) and susceptible (Igri and Uschi) parental lines were filtered in the resulting VCF file (Danecek et al., 2011). Variant sites were retained in case they presented a minimum SNP quality score of 40, minimum genotype quality of 5, and minimum number of homozygous/heterozygous reads covering a position per sample of 2/4.

Whole-Genome Re-Sequencing of Chikurin Ibaraki 1 and Data Analysis

In order to obtain the whole-genome sequencing data of resistance donor line Chikurin Ibaraki 1, a seed bulk of Chikurin Ibaraki 1 was grown for 7 days and dark treated for 48 h (INRA-CNRGV Plant Genomic Center, Toulouse, France). High molecular weight (HMW) DNA was isolated using a Qiagen G-100 DNA extraction kit following the manufacturer's protocol (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiagen-genomic-tips/). The DNA was quantified

TABLE 1 | Screening of F_2 plants for the construction of *rym15* high-resolution mapping populations.

Crosses	Number of analyzed F_2 plants	Number of recombinant F_2 plants	Recombination frequency	Number of segmental RILs (F_4)		χ^2 1:1 ($df = 1$, $p > 0.05$)
				Resistant	Susceptible	
Igri × Chikurin Ibaraki 1	2,174	162	3.72%	67	72	0.18 ($P = 0.6714$)
Chikurin Ibaraki 1 × Uschi	5,728	288	2.51%	140	144	0.056 ($P = 0.8129$)

on a QBit (Invitrogen) and the quality was checked by using NanoDrop One (Thermo Scientific) according to the A260/A280 and A260/A230 ratios. The fragment size estimation was conducted by using the FEMTO pulse (Agilent). Subsequently, lyophilized DNA samples were used for PacBio SMRT sequencing (Center for Genomic Analysis, University of Kiel). Library preparation was conducted using the HiFi SMRTbell Express 2.0 kit (Pacific Biosciences, Menlo Park, USA) including BluePippin (Sage Science Inc., Beverly Massachusetts) size selection with a lower cutoff of 10 kb. Sequencing was performed on the Sequel II instrument on 6 SMRTcell 8 M, movie time of 30 h (Pacific Biosciences, Menlo Park, USA). PacBio HiFi data was assembled with the HiFi read assembler hifiasm (Cheng et al., 2021). The HiFi reads were deposited under project ID PRJEB50079 at the European Nucleotide Archive (ENA).

Marker Saturation

Genomic DNA of the constructed 423 segmental homozygous F_4 RILs was extracted using the CTAB method according to Stein et al. (2001). DNA samples of RILs were adjusted to a final concentration of 20 ng/μl and subsequently used for marker saturation.

Based on the physical position of the previous flanking markers *rym15*_1 and *rym15*_8, a set of 28 SNPs derived from the 50 K Illumina Infinium iSelect SNP chip (8 SNPs), GBS (8 SNPs) and assembly data (12 SNPs) located in the target interval was converted to KASP markers using BatchPrimer3 and PolyMarker (You et al., 2008; Ramirez-Gonzalez et al., 2015) algorithms. Furthermore, another two KASP markers located between markers *rym15*_1 and *rym15*_8 were selected from a previous study (Wang et al., 2021; **Supplementary Table S1**).

The high-resolution mapping populations derived from crosses I × C and C × U were genotyped using 32 and 29 KASP markers, respectively (**Supplementary Table S1**). PCR amplification was conducted in a 5 μl reaction volume consisting of 2.5 μl PACE™ (PCR Allele Competitive Extension) Genotyping Master Mix (Part. No.001–0002, 3CR Bioscience), 0.08 μl of each allele-specific primer 1 and allele-specific primer 2 (10.0 pmol/μl), 0.2 μl common primer (10.0 pmol/μl) and 2.2 μl template DNA (20 ng/μl). For KASP analysis, DNA was amplified in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with the following conditions: 94°C for 15 min; followed by PCR with 9 cycles of 20 s at 94°C, 1 min at 61°C; and then 25 cycles with 20 s at 94°C, 1 min at 55°C, and a final cool down at 37°C for 1 min. If necessary, a re-cycle with the following conditions was performed: 94°C for 3 min; followed by PCR with 9 cycles of 20 s at 94°C, 1 min at 57°C and a final cool down at 37°C for 1 min. The fluorescence signals from HEX and FAM for the specific alleles were detected using the same Detection System (Bio-Rad, Hercules, CA, USA) at 37°C after thermal cycling was complete. The physical position of the KASP markers was determined by blasting primers against the barley reference genome sequences (Mascher et al., 2017, 2021; Monat et al., 2019) using blastN at the IPK barley blast server (https://galaxy-web.ipk-gatersleben.de).

Linkage Analysis

Linkage analysis was performed by setting the number of recombinant gametes in relation to the number of gametes analyzed (Pellio et al., 2005). The genetic resolution of the population (% recombination) was calculated by dividing the number 1 by the number of gametes. To correct for those plants which died during cultivation, a "Corrected genetic resolution" for the remaining RILs was applied by dividing the % recombination identified for the F_2 generation by the number of those remaining RILs (Lüpken et al., 2013).

Collinearity of the Target Region Between Resistant and Susceptible Cultivars

The physical position of the new flanking markers identified in the present study was determined according to the sequence assembly of Morex v3. In order to visually compare the target region between the genotypes Chikurin Ibaraki 1, Igri, and Morex (Jayakodi et al., 2020; Mascher, 2020), the flanking markers were blasted against the whole-genome sequence of Chikurin Ibaraki 1 and Igri by using the tool Multiple Alignment using Fast Fourier Transform (MAFFT; Katoh and Standley, 2013) in the Galaxy web server (Giardine et al., 2005; Blankenberg et al., 2010; Goecks et al., 2010). The target region was identified in these two genotypes according to the best hits of both flanking markers, and the alignments of the target region between the three genotypes were plotted and visualized as a dot-plot with D-GENIES webpage (Cabanettes and Klopp, 2018) by using the Minimap2 aligner (Li, 2018).

Identification and Re-Sequencing of Candidate Genes

In the target region, the HC and LC genes were identified according to the gene annotation of Morex v3 (Mascher, 2020).[1] In order to extract the corresponding genes from Chikurin Ibaraki 1 assembly data, the sequences of HC and LC genes in the target interval of Morex were used as query for a BLASTN (Altschul et al., 1997) search against the target region of Chikurin Ibaraki 1. For the susceptible parental line Igri, annotated genes in the target interval were identified according to the pan-genome database available on the IPK Galaxy Blast Suite (Deng et al., 2007; Jayakodi et al., 2020).[2]

In order to obtain the gene sequence of 6 HC and 2 LC genes in the second susceptible parental line Uschi, based on the gene sequences of Morex v3 and Igri, the corresponding primers for re-sequencing of all identified genes were developed by using the online tool primer3 (**Supplementary Table S2**).[3] PCR amplification was conducted in a 30 μl reaction volume consisting of 3 μl of template DNA (25-30 ng/μl), 3 μl of 10 × buffer BD (detergent-free buffer), 3 μl of 25 mM $MgCl_2$, 0.6 μl of

[1]https://wheat.pw.usda.gov/GG3/content/morex-v3-files-2021
[2]https://galaxy-web.ipk-gatersleben.de/
[3]http://primer3.ut.ee/cgi-bin/primer3/primer3web_results.cgi

10 mM dNTP-Mix, 0.75 µl of each forward primer (10.0 pmol/µl) and reverse primer (10.0 pmol/µl), 0.6 µl of HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) and 18.3 µl double distilled water. The DNA was amplified in a GeneAmp PCR System 9,700 (Applied Biosystems) under the following conditions: 94°C for 5 min; followed by touchdown PCR with 12 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C; and then 35 cycles with 30 s at 94°C, 30 s at 56°C, 30 s at 72°C; and a final extension at 72°C for 10 min. Amplified products (1 µl) were checked on an agarose gel (1.5%) and analyzed using the imaging system Gel Doce™ XR and the Quantity One® 1-D analysis software (4.6.2; Bio-Rad, Hercules, CA, USA). PCR products were purified and sequenced by the company Microsynth AG (Balgach, Switzerland). Obtained sequences were edited and the polymorphisms between parental lines (Chikurin Ibaraki 1, Igri, and Uschi) were identified using Sequencher 5.1 software (Gene Codes, Ann Arbor, MI, United States).

RESULTS

High-Resolution Mapping Populations for *rym15*

Two crosses were used for the construction of the high-resolution mapping populations. In total, 2,218 and 5,870 F_2 plants derived from I × C and C × U were sown, of which 2,174 and 5,728 germinated and were analyzed subsequently. From these, 162 (3.725% recombination) and 288 (2.514% recombination) segmental recombinant F_2 plants were identified, respectively (**Table 1**). Initially, for the population I × C, a total of 2,174 F_2 plants providing a genetic resolution of 0.0230% recombination was screened for recombination events between the previous flanking markers *rym15*_1 and *rym15*_8 and a genetic distance of 3.725% recombination was determined. Due to the non-survival of recombinant plants, the corrected genetic resolution provided by 139 remaining RILs equaled 0.02679% recombination. For population C × U, a total of 5,728 F_2 plants providing a genetic resolution of 0.0087% recombination were screened for recombination events between the flanking markers *rym15*_1 and *rym15*_8 and a genetic distance of 2.514% recombination was determined. Due to the non-survival of recombinant plants, the corrected genetic resolution provided by 284 remaining RILs equaled 0.00885% recombination.

BaMMV Phenotyping

The BaMMV infection experiment showed a segregation of 67 resistant and 72 susceptible, as well as 140 resistant and 144 susceptible RILs in the population I × C and C × U, respectively, which fit to the expected 1r:1 s ratio. Chi-square test in the population I × C (χ^2 1r:1 s = 0.180, df = 1, $p = 0.6714$) and C × U (χ^2 1r:1 s = 0.056, df = 1, $p = 0.8129$) for goodness of fit indicated that the resistance against BaMMV is controlled by a single gene (*rym15*) in both populations (**Table 1**).

Marker Saturation of the *rym15* Locus

GBS analysis of three parental lines identified 27,017 (Chikurin Ibaraki 1 and Igri) and 29,197 (Chikurin Ibaraki 1 and Uschi) polymorphisms. In total, 20,099 polymorphisms (74.39%) were identical among both comparisons. On the target chromosome 6H, a set of 3,388 (Chikurin Ibaraki 1 and Igri) and 3,813 (Chikurin Ibaraki 1 and Uschi) polymorphisms was identified, of which 2,488 (73.44%) were in common. In the target region between the previous flanking markers *rym15*_1 and *rym15*_8, a set of 365 (Chikurin Ibaraki 1 and Igri) and 396 (Chikurin Ibaraki 1 and Uschi) polymorphisms was identified, of which 301 (82.47%) were in common (**Supplementary Table S3**).

The *rym15* target region was saturated with a set of 32 KASP markers that span a 133 Mb interval on chromosome 6H in Morex v3. Out of these 32 markers, three polymorphisms (QBS134, QBS135, and QBS140) could not be reproduced in the population C × U (**Supplementary Table S1**). In the population I × C, mapping of all 32 markers reduced the target interval of *rym15* from 3.5 cM to a smaller region of 0.161 cM between markers QBS140 and QBS159, and 18 markers co-segregated with the target locus (**Figure 1**). In the population C × U, analysis of all 29 markers reduced the interval harboring *rym15* from 3.7 cM to 0.036 cM between markers QBS143 and QBS151, and 7 markers co-segregated with the target gene *rym15* (**Figure 1**).

BLASTN comparison of marker sequences against the barley reference sequence Morex v3 revealed that all mapped markers are co-linear genetically and physically in both mapping populations, and the physical size of the target region in the population I × C and C × U is 11.3 Mb and 0.28 Mb, respectively (**Figure 2**). The marker saturation revealed a large difference of recombination distribution between the two populations (**Figure 2**). In the population C × U, the recombination frequencies have been estimated from 1.51 to 190.19 Mb/cM, while the population I × C shows suppressed recombination, of which the physical/genetic ratio varies from 7.95 to 686.07 Mb/cM. In the population C × U, the recombination event between markers QBS143 and QBS144 (1.51 Mb/cM), as well as QBS150 and QBS151 (7.91 Mb/cM) are crucial for mapping the target gene *rym15* to a smaller interval of 0.28 Mb. In contrast, those markers co-segregated with *rym15* in the population I × C (**Figure 2**).

Similarity of Target Region Between Parental Lines

Taking advantage of the second population C × U, the physical size of the target region between markers QBS143 and QBS151 encompassed 281 kb according to Morex v3 (**Figure 2**). Blasting the flanking marker sequences against the Chikurin Ibaraki 1 and Igri genome assemblies revealed that the corresponding physical size of the target region is around 282 and 285 kb, respectively (**Supplementary Figure S1**). A dot-plot analysis comparing the target region between the parental lines Chikurin Ibaraki 1 and Igri, and between Chikurin Ibaraki 1 and Morex v3, revealed a substantial co-linearity and similarity with identity ranging from 75 to 100% (**Supplementary Figure S1**). The

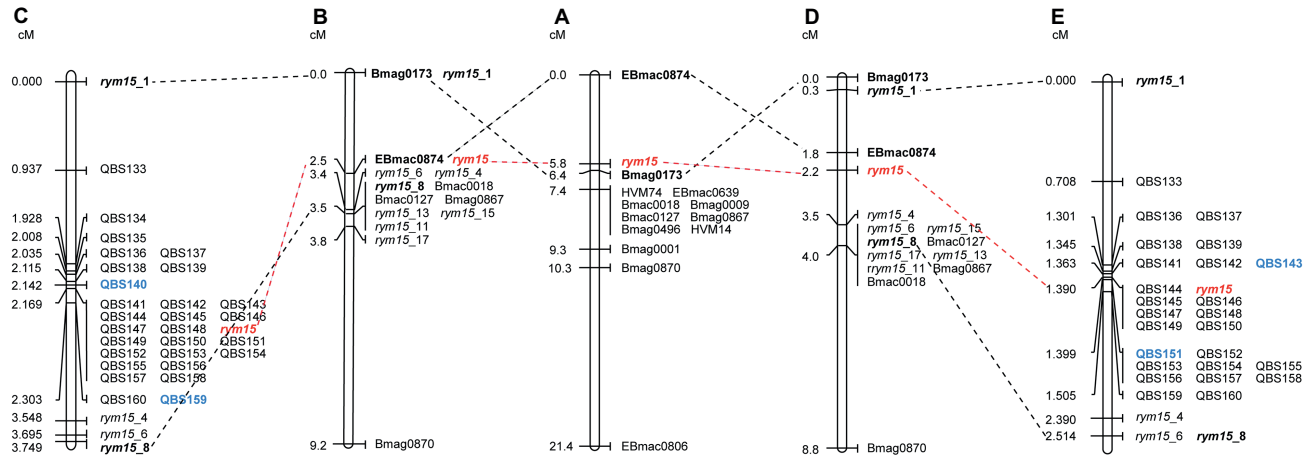

FIGURE 1 | Genetic maps of *rym15* and collinearity of common molecular markers. **(A)** An initial genetic map of *rym15* based on a set of 217 DH lines derived from the cross of Chikurin Ibaraki 1 × Plaisant (Le Gouis et al., 2004). **(B)** Medium-resolution map of *rym15* based on a set of 180 F_2 lines derived from the population Igri×Chikurin Ibaraki 1 (Wang et al., 2021). **(C)** High-resolution map of *rym15* based on a set of 139 F_4 segmental RILs in the population Igri ×Chikurin Ibaraki 1. **(D)** Medium-resolution map of *rym15* based on a set of 342 F_2 lines derived from the population Chikurin Ibaraki 1 × Uschi (Wang et al., 2021). **(E)** High-resolution map of *rym15* based on a set of 284 F_4 segmental RILs in the population Chikurin Ibaraki 1 × Uschi. The target gene *rym15* is highlighted in red, the bold font indicates previous flanking markers from the initial and medium-resolution maps, while the new flanking markers identified from high-resolution mapping are shown in blue.

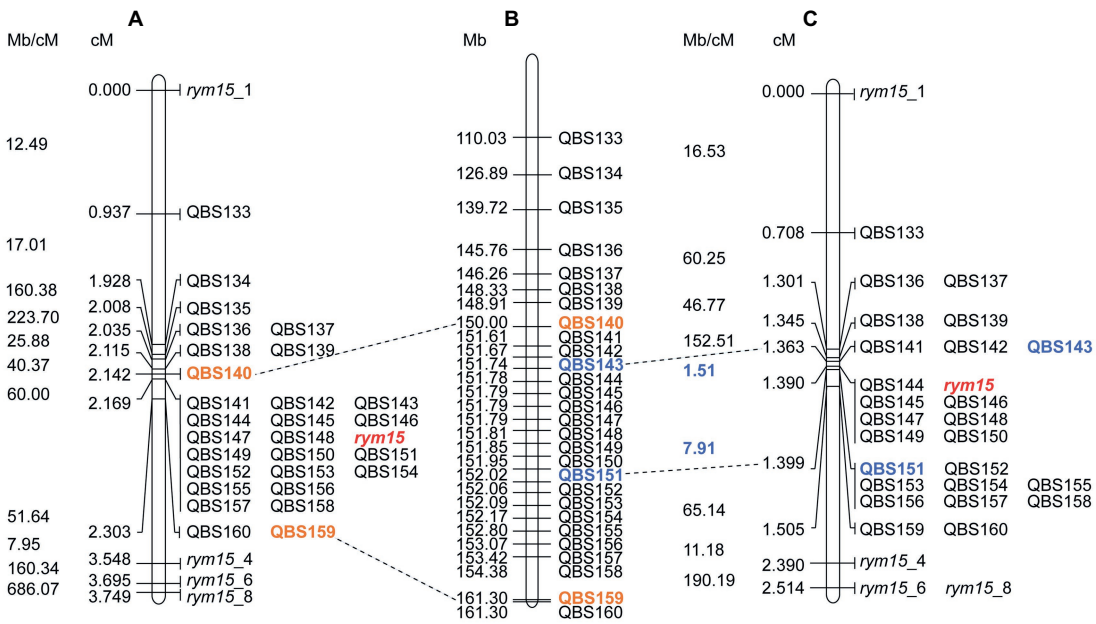

FIGURE 2 | High-resolution genetic maps of *rym15* and physical map of barley chromosome 6HS. **(A)** High-resolution map of *rym15* based on a set of 139 F_4 RILs in the population Igri×Chikurin Ibaraki 1. **(B)** Physical map of Morex on chromosome 6HS according to Morex v3. **(C)** High-resolution map of *rym15* based on a set of 284 F_4 RILs in the population Chikurin Ibaraki 1 × Uschi. The recombination rates (Mb/cM) are listed left of the genetic maps for both populations. The target gene *rym15* is highlighted in red and the flanking markers are shown in orange (Igri×Chikurin Ibaraki 1) and blue (Chikurin Ibaraki 1 × Uschi). Crucial recombination events in the population Chikurin Ibaraki 1 × Uschi are highlighted in blue.

micro co-linearity between physical and genetic order of all used markers was consistent. In the other pan-genome accessions, the physical size of the target region ranged from 0.26 (Golden Promise) to 0.34 Mb (HOR3365; **Supplementary Table S4**).

Candidate Gene Analysis at the *rym15* Locus

In our previous medium-resolution maps of *rym15*, the interval was mapped between the two markers *rym15*_1 and *rym15*_8

with a physical size of 133 Mb according to the Morex v3 reference genome. In this region, 620 HC genes and 1,025 LC genes are located (Wang et al., 2021). Due to extensive marker saturation in the present study, the physical size of the target region was reduced to 281 kb in the population C × U. In this region a set of 8 genes was identified, of which 6 are HC and two are LC genes. The annotation of two LC genes HORVU.MOREX.r3.6HG0573640 and HORVU.MOREX.r3.6HG0573660 are ATP-dependent DNA helicase and Retrovirus-related Pol polyprotein from transposon TNT 1–94, respectively. Out of the 6 HC genes, four encode zinc finger CCCH domain-containing proteins (HORVU.MOREX.r3.6HG0573600, HORVU.MOREX.r3.6HG0573610, HORVU.MOREX.r3.6HG0573620 and HORVU.MOREX.r3.6HG0573650). The other two HC genes are coding for non-structural maintenance of chromosome element 4 (NSE4) and D-alanine-D-alanine ligase family (HORVU.MOREX.r3.6HG0573590 and HORVU.MOREX.r3.6HG0573630; **Figure 3**). Meanwhile, according to the annotation data of Igri, in the target region, the same number of the HC genes was found with the same order and description as in Morex v3 (Horvu_IGRI_6H01G211100.1, Horvu_IGRI_6H01G211200.1, Horvu_IGRI_6H01G211300.1, Horvu_IGRI_6H01G211400.1, Horvu_IGRI_6H01G211500.1, and Horvu_IGRI_6H01G211600.1). Furthermore, the order of those 6 HC and two LC genes in Chikurin Ibaraki 1 was revealed to be the same as in Morex and Igri. Finally, the alignment analysis of the coding region of the 6 HC and two LC genes from three parental lines shows that three HC genes (HORVU.MOREX.r3.6HG0573620, HORVU.MOREX.r3.6HG0573630, and HORVU.MOREX.r3.6HG0573650) and two LC genes (HORVU.MOREX.r3.6HG0573640 and HORVU.MOREX.r3.6HG0573660) are monomorphic between resistant and susceptible genotypes. In contrast, for the remaining three HC genes, one functional SNP was identified for each of the genes (HORVU.MOREX.r3.6HG0573590, HORVU.MOREX.r3.6HG0573600, and HORVU.MOREX.r3.6HG0573610; **Table 2**).

Further analyses of the sequence of Golden Promise, which is susceptible to BaMMV, revealed the same three HC genes (Horvu_GOLDEN_6H01G188600, Horvu_GOLDEN_6H01G188700, and Horvu_GOLDEN_6H01G188800). The alignment of these three HC genes between Chikurin Ibaraki 1 and Golden Promise revealed that one HC gene (Horvu_GOLDEN_6H01G1887000) has the same coding sequence in both genotypes. For the remaining two HC genes Horvu_GOLDEN_6H01G188600 and Horvu_GOLDEN_6H01G188800, one functional SNP was detected in each gene between Chikurin Ibaraki 1 and Golden Promise. Thus, only two HC genes (HORVU.MOREX.r3.6HG0573590 and HORVU.MOREX.r3.6HG0573610) are promising candidates in the target region (**Table 2**). Meanwhile, it was shown that the functional SNPs-derived KASP markers QBS146 (located in HC gene HORVU.MOREX.r3.6HG0573590) and QBS148 (located in HC gene HORVU.MOREX.r3.6HG0573610) co-segregated with the target locus *rym15* in both populations.

DISCUSSION

In the present study, phenotypic analysis of 423 F_4 segmental RILs showed that the BaMMV resistance of Chikurin Ibaraki 1 is controlled by a single gene. This confirms results of previous studies (Le Gouis et al., 2004; Wang et al., 2021). By high-resolution mapping, the target region harboring *rym15* was narrowed down to 281 kb and 6 HC candidate genes were identified for the BaMMV resistance locus *rym15*. Functional SNPs between resistant and susceptible genotypes were detected in only two HC genes, representing a substantial step toward cloning of *rym15*.

It is well known that recombination rates are not fixed and a significant inter-individual variability has been reported for virtually every species, such as bacteria, fungi, plants, and animals (Simchen and Stamberg, 1969; Brooks, 1988; Fisher-Lindahl, 1991; Petes et al., 1991). Various studies about recombination rates and gene densities in barley show that gene density is not uniform along the chromosome and is usually correlated with recombination frequency (Han et al., 1998; Künzel et al., 2000; Rostoks et al., 2002). On chromosome 6HS, the calculated recombination frequency and gene density are not high in the region between markers *rym15*_1 and *rym15*_8 (Muñoz-Amatriaín et al., 2015). In the present study, the use of two different mapping populations reflects the different recombination rates within a defined interval. The population I × C showed a reduced recombination rate in this region compared with the population C × U. A set of 18 and 7 markers co-segregated with the target locus *rym15* in the population I × C and C × U, respectively. Four markers, which co-segregated with *rym15* in the population I × C revealed crucial recombination events between QBS143 and QBS144 (1.51 Mb/cM), as well as QBS150 and QBS151 (7.91 Mb/cM) in the population C × U, facilitating narrowing of the *rym15* interval to 281 kb.

The accuracy of genome sequence information in the target region is key to identifying candidate genes in a resistance donor. Previously, cloning of BaYMV/BaMMV recessive resistance genes *rym4*/*5* and *rym1*/*11* was assisted by bacterial artificial chromosome (BAC) clones, which is a cumbersome and time-consuming process (Stein et al., 2005; Yang et al., 2014). As third-generation sequencing technologies recently become achievable and affordable, a recent study comparing different long-read sequencing methods revealed that the PacBio HiFi sequencing method performed best for sequence assembly of barley (Mascher et al., 2021). In the present study, re-sequencing of the resistant donor Chikurin Ibaraki 1 was conducted using PacBio HiFi reads. Finally, a set of two HC genes was identified with the assistance of the whole-genome assembly of Chikurin Ibaraki 1. In future, this assembly may be used to map another recessive BaYMV resistance gene present in Chikurin Ibaraki 1, which is located on chromosome 5HS (Werner et al., 2003). The availability of the barley pan-genome, comprising a set of 20 diverse barley accessions including the population I × C susceptible parental line Igri (Jayakodi et al., 2020), was critically important for the *rym15* candidate gene identification.

It is well known that new pathogen variants may be virulent to major resistance genes. For example, the isolated resistance gene *rym4*/*5* has been overcome in different regions of Europe and East Asia, and another resistance gene *rym1*/*11* became susceptible to isolate BaYMV-CN_NY in China as well (Kühne

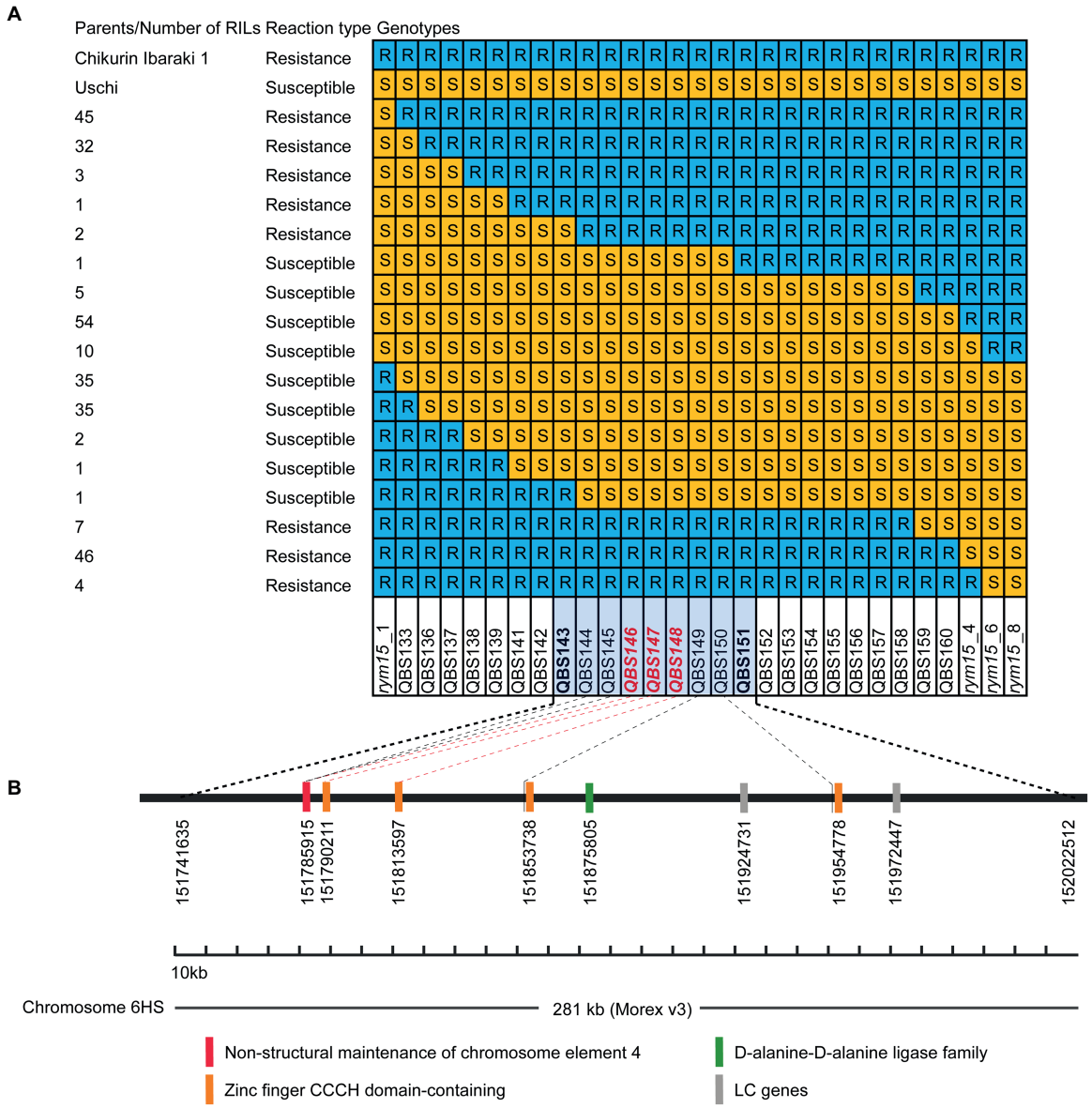

FIGURE 3 | Candidate genes in the *rym15* target region of 281 kb. **(A)** Graphical genotypes of 284 F_4 RILs derived from population Chikurin Ibaraki 1 × Uschi carrying recombination events between *rym15*_1 and *rym15*_8. Marked with red color are those located in the coding region of high confidence genes. **(B)** A set of six high confidence and two low confidence genes positioned in the target interval.

TABLE 2 | Functional SNPs between resistant (Chikurin Ibaraki 1) and susceptible (Igri, Uschi and Golden Promise) lines originated from three candidate genes.

Gene			SNP				Codon	Amino acid substitution
Morex v3	Igri	Golden Promise	Chikurin Ibaraki 1	Igri	Uschi	Golden Promise		
HORVU.MOREX.r3.6HG0573590.1	Horvu_IGRI_6H01G211100.1	Horvu_GOLDEN_6H01G188600.1	G	T	T	T	GCA->TCA	Ala(A)->Ser(S)
HORVU.MOREX.r3.6HG0573600.1	Horvu_IGRI_6H01G211200.1	Horvu_GOLDEN_6H01G188700.1	G	A	A	G	GAC->AAC	Asp(D)->Asn(N)
HORVU.MOREX.r3.6HG0573610.1	Horvu_IGRI_6H01G211300.1	Horvu_GOLDEN_6H01G188800.1	A	G	G	G	TGA->TGG	Ter(*)->Trp(W)

et al., 2003; Kanyuka et al., 2004; Habekuß et al., 2007; Nishigawa et al., 2008; Rolland et al., 2017; Jiang et al., 2022). These examples highlight the importance of identifying new genetic resources that are resistant to new virulent virus isolates. The two HC genes carrying functional SNPs between resistant and susceptible cultivars are NSE4 (HORVU.MOREX.r3.6HG0573590) and a zinc finger CCCH domain-containing protein (HORVU.MOREX.r3.6HG0573610), which have not yet been reported as resistance genes against BaMMV/BaYMV. According to the information obtained from UniProt (https://www.uniprot.org/), the candidate gene HORVU.MOREX.r3.6HG0573590 promotes sister chromatid alignment after DNA damage and facilitates double-stranded DNA break (DSBs) repair *via* homologous recombination between sister chromatids (Watanabe et al., 2009). In contrast, the other candidate gene HORVU.MOREX.r3.6HG0573610 encodes a zinc finger CCCH domain-containing protein. This kind of protein was reported to be involved in cell fate specification and developmental processes in plants, as well as in the response to biotic and abiotic stress (Ai et al., 2022). Several studies confirmed that the CCCH-type zinc finger protein is responsible for resistance against different pathogens in different plant species. For example, a novel CCCH-type zinc finger protein GhZFP1 derived from cotton (*Gossypium hirsutum*) positively regulates resistance to the fungal pathogen *Rhizoctonia solani* in tobacco (Guo et al., 2009). The study of rice CCCH-type zinc finger protein C3H12 concluded that this gene is positively regulated to mediate resistance against the bacterial pathogen *Xoo* (Deng et al., 2012). Another study shows that the pepper TZnF protein CaC3H14 is involved in the defense response of pepper to infection by *Ralstonia solanacearum* (Qiu et al., 2018). Furthermore, an Arabidopsis CCCH protein C3H14 is a positive regulator for basal defense against *Botrytis cinerea* mainly by WRKY33 signaling (Wang et al., 2020). Moreover, the predicted K homology (KH) domain in the gene HORVU.MOREX.r3.6HG0573610 usually has an RNA-binding function (Burd and Dreyfuss, 1994). Considering all the evidence, it seems that the gene HORVU.MOREX.r3.6HG0573610 is the most likely candidate for BaMMV resistance encoded by *rym15*. Functional analysis of the two candidate genes, for example by gene editing (Hoffie et al., 2021) will likely lead to cloning of the causal gene for *rym15*.

CONCLUSION

In the present study, two high-resolution mapping populations were constructed, comprising 423 F_4 segmental RILs from the crosses of I×C (139 RILs) and C×U (284 RILs). Phenotypic analysis revealed that the resistance against BaMMV encoded by *rym15* is controlled by a single gene. Using combinations of different whole-genome and targeted sequencing methods, detected polymorphisms between parental lines were converted to KASP markers and subsequently analyzed on all RILs. Combining the genetic and phenotypic data, two high-resolution maps were constructed. The physical size of the target region was reduced to a 0.28 Mb region containing six HC and two LC genes. Taking advantage of public genome assemblies including the susceptible cultivar Golden Promise and Igri assembly data, functional SNPs between resistant and susceptible parental lines were detected in only two HC genes. However, the functional analysis of these two genes is still needed to identify the causal gene for *rym15*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI with accession PRJEB50079.

AUTHOR CONTRIBUTIONS

DP and FO conceived the project, acquired the funding, and designed the experiments. AH provided the initial F_2 populations. YW performed the experiments and wrote the manuscript. JF carried out the re-sequencing of Chikurin Ibaraki 1. YW and DP analyzed the data. MJ and MM conducted the genome assembly of Chikurin Ibaraki 1. FO, RS, AS, and DP edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.908170/full#supplementary-material

Supplementary Figure S1 | Collinearity of **(A)** Chikurin Ibaraki 1 vs. Morex v3 and **(B)** Chikurin Ibaraki 1 vs. Igri genome assemblies in the target region between markers QBS143 and QBS151. Colored boxes with red and green indicate high confidence and low confidence genes, respectively.

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6. General Discussion

6.1. Barley genome resources greatly facilitate gene isolation in barley

The increased accessibility of genome sequence information and publicly available genomic databases have brought barley breeding to a "genomic" era (Riaz et al., 2021). Using genomics tools for accelerating crop improvement is referred to as genomics-assisted breeding (GAB, Varshney et al., 2005, 2021). During the last decades, many success stories demonstrated the potential of GAB in barley breeding, for example the isolation of the recessive resistance genes *rym4*/*5* and *rym1*/*11* against barley yellow mosaic disease (Kanyuka et al., 2005; Stein et al., 2005; Yang et al., 2014b), the major seed dormancy genes *QTL FOR SEED DORMANCY 1* (*Qsd1*) and *Qsd2* (Sato et al., 2016; Nakamura et al., 2016; Hisano et al., 2021), the gene *Uniculme4* (*Cul4*) that controls axillary bud differentiation and the development of the ligule (Pozzi et al., 2003; Druka et al., 2011; Tavakol et al., 2015), and a gene that encodes a P-type heavy metal ATPase 3 (*HvHMA3*) responsible for grain Cd accumulation in barley (Lei et al., 2020).

In the present study, barley reference assemblies, barley genomic databases and molecular tools, as well as bioinformatics- and genomics-based approaches were applied to accelerate the identification of candidate genes for the BaMMV resistance gene *rym15*. The genome assembly of the susceptible parental line Igri is included in the barley pan-genome (Jayakodi et al., 2020). In combination with re-sequencing data from the resistant donor Chikurin Ibaraki 1, this led to the identification of 12 SNPs flanking *rym15* in a 1.18 Mbp region between flanking markers QBS141 and QBS155. This was critical to downsizing the target region to 0.28 Mbp. The ability to develop and use KASP markers from these 12 SNPs for fine mapping reflected the importance of the availability of the barley references and pan-genome resources, due to the absence of polymorphisms in the mentioned region of 1.18 Mbp from GBS and SNP-array data. In addition, the BaMMV-susceptible barley accession Golden Promise is also included in the barley pan-genome. Combining data from this

genotype with WGS data of Chikurin Ibaraki 1, followed by blast analysis of HC genes in these two genotypes, decreased the number of candidate genes for the BaMMV resistance locus *rym15*.

During the study period, the use of the barley reference genomes Morex v1, v2 and v3 not only accelerated the identification of candidate genes for the target locus *rym15*, but also witnessed improvements in accuracy and completeness of each updated version of the Morex reference genome. Notably, in the step of medium-resolution mapping of *rym15*, no hits on chromosome 6H in Morex v1 were found for the SSR marker Bmag0173, while the blastN alignment of the reverse primer against Morex v2 revealed a hit on chromosome 6H. Thus, the physical position of Bmag0173 in Morex v2 could be used as reference information when comparing the order of the two previous flanking markers EBmac0874 and Bmag0173, which were inverted in previous studies (Le Gouis et al., 2004; Ramsay et al., 2000). However, it has to be noticed that the region between these two SSR markers is not within target region of 0.281 Mbp in the present study. This discrepancy emphasizes the importance of remapping the gene when using different mapping populations. Usually, after low-resolution mapping of the target locus, the following step is the construction of high-resolution mapping populations for the target locus by using the flanking markers identified in low-resolution maps. In the present study, medium-resolution maps were constructed in order to identity new flanking markers, based on SNP chip data with known precise positions compared to previous SSR markers. Unexpectedly, the results revealed that the locus *rym15* is not located in the previously identified region.

In addition, several map-based cloning studies also revealed the accuracy improvements of the updated version of the Morex reference genome. For example, fine mapping of barley male-sterility gene *msg26* (Qi et al., 2019) identified an inversion between the genetic and physical map of Morex v1. Based on the genetic analyses in barley and collinear studies in wheat and rice, the results indicated that the interval (ca. 45 kbp on chromosome 4HL) in barley was incorrectly assembled in the reference sequence Morex v1 (Mascher et al., 2017). Another study about fine

mapping of leaf rust resistance gene *Rph28* used Morex v1 and v2 as references (Mehnaz et al., 2021). The quality of genome assemblies within the target region on chromosome 5HL was determined for both Morex v1 and v2 using the genomic similarity search tool YASS, which revealed a putative 310 kbp inversion from 640.59 to 640.90 Mbp. In this region, only two HC genes were detected in Morex v1, while 10 HC genes were found in v2. Finally the Morex v2 was used as a road map in this study (Mehnaz et al., 2021).

6.2. Different recombination rates among the different populations

The recombination rate in the target region is one of the key factors for narrowing down the target region of the gene of interest. The big difference of recombination rates between the two populations C×U and I×C revealed the advantage of using population C×U for the identification of two HC genes for the BaMMV resistance locus *rym15* in the present study. However, it is not possible to determine which factors account for differences of the recombination rates among the two populations. It is known that crossover frequencies can be significantly different between male and female meiosis (Devaux et al., 1995; Cistue et al., 2011; Phillips et al., 2015). In these two crosses, the resistant parental line Chikurin Ibaraki 1 was used as male and female in I×C and C×U, respectively. However, the cross was conducted with different susceptible parental lines and the size of the generated population was different.

Mascher et al. (2017) reported that disease resistance genes are located in the distal regions with high recombination rates, while the genes involved in photosynthesis are located in interstitial regions with low recombination rates. Similarly, the gene density distribution is not uniform along the chromosomes. The centromeric regions are known to have a low gene density which increased towards the distal ends. However, there were also gene-dense regions with suppressed recombination regions identified on barley chromosomes 2H (approx. 200–250 Mbp) and 5H (approx. 100–150 Mbp) (Muñoz-Amatriaín et al., 2015). A total of six HC and two LC genes were located in

the target region of 0.281 Mbp. According to the released consensus map, the gene density and recombination frequency in this region is relatively low (Muñoz-Amatriaín et al., 2015).

6.3. Candidate genes for *rym15*

The HC genes found within the fine-mapping interval of *rym15*, HORVU.MOREX.r3.6HG0573590 and HORVU.MOREX.r3.6HG0573610, encode for the non-structural maintenance-of-chromosome element 4 (NSE4) and a zinc-finger Cysteine3Histidine (CCCH) domain containing protein, respectively. Up to now, neither gene has been reported as resistance gene against BaMMV/BaYMV. In plants, NSE4 plays a role in DNA double strand break repair, meiotic synapsis and recombination (Zelkowski et al., 2019). It also preserves genome stability and controls seed development (Díaz et al., 2019). The second candidate gene belongs to the *CCCH* gene family and exhibits RNA binding and processing activity in animals and plants (Bai and Tolias, 1996; Wang et al., 2008). Moreover, zinc-finger CCCH domain containing proteins are reported to be involved in plant growth, development, and adaptive responses to the environment (Bogamuwa and Jang, 2014).

Recently, a study provided a comprehensive characterization of barley CCCH transcription factors, their diversity and biological functions (Ai et al., 2022). In this study, a set of 53 protein-encoding CCCH genes was identified in barley, of which nine are located on chromosome 6H. In the target region of 0.281 Mbp, four zinc-finger domain containing proteins were identified. The second candidate gene (HORVU.MOREX.r3.6HG0573610) and another nearby located zinc-finger domain containing protein (HORVU.MOREX.r3.6HG0573620) are a pair of tandemly duplicated genes that cluster in the same clade of the phylogenetic tree (Ai et al., 2022). Both have two zf-CCCH domains and one K homology (KH) domain. However, the characterized cis-acting regulatory elements in the promoter region of the two zinc-finger domain containing protein genes are different. In HC gene HORVU.MOREX.r3.6HG0573610, three GC-motifs (CCCCCG) were identified that

are involved in anoxic specific inducibility. These are absent in the duplicated gene HORVU.MOREX.r3.6HG0573620. Moreover, the subcellular localization prediction showed that the candidate gene HORVU.MOREX.r3.6HG0573610 is located in the nucleus, while the HORVU.MOREX.r3.6HG0573620 is located in the chloroplast (Ai et al., 2022). Nevertheless, in addition to the two candidate genes, the gene expression analysis of gene HORVU.MOREX.r3.6HG0573620 may be conducted to examine whether this gene also functions in BaMMV resistance, even though no functional SNP could be identified between resistant and susceptible genotypes.

6.4. Outlook

To conclude, the medium-resolution maps identified new flanking markers *rym15*_1 and *rym15*_8. All markers showed the same order in two mapping populations and the barley reference assembly Morex v2 (Wang et al., 2021). By using the flanking markers *rym15*_1 and *rym15*_8, the high-resolution mapping populations were constructed and the target region was downsized to 11.3 Mbp and 0.281 Mbp in population I×C and C×U, respectively. In target region, a set of six HC and two LC genes was identified. Blast analysis revealed functional SNPs at two HC genes (Wang et al., 2022). Overall, the results obtained in the present study are the end point for mapping of the target locus *rym15*. The identified markers QBS146 and QBS148 may be used for MAS of the BaMMV resistance locus *rym15*. The segmental F_4 recombinant inbred lines (RILs) and sequence assembly data of Chikurin Ibaraki 1 that were generated in the present study can also be used for map-based cloning of another BaYMV resistance gene derived from Chikurin Ibaraki 1, which is located on chromosome 5HS. Meanwhile, this work represents the starting point for gene identification of the target gene *rym15*.

Usually the gene function has to be confirmed by complementation or mutant analysis once a candidate gene has been identified (Stein and Graner, 2005). Like the previously isolated BaMMV/BaYMV resistance genes *rym4/5* (Kanyuka et al., 2005; Stein et al., 2005) and *rym1/11* (Yang et al., 2014b), the target gene *rym15* is also

recessively inherited. The full-length cDNA of candidate genes HORVU.MOREX.r3.6HG0573590 or HORVU.MOREX.r3.6HG0573610 from susceptible genotype Igri/Uschi could be transformed to resistant genotype Chikurin Ibaraki 1, resulting in transgenic plants which possess both an endogenous resistance allele and the putative susceptibility allele (present as the transgene). In addition, the project partner IPK, who has created new alleles of *EIF4E* gene (*rym4*) by Cas9 endonuclease in Igri (Hoffie et al., 2021), is presently carrying out CRISPR-Cas9 validation studies for two HC genes in the BaMMV-susceptible cultivar Golden Promise. To date, genome editing in barley has been mostly carried out in this genotype due to the superior transformation efficiency (Murray et al., 2004; Ibrahim et al., 2010; Lim et al., 2018) and the availability of the genome reference sequence (Schreiber et al., 2020; Jayakodi et al., 2020). Results from this ongoing work, based on the map-based medium-resolution and fine-mapping of *rym15* from Chikurin Ibaraki 1, are likely to lead to cloning of the causal gene for *rym15* in the foreseeable future.

7. Reference

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8. Appendix

8.1. Appendix 1

Delineating the elusive BaMMV resistance gene *rym15* in barley by medium-resolution mapping

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Molecular breeding 41(12): 1–13.

Supporting information for this article is available on the internet as an online version:

https://link.springer.com/article/10.1007/s11032-021-01270-9#Sec13

8.2. Appendix 2

High-resolution mapping of *Barley mild mosaic virus* resistance gene *rym15*

Wang Y, Habekuß A, Jayakodi M, Mascher M, Snowdon RJ, Stahl A, Fuß J, Ordon F, Perovic D. 2022.

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Supporting information for this article is available on the internet as an online version:

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9. Contributions to meetings and conferences

Poster presentations

Wang Y, Habekuß A, Perovic D, Ordon F (2017) High-resolution and-density mapping of *Barley mild mosaic virus* (BaMMV) resistance gene *rym15*. In: JKI (ed.): 10th Young Scientists Meeting, 08-10.11.2017, Siebeldingen- Abstracts (Berichte aus dem Julius Kühn-Institut 192), pp76.

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Oral presentations

Wang Y, Habekuß A, Perovic D, Ordon F (2019) High-resolution mapping of BaMMV resistance gene *rym15*. GPZ, 09-10.12.2019, Fulda

Wang Y, Cai Y, Perovic D, Hoffie RE, Kumlehn J, Schondelmaier J, Eibel S, Schweizer G, Büttner B, Ordon F (2021) IdeMoDeResBar ll: dentification, Modification and Deployment of genetic factors controlling Resistance to important pathogens in Barley. PLANT 2030 Status Seminar 2021, 10-11. 03.2021

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11. Erklärung

gemäß der Promotionsordnung des Fachbereichs 09 vom 07. Juli 2004 § 17 (2)

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe.

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