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ORIGINAL PAPER

High-resolution genetic mapping and physical map construction for the fertility restorer *Rfm1* locus in barley

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Abstract

Key message **High-resolution genetic linkage mapping and BAC physical mapping narrowed the fertility restorer locus *Rfm1* in barley to a sub-centimorgan genetic interval and a 208-kb physical interval.**

Abstract *Rfm1* restores the fertility of *msm1* and *msm2* male-sterile cytoplasms in barley. The fertility restoration gene is located on the short arm of chromosome 6H (6HS), and we pursued a positional cloning of this gene. Starting from a previous result that has delimited *Rfm1* within a 10.8 cM region on 6HS, we developed novel CAPS and SSR markers tightly linked to the gene in barley using the sequence information from the syntenic region of rice and barley genome assemblies. Next, we performed fine mapping of the *Rfm1* locus. To isolate recombinants, we surveyed 3,638 F_2 plants derived from a cross between the CMS strain and the *Rf* strain with adjacent markers (*NAS2090* and *NAS1080*). This analysis identified 175 recombinant plants from the F_2 population to build a high-resolution map with nine markers tightly linked to the *Rfm1* locus. *Rfm1* was located within the 0.14 cM region delimited by two markers (*NAS9113* and *NAS9200*). Using these flanking markers as well as marker cosegregating with *Rfm1* (*NAS9133*), we screened the BAC libraries of the cultivar Morex, an *rfm1* carrier. We isolated 11 BAC clones and constructed a BAC physical map using their fingerprints. Finally, we delimited the *Rfm1* locus encompassing the *rfm1* allele on a 208-kb contig composed of three minimally overlapping BAC clones. This precise localization of the *Rfm1* locus in the barley genome is expected to greatly accelerate the future map-based cloning of the *Rfm1* gene by sequence analysis and its genetic transformation for the complementation of cytoplasmic male-sterile plants.

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that causes sterility of plants because of a defect in the

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male reproductive organs but does not affect female fertility. CMS is caused by lesions in or rearrangements of the mitochondrial genome. Nuclear-encoded "restorer of fertility (*Rf*)" genes can restore CMS (Hanson and Bentolila 2004). The phenotypes conferred by CMS and *Rf* genes underlie agronomic traits that are important for the production of hybrid seed at a commercial scale. CMS-*Rf* systems have been identified in many plants and are used for hybrid seed production of several crops such as maize, rapeseed, sorghum, and rice. In barley, F_1 hybrid seed production using balanced tertiary trisomics has been reported (Ramage 1983), and a CMS-Rf system has been commercially used (HYVIDO® from Syngenta, http://www3.syngenta.com/country/uk/en/Crops/Cereals/Hyvido/Pages/HybridBarley.aspx), although the molecular mechanisms underlying the barley CMS-Rf system is still unknown.

To date, several *Rf* genes have been cloned from different species. Most of them encode PPR proteins composed of 14 or 16 repeats of the 35-amino acid pentatricopeptide repeat (PPR) motif, which form a large multi-gene family in plant genomes. PPRs are thought to be RNA-binding proteins involved in posttranscriptional processes (RNA processing and translation) in mitochondria and chloroplasts (Lurin et al. 2004). Petunia *Rf* (Bentolila et al. 2002), radish *Rfk1* (*Rfo*) (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003), and rice *Rf-1* (Akagi et al. 2004; Kazama and Toriyama 2003; Komori et al. 2004) are *Rf*-PPR genes classified into the RFL type belonging to the P-subfamily of the PPR gene family (Fujii et al. 2011). In addition to PPR proteins, other types of *Rf* genes have been isolated. LD-CMS rice *Rf2* encodes a glycine-rich protein (GRP) (Itabashi et al. 2011), CW-CMS rice *Rf17* encodes an unknown protein (Fujii and Toriyama 2009), and sugar beet *Rf1* encodes a yeast Oma1-like peptidase (Matsuhira et al. 2012).

Ahokas (1979, 1982) found two male-sterile cytoplasms, designated *msm1* and *msm2*, in two accessions of wild barley, *H. vulgare* ssp. *spontaneum* (C. Koch) Thell. A single dominant gene, *Rfm1a*, which was carried in the *spontaneum* subspecies accession with the *msm1* cytoplasm, can restore fertility of a male-sterile plant with both *msm1* and *msm2* cytoplasms. Three RAPD-STS markers were located on the short arm of chromosome 6H and linked to the *Rfm1* locus. *Rfm1* was located within a 10.8 cM region delimited by 2 markers (*OPI-18/900* and *MWG2218*) (Matsui et al. 2001).

Colinearity between the barley and rice genomes was reported previously (Moore et al. 1995). Additionally, a reference sequence of the rice genome (International Rice Genome Sequencing Project 2005) and a draft barley genome assembly (The International Barley Genome Sequencing Consortium 2012) are resources for positional cloning in barley. By taking advantage of these genomic resources, we developed novel markers for the *Rfm1* locus and performed fine mapping of *Rfm1*. Moreover, we screened and fingerprinted barley BAC libraries and constructed a BAC physical map covering the *rfm1* allele based on flanking and cosegregating genetic markers.

Materials and methods

Plant materials and morphological observation

Maintainer (cv. Adorra), CMS and restorer lines were kindly provided by Dr. H. Ahokas (Agricultural Research Center, Finland). Phenotyping of male sterility (pollen fertility) was done at two stages, flowering and maturity. We observed the anthers of each plant at flowering (booting stage) for pollen fertility, and at the maturity stage, we counted the selfed (bagged) seed setting of each plant. Morphological observations of pistils, stamens and anthers were performed using a binocular stereomicroscope S2X12 (OLYMPUS, Japan) after removing the glume, lemma and palea from the florets. For mapping the *Rfm1* locus, we used an F_2 plant population that was derived from a single cross between a CMS line [*msm1*–(*rfm1/rfm1*)] and a restorer line [*msm1*–(*Rfm1a/Rfm1a*)] (Matsui et al. 2001).

DNA isolation

Total DNA was isolated from leaf tissues of individual plants using the CTAB extraction method (Murray and Thompson 1980) and was used to detect polymorphisms between the parent lines for the construction of the genetic map. A simpler DNA extraction method was used for the PCR-based screening of the recombinant plants: A small piece (approximately 1 cm) of leaf tip was homogenized in 400 µl of TE200 buffer [200 mM Tris–HCl (pH 8.0), 25 mM EDTA, 250 mM NaCl and 0.5 % SDS], and the samples were incubated at 37 °C for 1 h. The samples were centrifuged, and 300 µl of the supernatants were recovered. An equal volume of 2-propanol was then added to precipitate the total DNA.

Genotyping of F_2 plants using DNA markers

CAPS (cleaved amplified polymorphic sequences; Konieczny and Ausubel 1993) including dCAPS (derived CAPS;Neff et al. 1998) and SSR (simple sequence repeats) markers were used for the genotyping of F_2 plants. Details of the PCR conditions, primer sequences and restriction enzymes used for each marker are summarized in Table 1. The PCR was performed using a MyCycler thermal cycler (Bio-Rad, USA) or a GeneAmp PCR System 9700 (Applied Biosystems, USA). For SSR marker detection, polyacrylamide gel electrophoresis was used after PCR amplification. For CAPS markers, PCR products were digested with the

Table 1 Characterization of DNA markers linked to *Rfm1*

Marker	Marker type	Sequence (5′–3′)	Restriction enzyme
NAS2070	CAPS	GTTGACTTCAACGGGAGTTTCCTG CCTGTCTATGGTGAAGATGGACTC	*Hae*III
NAS2080	CAPS	AAGGAGTACTTTGCTCGTGAGCAG TTCTGCGGAAGATCTGCCAATGGC	*Xba*I
NAS2090	CAPS	ACGTCTACTTCATCAAGTTCGCGC GGTGTGGGTTCCCACTAGATGATC	*Msp*I
NAS2100	CAPS	TGTAGCTGTACAGACTGAGCATGG ACCTACATCAATCTGGTCATTCCTG	*Hpy*CH4IV
NAS9110	Gene targeted AFLP	TATGACTGGGGAAAGTTCTACGC TTTCACCACCGTGGTGCCTGG	
NAS9113	SSR	TTGGTAGCTAGAAGGGAAAA CAGTGTGTGGTATGACTTGG	
NAS9130	dCAPS	TTTGGCTTAATTTTTGTTCCGGAGGAAATGT TCATCTCTCCAATCGGTCAT	*Taq*αI
NAS9133	CAPS	CGATGGGATATGGACTACCA AGCCTTGTCATCTCTGATCG	*Csp*CI
NAS9200	SSR	GTACACTAGGGATGCCAGAA TGTGGCAATATTTTCCTTTT	
NAS9290	CAPS	CCTGTTATTTCCATCCACTCTCCT AGCAAGATCCCACAAACTATGACC	*Hae*III
NAS1080	CAPS	TTCAAGCATGTCCTGGTGGATCCG GACCTTTCGGTGAATGCAGAAACC	*Bbs*IA
NAS2120	CAPS	CGTCGAGGATGCACAAGGCGTC GCAGTTCTCCCAGAGCGTCTTG	*Hae*III

appropriate restriction enzymes (2 units/20 μl reaction) and then subjected to agarose gel electrophoresis.

Linkage map construction

Using the *Rfm1* phenotype and the DNA marker genotypes of F_2 individuals, we calculated the order of the markers and the distances between them using the genetic mapping software AntMap ver. 1.1 (Iwata and Ninomiya 2006). Kosambi's function was applied for the estimation of map distances (Kosambi 1943).

Physical mapping using a BAC library

Two BAC libraries, HVVMRXALLhA and HVVMRXALLmA, were used to screen the BAC clones for the physical map construction of the *rfm1* allele (Yu et al. 2000; Schulte et al. 2011). Those libraries were constructed for barley (*Hordeum vulgare* L.) cultivar Morex using different cloning sites, *Hin*dIII and *Mbo*I, respectively. BAC library HVVMRXALLhA was screened with PCR markers according to a method described elsewhere (Komatsuda et al. 2007). Positive BAC clones were identified, and their DNA was extracted. Each BAC DNA was individually digested with *Hin*dIII and then electrophoresed. The BAC contig was generated based on the *Hin*dIII fingerprinting image.

Results

Morphological observation of the flower organs

We observed the morphological changes in the flower organs induced by the integration of the *msm1* cytoplasm into barley (Fig. 1). The anther development differed between the maintainer (or restorer) and CMS lines. Anthers in the maintainer and restorer lines were yellow, fully bulged and contained fertile pollen, whereas anthers in the CMS line were white, thin and did not contain any pollen grains. The *msm1* cytoplasm had no significant effects on the glume, lemma, palea, pistil or overall plant growth.

Molecular marker development and basic genetic map construction for the *Rfm1* locus using synteny with the rice genome

We previously mapped the *Rfm1* locus to a ~10.8 cM region of the short arm of chromosome 6H (Matsui et al. 2001). Comparison of the maps of Matsui et al. (2001) and Künzel et al. (2000) indicated that *Rfm1* was linked to *MWG2318*, *MWG573*, and *MWG966*. Sequence information on these three "MWG" markers was used to identify their orthologous regions in rice genomes using BLASTN analysis. We

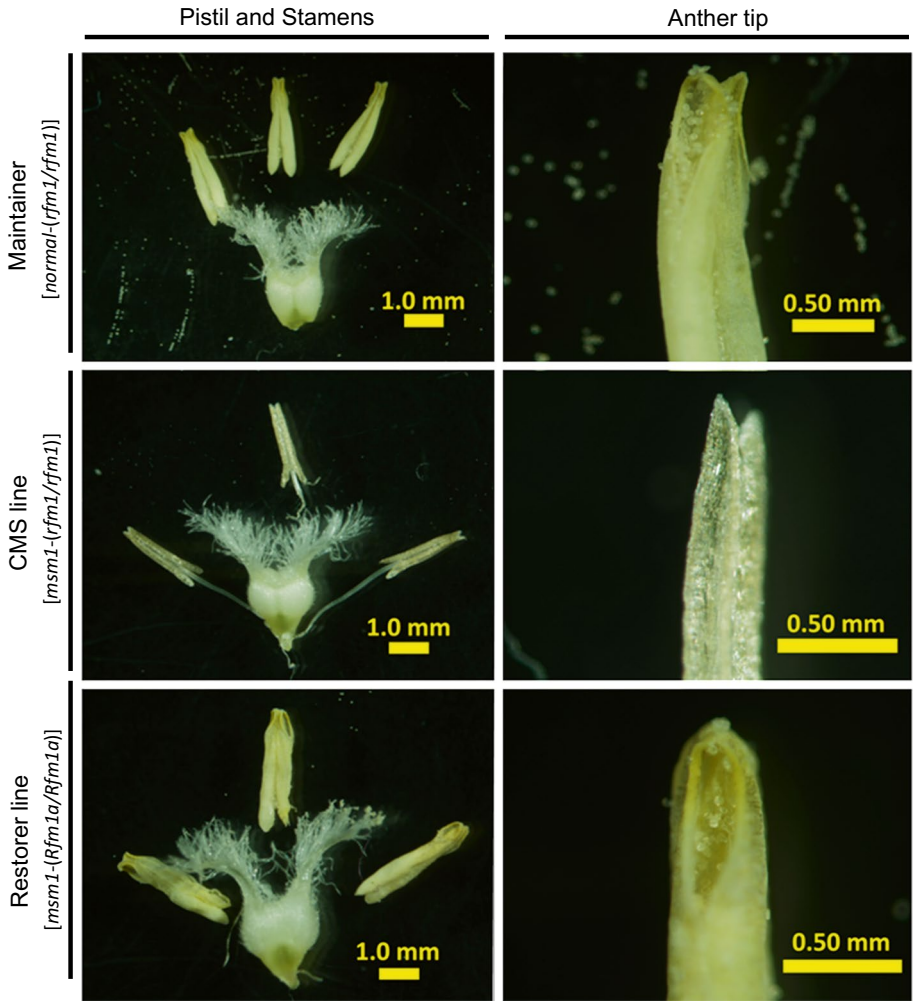

Fig. 1 Flower morphology of the three barley lines, maintainer (cv. Adorra), CMS and restorer. The *left column* shows the pistil and stamens, and the *right column* indicates enlarged images of the anther tip. *Bars* indicate 1 mm (*left column*) and 0.5 mm (*right column*)

identified the putatively orthologous sequences of these markers on the short arm of rice chromosome 2, which is syntenic to barley 6H (Moore et al. 1995), in the same order. This information enabled us to assign the *Rfm1* locus to the region between Os02g0103800 and Os02g0112100 on rice chromosome 2 (RAP-DB; http://rapdb.dna.affrc.go.jp/). Using the gene information encoded in this region as queries, we identified barley and wheat-expressed sequence tags (ESTs) using BLASTN analysis (Barley DB, http://www.shigen.nig.ac.jp/barley/index.html; NBRP-Komugi, http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp), and we analyzed the polymorphisms between the parental lines for those EST sequences. Finally, we developed nine novel markers in the *Rfm1* region, eight of which were CAPS or dCAPS (derived CAPS) markers. The remaining marker was an STS (sequence tagged site) PCR marker for detecting an insertion/deletion polymorphism (Table 1). Using the nine markers and a mapping population consisting of 186 F_2 plants derived from the cross between the CMS line and the restorer line, we constructed a genetic linkage map spanning 10.0 cM for the *Rfm1* region (Fig. 2). These novel markers allowed us to further narrow down the *Rfm1* locus to a genetic interval of 0.8 cM, which was defined by the flanking co-dominant markers *NAS2090* and *NAS9290* (Fig. 2). *NAS2100*, *NAS9110* and *NAS9130* co-segregated with *Rfm1*.

Comparative mapping between barley and other grasses

The orthologous relationship between barley 6HS and the short chromosome arms of rice 2, *Brachypodium* 3 and sorghum 4 is well known (Mayer et al. 2011). Therefore, we mapped our markers of the *Rfm1* locus on the syntenic regions of rice, *Brachypodium* and sorghum using the data from GenomeZipper (http://mips.helmholtz-muenchen.de/plant/barley/gz/index.jsp; Mayer et al. 2011) (Fig. 2). The comparative mapping revealed that colinearity was maintained between NAS2070 and NAS1080 among these grass species. We found several PPR genes in the orthologous regions of rice (http://rapdb.dna.affrc.go.jp/index.html), *Brachypodium* (http://jbrowse.brachypodium.org/JBrowse.html) and sorghum (http://www.phytozome.

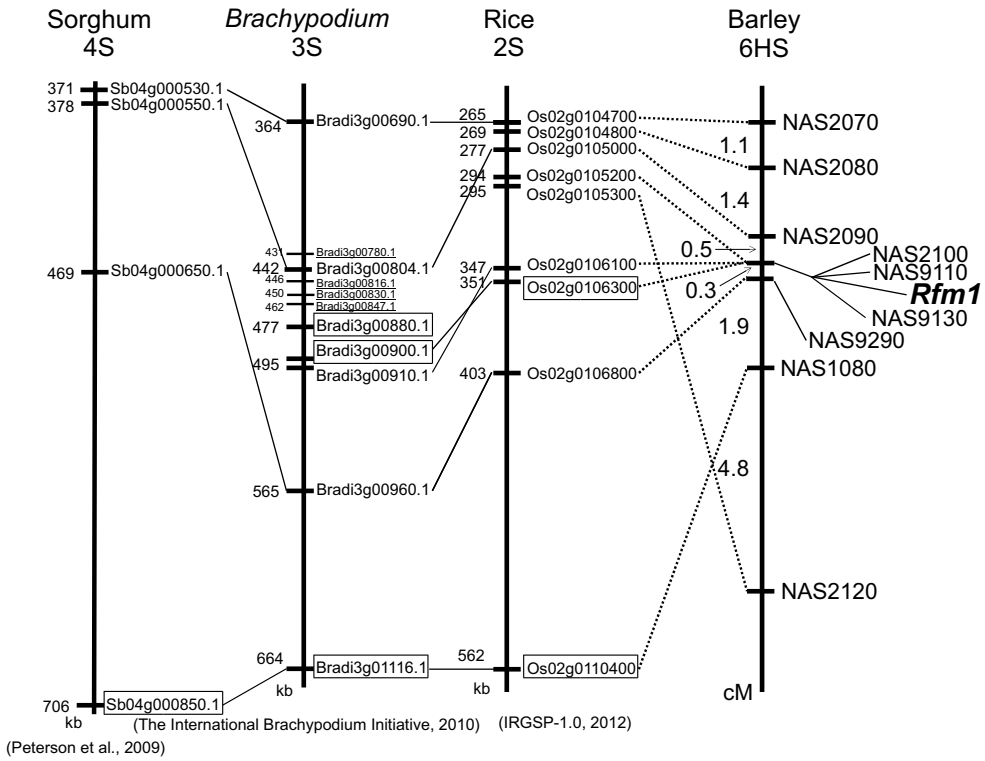

Fig. 2 Comparative mapping of the genetic map for the *Rfm1* region on barley 6HS and physical maps for the syntenic regions of rice, *Brachypodium* and sorghum. The *dashed lines* indicate rice genes used for the marker development in barley. The *solid lines* indicate the syntenic relationship reported in the barley GenomeZipper (Mayer et al. 2011). The loci encoding PPR proteins are *boxed*. The underlined loci encode the proteins with the mitochondrial initiation factor-related motif

Table 2 PPR proteins found in the syntenic regions

Plant	Locus	Length (aa)	No. of PPRs	Subclass
Rice	Os02g0106300	751	14	DYW
	Os02g0110400	788	15	P
Brachypodium	Bradi3g00880.1	302	3	P
	Bradi3g00900.1	750	14	DYW
	Bradi3g01116.1	753	16	P
Sorghum	Sb04g000850.1	757	16	P

net/cgi-bin/gbrowse/sorghum/), which could serve as possible candidates for *Rfm1* orthologues (Table 2). In addition to the PPR genes, we also identified several genes that encoded proteins with the mitochondrial initiation factor-related motif in *Brachypodium* (Fig. 2).

High-resolution genetic mapping of the *Rfm1* locus

Before high-resolution mapping, we developed three additional markers, *NAS9113*, *NAS9133* and *NAS9200* (Table 1) based on the sequence data of barley genome (The International Barley Genome Sequencing Consortium 2012; http://mips.helmholtz-muenchen.de/plant/barley/index.jsp). The three markers co-segregated with *Rfm1* in 92 F_2 plants, confirming that they were closely linked (data not shown). Then, we analyzed 3,639 F_2 plants (corresponding to 7,278 gametes) to separate the multiple markers from *Rfm1* (Fig. 3). We screened recombinants between *NAS2090* and *NAS1080* because these two markers flanked the *Rfm1* locus. A total of 175 plants were detected and further characterized using seven markers, including the three newly developed markers described above (Fig. 3). *Rfm1* was delimited to a 0.14 cM interval flanked by *NAS9113* and *NAS9200*. *Rfm1* did not recombine with *NAS9130* and *NAS9133* (Fig. 3).

Construction of a BAC contig spanning the *Rfm1* locus

The results from high-resolution genetic mapping allowed us to proceed to physical mapping of the *Rfm1* locus. Primer pairs derived from three adjacent markers, *NAS9130*, *NAS9133* or *NAS9200*, were used for PCR screening of the barley BAC library, HVVMRXALLhA, constructed from digestion by *Hin*dIII. We isolated 10 BAC clones by screening the library using these three primer pairs, and these BACs were divided into two groups (Fig. 3). The

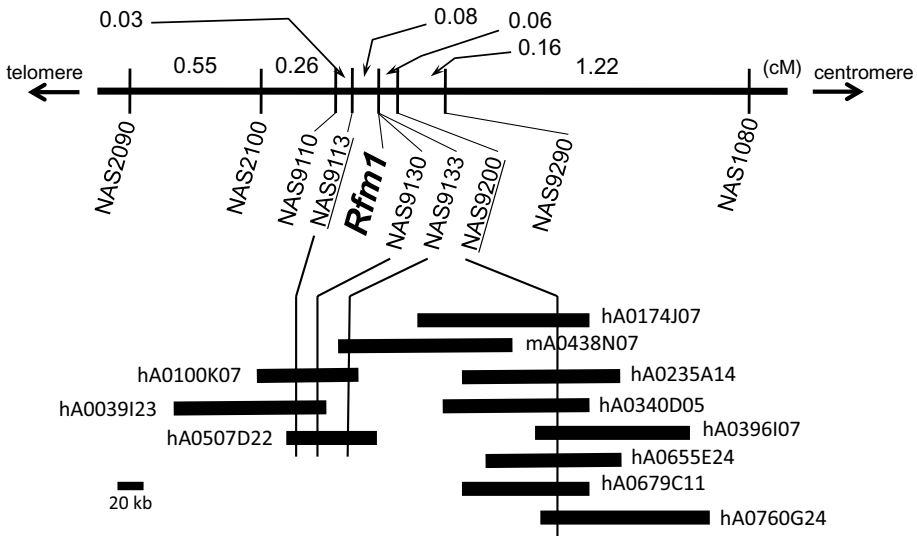

Fig. 3 High-resolution genetic and BAC physical maps encompassing the *Rfm1* locus on barley 6HS. The physical map consisting of 11 BAC clones is aligned with the high-resolution genetic map constructed using the mapping population of 3,639 F2 plants. The *vertical lines* show the positions of the respective markers on each clone. For the names of all BAC clones, "HVVMRXALL" should precede each name. *Bar* 10 kb

first group was composed of three BACs, hA0100K07, hA0039I23 and hA0570D22, all of which were tagged by *NAS9130*. Two of the three BACs in the first group were also tagged by *NAS9133*. The remaining seven BACs constituted the second group, and these clones were tagged with *NAS9200*. Fingerprinting of these 10 BACs confirmed this grouping. We obtained two assembled contigs anchored by *NAS9133* or *NAS9200*. To connect these two contigs, we consulted a progressive genome-wide physical map (Ariyadasa et al. 2014), and two BAC contigs (FPcontig_6870 and FPcontig_44212) were identified, comprising 6 of the above-mentioned BACs that were selected by PCR screening of the library HVVMRXALLhA. The BAC clone HVVMRXALLmA0438N07, the terminal BAC of Fpcontig_6870, was identified as a potential bridge clone between both contigs anchored by *NAS9133* or *NAS9200*. Based on the fingerprinting and probe amplification of *NAS9133*, we confirmed that this clone indeed bridged the two contigs. We thus succeeded in constructing a single BAC contig corresponding to approximately 208 kb between *Rfm1* flanking markers (Fig. 3). The estimate of the physical to genetic distance for this region is 1.49 Mb/cM, which is comparable to the data provided in previous studies for this region of barley chromosome 6H (Ariyadasa et al. 2014).

Discussion

Rf genes were isolated from various plants, and the map-based cloning technique was used to isolate most of them. In this study, we succeeded in delimiting the location of the barley *Rfm1* locus to a minimum region of four BAC clones spanning a distance of 208 kb (Fig. 3). These genetic and physical maps provide a framework for cloning the *Rfm1* gene, which serves as a prerequisite for studying the mechanisms of male sterility and fertility restoration in barley, as well as for marker-assisted selection of the trait. In fact, we found several PPR genes in the orthologous regions of rice, *Brachypodium* and sorghum (Table 2). The possible presence of PPR genes in the 208-kb region in barley indicates that these genes could serve as possible candidates for the *Rfm1* gene.

However, this physical map was constructed using the BAC library of cultivar Morex, which does not have a functional *Rfm1* gene in its genome. In other studies of cloned restorer loci, the functional restorer genes were identified as members of several gene copies with a similar gene structure. For example, three PPR genes are present at the radish *Rfo* locus, four PPR genes are at the rice *Rf-1* locus and four peptidase-like genes are found at the sugar beet *Rf1* locus in tandem. In all cases, only one of these genes restores pollen fertility (Brown et al. 2003; Komori et al. 2004; Matsuhira et al. 2012). In the non-restorer lines, the allelic homologous genes including the *Rf* gene were partially deleted. For example, one PPR gene corresponding to *Rf-1* is lacking and only three PPR genes are located at the rice *rf-1* locus. At *rf1* in sugar beet, only one peptidase-like gene is found (Komori et al. 2004; Matsuhira et al. 2012). These results suggest that gene clustering and copy number variation are common features of restorer gene loci (Touzet and Budar 2004).

The genes encoding PPR proteins are divided into two subfamilies, P and PLS subfamilies, based on differences in the organization of PPR and PPR-like motifs. The PLS subfamily is further classified into three main subclasses, PLS, E and DYW subclasses based on their C-terminal domain structure (Lurin et al. 2004; O'Toole et al. 2008). Fujii et al. (2011) discovered that all *Rf*-PPR genes and similar *Rf*-PPR-like (RFL) genes that clustered with *Rf*-PPR genes came from the same small clade of PPR genes

in the P-subfamily of the PPR gene family, which suggests functional differences between Rf proteins and other PPR proteins. They proposed that the distinction of RFL genes from the hundreds of other PPR genes in the genome is useful to identify candidate *Rf* genes.

In the case of *Rfm1*, the PPR genes found at syntenic regions of other grass species do not belong to the RFL gene clade, but they are classified into the DYW subclass in the PLS subfamily. These genes include Os02g0106300 and Bradi3g00900.1 (Table 2). As mentioned above, all *Rf*-PPR genes cloned so far have been classified into the RFL-type, and there are no reports that a DYW-subclass PPR gene is a functional *Rf* gene. Therefore, the PPR genes found in the *Rfm1*-syntenic regions may not represent orthologues of candidate genes for *Rfm1*, although we cannot rule out the possibility that *Rfm1* represents a novel type of *Rf*-PPR genes.

To test these possibilities, future work should focus on comparing the genomic sequences, including candidate genes for the *Rfm1* loci, between restorer and CMS lines to identify the *Rfm1* gene. We are currently investigating the genome organization of the corresponding region of the restorer line that has a functional *Rfm1* gene to determine its gene content and how *Rfm1* and *rfm1* diverged through the genome evolution of cultivated barley.

Author contributions HH, TK and HS designed the experiments. HU, MCC, MS, TK and HH performed the marker development and genetic mapping, and HU, MS, MP, TK and NS conducted the physical mapping. HH performed the comparative mapping. HH and TK wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest The authors have declared no conflict of interest.

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