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Molecular mapping of a fertility restoration locus (*Rfm1*) for cytoplasmic male sterility in barley (*Hordeum vulgare* L.)

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Abstract The *Rfm1a* gene restores the fertility of *msm1* cytoplasmic male-sterile lines in barley. We identified three RAPD markers linked to the *Rfm1* locus (CMNB-07/800, OPI-18/900, and OPT-02/700) using isogenic lines and segregating BC_1F_1 and F_2 populations. Using a previously developed linkage map of barley, we located CMNB-07/800 and OPT-02/700 beside MWG2218 on chromosome 6HS. The linkage between MWG2218 and the *Rfm1* locus was demonstrated using the segregating BC_1F_1 and F_2 populations. To confirm the chromosomal locations of these markers, we converted them to STSs and tested against two sets of wheat–barley chromosome addition lines. These STS markers, CMNB-07/800, OPT-02/700, and MWG2218, were amplified only in the addition lines possessing the chromosome 6H, thereby providing additional evidence the *Rfm1* locus is located on chromosome 6H. Homoeologous relationships among fertility restoration genes in Triticeae are discussed.

Keywords Barley · Randomly amplified polymorphic DNA (RAPD) · Sequence tagged site (STS) · Cytoplasmic male sterility (CMS) · Restorer gene · Isogenic lines

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Introduction

A cytoplasmic male-sterile (CMS) system is desirable for use in hybrid seed production as it eliminates the need for hand emasculation. CMS is a maternally inherited trait characterized by the inability to produce viable pollen but without affecting the female fertility, and it is often associated with mitochondrial DNA (mtDNA) rearrangements, mutations, and editing (e.g. Schardl et al. 1984; Dewey et al. 1986; Werner and Frank 1997). Hybrid seed production systems using CMS have been developed for several crops such as maize, *Brassica*, sorghum, rice, and wheat. In maize, Texas male-sterile cytoplasm was used to produce about 85% of the hybrid seed until Southern corn leaf blight struck the South and Corn Belt regions of the United States (Ullstrup 1972). In barley, F_1 hybrid seeds have been produced using balanced tertiary trisomics (Ramage 1983), but CMS has not yet been used commercially in this crop.

Two male-sterile cytoplasms, designated *msm1* and *msm2*, were found in two strains of the wild barley, *H. vulgare* ssp. *spontaneum* (C. Koch) Thell. (Ahokas 1979a, 1982a). The *spontaneum* subspecies strain with the *msm1* cytoplasm carried a dominant restorer gene, designated *Rfm1a* (Ahokas 1979a), which was also able to restore the male fertility of the *msm2* cytoplasm (Ahokas 1982a). Restorer materials have been found in wild barley, but cultivated barley carrying a restorer gene has not yet been found (Ahokas 1979b, 1980b). All of these restorer accessions had a single dominant restorer gene and, as far as examined, all of them were allelic to the *Rfm1a* gene (Ahokas 1980b). Despite the importance of the *Rfm1a* gene for hybrid barley production, there is no information on its location in the barley genome.

DNA markers tightly linked to the *Rfm1* locus enable the molecular study of the CMS system. Several molecular markers linked to restorer genes have been identified in many crops, such as maize (e.g. Sisco 1991; Kamps and Chase 1997; Schnable and Wise 1994; Wise and Schnable 1994), rice (Zhang et al. 1997), rye (Börner et al. 1998), *Brassica* (Delourme et al. 1994; Jean et al.

1997), and bean (He et al. 1995). In the study reported here we mapped the *Rfm1* locus of barley using molecular markers in order to apply the linked markers to marker-assisted selection and map-based cloning of the relevant gene.

Materials and methods

Plant materials

The CMS line (msm1/16*Adorra, BC_{15}), the restorer line (78–383/6*Adorra, BC_5), which was obtained by the recurrent backcrosses of Adorra to 80–414–10, and Adorra were kindly provided by Dr. H. Ahokas, Agricultural Research Centre, Finland. The 80–414–10 was produced by following the cross: msm1/4*Adorra/3/msm1/3*Adorra//Sel.77–1/4/4*Adorra (Ahokas 1982a). The CMS and restorer lines are near isogenic for the *Rfm1a* gene. In this paper, the cytoplasm type is designated by italics and the genotype of the restorer gene is designated by parenthesized italics. Therefore the restoration line is *msm1*-(*Rfm1a*/*Rfm1a*) and the CMS line is *msm1*-(*rfm1a*/*rfm1a*).

Isolation of genomic DNA and RAPD

Genomic DNA was isolated from leaf tissue following the procedure of Komatsuda et al. (1998). We used a total of 200 12-mer oligonucleotides, designated Common Primers, consisting of CMNA-00 through CMNA-99 and CMNB-00 through CMNB-99 (Bex, Tokyo), and 684 10-mer oligonucleotide primers, corresponding to OPA through OPZ and OPAA through OPAI (Operon Technologies, Alameda, Calif.). We used these primers to detect polymorphism between *msm1*-(*Rfm1a*/*Rfm1a*) and *msm1*-(*rfm1a*/*rfm1a*) in the random amplified polymorphic DNA (RAPD) profiles. Amplification by polymerase chain reaction (PCR) was performed as described by Komatsuda et al. (1997). Amplified fragments were separated by electrophoresis on 1.5% agarose gels prepared in 0.5×TBE (1×TBE: 89 mn Tris-borate plus 2 m*M* EDTA). Approximate sizes of the amplification products were determined by reference to the DNA molecular-weight marker VIII (Boehringer Mannheim).

Sequence-tagged site (STS) analysis

RAPD fragments were purified from the gel with Geneclean 2 (Bio 101). The fragments were cloned into the pCRII vector using a TA cloning Kit (Invitrogen). Nucleotide sequences were determined with Fluorescence Cycle Sequencing Kits and a 373A automated DNA sequencer (Applied Biosystems, Perkin-Elmer Corp). STS primers designed for CMNB-07/800 were 5´ATCATCCAACTTAAAGAACC3´ and 5´TAGTAAAAGTGT-TGCAAGCC 3´, and for OPT-02/700 these were 5´GGAGAGACTCACGGATTCAC3´ and 5´ACTCGATCGCGCCACTGAA 3´. After incubation at 95°C for 5 min, the samples were subjected to 30 cycles of 95°C for 1 min, either 60°C for CMNB-07/800 or 65°C for OPT-02/700 for 2 min, and 72°C for 2 min. Extension of the amplified product was then allowed to proceed at 72°C for 7 min. The Program Temp Control System PTC-100 (MJ Research) was used for these amplifications.

Linkage analysis

An F_2 family consisting of 233 plants was derived from the *msm1*-(*Rfm1a*/*Rfm1a*)×*N*-(*rfm1a*/*rfm1a*) cross. A BC_1F_1 family consisting of 242 plants was produced by backcrossing of the F_1 plants to *msm1*-(*rfm1a*/*rfm1a*). In order to map the RAPD markers on the known linkage group, recombinant inbred lines (RILs) of the Azumamugi (AZ)×Kanto Nakate Gold (KNG) cross and the molecular maps were used (Mano et al. 1999). Linkage maps were constructed using MAPMAKER/EXP 3.0 (Lander et al. 1987). Map distances were estimated after conversion by applying Kosambi's function (Kosambi 1944).

Chromosomal location

Two series of wheat–barley chromosome addition lines were used. One series contained a single pair of *H. vulgare* cv. Betzes chromosomes in Chinese Spring (CS) wheat (Islam and Shepherd 1981); the other contained a single pair of *H. vulgare* ssp. *spontaneum* (OUH602) chromosomes in Shinchunaga wheat (Taketa et al. unpublished).

Fertility scoring

Plants were grown in a growth chamber or in the field, and the spikes were bagged just before flowering to prevent outcrossing. The fertility was assessed by seed set of one bagged or at least 2 non-bagged spikes. In addition, we investigated anther morphology for confirmation of the fertility under the criterion reported by Ahokas (1978) that fertile anthers are large, but anthers of sterile plants are rudimentary.

Results

Fertility segregation in F_2 and BC_1F_1

Plants of F_2 and BC_1F_1 families were unambiguously classified as either sterile or fertile, because we observed no partial seed set or variation in anther morphology in the lines. The segregations of the F_2 and BC_1F_1 populations fitted the expected monogenic segregation ratios of 3:1 (χ^2=0.71, 0.25<*P*<0.50) and 1:1 (χ^2=0.0, 0.95<*P*), respectively.

Identification of RAPD fragments and linkage analysis

One 12-mer primer and 4 10-mer primers produced a total of five polymorphic fragments between the *msm1*-(*Rfm1a*/*Rfm1a*) and *msm1*-(*rfm1a*/*rfm1a*) isogenic lines. The BC_1F_1 population was used for mapping of the fragments generated from *msm1*-(*Rfm1a*/*Rfm1a*), while the F_2 population was used for mapping of the fragments derived from *msm1*-(*rfm1a*/*rfm1a*). The linkage of these fragments to the *Rfm1* locus was initially tested using 50 F_2 and 63 BC_1F_1 segregating plants. Three fragments generated by CMNB-07 (5´GGCAGATATCAT3´), OPI-18 (5´TGCCCAGCCT3´), and OPT-02 (5´GGAGAGACTC3´) were linked to the *Rfm1* locus, while the remaining two primers generated from *msm1*-(*rfm1a*/*rfm1a*) were not linked to the *Rfm1* locus. The three former fragments were generated from the *msm1*-(*Rfm1a*/*Rfm1a*) line. Approximate sizes of the polymorphic fragments of each primer were 800, 900, and 700 bp, and they were designated as CMNB-07/800, OPI-18/900 and OPT-02/700, respectively. Based on the population of 242 BC_1F_1 plants, OPI-18/900 was the most closely linked marker to the *Rfm1* locus (5.2 cM) (Fig. 1B);

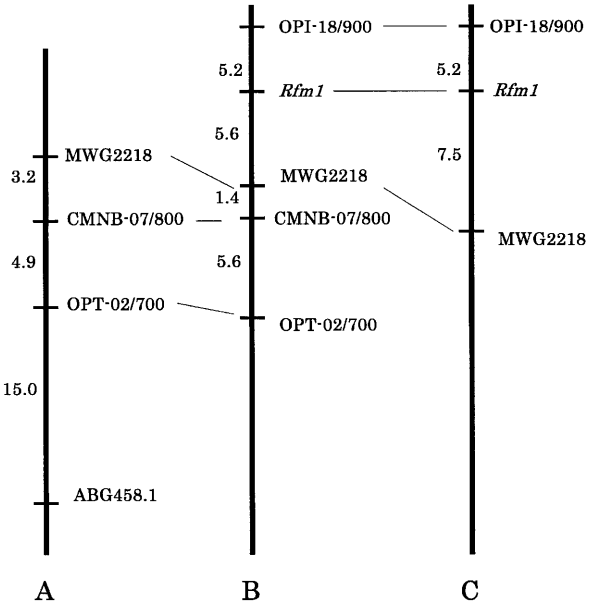

Fig. 1A–C Genetic maps of the *Rfm1* locus on the short arm of barley chromosome 6H. The maps were constructed using RILs from an Azumamugi×Kanto Nakate Gold cross (**A**), BC_1F_1 (**B**), and F_2 from a *msm1*–(*Rfm1a*/*Rfm1a*)×*N*-(*rfm1a*/*rfm1a*) cross (**C**). The first map (**A**) corresponds to the short arm of chromosome 6H (Mano et al. 1999). Map distances are given in centiMorgans

CMNB-07/800 and OPT-02/700 were located on the opposite side, at distances of 7.0 cM and 12.6 cM, respectively (Fig. 1B). The location of OPI-18/900 was similar to that found in the F_2 population (Fig. 1C).

Location of the RAPD fragments in a previously developed linkage map

To identify the map locations of the three RAPD markers, we utilized our previously developed molecular linkage maps of RILs constructed from the AZ×KNG cross (Mano et al. 1999). The CMNB-07 primer generated polymorphic fragments between AZ and KNG, producing the 800-bp fragment from AZ (Fig. 2). RAPD analysis using the 99 RILs showed that CMNB-07/800 was linked to MWG2218 on the short arm of chromosome 6H (Fig. 1A). To obtain additional evidence for the map locations, we attempted to convert these RAPD markers to STS markers and test them for polymorphism between AZ and KNG. CMNB-07/800 and OPT-02/700 could be converted from RAPD to STS markers, however OPI-18/900 could not be cloned, because some bands were produced when the excised band was used as template with the OPI-18 primer. Amplification of OPT-02/700 using the STS primers generated a monomorphic band of 700 bp from AZ and KNG, and the amplified products showed polymorphism after digestion by *Hae*III (Fig. 3A). STS primers of CMNB-07/800 generated an amplification product of 800 bp from *msm1*-(*Rfm1a*/*Rfm1a*) and AZ only, thus retaining the polymorphisms as dominant-recessive markers shown by RAPD (Fig. 2). Using the RILs, we mapped OPT-02/700 on chromosome 6HS at 4.9 cM from CMNB-07/800 (Fig. 1A). In addition, two STS markers, MWG2218 and ABG458.1, were tested for polymorphism between *msm1*-(*Rfm1a*/*Rfm1a*) and

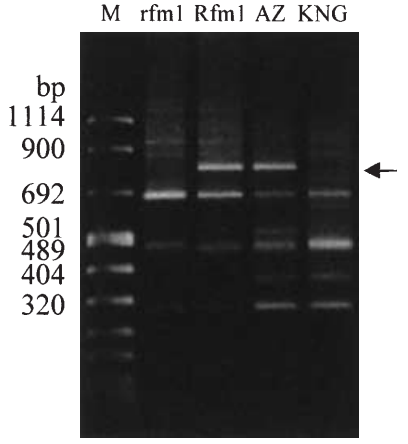

Fig. 2 PCR products using the CMNB-07 primer. *Lanes: rfm1 Rfm1, AZ, KNG msm1*-(*rfm1a*/*rfm1a*), *msm1*-(*Rfm1a*/*Rfm1a*), Azumamugi and Kanto Nakate Gold, respectively, *M* DNA size marker. The *arrow* indicates the polymorphic fragments

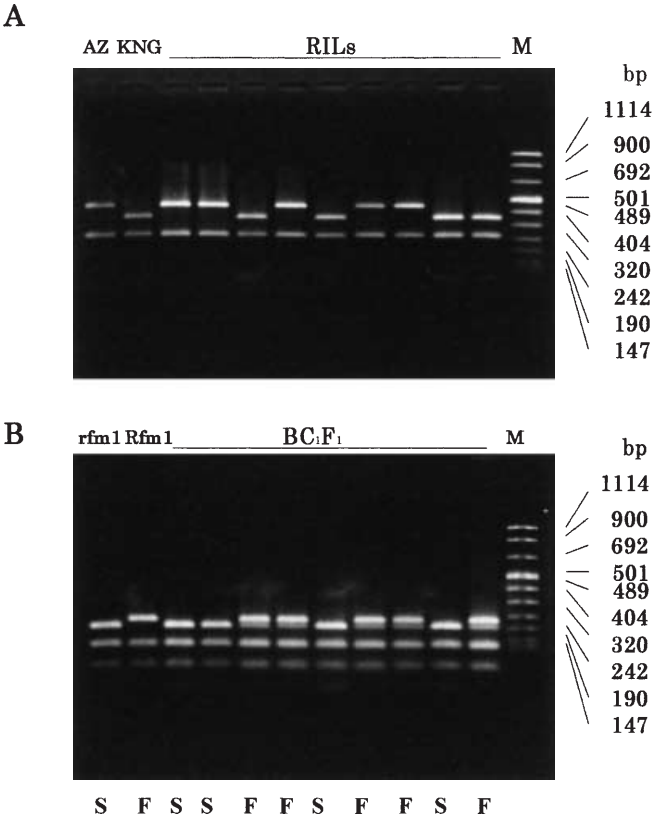

Fig. 3A, B PCR products using co-dominant markers digested by restriction enzymes. The fragments were amplified using STS-OPT-02/700 and digested with *Hae*III in BC_1F_1 (**A**) and using MWG2218 and digested with *Hae*III in RILs (**B**). *S* Sterility, *F* fertility, *M* DNA size marker

Table 1 Amplification of polymorphic fragment in *msm1*-(*rfm1a*/*rfm1a*), *msm1*-(*Rfm1a*/*Rfm1a*), parental lines, and the wheat-barley addition lines (*NT* not tested)

Fragment	Type of Marker	rfm1[a]	Rfm1[a]	Barley		Wheat		Addition lines	
				Betzes	OUH602	CS	Shinchunaga	CS-Betzes	Shinchunaga-OUH602
OPI-18/900	RAPD	–[c]	+	–	–	+	+	NT	NT
OPT-02/700	RAPD	–	+	–	–	–	–	NT	NT
CMNB-07/800	RAPD	–	+	–	+	–	–	NT	–
OPT-02/700	STS[b]	+	+	+	+	–	–	6H	6H
CMNB-07/800	STS	–	+	+	+	–	–	6H	6H
MWG2218/420	STS	+	+	+	+	–	–	6H	6H

[a] rfm1, *msm1*-(*rfm1a*/*rfm1a*); Rfm1, *msm1*-(*Rfm1a*/*Rfm1a*)
[b] The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AB045727 through AB045729
[c] + and – indicate that the DNA fragment was amplified or not amplified, respectively

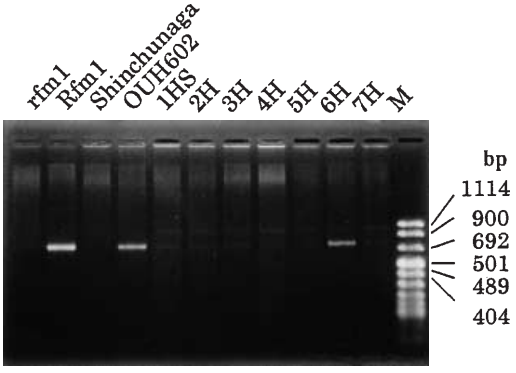

Fig. 4 PCR products using the STS primers of CMNB-07/800 in wheat–barley chromosome addition lines. *Lanes: rfm1* and *Rfm1 msm1*-(*rfm1a*/*rfm1a*) and *msm1*-(*Rfm1a*/*Rfm1a*), respectively, *1HS–7H* Shinchunaga wheat–OUH 602 barley addition lines 1HS to 7H, *M* DNA size marker

msm1-(*rfm1a*/*rfm1a*). MWG2218 showed polymorphism between the two lines after digestion by *Hae*III (Fig. 3B). Linkage analysis in the BC_1F_1 population showed that MWG2218 was located at 5.6 cM from the *Rfm1* locus (Fig. 1B). Comparison of the RIL and BC_1F_1 maps (Fig. 1A and B) showed that the *Rfm1* locus was located distal to MWG2218.

Analysis using wheat–barley addition lines

We used the wheat–barley chromosome addition lines to determine the chromosomal locations of the molecular markers linked to the *Rfm1* locus. First, electrophoretic patterns were compared between CS and Betzes as well as between Shinchunaga and OUH602. The study showed that CMNB-07/800 was amplified in OUH602 barley (Table 1). CMNB-07/800, however, did not amplify in any of the Shinchunaga–OUH602 chromosome addition lines, presumably reflecting the difficulty of RAPD amplification in the presence of wheat genomes. We therefore used STS-CMNB-07/800 and STS-OPT-02/700. The STS and RAPD fragments of the CMNB-07/800 were allelic based on 40 BC_1F_1 and 51 F_2 segregating plants (data not shown). The fragments of CMNB-07/800, OPT-02/700 and MWG2218 were amplified in barley but not in wheat (Table 1). Each of the STS fragments appeared only in chromosome 6H addition lines and was not generated in the other addition lines (Table 1, Fig. 4), indicating that the *Rfm1* locus is located on chromosome 6H.

Discussion

The CMS systems of barley have been intensively studied using genetic, biochemical, physiological, and cytological methods (e.g. Ahokas 1978, 1980a, 1982b; Hockett et al. 1989). These studies showed that CMS is caused by an overproduction of sporopollenin in the anthers and that fertility restoration is associated with a change in the quantity and composition of cytokinins in root exudates, where the total quantity of cytokinins is lower in the sterile line than in the fertile line. However, there is no information about the chromosomal location or linkage relationship of the restoration gene. We have therefore mapped the *Rfm1* locus using RAPD and STS markers. In this study, we inferred that the *Rfm1* locus is on the short arm of chromosome 6H based on linkage relationships to previously localized markers and amplification of linked markers with wheat–barley addition lines.

In wheat, six designated restorer genes for the *Triticum timopheevi* cytoplasm have been reported. These are *Rf1* on chromosome 1 A, *Rf2* on 7D, *Rf3* on 1B, *Rf4* on 6B, *Rf5* on 6D, and *Rf6* on 6 A and 6B (McIntosh et al. 1998). Some of these restorer genes are located near the nucleolar organizer region (NOR) in wheat (Tsunewaki 1974; Mukai and Tsunewaki 1979). In the present study, the restorer gene *Rfm1a* seems to be located on a satellite because the *Rfm1* locus was found to be distal to MWG2218, which is itself distal to the NOR in 6HS (Künzel et al. 2000). Considering the high degrees of synteny between barley and wheat (Linde-Laursen et al.

1997), the restorer gene *Rfm1a* of barley might be homoeologous to the wheat restorer genes *Rf4*, *Rf5*, or *Rf6* on homoeologous group 6 chromosomes. In wheat–rye addition lines, the rye restorer gene can restore the male fertility of wheat that has *T. timopheevi* cytoplasm (Curtis and Lukaszewski 1993). The rye restorer gene (*Rfg1*) was mapped on the long arm of chromosome 4, which is homoeologous to the short arm of homoeologous group 6 chromosome in Triticeae (Börner et al. 1998). These pieces of evidence indicate that CMS and the restoration system might be homoeologous among wheat, rye, and barley. Börner et al. (1998) suggested that some genes controlling CMS restoration have been conserved across the cereal species. If so, barley is a good material for cloning the homologous restoration genes in cereals. Cloning of the restorer gene provides us with important information about the mechanisms of CMS and the restoration of fertility, such as *Rf2* of T-cytoplasm in maize (Cui et al. 1996).

The RAPD and STS markers we identified in this study will be good tools for selecting and detecting lines that possess the *Rfm1a* gene. Because of the lack of restorer gene in cultivars, we have to introduce this gene into cultivars that show hybrid vigor in combination with CMS lines. Producing restorer lines with normal cytoplasm generally takes a longer time than does producing lines with male-sterile cytoplasm because of the necessity of test-crossing to the CMS lines. Using the RAPD and STS markers flanking the *Rfm1* locus that we identified in this study, the selection becomes easier. Marker-assisted selection (MAS) using RAPD markers is effective because a large number of samples can be handled with easy manipulation at one time. However, there are several problems associated with the reliability of the RAPD phenotype. The use of STS markers developed from RAPDs is effective in overcoming the problems and improving their utility for MAS, co-dominant STS-markers are especially very useful. In our case, co-dominant markers, such as MWG2218, were useful in identifying the homozygous *Rfm1aRfm1a* genotype after the backcrossing for production of restorer lines, and, in this way, the restorer could be produced 1 year earlier than by conventional methods. Restorer lines possessing a normal cytoplasm are better than those possessing the male-sterile cytoplasm, because with the wide distribution of a single type of cytoplasm there is obviously a danger of cytoplasm-specific diseases, as occurred with the T-cytoplasm in maize.

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