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 BC1 F1 and F2 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 BC1 F1 and F2 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

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, F1 hybrid seed 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. 1995). 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 BC1 F1 and F2 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 BC1 F1 and F2 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.

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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, BC16), the restorer line \((78\text--}383/6^*\)Adorra, BC6), 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. Therefore the restoration line is \(Rfm1\) and the genotype of the restorer gene is designated by \(rfm1a\). 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 \(msm1\)\(-\(Rfm1aRfm1a\)) and the CMS line is \(msm1\)\(-\(rfm1arfm1a\)).

**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 \(msm1\)\(-\(Rfm1aRfm1a\)) and \(msm1\)\(-\(rfm1arfm1a\)) 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 mM Tris-borate plus 2 mM 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´ATCATCCAACTTT-GAAGACCC 3´ and for OPT-02/700 these were 5´GGAGAGACTACGAGATTCA 3´ and 5´ACTGATCCGCGCCTGAA 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 F2 family consisting of 233 plants was derived from the \(msm1\)\(-\(Rfm1aRfm1a\))\(\times\)\(rfm1arfm1a\) cross. A BC1F1 family consisting of 242 plants was produced by backcrossing of the F1 plants to \(msm1\)\(-\(rfm1a\)). In order to map the RAPD markers on the known linkage group, recombinant inbred lines (RILs) of the

**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 \(H. vulgare\) sp. 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 F2 and BC1F1**

Plants of F2 and BC1F1 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 F2 and BC1F1 populations fitted the expected monogenic segregation ratios of 3:1 (\(\chi^2=0.71, 0.25<P<0.50\)) and 1:1 (\(\chi^2=0.0, 0.95<P\)), 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 \(msm1\)\(-\(Rfm1aRfm1a\)) and \(msm1\)\(-\(rfm1a\)) isogenic lines. The BC1F1 population was used for mapping of the fragments generated from \(msm1\)\(-\(Rfm1aRfm1a\)) while the F2 population was used for mapping of the fragments derived from \(msm1\)\(-\(rfm1a\)). The linkage of these fragments to the \(Rfm1\) locus was initially tested using 50 F2 and 63 BC1F1 segregating plants. Three fragments generated by CMNB-07 (5´GGCAGA TA T3´), OPI-18 (5´TGCCCAGCCT3´), and OPT-02 (5´GGAGAGA CTAC3´) were linked to the \(Rfm1\) locus, while the remaining two primers generated from \(msm1\)\(-\(rfm1a\)) were not linked to the \(Rfm1\) locus. The three former fragments were generated from the \(msm1\)\(-\(Rfm1aRfm1a\)) 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 BC1F1 plants, OPI-18/900 was the most closely linked marker to the \(Rfm1\) locus (5.2 cM) (Fig. 1B);