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ORIGINAL PAPER

A new fertility restorer locus linked closely to the *Rfo* locus for cytoplasmic male sterility in radish

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Abstract In this study, we have investigated a new fertility restorer (*Rf*) locus for cytoplasmic male sterility (CMS) in radish. We have obtained a CMS-*Rf* system consisting of sterile line '9802A1', maintainer line '9802B1' and restorer line '9802H'. F_1 plants from cross between sterile line '9802A1' and restorer line '9802H' were all male fertile, self pollination of F_1 plants produced an F_2 segregating population consisting of 600 individuals. The segregating population was found to fit a segregation ratio 3:1 for male fertile and sterile types, indicating that male fertility is restored by a single dominant gene (termed *Rfo2*) in the CMS-*Rf* system. Based on the DNA sequence of *Rfo/Rfk1* (AJ535623), just one full length gene in the sterile line '9802A1', in the restorer line '9802H' and in the male fertile line '2006H', was cloned, respectively. The three sequences correspond to the same gene with two alleles: *Rfob* in '9802H' and *rfob* in '9802A1' and '2006H'. These two alleles differ from *Rfo/Rfk1* and *rfk1* (AJ535624) alleles by two synonymous base substitutions, respectively. Based on the differences between the *Rfob* and *rfob* genes, one PCR-based marker was developed, and designated Marker 1, which is identical to the corresponding region of *Rfob* by sequence analysis. In the F_2 segregating population described above, the Marker 1 was present in 5 sterile plants and in 453 fertile plants, absent in 4 fertile plants and in 138 sterile plants, and was found to fit a segregation ratio 3:1 indicating that *Rfob* was single copy in '9802H'. Linkage analysis showed that the *Rfo2* locus for our CMS-*Rf* system was distant from the *Rfo* locus by about 1.6 cM. The sterile line '9802A1' was pollinated by the male fertile line '2006H' and the resulting F_1 plants were all male fertile. These results indicated that the male fertility of radish CMS can be restored by a new *Rf* locus, which linked tightly to the *Rfo* locus.

Y. J. Zhang, C. P. Xiang, S. Y. Mei, and T. Wang have equally contributed to this work.

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Introduction

Plant cytoplasmic male sterility (CMS) is a widespread, maternally inherited trait caused by an incompatibility between the nucleus and the cytoplasm that prevents the production of functional pollen, but maintains female fertility. Till now, all of the CMS systems characterized are attributed to chimeric open reading frames (ORFs) in the mitochondrial genome (Schnable and Wise 1998; Städler and Delph 2002). In many cases, specific dominant nuclear genes named restorers of fertility (*Rf*) can re-establish the male function of higher plants possessing the CMS mitochondria (Hanson and Bentolia 2004). CMS-restorer

Table 1 Radish materials used in this study

Strain	Abbrev.	Number of plants	
		Male-fertile	Male-sterile
9802A1		0	5
9802B1		5	0
9802H		5	0
2006H		1	0
9802A1 × 2006H		20	0
9802A1 × 9802H	F_1	20	0
(9802A1 × 9802H) × (9802A1 × 9802H)	F_2	457	143

systems have been identified in many higher plants and provide useful tools to exploit heterosis in several crop species such as maize, sunflower, sorghum, onion and sugar beet (Hanson 1991; Schnable and Wise 1998; Mackenzie 2005). Apart from their agronomic importance in hybrid seed production, detailed studies of CMS and *Rf* genes can be very helpful for lightening the interactions between the nucleus and the mitochondrion (Bentolila et al. 2002).

So far, several CMS-*Rf* systems have been well characterized. The *Rf* gene of maize, termed *Rf2*, was the first restorer allele cloned and encodes an aldehyde dehydrogenase (Cui et al. 1996; Liu et al. 2001). But the *Rf2* gene has no discernible effect on the expression of the maize CMS-associated mitochondrial gene *urf13*. The cloned *Rf* gene of Petunia was the first single dominant *Rf* gene affecting the expression of a CMS-associated mitochondrial DNA locus (Bentolila et al. 2002). The *Rf* allele encodes a protein containing 14 pentatricopeptide repeat (PPR) domains, and greatly reduces the abundance of the CMS-associated PCF protein. The radish *Rfo/Rfk1* was precisely positioned, cloned and encodes a predicted protein of 687 amino acids comprising 16 copies of the 35-amino acid PPR motif (Brown et al. 2003; Desloire et al. 2003; Imai et al. 2003; Koizuka et al. 2003), which reduces the amount of the CMS-associated mitochondrial protein (ORF138/ORF125) without changing the level of mRNA. The precise interactions between a mitochondrial locus that confers CMS and nuclear restorer genes were elucidated in rice. The research indicated that Boro II CMS inducing mitochondrial gene, *orf79*, encodes a cytotoxic peptide. Two *Rf* genes of mitochondrion transit signal, *Rf1a* and *Rf1b*, were identified, containing contiguous arrays of 18 and 11 PPR repeats, respectively, and can restore male fertility by blocking ORF79 production through endonucleolytic cleavage and degradation of dicistronic *B-atp6/orf79* mRNA, respectively (Wang et al. 2006). In addition to a single base variation, *Rf1a* is identical to the reported gene *Rf-1* gene, which also restores the male fertility disturbed by the rice BT-type male sterile cytoplasm (Komori et al. 2004).

In terms of the genetic control of restoration for radish Ogura/Kosena CMS, several models were introduced. The early study deduced that the male sterility was restored by one dominant gene (Humaydan and Williams 1976). Koizuka et al. (2000) reported that restoration for Kosena CMS radish is controlled by two unlinked dominant genes that act complementary to achieve restoration. A more complicated genetic model suggested that Ogura CMS is probably controlled by one dominant and two recessive independently acting genes, and several minor genes are involved in the control of fertility in Ogura CMS (Nieuwhof 1990). Through molecular marker mapping, three mutually unlinked restorer loci were identified by Bett and Lydiate (2004). The three genes each exhibited dominant restoring allele, and mutually acted complementary for restoration of male fertility. Furthermore, a fourth restorer locus could exist in the material investigated. Genetic studies cannot reveal the presence of loci at which an allele is homozygous in the population under investigation. This will simplify the true genetic control.

In this report, we have utilized molecular marker approach to identify and map a new *Rf* locus for radish CMS.

Materials and methods

Plant materials

Table 1 lists the CMS lines and fertile lines utilized in this experiment. '9802A1' is a male sterile strain. '9802B1' is a radish cultivar used as a maintainer of '9802A1'. '9802H' is a radish cultivar in China, and used as a restorer for the CMS system. The sterile line '9802A1' was pollinated by a single plant from another male fertile line '2006H', the resulting F_1 plants were all male fertile. The cross between '9802A1' and '9802H' yielded male fertile F_1 plants and self pollination of F_1 plants yielded an F_2 segregating population. Among the progenies involved in this study, two phenotypic classes were distinguished: male sterile individuals with yellow empty anthers and male fertile individuals with full and dehiscent anthers (Fig. 1). The male fertility was visually scored at flowering time.

Fig. 1 Anther morphology of the male sterile line '9802A1' and the maintainer line '9802B1': **a** anther of a male fertile plant; **b** anther of a male sterile plant. Observed at time 3 h after flowering

DNA extraction

Genomic DNA was isolated from young leaves according to the method of Murayama et al. (1999). The quantity and the quality of DNA extractions were determined with a spectrophotometer at a wavelength of 260 nm versus 280 nm. Final DNA concentration was 20 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0).

PCR-specific amplification

PCR-specific primers were designed using the Primer 3 program (http://fokker.wi.mit.edu/primer3/input.htm) and synthesized for the specific amplification (Table 2). Primer pairs, CMSF/R and NWB-F/R, were used to amplify the DNA fragments specific to the Ogura and NWB CMSs, respectively (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993; Nahm et al. 2005). The sequence-tagged site (STS) marker STS190 closely linked to the *Rfo* gene was amplified using primer pair AFLP190-F/R (Murayama et al. 2002). The sequences of the primers defined on AJ535623 accession to amplify the single radish nuclear *Rfo/Rfk1* gene are indicated in Table 3. The PCR reaction mixture (20 μl) contained 1× concentrate *Taq* DNA polymerase buffer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, 0.2 μM of each primer, 0.5 U *Taq* DNA polymerase (Promega) and 30 ng of plant genomic DNA or 1 μl reverse transcriptional products. Amplification was performed in a PTC-100 (MJ Research, USA) thermocycler and consisted of an initial denaturation step at 94°C for 3 min, followed by 36 cycles as follows: a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72°C for 2 min; ending with an extension period at 72°C for 5 min. All amplification products were separated on a 1% agarose, 1× TAE gel, stained with ethidium bromide and viewed under ultra-violet light. The three primer pairs (CMSF/R, F2/R3 and F2/R5) were used to amplify DNA from the parents and then the two primer pairs (CMSF/R and F2/R5) amplifying bright bands were used to screen the entire F_2 population, respectively.

Table 2 Nucleotide sequences of primers used for the specifc amplification

Name	Sequence
F1	ACAAGGAACTCAATCAATCAACTGG
F2	TCGATGTGATATATACAGCTTCAAT
R3	TGCGGATGGTAATGGTAT
R4	GACATTGAAGCTCTGCTGCGC
R5	GGAAATGGTCAAATTTATTAAGCCC
CMSF	TTCAAATCCTGTCCCCGCACC
CMSR	GCCTTACACCATTGGGATACTTC
AFLP190-F	GACAGCCCATTCGAGAGCTGCG
AFLP190-R	ATTGTGGATACATATACACAGC
NWB-F	CGCTTGGACTATGCTATGTATGA
NWB-R	TCATAGAGAAATCCAATCGTCAA

Table 3 Locations within the *Rf* gene (accession no. AJ535623) that correspond to these primer sequences

Primer	Location
F1	44–68
F2	553–557
R3	2171–2189
R4	2312–2332
R5	2472–2496

RT-PCR

Total RNA was isolated by TRIzol reagent (Invitrogen Life Technologies) and first-strand cDNA was synthesized using a SMART™ RACE cDNA amplification kit (Clontech) according to the user manual. After reverse transcription, the products were diluted 10× with distilled water. The PCR reaction mixture and conditions were carried out as described above.

Cloning and sequencing

The amplified products were separated on a 1.0% agarose gel. The target DNA band was cut out with a sterile cutter, purified with UNIQ-10 EZ Spin Column DNA Gel Extraction

Kit (Sangon, Shanghai) and cloned into the T/A-cloning vector pGEM-T Easy (Promega), as described by the manufacturer. The recombinant plasmids were transformed into *Escherichia coli* strain DH5α. The recombinant plasmids were screened using the colony PCR method (Innes et al. 1990). The complete sequence of each cloned fragment was obtained using an automated DNA sequencer (Shanghai Genecore Company, China).

Sequence analysis

Sequence homologies were analyzed using the BLAST program (Altschul et al. 1990). Multiple sequence alignment was carried out using CLUSTAL X (Jeanmougin et al. 1998).

Genetic mapping of the molecular markers

Linkage analysis was performed on Mapmaker/exp version 3.0b (Lincoln et al. 1992). A minimum LOD of 4.0 was chosen. The Kosambi mapping function (Kosambi 1944) was used in calculating genetic distances.

Database accession numbers: EU163282 (*Rfob* gene), EU163283 (*rfob* gene).

Results

Molecular identification of the CMS in the male sterile line '9802A1'

In order to determine whether the male sterile line '9802A1' possesses the *orf138* gene, the male sterility-inducing gene (Bonhomme et al. 1992), primer combination CMSF/R was designed on the basis of the DNA sequence reported by Krishnasamy and Makaroff (1993). Using PCR, the combination resulted in the amplification of a 1034-bp DNA fragment in the male sterile line '9802A1', but no PCR product was detected in the maintainer line '9802B1' (Fig. 2); sequence analysis showed that the DNA fragment contains the full length *orf138* gene. So, we deduced that the CMS of the male sterile line '9802A1' belongs to the Ogura CMS type.

Genetic control of the male fertility restorer

Of the 600 F_2 plants developed from the self pollination of the F_1 plants produced by the cross between '9802A1' and '9802H', the male sterile and the restorer lines, 457 were male fertile and 143 were male sterile. All F_2 plants had the specific DNA fragment amplified by the primer pair CMSF/R indicating that no cytoplasmic revertants occurred. The population thus displayed a ratio of male fertile to male

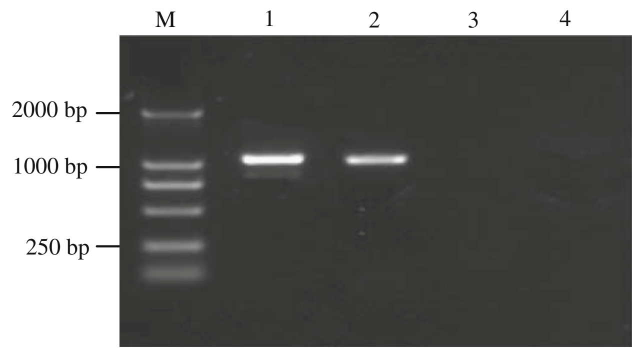

Fig. 2 Amplification of DNA fragments using primer pair CMSF/R. *M* DL2 000 marker. *Lanes 1*, *2*, *3* and *4* indicate the patterns of '9802A1', F_1, '9802B1'and '9802H', respectively

sterile plants that did not differ significantly from 3:1 ratio expected for a trait controlled by a single locus ($\chi^2 = 0.38$, $P > 0.05$), confirming that one dominant gene segregated in the F_2 population.

Cloning of the *Rfob* and *rfob* genes

In order to detect whether the *Rfo* gene, restoring male fertility for Ogura CMS radish, is present in the restorer line '9802H', we designed primer combination F1/R4 which can amplify the full length *Rfo* gene. Using the primer pair F1/R4 (Table 2), an about 2.0-kb fragment was amplified by PCR from genomic DNA isolated from the male sterile line '9802A1' and the restorer line '9802H', respectively (Fig. 3). The fragments from '9802A1' and '9802H' were cloned and designated *rfob* and *Rfob*, respectively. Compared with *Rfob*, *rfob* contained 11 base substitutions (Fig. 4). Sequence analysis revealed two synonymous base substitutions in *rfob* in comparison with non-restoring allele *rf* (AJ535624) (Fig. 4). *Rfob* was also identical to

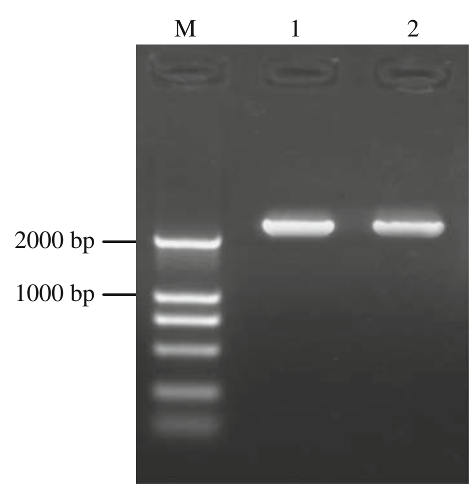

Fig. 3 Analysis of PCR fragments obtained with F1/R4 primers. *M* DL2 000 marker. *Lanes 1* and *2* indicate the patterns of '9802A1'and '9802H', respectively

Fig. 4 DNA sequence alignments of the *Rfo* alleles with non-identities indicated by *asterisks*

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Rfo        CTCTATCAGAAGATGGAAAGGAAACAGATTCGATGTGATATATACAGCTTCAATATTCTG 360
rfo        CTCTATCAGAAGATGGAAAGGAAACAGATTCGATGTGATATATACAGCTTCACCATTCTG 360
Rfob       CTCTATCAGAAGATGGAAAGGAAACAGATTCGATGTGATATATACAGCTTCAATATTCTG 360
rfob       CTCTATCAGAAGATGGAAAGGAAACAGATTCGATGTGATATATACAGCTTCACCATTCTG 360
                                                               **

Rfo        ATAAAATGTTTCTGCAGCTGCTCTAAGCTCCCCTTTGCTTTGTCTACATTTGGTAAGATC 420
rfo        ATAAAATGTTTCTGCAGCTGCTCTAAGCTCCCCTTTGCTTTGTCTACATTTGGTAAGATC 420
Rfob       ATAAAATGTTTCTGCAGCTGCTCTAAGCTCCCCTTTGCTTTGTCTACATTTGGTAAGATC 420
rfob       ATAAAATGTTTCTGCAGCTGCTCTAAGCTCCCCTTTGCTTTGTCTACATTTGGTAAGATC 420

Rfo        ACCAAGCTTGGACTCCACCCTGATGTTGTTACCTTCACCACCCTGCTCCATGGATTATGT 480
rfo        ACCAAGCTTGGACTCCACCCTGATGTTGTTACCTTCAACACCCTGCTCCACGGATTGTGC 480
Rfob       ACCAAGCTTGGACTCCACCCTGATGTTGTTACCTTCACCACCCTGCTCCATGGATTATGT 480
rfob       ACCAAGCTTGGACTCCACCCTGATGTTGTTACCTTCAACACCCTGCTCCACGGATTGTGC 480
                                                *            *     *  *

Rfo        GTGGAAGATAGGGTTTCTGAAGCCTTGGATTTTTTTCATCAAATGTTTGAAACGACATGT 540
rfo        GTGGAAGATAGGGTTTCTGAAGCTTTGAATTTGTTTCATCAAATGTTTGAAACGACATGT 540
Rfob       GTGGAAGATAGGGTTTCTGAAGCCTTGGATTTTTTTCATCAAATGTTTGAAACGACATGT 540
rfob       GTGGAAGATAGGGTTTCTGAAGCTTTGAATTTGTTTCATCAAATGTTTGAAACGACATGT 540
                                  *   *    *

Rfo        AGGCCCAATGTCGTAACCTTCACCACTTTGATGAACGGTCTTTGCCGCGAGGGTAGAATT 600
rfo        AGGCCCAATGTCGTAACCTTCACCACTTTGATGAACGGTCTTTGCCGCGAGGGTAGAATT 600
Rfob       AGGCCCAATGTCGTAACCTTCACCACTTTGATGAACGGTCTTTGCCGCGAGGGTAGAATT 600
rfob       AGGCCCAATGTCGTAACCTTCACCACTTTGATGAACGGTCTTTGCCGCGAGGGTAGAATT 600

Rfo        GTCGAAGCCGTAGCTCTGCTTGATCGGATGATGGAAGATGGTCTCCAGCCTACCCAGATT 660
rfo        GTCGAAGCCGTAGCTCTGCTTGATCGGATGATGGAAGATGGTCTCCAGCCTACCCAGATT 660
Rfob       GTCGAAGCCGTAGCTCTGCTTGATCGGATGATGGAAGATGGTCTCCAGCCTACCCAGATT 660
rfob       GTCGAAGCCGTAGCTCTGCTTGATCGGATGATGGAAGATGGTCTCCAGCCTACCCAGATT 660

Rfo        ACTTATGGAACAATCGTAGATGGGATGTGTAAGAAGGGAGATACTGTGTCTGCACTGAAT 720
rfo        ACTTATGGAACAATCGTAGATGGGATGTGTAAGAAGGGAGATACTGTGTCTGCATTGAAT 720
Rfob       ACTTATGGAACAATCGTAGATGGGATGTGTAAGAAGGGAGATACTGTGTCTGCATTGAAT 720
rfob       ACTTATGGAACAATCGTAGATGGGATGTGTAAGAAGGGAGATACTGTGTCTGCACTGAAT 720
                                                                 *

Rfo        CTGCTGAGGAAGATGGAGGAGGTGAGCCACATCATACCCAATGTTGTAATCTATAGTGCA 780
rfo        CTTCTGAGGAAGATGGAGGAGGTGAGCCACATCATACCCAATGTTGTAATCTATAGTGCA 780
Rfob       CTTCTGAGGAAGATGGAGGAGGTGAGCCACATCATACCCAATGTTGTAATCTATAGTGCA 780
rfob       CTGCTGAGGAAGATGGAGGAGGTGAGCCACATCATACCCAATGTTGTAATCTATAGTGCA 780
             *
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Rfo/Rfk1 (AJ535623) except for two base synonymous substitutions (Fig. 4). These results suggested that our CMS system contains a gene, *Rfob* which encodes identical putative protein encoded by the *Rfo* gene.

The male fertility of our CMS system can be restored without the presence of *Rfo* gene

Primer combination F1/R4 produced a fragment in a male fertile line '2006H'. The fragment was cloned and five positive clones were sequenced. Sequence analysis indicated that the five sequences were the same and identical to *rfob*, suggesting that '2006H' carried non-restoring allele at the *Rfo* locus. But the sterile line '9802A1' was pollinated by the male fertile line '2006H' and the resulting F_1 plants were all male fertile. These results showed that the male restoration of our CMS did not depend on the restoring allele at the *Rfo* locus and there is another *Rf* gene for our CMS system.

Identification of a new male fertility restorer locus

Based on the sequence differences between *rfob* and *Rfob*, specific primer (F2) for *Rfob* was designed (Table 2). Using primer pair F2/R5, a PCR product was found in the restorer line '9802H', but absent in the male sterile line '9802A1' (Fig. 5). The product was isolated, identical to the corresponding region of the *Rfob* and named as Marker 1. In the F_2 segregating population described above, the Marker 1 was absent in 4 fertile plants and in 138 sterile plants, present in 5 sterile plants and in 453 fertile plants. The segregation of the Marker 1 was found to fit 3:1 ratio ($\chi^2 = 0.50$, $P > 0.05$) suggesting that *Rfob* was single copy in '9802H'. Linkage analysis indicated that the *Rf* gene for our CMS-*Rf* system was linked in coupling phase to the *Rfo* gene at 1.6 cM in '9802H'. Here, the new *Rf* gene was termed *Rfo2*. These results showed that the male fertility of radish CMS can be restored by the *Rfo2* locus which linked tightly to the *Rfo* locus.

Transcription of *Rfob* in the restorer line '9802H'

The reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze expression of the *Rfob* gene. One set of primers (F2/R5) amplified RT-PCR products from root, stem, leaf, flower and young pod RNA of the restorer line '9802H' (Fig. 6). The presence of intron was detected by comparing genomic DNA PCR and RT-PCR products (Fig. 6). These RT-PCR fragments were cloned, sequenced and identical to the corresponding region of *Rfob* mRNA by sequence analysis. However, Brown et al. (2003) reported that different sets of primers internal to the *Rfo* ORF amplified no RT-PCR product from root RNA of homozygous

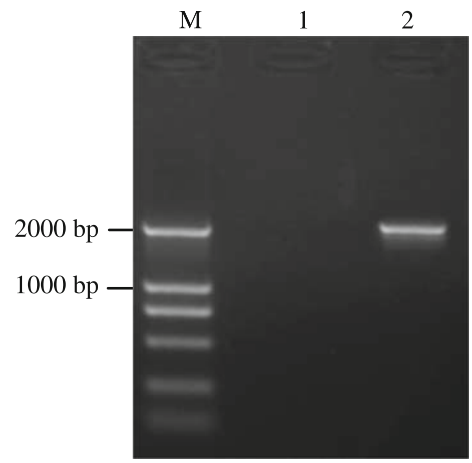

Fig. 5 PCR products obtained using F2/R5 primers. *M* DL2 000 marker. *Lanes 1* and *2* indicate the patterns of '9802A1'and '9802H', respectively

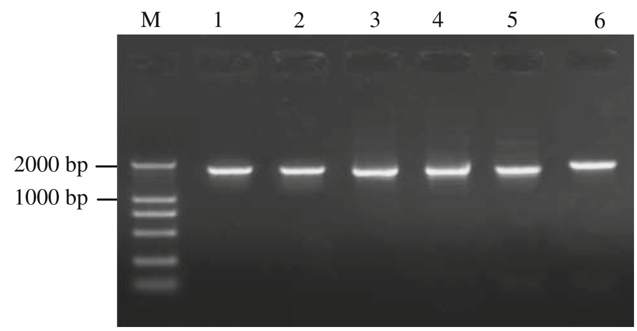

Fig. 6 Expression of the *Rfob* gene in different organs of radish restorer line '9802H' with specific primers F2/R5. *M* DL2 000 marker. *Lanes 1*, *2*, *3*, *4* and *5* products with the first strand cDNA of root, stem, leaf, flower and young pod as template, respectively. *Lane 6* product with the genomic DNA as template

fertile radish plants. These results indicate that the expression pattern of the *Rfob* gene is different from that of the *Rfo* gene.

The male sterile line '9802A1' includes the STS marker STS190

The STS marker STS190 was linked in coupling phase to the *Rfo* gene at 1.2 cM in the restorer line 'Comet' (Murayama et al. 2002). However STS190 was present in the male sterile line '9802A1' and absent in the restorer line '9802H', indicating that '9802H' has different genetic background compared with 'Comet' (Fig. 7).

DNA marker specific to NWB CMS is present in the maintainer line '9802B1'

A DNA marker specific to radish NWB CMS different from Ogura/Kosena CMS was developed (Nahm et al. 2005). Using the set of primers NWB-F/R reported by Nahm et al. (2005), a DNA fragment was amplified in maintainer line

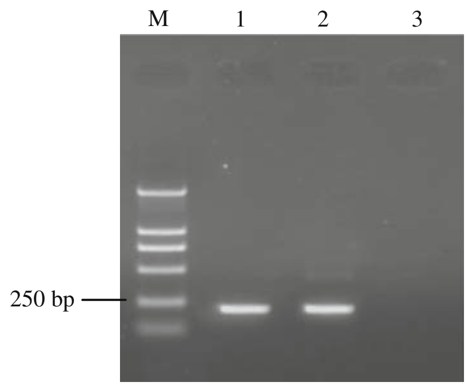

Fig. 7 PCR products obtained with AFLP190-F/R primers. *M* DL2 000 marker. *Lanes 1*, *2* and *3* indicate the patterns of '9802A1', F_1 and '9802H', respectively

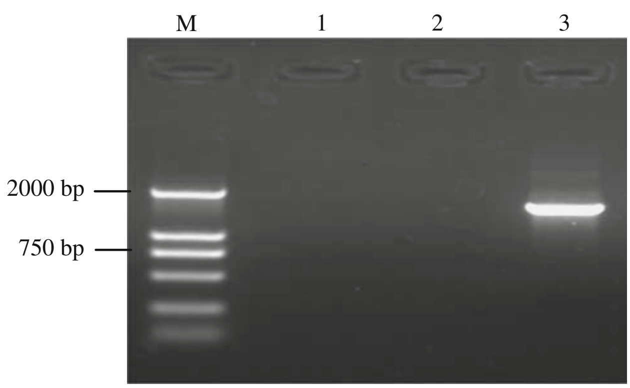

Fig. 8 Amplification of DNA fragment using primer pair NWB-F/R. *M* DL2 000 marker. *Lanes 1*, *2* and *3* indicate the patterns of '9802A1', F_1 and '9802B1', respectively

'9802B1', but not in male sterile line '9802A1' (Fig. 8). The fragment was cloned and sequenced (data not shown). Sequence analysis showed that the DNA fragment contained 3′ region of the *atp6* gene and the 5′ region of the *nad3* gene as described by Nahm et al. (2005).

Discussion

In the present study, PCR and sequence analysis demonstrated the presence of the sterility-inducing gene *orf138* in the male sterile line '9802A1' (Fig. 2) and that restorer line '9802H' contains *Rfob* gene which is single copy and identical to the *Rfo* gene except for two base synonymous substitutions. This result showed that the *Rfob* gene is allelic to the *Rfo* gene. However, *Rfob* gene cannot restore the male fertility in our CMS-*Rf* system by F_2 segregating population analysis. Why can the *Rfo* gene restore the male fertility for CMS, but *Rfob* cannot? Two reasons could be suggested. First, the sequences of the upstream regulating region could be different between *Rfob* and *Rfo*. The conjecture may be supported by the difference of expression profile between the *Rfob* gene and the *Rfo* gene which was not expressed in radish root (Brown et al. 2003). Recently, a study showed that the dominant allele, *Xa13* required for both bacterial growth and pollen development, and the fully recessive allele, *xa13* against bacterial blight in rice, can encode identical proteins, but have vital sequence differences in their promoter regions (Chu et al. 2006). However, both *Rfob* and *Rfo/Rfk1* are expressed in the radish flower. So, we would accept the suggestion that the difference of expression could be the cause of non-restoring ability of *Rfob* after *Rfob* would not be expressed in a specific part of the flower at different stages (especially in the anthers). The second hypothesis was as follows: irrespective of the *Rfo2* gene, the restoration of male fertility could be controlled by two complementary dominant genes, *Rfob* and another *Rf* genes, and the second gene is recessively homozygous in the male sterile line '9802A1' and restorer line '9802H'.

NWB CMS was crossed to 58 breeding male fertile lines collected from several countries, and the resulting F_1 plants were all male sterile (Nahm et al. 2005). The DNA marker specific to NWB CMS was present in maintainer line '9802B1' (Fig. 8) and the STS marker STS190 linked in coupling phase to the *Rfo* gene in the restorer line 'Comet' (Murayama et al. 2002) was amplified in the male sterile line '9802A1' (Fig. 7). These results suggested that our CMS-*Rf* system has different genetic background from other radish CMS-*Rf* systems and the maintainer line '9802B1' could possess the *Rf* gene for the NWB CMS.

The fertility restorer of CMS-BT of rice has long been consindered as the single locus *Rf-1* through classical genetic analysis (Akagi et al. 1996). The recent research showed that *Rf-1* is actually a complex locus consisting of at least two *Rf* genes as members of a multigene cluster that encode PPR proteins within an about 105-kb region, and each of the two *Rf* genes, *Rf1a* and *Rf1b*, can independently restore fertility to CMS-BT rice (Wang et al. 2006). Our present study revealed a similar result that the *Rfo2* gene is sufficient to restore fertility to Ogura CMS radish and closely linked to the *Rfo* locus.

All *Rf* genes cloned to date comprise PPR motifs with the exception of maize *Rf2* (Cui et al. 1996; Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Imai et al. 2003; Koizuka et al. 2003; Komori et al. 2004; Wang et al. 2006). And rice *Rf* genes, *Rf1a* and *Rf1b*, share 70% identity between their protein sequences (Wang et al. 2006). These results offer compelling impetus for studies of possible genetic relationships between the PPR gene family and the new *Rf* locus in radish.

In radish, 0.3 cM would correspond to physical distance of 317–416 kb (Imai et al. 2003). Thus 1.6 cM would cover 1,690–2,218 kb. A genomic DNA insert size of a bacterial artificial chromosome (BAC) library is approximately

100 kb. So, the Marker 1 is not suitable for screening BAC library. For map-based cloning, we are developing more closely linked markers to the new *Rf* gene.

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