Journal of Experimental Botany, Vol. 64, No. 7, pp. 2041–2048, 2013
doi:10.1093/jxb/ert065 Advance Access publication 4 March, 2013
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Heterozygous alleles restore male fertility to cytoplasmic male-sterile radish (*Raphanus sativus* L.): a case of overdominance

Zhi Wei Wang[1,†], Chuan Wang[1,2,†], Lei Gao[1,†], Shi Yong Mei[3], Yuan Zhou[1], Chang Ping Xiang[4,*] and Ting Wang[1,*]

[1] CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, PR China
[2] Graduate University of the Chinese Academy of Sciences, Beijing 100049, PR China
[3] Hubei Academy of Agricultural Sciences, Wuhan 430064, PR China
[4] Key Laboratory of Ministry of Education for Horticultural Plant Biology, Huazhong Agricultural University, Wuhan 430070, PR China

*To whom correspondence should be addressed. E-mail: chpxiang@mail.hzau.edu.cn or tingwang@wbgcas.cn
†These authors contributed equally to this work.

Received 11 October 2012; Revised 18 January 2013; Accepted 18 February 2013

Abstract

The practice of hybridization has greatly contributed to the increase in crop productivity. A major component that exploits heterosis in crops is the cytoplasmic male sterility (CMS)/nucleus-controlled fertility restoration (*Rf*) system. Through positional cloning, it is shown that heterozygous alleles (*RsRf3-1*/*RsRf3-2*) encoding pentatricopeptide repeat (PPR) proteins are responsible for restoring fertility to cytoplasmic male-sterile radish (*Raphanus sativus* L.). Furthermore, it was found that heterozygous alleles (*RsRf3-1*/*RsRf3-2*) show higher expression and RNA polymerase II occupancy in the CMS cytoplasmic background compared with their homozygous alleles (*RsRf3-1*/*RsRf3-1* or *RsRf3-2*/*RsRf3-2*). These data provide new insights into the molecular mechanism of fertility restoration to cytoplasmic male-sterile plants and illustrate a case of overdominance.

Key words: Cytoplasmic male sterility, fertility restoration, heterozygous alleles, overdominance, radish.

Introduction

Plant cytoplasmic male sterility (CMS), a maternally inherited trait that prevents plants from producing functional pollen, has been identified in many higher plants, including rice, cotton, maize, and sorghum (Liu *et al*., 2001; Bentolila *et al*., 2002; Klein *et al*., 2005; Yin *et al*., 2006; Fujii *et al*., 2011). CMS restorer systems have been widely exploited to produce hybrids that outperform their inbred parents in yield, biomass, or other traits. CMS is usually attributed to an unusual chimeric gene in the mitochondrial genome (Schnable and Wise, 1998; Ivanov and Dymshits, 2007). In many cases, a nuclear-encoded fertility restorer gene (*Rf*) can restore fertility of the cytoplasmic male-sterile plants (Hanson and Bentolila, 2004). Therefore, the CMS/*Rf* system is an ideal model for dissecting the interaction between mitochondrial and nuclear genomes.

A variety of mechanisms of fertility restoration by the *Rf* genes have been reported for different CMS systems. T-*urf13*, a mitochondrial gene encoding a 13 kDa protein, has been detected only in maize carrying T male-sterile cytoplasm (Dewey *et al*., 1987; Fujii and Toriyama, 2008). The first restorer allele cloned, the maize *Rf2* gene, does not affect the expression of *urf13* and encodes aldehyde dehydrogenase (ALDH), which is located in the mitochondrial matrix in a homotetrameric form (Cui *et al*., 1996; Liu *et al*., 2001; Liu and Schnable, 2002). The cloned petunia *Rf-PPR592* gene is the first single dominant *Rf* gene encoding a pentatricopeptide

repeat-containing (PPR) protein that can eliminate the CMS-associated protein PCF (Bentolila *et al*., 2002). The Rf-PPR592 protein contains 11 continuous PPR motifs and is a part of the mitochondrial inner membrane-associated, RNase-sensitive large soluble high molecular weight complex that interacts with *pcf* mRNA (Gillman *et al*., 2007). The radish *Rfo*/*Rfk1* gene for Ogura/Kosena CMS also encodes a mitochondrially targeted PPR protein of 687 amino acids comprising 16 repeats of the 35 amino acid PPR motif (Brown *et al*., 2003; Desloire *et al*., 2003; Imai *et al*., 2003; Koizuka *et al*., 2003) that reduces the protein level of ORF138 without changing the level of mRNA (Bellaoui *et al*., 1999; Uyttewaal *et al*., 2008). Molecular components responsible for fertility restoration processes were deduced from various types of CMS/*Rf* systems in rice. Either of two tightly linked genes, *Rf1a* and *Rf1b*, can independently restore fertility to Boro II rice and have been identified as encoding PPR proteins, containing contiguous arrays of 18 and 11 PPR repeats, respectively (Komori *et al*., 2004; Wang *et al*., 2006). Rf1a blocks cytotoxic peptide ORF79 through endonucleolytic cleavage of the dicistronic *B-atp6*/*orf79* mRNA, whereas Rf1b degrades *B-atp6*/*orf79* mRNA (Wang *et al*., 2006; Kazama *et al*., 2008). *Rf17* and *Rf2* have been isolated as the *Rf* genes for CW-type and Lead Rice-type CMS, and have been shown to encode mitochondrial proteins, acyl-carrier protein synthase-like protein, and glycine-rich protein (GRP) that are not PPR proteins (Fujii and Toriyama, 2009; Itabashi *et al*., 2011). Interestingly, a recent work demonstrates that RF5, a PPR protein, physically interacts with GRP162, a GRP encoding 162 amino acids that was identified to bind to *atp6-orfH79* (Hu *et al*., 2012).

Here the map-based cloning of heterozygous alleles controlling male fertility of CMS in radish is reported.

Materials and methods

Plant materials

9802A1 is a male-sterile strain. 9802B1 is a radish cultivar used as a maintainer of 9802A1. 9802A1 contains the same nuclear genome as 9802B1. 9606H is a Chinese radish cultivar. 0107H is a male-fertile strain carrying *Rfob* (EU163282). The cross between 9802A1 and 9606H yielded male-fertile F_1 plants. Self-pollination of F_1 plants yielded an F_2 segregating population. The BC1F1 population was produced from the backcross between 9802A1 (the acceptor parent) and 9606H (the donor parent) (Supplementary Fig. S1 available at *JXB* online). Among the progeny involved in this work, two phenotypic classes were distinguished: male-fertile plants with full and dehiscent anthers and male-sterile plants with empty yellow anthers. To ensure the accuracy of the genetic position, the phenotypes of male-fertile recombinant F_2 plants were confirmed by phenotyping the F_3 progeny in a glasshouse.

Microscopic observation

Floral buds at different developmental stages were fixed overnight in FAA [ethanol 50% (v/v), acetic acid 5.0% (v/v), and formaldehyde 3.7% (v/v)]. Fixed floral buds were dehydrated with a 50–100% ethanol series and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim/Ts., Germany) according to the manufacturer's manual. Transverse sections (1.5 μm thickness) were cut from the polymerized blocks on an ultramicrotome (Leica Ultracut R; Leica Microsystems) using glass knives, heat fixed to glass slides, and stained with 2% toluidine blue. Semi-thin sections were stained with 0.05% analine blue to detect callose. Slides were inspected and photographed using an Olympus BX61 microscope equipped with a colour CCD camera.

Macroarray

The radish bacterial artificial chromosome (BAC) library consists of 120 000 clones and represents the haploid radish genome at least 23 times over (Desloire *et al*., 2003). The macroarrays are produced by spotting thousands of bacterial clones on the nylon membrane with a high-throughput machine (Q-Bot). The macroarray in pre-hybridization buffer (6× SSC, 5×Denhardt's, 100 mg ml^{-1} salmon sperm DNA) was pre-hybridized for at least 2 h at 68 ºC in a hybridization oven. The probe was denatured for 10 min at 100 ºC and immediately placed on ice. The pre-hybridization buffer was removed from the macroarrays. Then, the probe was added to hybridization buffer and hybridized overnight at 68 °C. Hybridized macroarrays were washed twice for 30 min in wash buffer (2× SSC, 0.1% SDS) at 50 ºC. After drying, macroarrays were wrapped in cling film, avoiding air bubbles. The level of radioactivity from a macroarray was checked, followed by fixation in a cassette. After scanning the macroarray with PhosphorImager, the analysis of the macroarray was carried out using high density filter reader (HDFR) software.

DNA extraction for genotyping

About 5 mm×5 mm from young seedlings was harvested into 96-well PCR plates with 50 μl of NaOH solution (0.25 M) in each well, and plates were sealed. Plates were incubated at 96° C for 10 min in the PCR block, cooled on a bench, and spun down. A 50 μl aliquot of 0.25 M HCl and 25 μl of buffer (0.5 M TRIS-HCl, pH 8.0; 0.25% NP-40) were added. After mixing by inverting and spinning down, plates were incubated at 96° C for 1 min in a PCR block. A 0.6 μl aliquot of DNA was used for each 12.5 μl PCR.

RT–PCR

Total RNA was isolated from floral buds using TRIzol reagent (Invitrogen Life Technologies) and first-strand cDNA was synthesized using a SMART™ RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. After reverse transcription, the products were diluted 10-fold with distilled water. The PCR mixture and conditions as described above were followed. The primers used for 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE are listed in Supplementary Table 1 at *JXB* online.

Real-time RT–PCR

The expression patterns of the PPR transcripts were examined through strand-specific RT–PCR, in which 1.5 μg of total RNA was used for the first-strand cDNA synthesis with the SuperScript III reverse transcriptase (Invitrogen) using the mixture of gene-specific primers. The cDNA reaction mixture was then diluted 10 times and 4 μl was used as a template in a 10 μl PCR with SYBR Green Supermix. PCR included a pre-incubation at 95 ºC for 5 min followed by 45 cycles of denaturation at 95 ºC for 15 s, annealing at 60 ºC for 20 s, and extension at 72 ºC for 30 s. The comparative threshold (Ct) cycle method was used for determination of relative transcript levels with Actin 2/7 as an internal control.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described by Wierzbicki *et al*. (2008) with minor modifications. A 2 g aliquot of above-ground tissue of seedlings was cross-linked with 1% formaldehyde by vacuum infiltration at room temperature three times, 5 min each time, followed by the addition of glycine to 125 mM vacuum infiltrate for an additional

5 min. Plants were rinsed with distilled water, frozen in liquid nitrogen, ground into powder, suspended in 35 ml of Honda buffer [20 mM HEPES, 0.44 M sucrose, 1.25% ficoll, 2.5% dextran T40, 10 mM $MgCl_2$, 0.5% Triton X-100, 5 mM dithiothreitol (DTT), 1% plant protease inhibitors], filtered through two layers of Miracloth twice, and centrifuged at 3100 *g* for 20 min. Nuclear pellets were resuspended in 1 ml of Honda buffer, centrifuged at 3100 *g* for 10 min at 4° C, resuspended in Nuclei Lysis Buffer (50 mM TRIS-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1% plant protease inhibitors), and sonicated three times, 5 min each time (30 s on/off intervals) at the 'Middle' setting. After centrifugation at 16 000 *g* for 7.5 min, the supernatant was diluted 11-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM TRIS-HCl pH 8.0, 167 mM NaCl, protease inhibitor cocktail). Immunoprecipitation was performed using 20 µl of Dynabeads protein G (Invitrogen) and 5 µl of Pol II antibody (Abcam ab5408). After reversion of cross-linking, samples were incubated with 40 µg of proteinase K (Invitrogen) at 50 ºC for 1 h, followed by heat inactivation at 95 ºC for 10 min. The resulting DNA was subjected to quantitative PCR in triplicate.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers JX521806 (*RsRf3-1* gene) and JX521807 (*RsRf3-2* gene).

Results

Histological analysis of male-sterile and male-fertile flowers of radish

No cytological differences were observed between the fertile line 9802B1 and the male-sterile line 9802A1 in the anther tissues until the last stages of the tetrad (data not shown). At the tetrad stage, individual tetrads were completely encased by a thick callose wall in 9802B1 and 9802A1 (Fig. 1A, B). After the tetrad stage, the microspores from male-fertile plants increased in size and the microspore exine was quite evident (Fig. 1C). In contrast, although microspore exines were found in the male-sterile plants, microspores were malformed. At the same time, the tapetal cells grew abnormally large (Fig. 1D). At anthesis, the tapetal cells degenerated completely in both male-fertile and male-sterile anthers (Fig. 1E, F). Many fully mature pollen were observed in the locules of male-fertile anthers. In contrast, many remnants of microspore exine were found in the locules of male-sterile anthers (Fig. 1F).

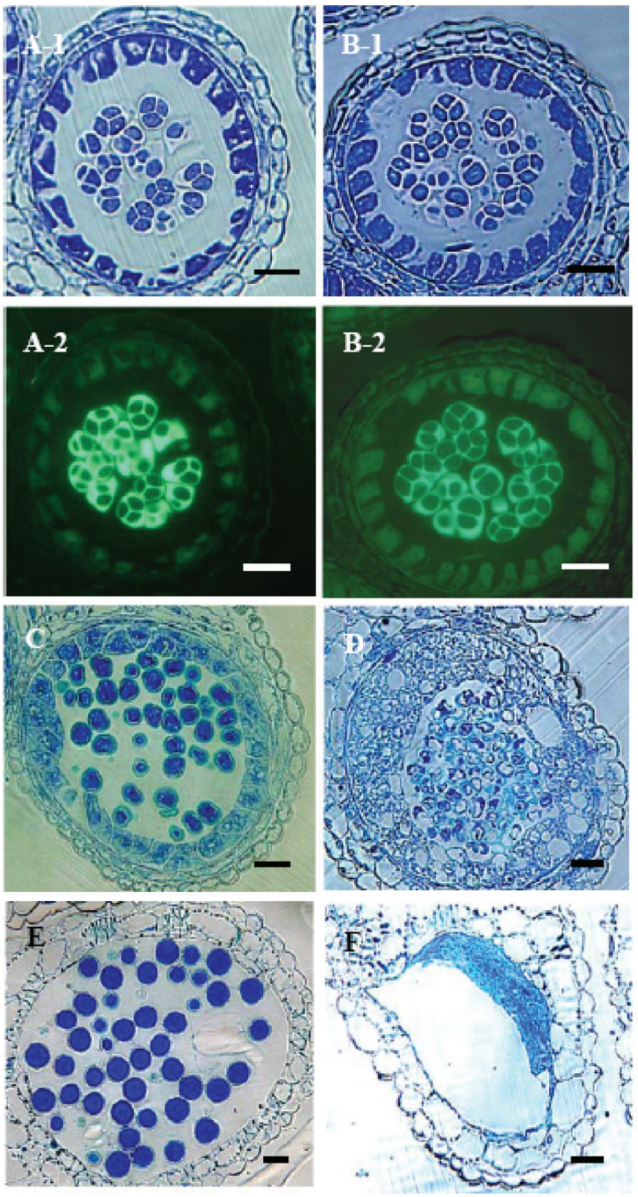

Fig. 1. Comparative study of anthers through different developmental stages in the male-fertile and male-sterile plants from 9802A1 and 9802B1. (A, C, E) Semi-thin sections from male-fertile anthers. (B, D, F) Semi-thin sections from male-sterile anthers. A-2 and B-2 were stained with aniline blue; the others were stained with toluidine blue. (This figure is available in colour at *JXB* online.)

The male fertility restorer is controlled by heterozygous alleles in an F_2 segregating population

An F_2 population was derived from a cross between 9802A1 and 9606H. In total, 600 plants were examined from the population, of which 296 male-fertile and 304 male-sterile plants were identified by investigation of male fertility. The segregation ratio of fertile to sterile plants in this F_2 population best fitted a 1:1 ratio ($P > 0.05$), but not a 3:1 ratio ($P < 0.05$), indicating control by heterozygous alleles at a single locus (designated as *RsRf3*). In addition, the single locus control of male fertility was also strongly supported by a 1:1 BC1F1 ratio of male-fertile to male-sterile plants in the backcross population between 9802A1 (the acceptor parent) and 9606H (the donor parent) (Supplementary Fig. S1, Supplementary Table S2 at *JXB* online). The data also demonstrated that fertility of the cytoplasmic male-sterile plant was restored by heterozygous alleles in a sporophytic manner.

The RsRf3 locus was limited to a locus encoding PPR proteins

Most *Rf* genes cloned to date have PPR motifs (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). Rice *Rf* genes, *Rf1a* and *Rf1b*, share 70% identity between their protein sequences (Wang *et al.*, 2006). It is possible that some

radish *Rf* genes are descendents of a progenitor restorer *PPR* gene (Brown *et al*., 2003), and that these multiple genes arose in response to the appearance of new forms of CMS. These results offered compelling reasons for studies of possible genetic relationships between the PPR-coding genes and new *Rf* loci in radish. Consequently, a BAC radish library screening was performed by macroarray hybridization. This method allows fast access to homologues of the *Rfo* gene in the radish genome. Based on the radish *Rfo* gene (accession no. AJ535623), a pair of PCR primers (78F/79R) were designed (Supplementary Table S1 at *JXB* online) to amplify a PCR product of 469 bp corresponding to the 1152–1620 bp region of *Rfob*. One PCR product (~500 bp) from the male-fertile line 0107H carrying *Rfob* (EU163282) was amplified using 78F/79R, purified, and used as a probe for a screening of the macroarray. Twenty-eight positive BAC clones were identified on macroarrays (Supplementary Fig. S2, Supplementary Table S3). Based on PCR by 78F/79R and sequencing, 18 clones corresponded to the *Rfo* locus. Primer pair 78F/79R did not amplify any product using the other 10 clones as templates, suggesting that these clones contain similar, but not identical, sequences to the *Rfo* gene. Then another 10 primer pairs were designed to amplify the corresponding *Rfo* amplicons from the 10 clones. The primer pair P1/P2 amplified ~300 bp products in BAC clones 48H19 and 158K3. The sequencing result showed that the 296 bp PCR product from 48H19 was identical to that from 158K3 and shared 96% identity with the corresponding region of *Rfo*. Pulsed field gel electrophoresis showed that 48H19 is ~145 kb and 158K3 is ~130 kb (Supplementary Fig. S3). Thus the BAC clone 48H19 was sequenced using 454 sequencing technology and 10 contigs were obtained. A contig, composed of 42 567 bp, assembled by 8205 reads, contained two predicted PPR genes. One predicted PPR gene of 1440 bp showed 90% identity with the *Rfo* gene. Based on previous genetic analysis, there should be polymorphism between the sterile line 9802A1 and the fertile line 9606H. Brief sequencing detected two polymorphic sites in predicted promoter and exon regions. Based on these polymorphic sites, two molecular markers were developed. Using the F_2 population described above, linkage analysis showed that this *PPR* locus co-segregated with the *RsRf3* locus (Supplementary Table S4). Based on the above contig sequence, a series of primers were designed to amplify the corresponding regions in 9802A1 and 9606H. The alignment of the corresponding sequences between 9802A1 and 9606H revealed a few more polymorphic sites. Additional molecular markers were developed and *RsRf3* was resolved to a 4 kb region containing only one gene using a large F_2 population with 20 000 plants (Fig. 2). Mapping analysis using the BC1F1 population with 18 000 plants yielded the same result (data not shown).

Characterization of the RsRf3 locus

The 5' and 3' RACE experiments showed that the *RsRf3* locus has an intron and encodes a PPR protein with 479 amino acids comprising 11 copies of the 35 amino acid PPR motif. The RsRf3 protein shows 85% similarity with the Rfo protein with 16 PPR domains (Fig. 3). Compared with the *RsRf3-1* allele in 9802A1, the *RsRf3-2* allele in 9606H contained one base substitution (C to T) and a CTT insertion at the promoter region. In the coding region, a single nucleotide polymorphism (SNP) results in a single amino acid replacement of arginine (AGG) in 9802A1 by lysine (AAG) in 9606H. Using a strand-specific RT–PCR method, the expression profile of *RsRf3-2* in 9606H was assayed using RNA from five tissues. *RsRf3-2* was detected in all of the tissues at the same expression level (Fig. 4). Interestingly, higher *RsRf3* expression and RNA polymerase II (Pol II) enrichment were detected at the *RsRf3* chromatin in F_1 seedlings compared with its parents (Figs 5, 6). Furthermore, the increase in expression and Pol II enrichment was found only in plants carrying the cytoplasm from 9802A1 (Figs 5, 6).

Discussion

In radish, several types of CMS/*Rf* systems have been reported. Ogura CMS is found to be controlled by *orf138* (Bonhomme *et al*., 1992; Krishnasamy and Makaroff, 1993), and the Kosena CMS-associated gene *orf125* shares extensive similarity with *orf138* (Iwabuchi *et al*., 1999). Both Kosena and Ogura CMS are reversed by the same gene *orf687* (Brown *et al*., 2003; Desloire *et al*., 2003; Koizuka *et al*., 2003). The male-sterile line 9802A1 contains the full-length *orf138* gene; the male-sterile line 9802A1 and its male fertility-restoring line 9606H contain the *rfob* gene that encodes the same predicted protein as the non-restoring allele *rf* (AJ535624) (Wang *et al*., 2008), suggesting that the CMS/*Rf* system used here (9802A1/9606H) is different from the Ogura/Kosena system. A recent report shows that the Ogura-type mitochondrial genome has been highly rearranged compared with the normal type genome and the rearrangement has resulted in four unique regions containing six open reading frames, including *orf138* specific to the

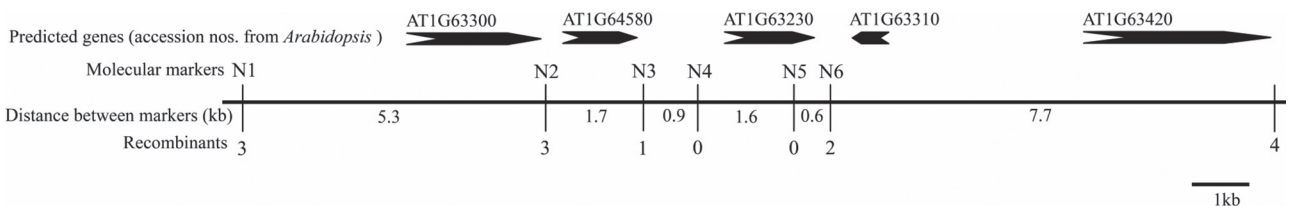

Fig. 2. Genetic and physical maps of *RsRf3*. *RsRf3* is mapped to the region between marker N3 and N6, which contains only one gene with homology to At1g63230.

Fig. 3. Amino acid sequence alignment of the *Rfo* gene and the *RsRf3-2* allele in 9606H.

Ogura-type mitochondrial genome (Tanaka *et al*., 2012). After the tetrad stage, microspores of Ogura CMS began to degenerate, resulting in empty anthers with no pollen (Lee *et al*., 2008). In 9802A1, many remnants of microspore exine were found in anthers. Both 9802A1 and the fertility-restored hybrid (9802A1 × 9606H) show comparable levels of *orf138* transcripts (Supplementary Fig. S4 at *JXB* online). The fact that the F_1 plants of the cross between 9802A1 and 0107H, carrying *Rfo* and *RsRf3-1*, were male fertile indicates that male sterility in 9802A1 is due to *orf138* but the restoration is achieved by another set of alleles (*RsRf3*). The male-sterile phenotype in 9802A1 is also different from other identified cytoplasmic male-sterile radish plants: a small number of pollen grains in the dehiscing anthers are visible in the NWB and DCGMS cytoplasmic male-sterile radish plants (Nahm *et al*., 2005; Lee *et al*., 2008). A new cytoplasmic male-sterile radish 805A has been identified that possesses two different abortion types in the locules during anther development (Shi *et al*., 2010).

To date, all the PPR-encoding *Rf* genes function dominantly against their non-functional *rf* allele. In this study, positional cloning of *RsRf3* was performed and it was found

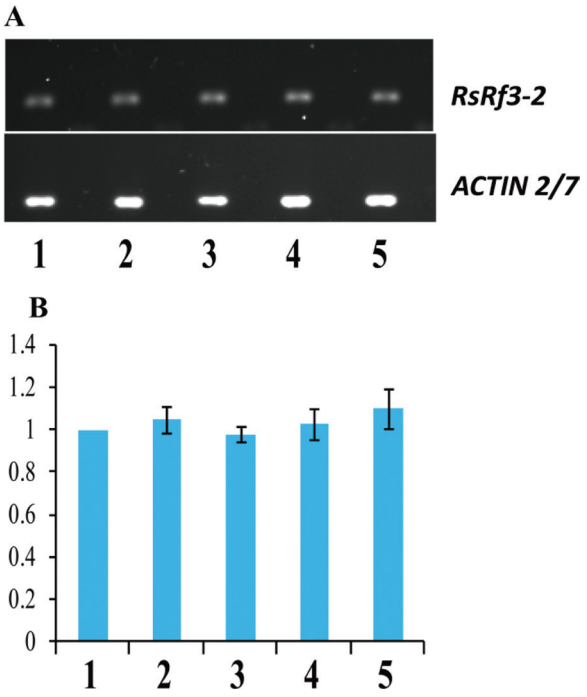

Fig. 4. Expression of the *RsRf3-2* allele in different organs of the radish restorer line 9606H with specific primers F2/R5. (A) Expression profile of transcripts in different tissues. (B) Comparative expression analysis of the transcripts in different tissues. 1, 2, 3, 4, and 5: products with the first-strand cDNA of root, stem, leaf, flower, and young pod as template, respectively.

that heterozygous *PPR* alleles are required for fertility restoration, suggesting that the *rf* allele in the CMS line is functional. How can heterozygous *PPR* alleles restore male fertility for CMS, but the homozygous allele cannot? Two possibilities

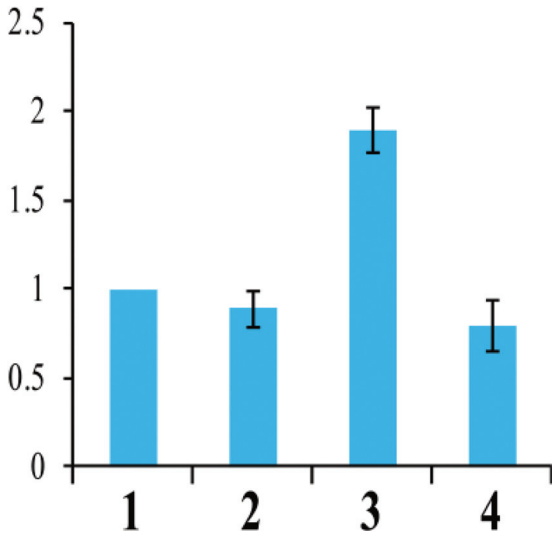

Fig. 5. Quantitative RT–PCR analysis of *Rf3* expression levels in the CMS/*Rf* system. The data are shown as mean ±SD (n=3). Columns 1, 2, 3, and 4 indicate the patterns of 9802A1, 9606H, F_1 from 9802A1 (female parent) and 9606H, and F_1 from 9802B1 (female parent) and 9606H, respectively.

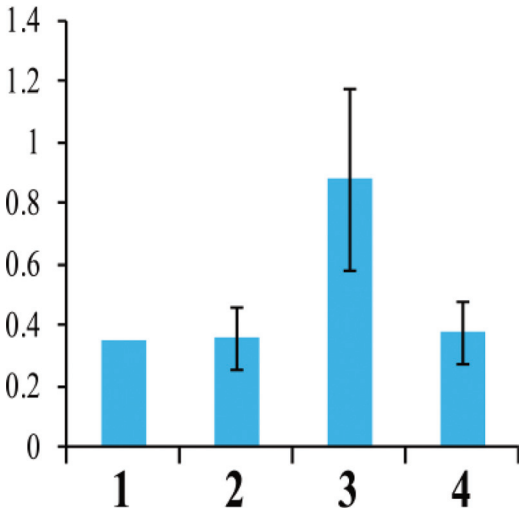

Fig. 6. ChIP analysis of Pol II on the *RsRf3* locus. Columns 1, 2, 3, and 4 indicate the patterns of 9802A1, 9606H, F_1 from 9802A1 (female parent) and 9606H, and F_1 between from 9802B1 (female parent) and 9606H, respectively.

are suggested to account for this observation. First, the two PPR proteins encoded by the different alleles at the same locus could show different functions, or a combination of their functions, probably forming a heterodimer, is sufficient to prevent the accumulation of CMS-associated gene products, which is supported by reports that PPR proteins might work as dimers (Nakamura *et al.*, 2003; Okuda and Shikanai, 2012). The second hypothesis is based on the fact that the present work showed that the expression of homozygous alleles is lower than that of the heterozygous alleles. So, the elevated expression of heterozygous *PPR* alleles may be the reason for reversing the male-sterile phenotype. Both explanations warrant further investigation.

Alignment of the BAC clone Bac64 (127 kb) containing the *Rfo* locus (rep) and the contig (42 kb) carrying the *RsRf3* locus from BAC clone 48H19 demonstrated that the *Rfo* and *RsRf3* loci are located in different regions. A BLAST search against the *Brassica rapa* genome using the two clones indicated that the *Rfo* BAC clone and the *RsRf3* contig correspond to 7059–7139 kb and 7205–7256 kb on chromosome 9 from *B. rapa*, respectively, suggesting tight linkage between the *Rfo* and *RsRf3* loci (Supplementary Fig. S5 at *JXB* online).

The heterozygous *RsRf3-1*/*RsRf3-2* alleles conditioning gain of function is a rare case which supports the 'overdominance' hypothesis of heterosis proposing that loci exhibit a heterozygote advantage (Crow, 1948). Recent research found that *cis* polymorphisms at the *FLC* locus and the *LDMAR* locus influence the accumulation of H3K27me3 and DNA methylation, respectively (Coustham *et al.*, 2012; Ding *et al.*, 2012). In this work, two polymorphic sites were detected between 9802A1 and 9606H, and higher expression and Pol II enrichment were found in F_1 plants with the CMS background, suggesting that retrograde regulation, DNA or histone methylations could be involved in transcriptional regulation of *RsRf3*.

Supplementary data

Figure S1. Crossing scheme for the backcross.

Figure S2. Result of the macroarray hybridization. Positive BAC clones identified on the macroarray.

Figure S3. Pulsed field gel electrophoresis for BAC clones 48H19 (20) and 158K3 (22).

Figure S4. Expression of *orf138* in 9802A1 (1) and F_1 (2) from a cross between 9802A1 and 9606H.

Figure S5. BLAST of the *RsRf3* contig (A) and the *Rfo* BAC clone (B) against the *Brassica rapa* genome.

Table S1. Primers used in this study.

Table S2. Backcross population of BC1F1.

Table S3. Positive BAC clones identified on macroarrays.

Table S4. F_2 population crossed from 9802A1 and 9606H.

Acknowledgements

We thank members of Professor Caroline Dean's lab at the John Innes Centre in Norwich, UK, for helpful suggestions and the anonymous reviewers for their valuable comments. This work was supported by the National Natural Science Foundation of China (31100231), the Natural Science Foundation of Hubei Province, China (2007ABA004, 2009CDB221, and 2011CDB408), the Youth Chenguang Project of Science and Technology of Wuhan, China (200850731402), and the fund of the CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture (Y152081t01).

References

Bellaoui M, Grelon M, Pelletier G, Budar F. 1999. The restorer *Rfo* gene acts post-translationally on the stability of the ORF138 Ogura CMS-associated protein in reproductive tissues of rapeseed cybrids. *Plant Molecular Biology* **40,** 893–902.

Bentolila S, Alfonso AA, Hanson MR. 2002. A pentatricopeptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. *Proceedings of the National Academy of Sciences, USA* **99,** 10887–10892.

Bonhomme S, Budar F, Lancelin D, Small I, Defrance MC, Pelletier G. 1992. Sequence and transcript analysis of the Nco2.5 Ogura-specific fragment correlated with cytoplasmic male sterility in *Brassica* cybrids. *Molecular and General Genetics* **235,** 340–348.

Brown GG, Formanova N, Jin H, Wargachuk R, Dendy C, Patil P, Laforest M, Zhang J, Cheung WY, Landry BS. 2003. The radish *Rfo* restorer gene of Ogura cytoplasmic male sterility encodes a protein with multiple pentatricopeptide repeats. *The Plant Journal* **35,** 262–272.

Coustham V, Li P, Strange A, Lister C, Song J, Dean C. 2012. Quantitative modulation of polycomb silencing underlies natural variation in vernalization. *Science* **337,** 584–587.

Crow JF. 1948. Alternative hypotheses of hybrid vigor. *Genetics* **33,** 477–487.

Cui X, Wise RP, Schnable PS. 1996. The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. *Science* **272,** 1334–1336.

Desloire S, Gherbi H, Laloui W, *et al.* 2003. Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. *EMBO Reports* **4,** 588–594.

Dewey RE, Timothy DH, Levings CS. 1987. A mitochondrial protein associated with cytoplasmic male sterility in the T cytoplasm of maize. *Proceedings of the National Academy of Sciences, USA* **84,** 5374–5378.

Ding J, Lu Q, Ouyang Y, Mao H, Zhang P, Yao J, Xu C, Li X, Xiao J, Zhang Q. 2012. A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. *Proceedings of the National Academy of Sciences, USA* **109,** 2654–2659.

Fujii S, Bond CS, Small ID. 2011. Selection patterns on restorer-like genes reveal a conflict between nuclear and mitochondrial genomes throughout angiosperm evolution. *Proceedings of the National Academy of Sciences, USA* **108,** 1723–1728.

Fujii S, Toriyama K. 2008. Genome barriers between nuclei and mitochondria exemplified by cytoplasmic male sterility. *Plant and Cell Physiology* **49,** 1484–1494.

Fujii S, Toriyama K. 2009. Suppressed expression of RETROGRADE-REGULATED MALE STERILITY restores pollen fertility in cytoplasmic male sterile rice plants. *Proceedings of the National Academy of Sciences, USA* **106,** 9513–9518.

Gillman JD, Bentolila S, Hanson MR. 2007. The petunia restorer of fertility protein is part of a large mitochondrial complex that interacts with transcripts of the CMS-associated locus. *The Plant Journal* **49,** 217–227.

Hanson MR, Bentolila S. 2004. Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *The Plant Cell* **16,** S154–S169.

Hu J, Wang K, Huang WC, *et al.* 2012. The rice pentatricopeptide repeat protein RF5 restores fertility in Hong-Lian cytoplasmic male-sterile lines via a complex with the glycine-rich protein GRP162. *The Plant Cell* **24,** 109–122.

Imai R, Koizuka N, Fujimoto H, Hayakawa T, Sakai T, Imamura J. 2003. Delimitation of the fertility restorer locus *Rfk1* to a 43-kb contig in Kosena radish (*Raphanus sativus* L.). *Molecular Genetics and Genomics* **269,** 388–394.

Itabashi E, Iwata N, Fujii S, Kazama T, Toriyama K. 2011. The fertility restorer gene, *Rf2*, for Lead Rice-type cytoplasmic male sterility of rice encodes a mitochondrial glycine-rich protein. *The Plant Journal* **65,** 359–367.

Ivanov MK, Dymshits GM. 2007. Cytoplasmic male sterility and restoration of pollen fertility in higher plants. *Russian Journal of Genetics* **43,** 354–368.

Iwabuchi M, Koizuka N, Fujimoto H, Sakai T, Imamura J. 1999. Identification and expression of the kosena radish (*Raphanus sativus* cv. Kosena) homologue of the ogura radish CMS-associated gene, *orf138*. *Plant Molecular Biology* **39,** 183–188.

Kazama T, Nakamura T, Watanabe M, Sugita M, Toriyama K. 2008. Suppression mechanism of mitochondrial ORF79 accumulation by Rf1 protein in BT-type cytoplasmic male sterile rice. *The Plant Journal* **55,** 619–628.

Klein R, Klein P, Mullet J, Minx P, Rooney W, Schertz K. 2005. Fertility restorer locus *Rf1* of sorghum (*Sorghum bicolor* L.) encodes

a pentatricopeptide repeat protein not present in the colinear region of rice chromosome 12. *Theoretical and Applied Genetics* **111,** 994–1012.

Koizuka N, Imai R, Fujimoto H, Hayakawa T, Kimura Y, Kohno-Murase J, Sakai T, Kawasaki S, Imamura J. 2003. Genetic characterization of a pentatricopeptide repeat protein gene, *orf687*, that restores fertility in the cytoplasmic male-sterile Kosena radish. *The Plant Journal* **34,** 407–415.

Komori T, Ohta S, Murai N, Takakura Y, Kuraya Y, Suzuki S, Hiei Y, Imaseki H, Nitta N. 2004. Map-based cloning of a fertility restorer gene, *Rf-1*, in rice (*Oryza sativa* L.). *The Plant Journal* **37,** 315–325.

Krishnasamy S, Makaroff CA. 1993. Characterization of the radish mitochondrial *Orfb* locus—possible relationship with male-sterility in Ogura radish. *Current Genetics* **24,** 156–163.

Lee YP, Park S, Lim C, Kim H, Lim H, Ahn Y, Sung SK, Yoon MK, Kim S. 2008. Discovery of a novel cytoplasmic male-sterility and its restorer lines in radish (*Raphanus sativus* L.). *Theoretical and Applied Genetics* **117,** 905–913.

Liu F, Cui XQ, Horner HT, Weiner H, Schnable PS. 2001. Mitochondrial aldehyde dehydrogenase activity is required for male fertility in maize. *The Plant Cell* **13,** 1063–1078.

Liu F, Schnable PS. 2002. Functional specialization of maize mitochondrial aldehyde dehydrogenases. *Plant Physiology* **130,** 1657–1674.

Nahm SH, Lee HJ, Lee SW, Joo GY, Harn CH, Yang SG, Min BW. 2005. Development of a molecular marker specific to a novel CMS line in radish (*Raphanus sativus* L.). *Theoretical and Applied Genetics* **111,** 1191–1200.

Nakamura T, Meierhoff K, Westhoff P, Schuster G. 2003. RNA-binding properties of HCF152, an *Arabidopsis* PPR protein involved in the processing of chloroplast RNA. *European Journal of Biochemistry* **270,** 4070–4081.

Okuda K, Shikanai T. 2012. A pentatricopeptide repeat protein acts as a site-specificity factor at multiple RNA editing sites with unrelated cis-acting elements in plastids. *Nucleic Acids Research* **40,** 5052–5064.

Schnable PS, Wise RP. 1998. The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends in Plant Science* **3,** 175–180.

Shi S, Ding D, Mei S, Wang J. 2010. A comparative light and electron microscopic analysis of microspore and tapetum development in fertile and cytoplasmic male sterile radish. *Protoplasma* **241,** 37–49.

Tanaka Y, Tsuda M, Yasumoto K, Yamagishi H, Terachi T. 2012. A complete mitochondrial genome sequence of Ogura-type male-sterile cytoplasm and its comparative analysis with that of normal cytoplasm in radish (*Raphanus sativus* L.). *BMC Genomics* **13,** 352.

Uyttewaal M, Arnal N, Quadrado M, Martin-Canadell A, Vrielynck N, Hiard S, Gherbi H, Bendahmane A, Budar F, Mireau H. 2008. Characterization of Raphanus sativus pentatricopeptide repeat proteins encoded by the fertility restorer locus for Ogura cytoplasmic male sterility. *The Plant Cell* **20,** 3331–3345.

Wang Z, Zou Y, Li X, *et al.* 2006. Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *The Plant Cell* **18,** 676–687.

Wang ZW, Zhang YJ, Xiang CP, Mei SY, Zhou Y, Chen GP, Wang T. 2008. A new fertility restorer locus linked closely to the *Rfo* locus for cytoplasmic male sterility in radish. *Theoretical and Applied Genetics* **117,** 313–320.

Wierzbicki AT, Haag JR, Pikaard CS. 2008. Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. *Cell* **135,** 635–648.

Yin J, Guo W, Yang L, Liu L, Zhang T. 2006. Physical mapping of the *Rf1* fertility-restoring gene to a 100 kb region in cotton. *Theoretical and Applied Genetics* **112,** 1318–1325.