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ORIGINAL PAPER

Introgression and molecular tagging of Rf_4, a new male fertility restoration gene from wild sunflower *Helianthus maximiliani* L.

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Abstract Cytoplasmic male sterility (CMS) and its fertility restoration (Rf) genes are critical tools for hybrid seed production to utilize heterosis. In sunflower, CMS PET1 and the associated Rf gene Rf_1 is the only source extensively used in commercial hybrid production. The objective of this research was to develop new sources of CMS and fertility restorers to broaden the genetic diversity of hybrid seed production. We identified a new type of CMS, named as CMS GIG2, from an interspecific cross between *Helianthus giganteus* accession1934 and *H. annuus* cv. HA 89. Based on reactions to a set of standard Rf testers, CMS GIG2 is different from all previously reported CMS types, including the CMS GIG1 from another *H. giganteus* accession. We also identified an Rf gene for CMS GIG2 from wild species *H. maximiliani* accession 1631. The CMS GIG2 and its restoration gene were introduced into HA 89 background through recurrent backcross and single plant selection techniques. Genetic analysis revealed that the CMS GIG2-Rf system is controlled by a completely dominant gene, named as Rf_4, and the gene additive and dominance effects were estimated as 39.9 and 42.2%, respectively, in the HA 89 background. The gene Rf_4 was mapped onto linkage group 3 with simple sequence repeat (SSR) markers and RFLP-derived STS-marker, and is about 0.9 cM away from the SSR marker ORS1114 based on a segregation population of 933 individuals. The CMS GIG2-Rf_4 system tagged by molecular markers provides an alternative genetic source for hybrid breeding in the sunflower crop.

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Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil-producing crops worldwide. High seed and oil yields are the main objectives of sunflower breeding programs, and the utilization of heterosis has been one of the most important tools for breeding high yield. Heterosis or hybrid vigor is often obtained through the use of complete sets of stable male-sterile lines (A line), maintainers (B line) and efficient restorer lines (R line) in higher plants. Use of cytoplasmic male sterility (CMS) eliminates the need for hand emasculation and ensures large-scale hybrid seed production. Since the first CMS reported in sunflower in 1969, 72 CMS sources have been identified (Serieys 2005). However, only the first CMS PET1 developed by Leclercq (1969) from *H. petiolaris* Nutt. has been extensively used to produce almost all commercial sunflower hybrids. Inheritance studies indicated that almost all restoration lines for CMS PET1 in sunflower breeding carry the same restorer gene Rf_1 (Serieys 1996, 2005). The lack of new CMS and fertility restoration (Rf) genes for use in commercial hybrid production is partly due to the limited number of stable CMS cytoplasms and their corresponding Rf genes, as well as the time-consuming effort of

converting lines to CMS and the incorporation of new Rf genes into adapted cultivated lines. In order to prevent cytoplasmic uniformity and reduce genetic vulnerability of sunflower hybrids to everchanging environmental stress and disease, alternative CMS and restorer sources need to be utilized in sunflower breeding programs.

The CMS phenotype can occur spontaneously or from induced mutation, as well as from the progenies of inter- or intraspecific crosses (Kaul 1988). The CMS system is a common phenomenon found in over 150 plant species (Laser and Lersten 1972). While a CMS line can be relatively easily produced, finding restorer genes has been proven much more difficult, especially when using the currently available cultivated sunflower. However, fertility restorer genes have been isolated from the same wild species population which provided the sterile cytoplasm or from other wild species (Leclercq 1969; Vranceanu and Stoenescu 1971, 1978). Kinman (1970) discovered the male fertility restorer gene Rf_1 in the line T66006-2-1-B, which was selected from a composite cross involving the wild annual sunflower. Since then, many restoration lines, such as all the USDA-ARS RHA lines, RHA 271, 272, 273, 274, 275, 276, 278, 279 and 296 carry the Rf_1 gene derived from T66006-2-1-B (Korell et al. 1992; Serieys 2005; Jan et al. 2002). An allelic test between T66006-2-1-B and MZ01398, an obsolete local cultivar, led to the discovery of a second major dominant gene Rf_2 from MZ01398 (Vranceanu and Stoenescu 1971, 1978).

Numerous studies have indicated that sunflower male fertility restoration is generally controlled by a limited number of major restoration genes. In most cases, the Rf is under the control of a single dominant gene (Rf_1), or two complementary dominant genes (Rf_1 and Rf_2) and modifiers (for review, see Serieys 1996, 2005). Since Rf_2 is present in nearly all the inbred lines, including maintainer lines of CMS, only the Rf_1 gene was considered to be the Rf gene needed in sunflower hybrid production (Dominguz-Gimeneze and Fick 1975; Leclercq 1984; Horn et al. 2003; Jan and Vick 2007). There has been confusion about the nomenclature of restoration genes in some of the early sunflower work where the "Rf_2" was named for different restorers in different publications (Fick and Zimmer 1974; Vranceanu and Stoenescu 1978; Leclercq 1984). Recently, a Rf gene Rf_3, which is different from both Rf_1 and Rf_2, was assigned to the confection restorer RHA 280 (Jan and Vick 2007).

The most studied CMS/Rf system in sunflower is the CMS PET1 and the corresponding restoration gene Rf_1. CMS PET1 is associated with recombination events in the mitochondrial (mt) genome which involved an inversion/insert rearrangement resulting in the creation of a novel mt gene, *orfH522* (Horn et al. 1991; Köhler et al. 1991; Laver et al. 1991), which encodes a ~16-kDa polypeptide. The level of ~16-kDa protein is significantly reduced in the presence of the dominant restorer genes (Monéger et al. 1994; Gagliardi and Leaver 1999). Although a number of mt genes associated with CMS have been characterized, the identification of Rf genes has proven elusive. To date, the only Rf genes to be identified and cloned are: maize (*Zea mays* L.) Rf_2 for restoration of the *cms*-T-type male sterility (Cui et al. 1996; Liu et al. 2001); *Rf* of petunia (*Petunia* sp.) for the RM cytoplasm (Bentolila et al. 2002); Rfk_1(*Rfo*) of radish (*Raphanus sativus* L.) for the restoration of Ogura (*ogu*) CMS in *Brassica napus* (Brown et al. 2003; Desloire et al. 2003); and rice (*Oryza sativa* L.) *Rf*-1 for restoration of CMS-Boro II cytoplasm (Komori et al. 2004; Wang et al. 2006). However, map-based cloning of Rf genes has not been successful in sunflower because of the lack of precise mapping of the gene region.

In sunflower the gene Rf_1, for restoration of PET1-CMS cytoplasm, was mapped to linkage group 6 of the RFLP map and the consensus map in 130 F_2 individuals from the cross CX × RHA 266 (Gentzbittel et al. 1995, 1999), to linkage group 2 of the RFLP genetic map in 93 F_2 individuals from the cross RHA 271 × HA 234 (Jan et al. 1998), and to linkage group 13 of the simple sequence repeat (SSR) map (Tang et al. 2002) using RAPD, AFLP and SSR markers with a 183-plant F_2 population of RHA 325 × HA 342 (Horn et al. 2003; Kusterer et al. 2005). All these markers linked with Rf_1 provide an essential basis for genetic analysis of restoration genes and marker-assisted selection in sunflower breeding.

In 1995, an interspecific hybridization program was started in our lab with the objective of incorporating useful traits from wild *Helianthus* species into cultivated sunflower. A wild species *H. giganteus* was crossed with HA 89. After backcrossing five cycles with HA 89, a new alloplasmic CMS line with *H. giganteus* cytoplasm and HA 89 nucleus was developed. Subsequently, the search for its corresponding Rf gene and the incorporation into cultivated sunflower were undertaken. This study reports the discovery of the new CMS and its corresponding restoration gene, and the molecular tagging of the Rf gene on existing molecular maps. This work will ultimately lead to the cloning of the restorer allele, as well as facilitating the understanding of the genetics of the restoration of the male fertility and nuclear–cytoplasm interaction.

Materials and methods

Plant materials and breeding scheme

The perennial wild sunflower *H. giganteus* accessions 1934 (Ames 1934, PI 503250) and *H. maximiliani* accession 1631 and the cultivated sunflower variety HA 89 and its nuclear male sterility (NMS) mutant NMS HA 89 were

used as parents in this research. These parental materials were obtained from the germplasm collections in our laboratory. Diploid *H. giganteus* 1934 was crossed with HA 89 (Fig. 1), and 12 F_1 seedlings were recovered through embryo rescue. All F_1 plants had $2n = 34$ chromosomes and were treated with colchicine to induce chromosome doubling. At flowering, one of the 12 F_1 plants was identified as male-sterile, while the others were male-fertile, based on the morphology of the anthers and pollen stainability. The male-sterile F_1 plant was backcrossed with HA 89. All ten BC_1F_1 progenies from this cross were male-sterile and had $2n = 51$–52 chromosomes, suggesting that chromosome doubling was successful. The BC plants were backcrossed with HA 89 repeatedly to reduce the chromosome number to $2n = 34$, the chromosome number of the cultivated sunflower. All plants from the BC_1 to BC_5 generations were male-sterile, indicating that the CMS trait from *H. giganteus* 1934 was introduced into the HA 89 background. To search for the Rf gene for the CMS from *H. giganteus* 1934, BC_5F_1 plants were crossed with seven interspecific amphiploids (Amp) including NMS HA 89 × *H. maximiliani* 1631, *H. cusickii* × P21, *H. atrorubens* × HA 89, *H. mollis* × P21, *H. grosseserratus* × P21, *H. pumilus* × P21, and *H. angustifolius* × P21.

In order to test the relationship of the CMS from *H. giganteus* 1934 with the reported types of CMS, selected BC_4F_1 plants were crossed with a set of 19 standard lines (Armavir, HA 89, HA 290, P21, HA 821, Hopi Dye, Issanka, Luch, RCMG1, RCMG2, RCMG3, RHA 266, RHA 274, RHA 280, RHA 294, RHA 801, Seneca, Smena, and

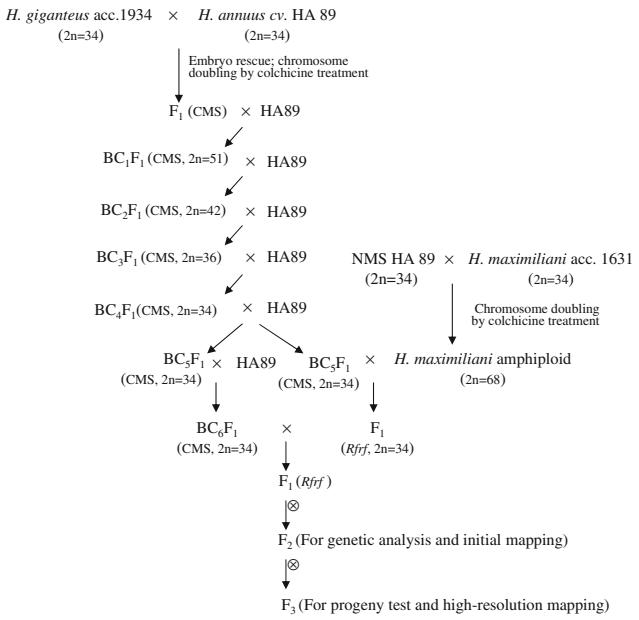

Fig. 1 Breeding scheme for introduction of the male sterile cytoplasm (CMS) and its fertility restoration (Rf) gene from the wild species into the HA 89 background

VNIIMK) in 2001. An average of 30 progeny plants from each cross were grown in the field in 2003 and were examined for pollen fertility at the flowering stage.

Genetic analysis

To understand the genetic control of the new CMS-Rf system, an initial F_2 segregation population of 113 individuals was generated from one single F_1 plant from the cross *H. giganteus*/7*HA 89/3/*H. giganteus*/6*HA 89//*H. maximiliani* 1631 (Amp) in 2005 (Fig. 1). Some of the male-fertile F_2 plants were selected for progeny testing to corroborate the genetic model developed based on the initial F_2 population. About 30 plants from each F_3 line grown under field conditions were identified for pollen fertility in 2006.

Pollen fertility for individual F_2 or F_3 plants was first identified visually after during flowering. The plants with normal, dehiscent anthers and abundant pollen were scored as male-fertile (F); otherwise, they were identified as male-sterile (S). To confirm the visual pollen fertility, all individuals were also microscopically examined for pollen stainability using Alexander's method (Alexander 1969). Pollen grains from dehiscent anthers were suspended in a drop of the stain solution for 2–3 h, and normally stained pollen grains in three fields per slide were counted. The well filled fertile pollen grains will be stained red and the non-filled aborted pollen grains will be stained green. Approximately 400–500 pollen grains from each plant were examined to estimate the percentage of fertile pollen. Plants having an average of <5 and >30% pollen stainability were classified as male-sterile and male-fertile, respectively. Most male-sterile individuals were completely sterile with severe atrophy of anthers and non-stainable (aborted) pollens. The Chi-square test was used to determine the segregation pattern of male fertility phenotypes in the F_2 and F_3 populations.

Molecular mapping of the fertility restoration gene

The above F_2 population of 113 individuals was used to map the restoration gene on a low-resolution genetic map with SSR markers. Genomic DNA was extracted from fresh young leaves from individual plants according to the protocol described in Zhang et al. (2006). The bulked segregant analysis strategy (Michelmore et al. 1991) was used to screen for polymorphic SSR markers for the mapping population. Equal amounts of DNA from each of ten male-sterile and male-fertile F_2 individuals were bulked to develop the sterile and fertile pools. In total 200 SSR markers randomly selected from the 17 linkage groups (Tang et al. 2002; Yu et al. 2002, 2003) were screened for polymorphism between male-fertile and male-sterile pools.

The polymorphic markers were used to genotype the 113 F_2 plants. In the genomic region containing the restoration gene, 11 single- or low-copy RFLP markers from linkage group 11 (Jan et al. 1998), which cross-referenced linkage group 3 of the SSR genetic map (Yu et al. 2003), were also selected to design sequence tagged sites (STS) primers to screen for more polymorphic markers. The STS primers were designed using PRIMER 3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). These STS markers converted from RFLP sequences, tentatively designed using the "STS" prefix and then the original RFLP marker name. The sequence of the RFLP-based STS primer which produced polymorphic markers was as follows: STS10B1, 5′-AAGATCCCATCAAACCACCA-3′ and 5′-GGTCCAC CTTGCGAAGATAA-3′.

PCR amplification was performed according to Tang et al. (2002) with minor modifications. The 15-μl mixture contained 1.5 μl of 10× PCR buffer, 0.6 μl of 25 mM $MgCl_2$, 1.2 μl of 2.5 μM dNTPs, 0.4 μl of 10 pmol each primer, 2 μl of 10 ng/μl DNA sample, 0.2 μl (5 unit/μl) *Taq* DNA polymerase (Qiagen, Valencia, CA, USA), and 0.1% Tween-20. An MJ Research single or a Bio-Rad dual 96-well thermal cycler was used along with the following "touchdown" profile: 95°C for 3 min; then 12 cycles of 94°C for 45 s, 64°C for 45 s, −0.5°C/cycle, and 72°C for 1 min; followed by 35 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min, with a final extension step at 72°C for 20 min. The amplified product was electrophoresed on a 6.5% denaturing polyacrylamide gel at 60 W for 2.0 h (0.5× TBE), and scanned with a Typhoon 9410 variable mode imager (Molecular Dynamics Inc., CA, USA) after staining with GelRed nucleic acid gel stain (Biotium, Inc., CA, USA).

An F_2-derived F_3 population of 1173 plants was grown in the field in 2007 to estimate the genetic distance of the restoration gene from the cosegregating markers detected with the initial mapping population. The selected F_2 plants were heterozygous for the Rf gene based on the progeny test. All F_3 plants were also visually and microscopically examined for pollen fertility as described above.

A partial linkage map for the restoration gene region was constructed using the software MAPMAKER/EXP version 3.0b (Whitehead Institute, Cambridge, MA, USA) (Lander et al. 1987). A constant LOD score of 3.0 and a maximum recombination frequency of 0.40 were used. The "error detection" command was used to check for mistakes in scoring and data entry, and for double crossover events. The Kosambi function was used to obtain the genetic distances in centiMorgan (cM) (Kosambi 1944). The linkage group and RFLP loci were named according to Tang et al. (2002) and Jan et al. (1998). Linkage group maps were drawn using the MapChart software (Voorips 2002).

The restoration gene, named as Rf_4, was estimated for gene additive (a) and dominance (d) effects on pollen fertility based on the F_2-derived F_3 population. The estimations were made using the method described by Kearsey and Pooni (1996):

$$a = 1/2\mathrm{M}_{Rf4Rf4} - 1/2\mathrm{M}_{rf4rf4},$$

$$d = \mathrm{M}_{Rf4rf4} - 1/2\mathrm{M}_{rf4rf4} - 1/2\mathrm{M}_{Rf4Rf4},$$

where, M_{Rf4Rf4}, M_{Rf4rf4}, and M_{rf4rf4} are mean percentages of pollen stainability for the genotypes Rf_4Rf_4, Rf_4rf_4, and rf_4rf_4, respectively. Standard errors for the parameters a and d were estimated as:

$$s_a = 1/2\left(s^2_{Rf4Rf4} + s^2_{rf4rf4}\right)^{1/2},$$

$$s_d = \left(s^2_{Rf4rf4} + 1/4s^2_{Rf4Rf4} + 1/4s^2_{rf4rf4}\right)^{1/2},$$

where, s^2_{Rf4Rf4}, s^2_{Rf4rf4}, and s^2_{rf4rf4} are variances of the means M_{Rf4Rf4}, M_{Rf4rf4}, and M_{rf4rf4}, respectively. Significance of the a and d estimates was determined by Student's t test.

Results

Isolation of a male sterility cytoplasm and screening the corresponding Rf gene

The initial CMS plant was obtained from the F_1 hybrid from the cross between *H. giganteus* 1934 and HA 89 to isolate the male sterility cytoplasm (Fig. 1). The F_1 hybrid was backcrossed with the recurrent parent HA 89 for five generations. All the BC progenies remained male-sterile. An alloplasmic CMS line with mostly HA 89 genome was recovered in the BC_5 generation, suggesting that a CMS source derived from *H. giganteus* 1934 had been isolated and incorporated into the cultivated sunflower HA 89 background.

BC_5F_1 plants were pollinated by a set of 19 genetically diverse cultivated lines to test the Rf pattern for the CMS from *H. giganteus* 1934. All the progenies of the crosses were male-sterile, suggesting the lack of restorer genes for the CMS *H. giganteus* 1934 and also indicating that this CMS from *H. giganteus* 1934 is different from all previously reported CMS types, including CMS GIG1 which can be restored by RHA 280, RHA 294 and RHA 801 (Serieys 1996). Therefore, it was concluded that this CMS derived from *H. giganteus* 1934 was a new CMS source. According to the FAO codification, the new CMS source was designated as CMS GIG2.

To further search for the Rf gene for the CMS GIG2, BC_5F_1 plants were crossed with seven interspecific Amp (see "Materials and methods"). The results showed that Rf genes were identified in four Amp involving wild species

H. maximiliani 1631, *H. atrorubens*, *H. grosseserratus*, and *H. angustifolius*, but the other Amp behaved as a CMS GIG2 maintainer. Surprisingly, a single male-fertile plant with $2n = 34$ was obtained after crossing a BC_5F_1 plant with the Amp NMS HA 89 × *H. maximiliani* 1631 (Fig. 1), while all other nine progenies of the same cross were also male-fertile but had $2n = 51$ chromosomes, indicating the presence of Rf genes in this amphiploid. Because of the time-consuming process of continuous backcrossing of the $2n = 51$ male-fertile progenies to reduce the $2n$ chromosome number and to maintain male-fertility, we focused on the $2n = 34$ male-fertile plant to further study the inheritance of the restoration genes. As mentioned above, the standard set of 19 cultivated lines carrying different Rf genes for various CMS sources, such as RHA 266 and RHA 274 carrying the Rf_1 gene, HA 89 and HA 821 carrying the Rf_2 gene, and RHA 280 carrying the Rf_3 gene (Jan and Vick 2007) were not able to restore CMS GIG2. This indicated that the Rf gene derived from amphiploid *H. maximiliani* 1631 is a novel Rf gene and is different from Rf_1, Rf_2, and Rf_3.

Inheritance of the male fertility restoration gene

A single F_1 plant with normal male fertility was grown in a greenhouse to develop a 113-plant F_2 segregation population to understand the inheritance of CMS GIG2 and its Rf gene. All individual plants were classified as fertile or sterile based on the quantity of stainable pollen from dehiscent anthers. In some cases, no pollen was visible to the naked eye, but under the microscope, pollen grains were observed after staining with Alexander's stain. Therefore, we examined all individuals visually and microscopically. Of the 113 F_2 plants, the segregation of 32 homozygous male-fertile, 49 heterozygous male-fertile and 32 male-sterile individuals fit the expected 1:2:1 ratio ($0.3 < P < 0.5$) (Table 1). In addition, progeny tests conducted with 48 F_2 male-fertile lines in the field showed that 30 families were heterozygous for male fertility and 18 families were homozygous, suggesting their parental F_2 plants were heterozygous and homozygous, respectively (Table 1). The 30 F_3 families derived from heterozygous male-fertile F_2 plants segregated into 607 male-fertile and 207 male-sterile, fitting an expected 3:1 ratio closely ($0.7 < P < 0.8$), while 18 F_3 families derived from the homozygous male-fertile F_2 plants produced 492 male-fertile plants and no male-sterile plant, fitting an expected 1:0 ratio. All the results from the F_2 and $F_{2:3}$ generations indicated that a single dominant gene, named as Rf_4, controls the Rf of CMS GIG2.

Molecular mapping and genetic effects of Rf_4

Two hundred ORS SSR primer pairs randomly chosen from 17 linkage groups of the SSR genetic map (Tang et al. 2002; Yu et al. 2002, 2003) were used for screening polymorphisms between male-fertile and male-sterile bulks. After PCR amplification, 182 primer pairs produced clear bands, 18 primer pairs produced unclear bands or no band, and only nine gave polymorphic bands between the two bulks. The lower polymorphism obtained between the two bulks compared with other strategies may be due to their similar genetic backgrounds after repeated backcrosses. However, those identified polymorphic markers are expected to be more likely linked to the target gene Rf_4. Our results through genotyping the F_2 population indicated that ORS1114 on linkage group 3 of the SSR genetic map was linked to the Rf_4 locus. Subsequently, 15 ORS markers from linkage group 3 and 11 RFLP-derived STS primers from linkage group 11 (Jan et al. 1998) were selected to screen two bulks and F_2 individuals. In total, four DNA markers near the Rf_4 locus were identified, including two codominant markers (ORS1114 and ORS13) and two dominant markers (ORS822-3 and STS10B1). All the polymorphic markers were mapped to the distal region of linkage group 3 of the SSR genetic map (Tang et al. 2002; Yu et al. 2002, 2003) (Fig. 2). The order of loci in this region is similar to the SSR genetic map. SSR marker

Table 1 Fitness test for segregation ratios of the pollen sterility/fertility trait controlled by the Rf_4/rf_4 locus in the F_2 and advanced F_3 populations (Fig. 1)

Population (year)	Number of plants observed				Expected ratio	χ^2 value (probability)
	Number of total plants	Sterile[a]	Fertile[a]			
		rf_4rf_4[b]	Rf_4rf_4[b]	Rf_4Rf_4[b]		
F_2 (2005)	113	32	49	32	1:2:1	1.992 ($0.3 < P < 0.5$)
30 F_2 Rf_4rf_4-derived F_3 lines (2006)	814	207	607		1:3	0.080 ($0.7 < P < 0.8$)
18 F_2 Rf_4Rf_4-derived F_3 lines (2006)	492	0	492		0:1	0 ($P = 1$)
F_2 Rf_4rf_4-derived F_3 lines (2007)	1,173	290	883		1:3	0.048 ($0.8 < P < 0.9$)

[a] Detected by microscopically pollen stainability

[b] Estimated by the markers OSR1114 and ORS13

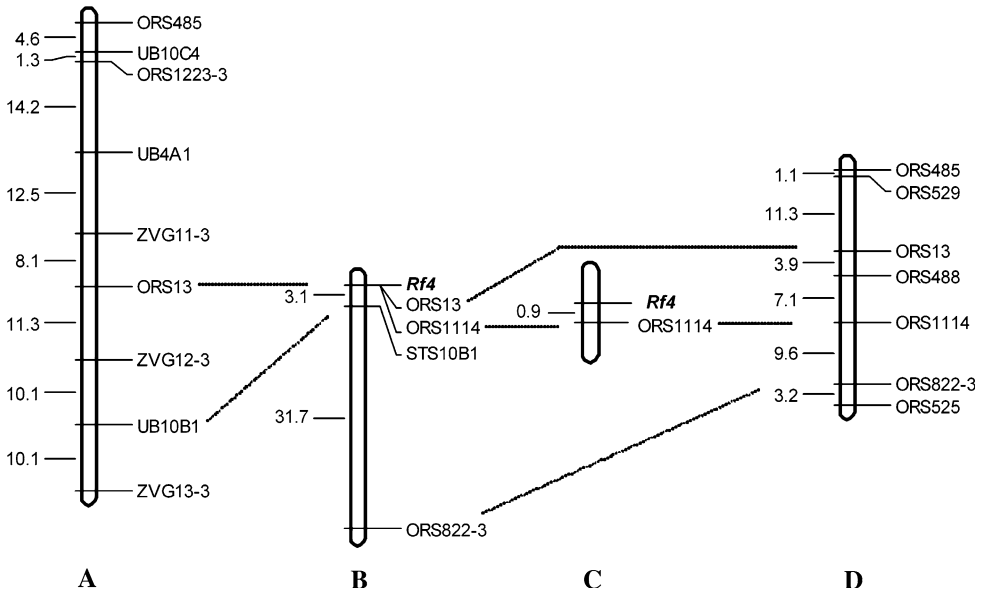

Fig. 2 The map position of the male fertility restoration gene Rf_4. The linkage groups constructed with different mapping populations were aligned based on the shared markers. **a** Part of linkage group 3 of the RFLP-SSR map (Yu et al. 2003); **b** partial linkage map for the Rf_4 region developed based on a population of 113 F_2 plants; **c** partial linkage map for the Rf_4 region based on a segregation population of 933 plants; and **d** part of linkage group 3 of the SSR map (Tang et al. 2003). Genetic distances are shown in centiMorgan (*cM*)

ORS13 and ORS1114 cosegregated with the Rf_4 locus, while STS10B1 and ORS822-3 mapped 3.1 cM and 31.7 cM, respectively, from the Rf_4 locus on the same side.

Since no recombinants between the Rf_4 locus and ORS1114 marker were detected using 113 F_2 plants, a large population of 1173 F_3 plants derived from heterozygous F_2 plants was genotyped using ORS1114. The pollen stainability of 1173 individuals showed a bimodal distribution with a clear valley from 5 to 30% (Fig. 3). The classification of male-fertile and male-sterile plants followed the standard of <5 and >30% as male-sterile and male-fertile, respectively. The segregation ratio of 883 male-fertile and 290 male-sterile agreed with the expected 3:1 ratio ($0.8 < P < 0.9$) (Table 1). These results further confirmed a single dominant gene control of the Rf of CMS GIG2. The recombinant frequency between ORS1114 and Rf_4 was low, with seven recombinants among 933 F_3 individuals identified. As a result, the SSR marker ORS1114 was 0.9 cM away from the Rf_4 locus (Fig. 2).

Gene additive and dominance effects of the Rf_4 locus on pollen fertility were about 40 and 42%, respectively, as estimated based on the tightly linked marker ORS1114 in the F_3 population (Table 2). Both the gene additive and dominance effects were similar in magnitude, indicating that Rf_4 is a completely dominant gene controlling pollen fertility restoration in the HA 89 genetic background.

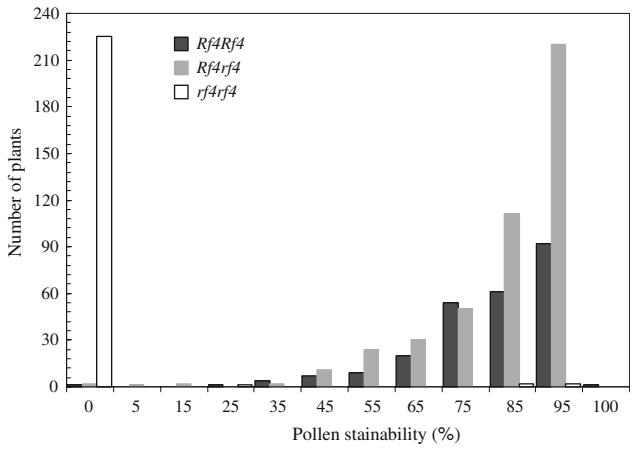

Fig. 3 Distribution of pollen stainability for three genotypes of the Rf_4 fertility restoration locus in the F_2-derived F_3 population. The Rf_4 locus was represented by its tightly linked marker ORS1114 (Fig. 2d)

Discussion

Enrichment of CMS sources and male fertility restorers

To broaden the genetic base and reduce the vulnerability of the cultivated sunflower to diseases and environmental stress, identification of new CMS sources and the associated restorers has been a continuous objective for commercial sunflower production in the world (Serieys 1996). Even though 72 CMS sources have been reported in

Table 2 Gene additive (*a*) and dominance (*d*) effects of the Rf_4 locus on pollen fertility estimated based on the tightly linked marker ORS1114 in the F_2-derived F_3 population grown in 2007

Parameter	Genotypes of the Rf_4 locus		
	Rf_4Rf_4	Rf_4rf_4	rf_4rf_4
Mean	81.5%	83.8%	1.7%
Variance	2.3831%	2.4579%	1.5069%
Number	250	453	230
Genic effects	$a = 39.9\%$ ($P < 0.001$); $d = 42.2\%$ ($P < 0.025$)		

the literature, inheritance studies of restoration were performed for only 19 CMS sources (Serieys 2005). Moreover, characterization and molecular mapping of the Rf genes was conducted only for the Rf_1 gene of CMS PET1, which is extensively used in hybrid sunflower production.

The newly identified CMS GIG2 is clearly different from CMS PET1, and represents a new source of male-sterile cytoplasm. More importantly, a single dominant Rf gene Rf_4 derived from a diploid perennial wild species *H. maximiliani* 1631 can efficiently restore the male fertility of CMS GIG2. Field experiments in 2007 showed that the pollen stainability of the enlarged population ranged from 33 to 100%, with 86% of the plants possessing pollen stainability >70%, and nearly half (47%) of the fertile plants possessing >90% pollen stainability (Fig. 3). However, due to environmental factors, some individuals showed poor pollen fertility both in the greenhouse or field.

While CMS GIG2 cytoplasm was crossed with 19 cultivated lines involving different restoration genes, all the progenies were male-sterile, suggesting that the restorer of CMS GIG2 cytoplasm did not exist in the cultivated germplasms evaluated. However, such genes were present in four Amp involving wild species. This is consistent with the conclusion that male fertility restoration of CMS is present at a higher frequency in wild species or their related hybrids than in current cultivars (Vranceanu and Stoenescu 1978). The reasons might be due to alloplasmic CMS lines containing the cytoplasm from wild species and the nucleus from the cultivated species, and this nuclear–cytoplasm conflict can most likely be eliminated by introducing specific nuclear genes from wild species.

Previous studies indicated that the pollen fertility restoration in sunflowers is genetically controlled by a single Rf gene or a series of two independent complementary genes in different crosses (Serieys 1996). However, polygenic restorers, which have two or more Rf genes, should be avoided in breeding programs because of their instability in different environments and their complicated inheritance (Vranceanu and Stoenescu 1978). Thus, a single dominant *Rf* gene is highly preferred for sunflower breeding. Rf_4 is a single major gene for restoration of CMS GIG2 and pollen fertility can be recovered to 100%. It can be concluded that CMS GIG2 and the Rf_4 gene are a new potential system that could be used to substitute for the current PET1 cytoplasm, if necessary, for hybrid sunflower production because of its stable male sterility, efficient male fertility restoration, and a single dominant gene controlling fertility restoration. In addition, closely linked codominant SSR marker ORS1114 can easily distinguish the homozygous and heterozygous alleles, and will facilitate marker-assisted selection for the Rf_4 gene in breeding programs. Nevertheless, additional studies of the CMS GIG2 cytoplasm for agronomic traits such as seed yield, oil quality, and disease resistance would be necessary to assess its suitability for commercial sunflower hybrids.

In the search for the restoration gene for CMS GIG2, BC_5F_1 plants with $2n = 34$ were crossed as female with several Amp ($2n = 68$). Theoretically, all their progenies should be $2n = 51$. However, a single male-fertile plant was obtained after crossing with an amphiploid NMS HA 89 × *H. maximiliani* 1631, and the chromosome number was $2n = 34$. At present, we do not have adequate data to explain how or why this happened. Whether the restoration gene from the amphiploid *H. maximiliani* was incorporated into the CMS BC_6 generation through a rare recombination event or chromosome translocation or others is unknown. Further cytogenetic research will be needed to understand the mechanism.

Molecular tagging of Rf_4

The fertility restorer gene Rf_1 of CMS PET1was mapped to linkage group 13 of the SSR genetic map (Tang et al. 2002) by using SSR marker ORS1030 and other markers (Horn et al. 2003; Kusterer et al. 2005), and to linkage group 2 of the RFLP genetic map (Jan et al. 1998), which is cross-referenced to linkage group 13 of the SSR genetic map (Gedil et al. 2001; Yu et al. 2003). Thus, Rf_1 was assigned to linkage group 13 of the SSR genetic map. In the present studies, the Rf_4 locus was mapped to linkage group 3 of the SSR genetic map, which also cross-referenced to linkage group 11 of the RFLP genetic map (Jan et al. 1998). Therefore, the Rf_4 gene needed for fertility restoration of CMS GIG2 is the first fertility restorer gene to be identified on linkage group 3 of the SSR genetic map. It is obviously different from Rf_1 of CMS PET1 based on mapping analysis. This further explains why all the restorer lines for the CMS PET1 do not restore the pollen fertility for CMS GIG2.

The isolation of the Rf genes through a map-based cloning strategy has been successfully used in various plant species (Bentolila et al. 2002; Brown et al. 2003; Wang et al. 2006). Fine mapping of the target gene region and constructing a large insert DNA library are two essential steps. BAC/BIBAC libraries with 8.9 × genome coverage have recently been constructed by Feng et al. (2006). A closely linked SSR marker ORS1114 was mapped <1 cM from Rf_4 with 933 individuals. It can be expected that the use of closely linked marker ORS1114 with backcross breeding will produce near isogenic lines (NILs) of the Rf_4 gene. Further fine mapping covering the Rf_4 gene region and screening the BAC/BIBAC libraries will greatly facilitate cloning of the Rf_4 gene in the near future.

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References

Alexander MP (1969) Differential staining of aborted and non-aborted pollen. Stain Technol 44:117–122

Bentolila S, Alfonso AA, Hanson MR (2002) A pentatrico-peptide repeat-containing gene restores fertility to cytoplasmic male-sterile plants. Proc Natl Acad Sci USA 99:10887–10892

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. Plant J 35:262–272

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, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide-repeat protein family. EMBO Rep 4:588–594

Dominguez-Gimeneze J, Fick GN (1975) Fertility restoration of male-sterile cytoplasm in wild sunflowers. Crop Sci 15:724–726

Feng JH, Vick BA, Lee MK, Zhang HB, Jan CC (2006) Construction of BAC and BIBAC libraries from sunflower and identification of linkage group-specific clones by overgo hybridization. Theor Appl Genet 113:23–32

Fick GN, Zimmer DE (1974) Fertility restoration in confectionery sunflowers. Crop Sci 14:603–604

Gagliardi D, Leaver CJ (1999) Polyadenylation accelerates the degradation of the mitochondrial mRNA associated with cytoplasmic male sterility in sunflower. EMBO J 18:3757–3766

Gedil MA, Wye C, Berry S, Segers B, Peleman J, Jones R, Leon A, Slabaugh MB, Knapp SJ (2001) An integrated restriction fragment length polymorphism-amplified fragment length polymorphism linkage map for cultivated sunflower. Genome 44:213–221

Gentzbittel L, Vear F, Zhang YX, Bervillé A, Nicolas P (1995) Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). Theor Appl Genet 90:1079–1086

Gentzbittel L, Mestries E, Mouzeyar S, Mazeyrat F, Badaoui S, Vear F, Tourvieille de Labrouhe D, Nicolas P (1999) A composite map of expressed sequences and phenotypic traits of the sunflower (*Helianthus annuus* L.). Theor Appl Genet 99:218–234

Horn R, Kusterer B, Lazarescu E, Prüfe M, Friedt W (2003) Molecular mapping of the Rf_1 gene restoring pollen fertility in PET1-based F1 hybrids in sunflower (*Helianthus annuus* L.). Theor Appl Genet 106:599–606

Horn R, Köhler RH, Zetsche K (1991) A mitochondrial 16 kDa protein is associated with cytoplasmic male sterility in sunflower. Plant Mol Biol 7:26–33

Jan CC, Vick BA, Miller JF, Kahler AL, Butler ET III (1998) Construction of an RFLP linkage map for cultivated sunflower. Theor Appl Genet 96:15–22

Jan CC, Zhang TX, Miller JF, Fick GN (2002) Inheritance of fertility restoration for two cytoplasmic male sterility sources of *Helianthus pauciflorus* (*rigidus*). Nutt Crop Sci 42:1873–1875

Jan CC, Vick BA (2007) Inheritance and allelic relationships of fertility restoration genes for seven new sources of male-sterile cytoplasm in sunflower. Plant Breed 126:213–217

Kaul MLH (1988) Male sterility in higher plant. Springer, Berlin

Kearsey MJ, Pooni HS (1996) The genetical analysis of quantitative traits. Chapman & Hall, London

Kinman ML (1970) New development in the USDA and state experiment station sunflower breeding programs. In: Proc. 4th int. sunflower conf., Memphis, TN, 23–25 June 1970, Int. Sunflower Assoc., Paris, France, pp 181–183

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.). Plant J 37:315–325

Korell M, Mösges G, Friedt W (1992) Construction of a sunflower pedigree map. Helia 15:7–16

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 112:172–175

Köhler RH, Horn R, Lössl A, Zetsche K (1991) Cytoplasmic male sterility in sunflower is correlated with the co-transcription of a new open reading frame with the *atpA* gene. Mol Gen Genet 227:369–376

Kusterer B, Horn R, Friedt W (2005) Molecular mapping of the fertility restoration locus *Rf*1 in sunflower and development of diagnostic markers for the restorer gene. Euphytica 143:35–42

Lander ES, Green J, Abrahamson J, Batlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Laser KD, Lersten NR (1972) Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms. Bot Rev 38:425–454

Laver HK, Reynolds SJ, Moneger F, Leaver CJ (1991) Mitochondrial genome organization and expression associated with cytoplasmic male sterility in sunflower (*Helianthus annuus* L.). Plant J 1:185–193

Leclercq P (1969) Une stérilité cytoplasmique chez le tournesol. Ann Amelior Plant 19:99–106

Leclercq P (1984) Identification de genes de restauration de fertilité sur cytoplasms stérilisants chez le tournesol. Agronomie 4:573–576

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

Michelmore RW, Paran I, Kessli RV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Monéger M, Smart C J, Leaver CJ (1994) Nuclear restoration of cytoplasmic male sterility in sunflower is associated with the tissue-specific regulation of a novel mitochondrial gene. EMBO J 13:8–17

Serieys H (1996) Identification, study and utilisation in breeding programs of new CMS sources. FAO progress report. Helia 19(special issue):144–160

Serieys H (2005) Identification, study and utilization in breeding programs of new CMS sources, in the FAO Subnetwork. In: Proc. 2005 sunflower subnetwork progress report, 17–20 July 2005, FAO, Rome, Italy, pp 47–53

Tang S, Yu JK, Slabaugh MB, Shintani DK, Knapp SJ (2002) Simple sequence repeat map of the sunflower genome. Theor Appl Genet 105:1124–1136

Tang S, Kishore VK, Knapp SJ (2003) PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theor Appl Genet 107:6–19

Voorips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTL. J Hered 93:77–78

Vranceanu VA, Stoenescu FM (1971) Pollen fertility restorer gene from cultivated sunflower (*Helianthus annuus* L.). Euphytica 20:536–541

Vranceanu VA, Stoenescu FM (1978) Gene for pollen fertility restoration in sunflowers. Euphytica 27:617–627

Wang ZH, Zou YJ, Li XY, Zhang QY, Chen LT, Wu H, Su DH, Chen YL, Guo JX, Luo D, Long YM, Zhong Y, Liu YG (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. Plant Cell 18:676–687

Yu JK, Mangor J, Thompson L, Edwards KJ, Slabaugh MB, Knapp SJ (2002) Allelic diversity of simple sequence repeat markers among elite inbred lines in cultivated sunflower. Genome 45:652–660

Yu JK, Tang S, Slabaugh MB, Heesacker A, Cole G, Herring M, Soper J, Han F, Chu WC, Webb DM, Thompson L, Edwards KJ, Berry S, Leon AJ, Grondona M, Olungu C, Maes N, Knapp SJ (2003) Towards a saturated molecular genetic linkage map for cultivated sunflower. Crop Sci 43:367–387

Zhang ZY, Ober JA, Kliebenstein DJ (2006) The gene controlling the quantitative trait locus *EPITHIOSPECIFIER MODIFIER*1 alters glucosinolate hydrolysis and insect resistance in *Arabidopsis*. Plant Cell 18:1524–1536