Identification of a new CMS cytoplasm and localization of its fertility restoration gene in sunflower

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ABSTRACT

Cytoplasm male sterility (CMS) and its fertility restoration (Rf) genes are critical tools for hybrid seed production. To broaden the genetic diversity of sunflower hybrid breeding materials, a new CMS, designated as CMS GIG2, was identified from an interspecific cross between *Helianthus giganteus* accession 1934 and *H. annuus* cv. HA 89. We also identified the fertility restoration gene for CMS GIG2 from an amphiploid involving *H. maximiliani* accession 1631. The CMS GIG2 and its fertility restoration gene were introduced into HA 89 background through recurrent backcross and single plant selection techniques. Genetic analysis revealed that the CMS GIG2-fertility restoration system is controlled by a completely dominant gene, designated as Rf_4, in a HA 89 background. The gene Rf_4 was mapped onto linkage group 3 and tightly links with the markers ORS1114 and ORS13, based on 576-plant F_2 and F_3 populations. The CMS GIG2-Rf_4 system tagged by molecular markers provides an alternative genetic source for hybrid sunflower breeding.

Key words: cytoplasm male sterility – interspecific hybridization – male fertility restoration – sunflower.

INTRODUCTION

Cytoplasm male sterility (CMS) has been reported for over 150 plant species (Kaul, 1988) and used to produce commercial hybrid seed for several crops. Since the first sunflower CMS was reported in 1969, 72 CMS sources have been identified in the *Helianthus* genus (Serieys, 2005). So far, the CMS PET1 developed by Leclercq (1969) from *H. petiolars* Nutt. is the only one that has been extensively used in commercial hybrid sunflower production. Genetic analyses indicated that almost all CMS restoration lines currently used in sunflower breeding carry the same restorer gene Rf_1 (Serieys, 1996, 2005). In order to prevent cytoplasmic uniformity and reduce genetic vulnerability, alternative CMS and restorer sources are needed for sunflower breeding programs.

Sunflower male fertility restoration is generally controlled by a single dominant gene (e.g., Rf_1), or two complementary dominant genes (e.g., Rf_1 and Rf_2) plus modifiers (Serieys, 1996, 2005). Kinman (1970) discovered the male fertility restorer gene Rf_1 in the line T66006-2-1-B. 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 (Vrânceanu and Stoenescu, 1971, 1978). Recently, a fertility restoration 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 current research was devoted to identification of a new CMS and fertility restoration genes, and also localization of the fertility restoration genes on the sunflower genetic map.

MATERIALS AND METHODS

Plant materials

The wild sunflower *H. giganteus* accession 1934 (Ames 1934, PI 503250), *H. maximiliani* accession 1631 (PI 468750), cultivated *H. annuus* cv. HA 89, and HA 89 nuclear male sterility mutant NMS HA 89 were used as parents for this study. An initial F_2 population of 113 individuals was generated from a single F_1 plant from the cross *H. giganteus*/7*HA 89/3/*H. giganteus*/ 6* HA 89// *H. maximiliani* 1631 (amphiploid) and this population was used to develop the genetic model for the new CMS and fertility restoration system. Some of F_2 heterozygous plants were selected to produce F_3 lines to confirm the genetic model and to map the Rf gene. Plants were grown in the field or greenhouse conditions in Fargo, USA.

Phenotype identification
Male fertility or sterility for individual plants was identified visually examining anther morphology and evaluating pollen stainability (Alexander, 1969). Plants with normal, dehiscent anthers and abundant pollen were scored as male-fertile, otherwise as male-sterile. To evaluate stainability, about 500 pollen grains were counted under a microscope to estimate the percentage of fertile pollen grains.

Marker genotyping
Genomic DNA was extracted from fresh young leaves of individual F_2 and F_3 plants according to the protocol described by Zhang et al. (2006). The bulked segregant analysis strategy (Michelmore et al., 1991) was used to screen for markers polymorphic for the segregation populations. Equal amounts of DNA from 10 male-sterile and 10 male-fertile F_2 plants were bulked to form the sterile and fertile pools, respectively. In total, 200 simple sequence repeat (SSR) markers relatively evenly distributed among the 17 linkage groups (Tang et al., 2002; Yu et al., 2002, 2003) were selected to screen for polymorphism between male-fertile and male-sterile pools. The polymorphic markers were used to genotype F_2 and F_2-derived F_3 populations. PCR amplification was performed according to Tang et al. (2002). PCR products were displayed on 6.5% polyacrylamide denaturing gel at 60 W for 2.0 h (0.5× TBE) and then 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).

RESULTS

Isolation of a male sterility cytoplasm
The wild sunflower *H. giganteus* acc.1934 was crossed with HA 89 and the resulting F_1 seedlings were recovered through an embryo rescue technique. All F_1 plants had 2n=34 chromosomes and were treated with colchicine to induce double haploid progenies (2n = 68). One of the F_1 plants was male-sterile and the remaining were male-fertile, based on morphology of the anthers and pollen stainability. The male-sterile F_1 plant was backcrossed with HA 89 repeatedly and the chromosome numbers of the hybrids were reduced with each successive backcross. After five cycles of backcrossing, plants with 2n=34 were selected from the BC_5F_1 generation. All plants from the BC_1 to BC_5 generations were male-sterile, indicating that the CMS trait from *H. giganteus* acc. 1934 was introduced into the HA 89 background.

BC_5F_1 plants were pollinated by a set of 19 cultivars, i.e., 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 VNIIMK. This standard set of restoration lines was used to test the fertility restoration pattern for the new CMS from *H. giganteus* because of their diverse genetic background. Hybrids from all these crosses were male-sterile, suggesting that the tester lines had no restorer genes for the new CMS from *H. giganteus* acc. 1934. Thus, the new CMS is different from all previously reported CMS types, including CMS GIG1 which can be restored by RHA 280 and RHA 801 (Serieys, 1996). Therefore, we concluded that the *H. giganteus* acc. 1934-derived CMS was a new type. According to the FAO codification, the new CMS source was designated as CMS GIG2.

Identification of the CMS GIG2 fertility restoration gene
To search for the fertility-restoration gene for CMS GIG2, we crossed the BC_5F_1 plants with seven interspecific amphiploids: 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. Four amphiploids involving wild species *H. maximiliani* 1631, *H. atrorubens*, *H. grosseserratus*, and *H. angustifolius* restored fertility of CMS GIG2. It was noticed that a single male-fertile plant with 2n=34 was obtained after crossing a BC_5F_1 plant with the amphiploid NMS HA 89 × *H. maximiliani* 1631, while all other progenies of the same cross were also male-fertile, but had 2n=51 chromosomes. This particular male-fertile plant was used to pollinate a male-sterile BC_6F_1 plant, and the resulting F_1 plant was used to develop an F_2 mapping population. The standard set of 19 cultivated lines used above, including 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, indicating that this fertility restoration gene derived from amphiploid *H. maximiliani* 1631 is different from Rf_1, Rf_2, and Rf_3.

Inheritance of the CMS GIG2 fertility restoration system
The population of 113 F_2 plants was classified into fertile and sterile groups (Fig. 1) and the segregation ratio fit a monogenic ratio of 3:1 (Table 1). The segregation pattern was confirmed by the F_3 population

(Fig. 1 and Table 1). The data from the two generations demonstrated that the CMS GIG2 fertility restoration system is controlled by one locus. This new fertility restoration locus was designed as *Rf4*.

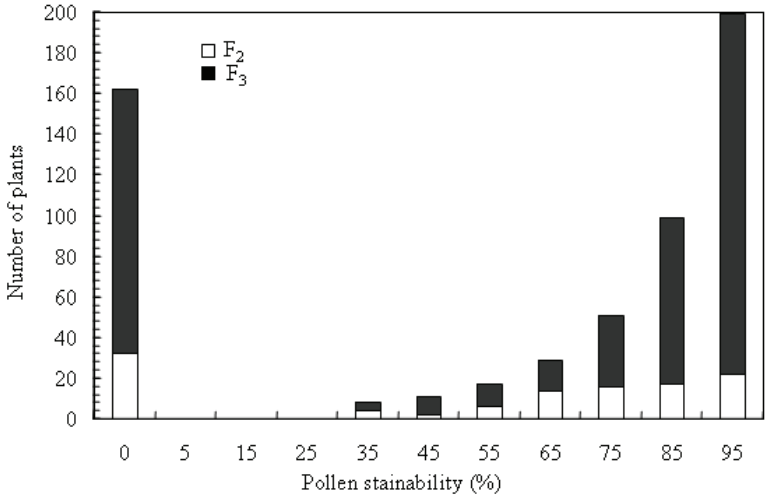

Fig. 1. Distribution of pollen stainability in the F_2 and F_2 heterozygote-derived F_3 populations.

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Table 1. Fitness test for segregation ratios of pollen fertility and its associated marker OSR1114 or ORS13 in the initial F_2 and F_2 heterozygote-derived F_3 populations

Population	Trait or marker	Number of plants[1]			Expected ratio	χ^2 - value	Probability
		Fertile		Sterile			
		Rf4Rf4	*Rf4rf4*	*rf4rf4*			
F_2	Pollen fertility	81		32	3:1	0.6	0.42
	ORS1114/ORS13	32	49	32	1:2:1	2.0	0.37
F_3	Pollen fertility	333		130	3:1	2.3	0.13
	ORS1114/ORS13	106	226	131	1:2:1	2.9	0.23

[1]Individual plants were visually and microscopically examined for pollen fertility/sterility based on the morphology of the anthers and the pollen stainability at flowering time. The *Rf4/rf4* locus is the proposed fertility restoration gene based on the expected segregation pattern and this locus co-segregated with the marker ORS1114 or ORS13 in the F_2 population.

Mapping of the Rf4 locus

Of 200 simple sequence repeat (SSR) markers selected from the linkage maps (Tang et al., 2002; Yu et al., 2002, 2003), seven (ORS13, ORS294, ORS349, ORS502, ORS822-3, ORS1114, ORS1146) were polymorphic between the fertile and sterile pools, respectively, and were used to genotype the whole F_2 population. The genotyping data showed that only ORS13 and ORS1114 (Fig. 2) on linkage group 3 were associated with pollen fertility and co-segregate with the *Rf4* locus. There were no recombinants between the markers ORS13 and ORS1114, and both fit a monogenic segregation ratio of 1:2:1 in the F_2 population (Table 1). The marker-trait association demonstrated that *Rf4* is a completely dominant gene, because there was no difference in pollen stainability between the *Rf4rf4* and *Rf4Rf4* genotypes (Fig. 1).

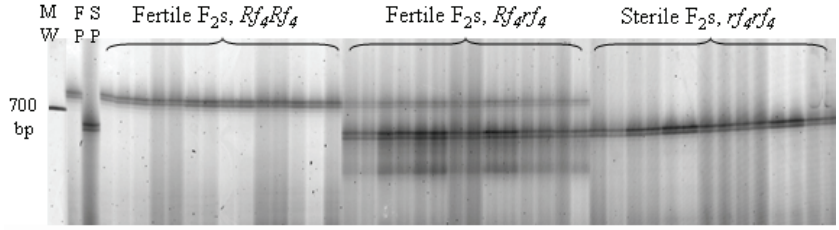

Fig. 2. Co-segregation of the marker ORS1114 with pollen fertility and the fertility restoration locus (*Rf4/rf4*) in the F_2 population. The letters MW indicate molecular weight (base pair or bp) ladder, and FP (fertile pool) and SP (sterile pool) indicate DNA samples bulked from 10 fertile and 10 sterile F_2 plants, respectively.

Of the 463 F_3 plants genotyped with ORS13 and ORS1114, no recombinants were detected between the two markers, while four recombinants were identified between the marker and *Rf4* loci. Combining the two populations of 576 individuals, it is estimated that the genetic distance between *Rf4* and ORS13 or ORS1114 loci is 0.69 cM.

DISCUSSION

The fertility restorer gene *Rf1* of CMS PET1 is universally used in hybrid seed production and this gene was assigned to linkage group 13 of the public SSR genetic map (Gedil et al., 2001; Yu et al., 2003; Horn et al., 2003; Kusterer et al., 2005), or linkage group 2 of the RFLP genetic map (Jan et al., 1998). We identified CMS GIG2 from wild species *H. giganteus* and incorporated the CMS into cultivated sunflower. It represents a new source of male-sterile cytoplasm different from CMS PET1. We also identified the fertility restoration gene *Rf4* for CMS GIG2 from *H. maximiliani* 1631. The *Rf4* gene is completely dominant and tightly links to the markers ORS13 and ORS1114 on linkage group 3 of the public SSR genetic map. ORS13 and ORS1114 were reported to be 11 cM apart (Tang et al., 2003), but co-segregated in our F_2 and F_3 populations of 576 plants. The possible reason for the difference could be the multi-allelic differentiation at one of the marker loci.

The estimated genetic distance between *Rf4* and the cosegregated markers is less than 1 cM. Therefore, the markers ORS13 and ORS1114 can be used to track the linked *Rf4* gene in sunflower hybrid breeding programs. Further fine mapping covering the *Rf4* gene region and screening of the BAC/BIBAC libraries will allow us to clone the *Rf4* gene in the future (Feng et al., 2006).

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