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ORIGINAL PAPER

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Molecular mapping of a nuclear male-sterility gene in sunflower (*Helianthus annuus* L.) using TRAP and SSR markers

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Abstract A nuclear male-sterile mutant, NMS 360, induced by streptomycin from an inbred maintainer line HA 89, possesses a single recessive gene, *ms9*, controlling male sterility. The present study identified DNA markers linked to the *ms9* gene in an F_2 population derived from the cross of NMS 360 × RHA 271 and maps the *ms9* gene to an existing sunflower SSR linkage map. Bulked segregant analysis was performed using the target region amplification polymorphism (TRAP) marker technique and the simple sequence repeats (SSR) technique. From 444 primer combinations, six TRAP markers linked with the *ms9* gene were amplified. Two markers, Ts4p03-202 and Tt3p09-529, cosegregated with the *ms9* gene. The other four markers, To3d14-310, Tt3p17-390, Ts4p23-300, and Tt3p09-531, linked with *ms9* at a distance of 1.2, 3.7, 10.3, and 22.3 cM, respectively. Thirty SSR primers from 17 linkage groups of a PHA × PHB cultivated sunflower linkage map were screened among the two parents and the F_2 population. SSR primer ORS 705 of linkage group 10 was tightly linked to *ms9* at a distance of 1.2 cM. The *ms9* gene was subsequently mapped to linkage group 10 of the public sunflower SSR linkage map. The markers that were tightly linked with the *ms9* gene will be useful in marker-assisted selection of male-sterile plants among segregating populations, and will facilitate the isolation of the *ms9* gene by map-based cloning.

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Introduction

Male sterility is defined as the failure of plants to produce functional anthers, pollen, or male gametes while the female reproduction is normal (Kaul 1988). Based on its inheritance or origin, male sterility may be divided into nuclear male sterility (NMS), also called genic male sterility (GMS), and cytoplasmic male sterility (CMS). Both types of male sterility have been found in sunflower. NMS in sunflower (*Helianthus annuus* L.) was first reported in the Soviet Union by Kuptsov in 1934 (Gundaev 1971) and since then, investigators have reported numerous incidences of NMS in sunflower. Leclercq (1969) reported the discovery of male-sterile plants among the offspring of an interspecific cross between *H. petiolaris* and cultivated *H. annuus*. Vranceanu (1970) isolated more than 30 NMS sources, most of which were controlled by single recessive genes, from Romanian sunflower breeding lines. Inheritance studies involving ten of these lines indicated the presence of five NMS genes, designated *ms1–ms5*. Seven mitomycin-C and streptomycin induced NMS mutants from cultivated line HA 89 were placed in four different allelic groups, each representing a unique NMS gene, designated *ms6–ms9* (Jan and Rutger 1988; Jan 1992). NMS genes from two released male sterility lines, NMS B11A3 and P21, have been designated *ms10* and *ms11*, respectively (Jan 1992).

Molecular marker technology provides the opportunity to map a gene of agronomic importance in segregating generations by comparison of near isogenic lines (Muehlbauer et al. 1988) or by bulked segregant analysis (BSA) (Michelmore et al. 1991). Using different molecular marker techniques, several NMS genes in plants have been mapped. Examples include the rice (*Oryza sativa* L.) photoperiod-sensitive NMS genes (Subudhi et al. 1997; Wang et al. 1995; Zhang et al. 1994; Koh et al. 1999), rice thermo-sensitive NMS gene *tms*5 (Wang et al. 2003), Chinese cabbage (*Brassica campestris* L. *ssp. chinensis*) NMS gene *gms* (Miao et al. 2003), soybean (*Glycine max L. Merr.*) NMS gene *ms* (Jin et al. 1998), tomato (*Lycopersicon esculentum* Mill.) NMS gene *ms14* (Gorman et al. 1996), and

sunflower (*H. annuus* L.) NMS genes *ms10* and *ms11* (Pérez-Vich et al. 2005).

Target region amplification polymorphism (TRAP) is a technique which uses expressed sequence tag (EST) sequences and bioinformatics tools to generate polymorphic markers around targeted candidate gene sequences (Hu and Vick 2003). The density of the cultivated sunflower simple sequence repeats (SSR) genetic linkage map has been greatly increased by the mapping of several hundred SSR markers (Tang et al. 2002; Yu et al. 2003). In this study, we identified a set of TRAP markers flanking the *ms9* locus and reported the mapping of the *ms9* gene to a sunflower linkage map constructed using SSR markers (Yu et al. 2003). This information will facilitate the transfer of the *ms9* allele in sunflower breeding programs through the use of markers closely linked to *ms9* that will distinguish *Msms* from *MsMs* plants, and will help to isolate the *ms9* gene by map-based cloning.

Materials and methods

Materials

An F_2 population of 93 plants was established by selfing an F_1 progeny of NMS 360 × RHA 271. NMS 360 is an NMS mutant produced by chemical mutagenesis using streptomycin on an inbred maintainer line HA 89 (Jan and Rutger 1988). NMS 360 possess a single recessive gene, designated *ms9*, controlling complete male sterility (Jan 1992). The male parent, RHA 271, is an oilseed restorer line released by the Texas Agricultural Experiment Station and the USDA-ARS in 1973. F_3 families from selfed male-fertile F_2 plants were grown in the field to differentiate heterozygous from homozygous F_2 plants.

Chi-square tests were used to determine the goodness of fit of the phenotype of the F_2 generation to a 3:1 ratio of male-fertile, to male-sterile progeny, and to a 1:2:1 ratio of homozygous male-fertile, heterozygous male-fertile, and male-sterile genotypes after F_3 progeny verification. The F_2 and F_3 plants were grown, respectively, in the greenhouse and field at Fargo, ND, and visually scored for male fertility at flowering. Plants with full anther extrusion and pollen production were classified as male-fertile (*MsMs* or *Msms*), and plants without anther extrusion or no pollen were classified as male-sterile (*msms*).

DNA preparation

Total DNA was isolated from freeze-dried powdered leaf tissue of individual F_2 plants and the parents using the DNeasy Plant Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. The concentration of DNA was determined using a DU7400 spectrophotometer (Beckman Coulter) and adjusted to 10–20 ng/μl for PCR amplification. Equal amounts of DNA from 12 homozygous male-sterile (*msms*) and 12 homozygous male-fertile (*MsMs*) F_2 individuals were pooled for BSA.

Primer design

Seventy-four primers used as fixed primers in this study belonged to two groups. The first group, the male-sterile primer (MSP) group, contained 26 primers that were designed based on published male-sterile genes using the web-based software Primer3 (http://www.basic.nwu.edu/biotools/Primer3.html). The second group, the non-MSP group, was comprised of 48 fixed primers that were designed for other mapping projects in our laboratory using EST sequences in the Compositae Genomics Initiative database (http://www.cgpdb.usdavis.edu). Arbitrary primers were designed to contain an AT- or GC-rich core that anneals to introns or exons within the targeted gene, respectively, and were 5′ end-labeled with either IRD 700 dye or IRD 800 dye. Sequence data of five fixed primers and three arbitrary primers which produced polymorphic markers in this study are listed in Table 1.

TRAP analysis

TRAP marker generation followed the protocol of Hu and Vick (2003), with slight modifications.

PCR was performed with a final reaction volume of 15 μl in 96-well microliter plates in a GenAmper 9700

Table 1 Sequence data of five fixed primers and three arbitrary primers which produced linked polymorphic markers in this study

Fixed primers	Sequences (5′–3′)	Sequence ID
MSP		
MSP03	GTTGCCATGGACATCAACAC	Silic-3 forward primer of wheat putative male-sterile gene (Chen et al. 2005)
MSP09	AAGAAAAGGAAGATGATTT	Arabidopsis ATA7 gene (Rubinelli et al. 1998)
MSP17	AGACCGGCGAGCTCTAC	Zea mays *Ms45* gene (Albertsen et al. 1993)
MSP23	TCCGTGTAGCCAACAAC	Arabidopsis 12-oxo-phytodienoate reductase (OPR3) mRNA (Stintzi 2000)
Non-MSP		
A11D14a	GTCAGTTTAATGAAGTTG	*H. annuus* delta-12 oleate desaturase (FAD2-2) mRNA (Martínez-Rivas et al. 2001)

Arbitrary primers	Sequences (5′–3′)	Labeled with
Trap3	CGTAGCGCGTCAATTATG	IR 700
Sa4	TTCTTCTTCCCTGGACACAAA	IR 700
Odd3	CCAAAACCTAAAACCAGGA	IR 800

thermal cycler (Applied Biosystems, Foster City, CA) with the following components: 2 µl of the 10–20 ng/µl DNA sample, 1.5 µl of 10 × reaction buffer, 1 µl of 25 mM $MgCl_2$, 1 µl of 5 mM dNTPs, 0.3 pmol each of IRD 800 dye and IRD 700 dye labeled arbitrary primers, 1.0 pmol of the fixed primer, and 1.5 units of *Taq* DNA polymerase (QIAGEN, Valencia, CA). The PCR was performed by initially denaturing template DNA at 94°C for 2 min; then five cycles at 94°C for 45 s, 40°C for 45 s, and 72°C for 1 min; followed by 35 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min; then a final extension step at 72°C for 7 min.

Upon completing the PCR cycles, 0.8 µl of PCR product of each sample was loaded onto a 6.5% polyacrylamide sequencing gel in a Li-Cor Global DNA Sequencer (Li-Cor Biosciences, Lincoln, NE), and electrophoresed at 1,500 V for 3.5 h.

Simple sequence repeats analysis

All SSR forward primers were 5′ end-labeled with either IRD 700 dye or IRD 800 dye. The PCR procedure for SSRs was performed with a final reaction volume of 15 µl in 96-well microliter plates as described above, with 1 µl of 10 mM each of labeled forward primer and the unlabeled reverse primer. The amplification reactions were conducted using the following touchdown profile: 94°C for 3 min; then ten cycles at 94°C for 45 s, 61°C for 45 s, −0.5°C/cycle, and 72°C for 1 min; followed by 35 cycles at 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min, with a final extension step at 72°C for 7 min.

The amplification products were separated on a 6.5% polyacrylamide sequencing gel in a Li-Cor Global DNA Sequencer (Li-Cor Biosciences). Electrophoresis was conducted at 1,500 V for 1.5 h.

Data analysis

Gel images were collected with SAGA Genotyping software (Li-Cor Biosciences). The polymorphic fragments were visually scored from the printed images. The scoring codes were 1 for present, 0 for absent, and a dash "–" for missing.

The name of each scored TRAP marker consists of four parts: prefix "T", two letters of arbitrary primer, three letters of fixed primer, and fragment size. For example, marker Ts4p03-202 is a 202 bp fragment which was amplified by the arbitrary primer Sa4 and the fixed primer MSP03.

Chi-square analyses were carried out on each locus to identify deviations from the expected Mendelian ratios for codominant (1:2:1) or dominant (3:1) markers. Linkage analysis was conducted using MAPMAKER/EXP version 3.0b (Lander et al. 1987). Marker order was determined with a LOD threshold of 3.0, and map distances were estimated by the Kosambi function (Kosambi 1944). The linkage map was produced using MapChart 2.0 (Voorrips 2002).

Results

Segregation of the *ms9* gene in the F_2 population

The F_2 population of 93 plants consisted of 79 male-fertile plants and 14 male-sterile plants. The segregation of fertile to sterile plants in this F_2 population best fit a 3:1 ratio ($\chi^2 = 4.89$, $0.05 < P < 0.01$), indicating single recessive gene control of male-sterility. Results of the F_3 progeny tests further classified the 79 male-sterile F_2 plants into 22 homozygous male-fertile plants and 57 heterozygous male-fertile plants, indicating that the segregation ratio best fit the 1:2:1 ratio for single recessive gene control of male-sterility ($\chi^2 = 6.12$, $0.05 < P < 0.01$) (Table 2). The slight deviations from either 3:1 or 1:2:1 ratio was assumed to have minimal effect on the conclusion of this particular study. In addition, the single recessive gene control of male-sterility was strongly supported by 3:1 F_2 and 1:1 BC_1F_1 ratios of male-fertile to male-sterile plants in crosses of NMS 360 with HA 89 (Jan 1992).

Identification of TRAP markers linked to the *ms9* gene

Separate bulks of DNA from 12 homozygous male-sterile (*msms*) and 12 homozygous male-fertile (*MsMs*) individuals were established for BSA. For BSA, PCR amplifications with 444 primer combinations generated TRAP markers differentiated by the male-sterile bulk and male-fertile bulk. Each primer combination amplified 30–60 scorable fragments ranging in size from 50 to 900 bp. Primer combinations which produced polymorphic markers were used to amplify the whole F_2 population and the parents. Twenty-two polymorphic fragments from 19 primer combinations distinguished the two bulks, and six fragments were confirmed to be linked to either male-sterile or to male-fertile phenotypes. Markers Ts4p03-202, Tt3p09-529, To3d14-310, Tt3p17-390, and Ts4p23-300 were linked with the male-fertile allele (*Ms9*), while marker Tt3p09-531 linked with the male-sterile (*ms9*) allele. As expected, segregation ratios of these markers either fit the 3:1 ratio or deviated slightly, depending on their relationship with the *ms9* gene. All these polymorphic fragments were considered as candidate markers for the *ms9* gene.

Figure 1 shows marker Ts4p03-202 amplified by the primer combination of fixed primer MSP03 and arbitrary primer Sa4. The marker was present in all 22 homozygous male-fertile and 57 heterozygous male-fertile plants, but absent in all 14 homozygous male-sterile plants. Primer MSP03 was designed based on a wheat putative male-sterile gene (Chen et al. 2005).

Mapping the ms9 gene by SSR markers

To map the *ms9* gene, 30 SSR primers randomly chosen from 17 linkage groups (LG) of the public sunflower SSR linkage map (Yu et al. 2003; Tang et al. 2002) were used to identify polymorphisms between the parents: HA 89 (NMS 360) and RHA 271. Fifteen SSR primers were

Table 2 Segregation of the *ms9* locus, six TRAP markers, and six SSR markers in an F_2 population from NMS 360 × RHA 271

Traits or markers	Number of F_2 plants[a]	Observed number[b]					χ^2	
		AA	HH	BB	CC	DD	1:3	1:2:1
ms9	93	14	57	22				6.12*
ORS 749	87	15	49	23				2.85
ORS 613	91	20	44	27				1.17
ORS 691	92	21	53	18				2.32
Ts4p03-202	93	14			79		4.89*	
Tt3p09-529	93	14			79		4.89*	
To3d14-310	93	13			80		6.00*	
Tt3p17-390	92	13			80		6.00*	
Ts4p23-300	92	10			83		10.00**	
ORS 705	93	15			78		3.87*	
Tt3p09-531	88			21		67	0.06	
ORS 595	93			22		71	0.09	
ORS 853	93			23		70	0.003	

[a] For ORS 749, six plants were not scorable; for ORS 613, two plants were not scorable; for ORS 691, Tt3p17-390, and Ts4p23-300, one plant was not scorable; for Tt3p09-531, five plants were not scorable

[b] Genotypes: *AA*, NMS 360 (*msms*); *HH* , heterozygous (*Msms*); *BB*, RHA 271 (*MsMs*); *CC* , not AA (*MsMs* or *Msms*); *DD* , not BB (*Msms* or *msms*)

*Significant at the 0.05 level probability

**Significant at the 0.01 level probability

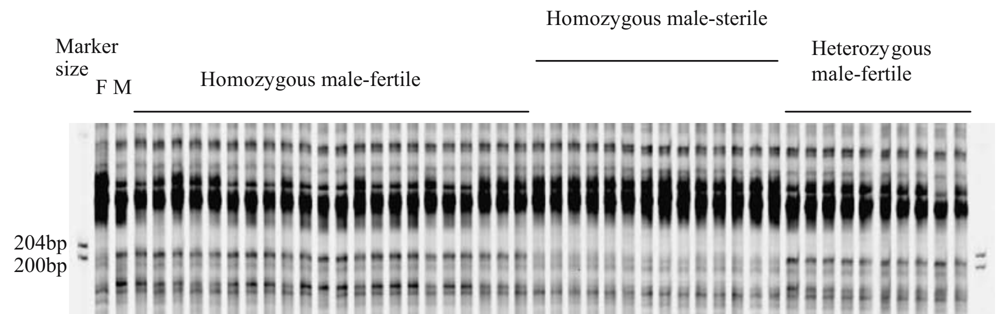

Fig. 1 Marker Ts4p03-202 amplified by fixed primer MSP03 and arbitrary primer Sa4. The marker was present in all 22 homozygous male-fertile and 57 heterozygous male-fertile (only ten are shown in this figure) plants but absent in all 14 homozygous male-sterile plants

polymorphic, and one SSR primer on LG 10, ORS 749, showed a weak linkage with the *ms9* gene in the F_2 population. To confirm that the *ms9* gene was located on LG 10, nine additional SSR primers around ORS 749 were examined in the F_2 population. Five primers, ORS 705, ORS 613, ORS 691, ORS 595, and ORS 853, were linked with the *ms9* gene. However, some SSR primers (ORS 1118 and ORS 185) did not work well in the amplification for unknown reason. ORS 705 was present in all 22 homozygous male-fertile and 56 heterozygous male-fertile plants, but was absent in all 14 homozygous male-sterile plants and absent in only one heterozygous male-fertile plant. Segregation ratios of nearly all SSR markers Wt a 1:2:1 or 3:1 ratio. Only those markers linked with ms9 are shown in Table 2.

Linkage analysis and map construction

Based on the six segregating TRAP markers and six SSR markers, a linkage map of the *ms9* gene-encompassing region was constructed using computer software MAPMAKER/EXP 3.0b with LOD > 3.0 (Fig. 2). All marker loci could be placed on the linkage group with the *ms9* locus in the center. The total genetic distance covered by those markers was 119.5 cM. TRAP markers Ts4p03-202 and Tt3p09-529 cosegregated with *ms9*. TRAP markers To3d14-310 and Tt3p17-390 were tightly linked with the *ms9* gene at distances of 1.2 and 3.7 cM, respectively. TRAP markers Ts4p23-300 and Tt3p09-531 were loosely linked with the *ms9* gene at distances of 10.3 and 22.6 cM, respectively. SSR marker ORS 705 was tightly linked with the *ms9* gene with a distance of 1.2 cM. The other five SSR markers were loosely linked with the *ms9* gene at distances of more than 30 cM. Map construction placed five SSR markers on one side of the *ms9* gene and one SSR marker, ORS 691, on another side. Therefore, it is reasonable to conclude that the *ms9* gene is located on LG 10 of the public sunflower SSR linkage map (Fig. 2).

Discussion

TRAP is a powerful technique to develop gene-specific markers

TRAP is a powerful molecular technique which uses bioinformatics tools and EST database information to

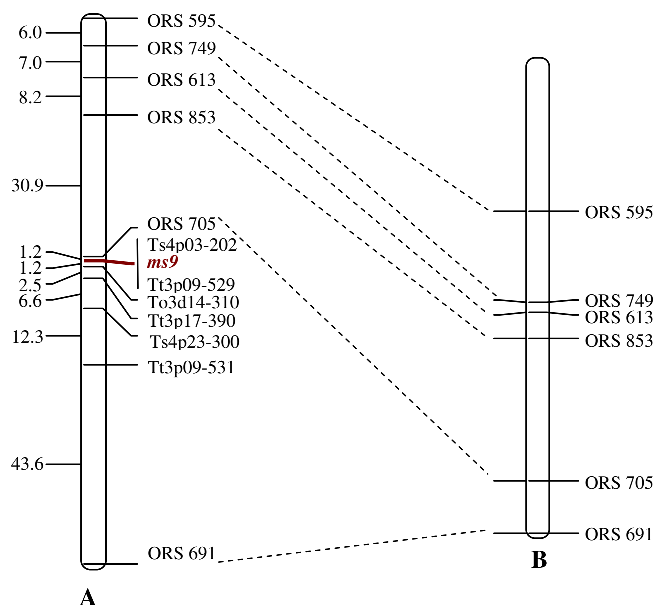

Fig. 2 **a** Mapping results of the *ms9* gene on LG 10 of sunflower consisting of six TRAP markers, six SSR markers, and the *ms9* gene. **b** A corresponding partial linkage map of the region surrounding ORS 705 on LG 10 of the public sunflower SSR linkage map (Yu et al. 2003). Distances are shown in centimorgans

generate polymorphic markers around targeted candidate gene sequences (Hu and Vick 2003). The TRAP technique has been successfully used for constructing a genetic linkage map in wheat (Liu et al. 2005). The results reported in this paper clearly demonstrated that the TRAP technique was reliable, stable, and rapid in molecular-marker screening. Coupled with BSA, it allowed us to identify 22 polymorphic fragments differentiating the male-fertile from the male-sterile bulks.

TRAP is probably more specific than random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and amplified fragment length polymorphism (AFLP) (Vos et al. 1995) techniques because it uses specific primers rather than arbitrary primers. In this study, 26 MSP primers designed based on the published male-sterile genes combined with six arbitrary primers produced 156 primer combinations, from which 14 primer combinations showed polymorphism between the male sterility and male fertility bulks. Five of the polymorphic markers were confirmed in the 93 individual plants of the F_2 population. On the other hand, 48 non-MSP primers combined with the same six arbitrary primers to produce 288 primer combinations, but only eight of these primer combinations were polymorphic. Of the eight primer combinations, only one marker was linked with the *ms9* gene in the F_2 population. Thus, five out of 156 MSP primer combinations produced useful polymorphic markers, but only one of 288 non-MSP primer combinations resulted in a useful polymorphic marker. Both markers, Ts4p03-202 and Tt3p09-529, cosegregated with the male-sterile phenotype and were amplified with MSP fixed primers. Future cloning and sequencing of these two fragments are expected to provide direct support to the aforementioned argument.

Mapping and utilization of the polymorphism markers

In this study, we mapped the nuclear male-sterile gene, *ms9*, to LG 10 on the public sunflower SSR linkage map. The gene was flanked by six TRAP markers and six SSR markers. Unfortunately, all TRAP markers and three of the six SSR markers linked to the *ms9* gene identified in the present study followed a dominant mode of inheritance, as indicated by the presence or absence of a specific fragment in the gel images. This will limit their applications since they cannot distinguish the homozygous genotype *MsMs* from the heterozygous genotype *Msms*. Therefore, it is highly desirable to convert these TRAP markers into codominant markers that are simple and reliable.

Since male sterility in NMS 360 is controlled by a single recessive gene, *ms9*, the presence of the male-sterile allele in F_2 (heterozygous) plants can only be detected by progeny testing. DNA markers linked to the *ms9* gene provide a useful approach for early and accurate identification of lines carrying the male-sterile allele, without the need of progeny testing. Sunflower breeders will greatly benefit from this high accuracy of marker-based selection for plants carrying *Ms* because it can greatly reduce the size of a breeding population and shorten the time needed in genotype determination.

Floral development and reproduction in higher plants is a complex developmental process involving a diverse range of gene interactions (McCormick 1993). Several male-sterile genes have been cloned (Aarts et al. 1993; Gorman et al. 1996; Moffatt and Somerville 1988), but none in sunflower. It will be essential to clone and characterize a large number of male-sterile gene products from a number of species to understand the myriad functions of male-sterile genes. Mapping and cloning of the *ms9* gene will promote the molecular study of NMS in sunflower. Since the late 1980s, map-based cloning via BAC landing (Tanksley et al. 1995) has been the most efficient approach to clone specific target genes and QTLs that are known only by phenotype. Map-based gene cloning is based on prior knowledge of the location of a target gene. The molecular markers closely linked to the *ms9* gene, such as Ts4p03-202, Tt3p09-529, To3d14-310, and ORS 705 can be used for isolating the *ms9* gene by means of a map-based cloning strategy. A sunflower bacterial artificial chromosome (BAC) library has been constructed in our laboratory (Feng et al. 2006). Based on the cosegregated markers identified for the *ms9* gene in this study, by screening this BAC library it is possible to isolate the *ms9* gene, to select BAC clones encompassing the ms9 gene, or to generate additional markers in the targeted regions.

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