IDENTIFICATION OF RAPD MARKERS LINKED TO A FERTILITY RESTORER GENE FOR PET1 CYTOPLASM OF SUNFLOWER (*HELIANTHUS ANNUUS*)

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Abstract

Fertility restoration ability of a restorer was controlled by a single dominant gene. Bulked segregant analysis (BSA) was applied to identify molecular markers linked to a major restorer gene (*Rf*) using the F2 population of CMS 234 A × RHA 272. A total of 144 random oligonucleotide primers were surveyed. The primer OPAM 06-1800 was found to produce putative markers, which differentiate fertile parent and bulk from sterile parent and sterile bulk. The cosegregation analysis of the putative marker on the F2 population confirmed the association of OPAM 06-1800 produced by the primer OPAM 06 with the fertility restoration gene. This will help in transfer of fertility restorer genes to the inbreds if they lack restoration genes.

Introduction

Sunflower (*Helianthus annuus* L.) is the third major supplier of edible oil in the world after soybean and groundnut. The identification and development of male sterile and fertility restorer lines was a major step in the success of hybrid breeding programmes. Modern sunflower breeding began with the development of F1 hybrids after the discovery of cytoplasmic male sterility (Leclercq, 1970) and fertility restorer genes (Kinman, 1970). The first reliable cytoplasmic male sterile source was isolated by Leclercq (1969) from the interspecific cross *Helianthus petiolaris* Nutt. × *H. annuus* and designated as PET1 cytoplasm (Serieys, 1987). The restoration of pollen fertility in PET1 cytoplasm has been reported to be controlled by a single dominant gene (Enns, 1972; Leclercq, 1972; Jan, 2000; and Seiler, 2000). In some cases restoration by two independent complementary dominant genes is also reported (Dominguez-Gimenez and Fick, 1975; Horn and Friedt, 1977; and Jan et al., 2000).

The development of a new fertility restorer by traditional backcrossing is costly and time-consuming because it requires extensive crossing. Hence, identification of molecular markers closely linked to fertility restoring genes will facilitate the breeding of new restorer lines by reducing the time requirement. Recently, random amplified polymorphic DNA (RAPD) markers have been developed (Williams et al., 1990; Welsh and McClelland, 1990). RAPDs are generated by the amplification of genomic DNA using a single primer of arbitrary nucleotide sequence to drive the amplification reaction. Several research groups are now using RAPDs to construct genetic maps. The most useful application of RAPD markers is,

however, to quickly generate markers within a genomic region of interest using near-isogenic lines (NILs) (Martin et al., 1991; Penner et al., 1993). However, several generations of backcrosses are required to create NILs and several regions of the donor genome can be co-introgressed into the NIL. An alternative method called bulked segregant analysis has been proposed by Michelmore et al., 1991. It aims at replacing the NILs by two bulked DNA samples collected from individuals identical for alleles at a specific locus in a single population, each bulk being homozygous for one or the other allele of the gene of interest. The advantage of this technology is that markers are targeted to a smaller region within the genome and likelihood of identifying false positive markers is small (Michelmore et al., 1991).

Using near isogenic line (NIL) or bulked segregant analysis (BSA) strategies (Michelmore et al., 1991) a number of RFLP or RAPD markers linked to *Rf* genes have been identified in some important crops, such as rapeseed (Delourme et al., 1994), rice (Zhang et al., 1997) and rye (Borner et al., 1998). These markers may facilitate the development of restorer lines. Similarly molecular markers closely linked to major fertility restoring genes and other fertility related genes will facilitate the breeding of new restorer lines used in the sunflower CMS system. Here we report on the identification of RAPD markers associated with a fertility gene for PET 1 cytoplasm of sunflower using the bulked segregant analysis approach.

Materials and Methods

Plant Materials. Parents, namely CMS 234A x RHA 272 of commercial hybrid 'TCSH 1,' were crossed. The F1 and F2 generations were subsequently selfed to raise F3 progeny rows. A mapping population of 114 F2 plants was used. The female parent 234A is sterile whereas the male parent RHA 272 is fertile.

Phenotypic Classification. The F2 individuals were classified as sterile and fertile based on the presence of pollen and stainability of pollen. Among 109 individuals observed, 26 and 83 were sterile and fertile, respectively. Seeds from each fertile F2 were planted to establish 50 plants for each F3 progeny row in Rabi (winter) 2001, in order to identify homozygous F2 plants for dominant fertility restorer alleles. Progeny rows of an F3 population with 100 percent fertile plants were concluded to have come from F2 plants homozygous for fertility.

Leaf Collection and DNA Extraction. Leaves were harvested from 114 F2 plants in the field, freeze-dried and ground to powder. DNA extraction was performed according to the cetyltrimethylammonium bromide (CTAR) method (Hoisington et al., 1994).

DNA Bulk and Polymorphism Evaluation. Equal quantities of DNA were bulked from seven homozygous fertile and from eight homozygous sterile F2 plants according to the method suggested by Michelmore et al. (1991) to give two DNA bulks. The fertile and sterile bulks along with parents were screened with a total of 144 random primers to identify the polymorphic marker which is present in the fertile parent, as well as the fertile bulk and not in the sterile parent and sterile bulk. The linkage of the polymorphic marker is confirmed using the individual segregating population from which the bulks were generated.

RAPD Analysis. Genomic DNA was used as a template for PCR amplification as described by Williams et al. (1990). A set of 144 arbitrary primers (OPERON Technologies, Inc. California, USA) was used (Table 1).

Table 1. List of random primers used in the parental survey.

Primer series	Name of the primer	Total no. of primer
OPA	OPA 01, OPA 02, OPA 03, OPA 04, OPA 05, OPA 06, OPA 11, OPA 12, OPA 13, OPA 14, OPA 15, OPA 16	12
OPF	OPF 04, OPF 10, OPF 15, OPF 18	4
OPD	OPD 10, OPD 20	2
OPS	OPS 01, OPS 02, OPS 03, OPS 04, OPS 05, OPS 06, OPS 07, OPS 08, OPS 09, OPS 10, OPS 11, OPS 12, OPS 13, OPS 14, OPS 15, OPS 16, OPS 17, OPS 18, OPS 19, OPS 20	20
OPT	OPT 01, OPT 02, OPT 03, OPT 04, OPT 05, OPT 14, OPT 15, OPT 16, OPT 17, OPT 18, OPT 19, OPT 20	12
OPAB	OPAB 02, OPAB 03, OPAB 04 , OPAB 05, OPAB 06, OPAB 07, OPAB 08, OPAB 09, OPAB 10, OPAB 11, OPAB 12, OPAB 13, OPAB 14, OPAB 15, OPAB 16, OPAB 17, OPAB 18, OPAB 19, OPAB 20,	19
OPAK	OPAK 01, OPAK 03, OPAK 04, OPAK 05, OPAK 06, OPAK 07, OPAK 08, OPAK 09, OPAK 10, OPAK 11, OPAK 12, OPAK 13, OPAK 14, OPAK 15, OPAK 17, OPAK 18, OPAK 19, OPAK 20,	18
OPAL	OPAL 01, OPAL 02, OPAL 03, OPAL 04, OPAL 05, OPAL 06, OPAL 07, OPAL 08, OPAL 09, OPAL 10, OPAL 11, OPAL 12, OPAL 13, OPAL 14, OPAL 15, OPAL 17, OPAL 18, OPAL 19, OPAL 20	19
OPAM	OPAM 01, OPAM 02, OPAM 03, OPAM 04, OPAM 06, OPAM 07, OPAM 08, OPAM 09, OPAM 10, OPAM 11, OPAM 12, OPAM 13, OPAM 14, OPAM 15, OPAM 16, OPAM 17, OPAM 18, OPAM 19, OPAM 20,	19
OPAW	OPAW 01, OPAW 02, OPAW 03, OPAW 05, OPAW 06, OPAW 07, OPAW 08, OPAW 09, OPAW 10, OPAW 11, OPAW 12, OPAW 13, OPAW 14, OPAW 15, OPAW 16, OPAW 17, OPAW 18, OPAW 19, OPAW 20	19

Amplification reactions were in the volumes of 20 µl containing 10 mM *Tris* HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.001 per cent gelatin, dATP, dCTP, dGTP and dTTPs each at 0.1 mM, 0.2 mM primer, 25-30 ng of genomic DNA mol 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplification was performed with Thermal controller (MJ Research, Inc.) programmed for 40 cycles. After initial denaturation for two minutes at 94C, each cycle consisted of one minute at 94C, one minute at 36C and two minutes at 72C. The 40 cycles were followed by seven minutes of final extension at 72C. PCR-amplified products were subjected to electrophoresis on a 1.5 percent agarose gel in 1X TBE buffer at 120 V for 4 hours using a Hoefer Super Submarine electrophoresis unit (Pharmacia Biotec, USA). The electronic images of ethidium bromide stained gels were captured using a Kodak Digital Science DC 120 Digital Camera (Eastern Kodak Company, Rochester, USA) and the gels were documented using an Electrophoresis Documentation and Analysis System (EDAS 120).

Results and Discussion

Commercial exploitation of cytoplasmic male sterility depends on the availability of good restorers. The restorer should possess effective restorer genes for complete restoration of fertility in the CMS line. Knowledge of genetic control of male fertility restoration is useful for transferring fertility restoring genes to promising breeding lines. In the present study, genetics of fertility restoration was studied by crossing CMS 234A with the restorer RHA 272. The F2 population of 109 segregated into 83 fertile and 26 sterile. This segregation ratio fits well with the expected ratio of 3 fertile: 1 sterile and thus it confirms that the restoration was controlled by a single dominant gene. These results confirmed the earlier findings of Horn and Friedt (1997), Jan (2000), and Seiler (2000).

Tight linkage of a marker to a gene can be exploited for indirect selection of traits. In crops where the seed is used as the economic part, the exploitation of a CGMS system is required for the restoration of the CGMS line. Hence, tagging of a fertility restoring gene with a molecular marker will help in the screening of genotypes for the presence of fertility restorer genes. It will also help in transfer of fertility restorer genes to the inbreds if they lack the restoration genes.

RAPD markers have the advantages of cost effectiveness, technical simplicity and non-requirement of sequence information of template DNA (Welsh and McClelland, 1990). In this study, bulked segregant analysis (Michelmore et al., 1991) was followed to identify the markers linked to fertility restoration in the F2 populations of CMS 234A x RHA 272. This approach provides information simultaneously on polymorphism of parents and possible linkage between the marker and targeted gene using only the parents and extreme genotype bulks, thereby reducing the cost and workload by several fold.

A total of 144 random oligonucleotide primers were surveyed for potential polymorphism between the DNA bulks of sterile and fertile lines and their parents. The primer OPAM 06 was found to produce putative markers, which differentiated fertile parent and fertile bulk from sterile parent and sterile bulks. The cosegregation analysis of the putative marker in the F2 population confirmed the association of OPAM 06-1800 produced by the primer OPAM 06 with the fertility restoration gene (Figure 1).

The results obtained from this study proved that RAPD analysis in combination with bulk segregant analysis of an F2 population provides a highly efficient strategy to tag the gene of interest i.e., fertility restoration. This was already reported in a barley leaf rust resistant gene by Borovkova et al. (1997); R1H gene restoring male fertility in *Beta vulgaris* L. by Laporte et al. (1998), in pepper by Zhang et al. (2000), and in *Secale cereale* L. by Miedaner et al. (2000).

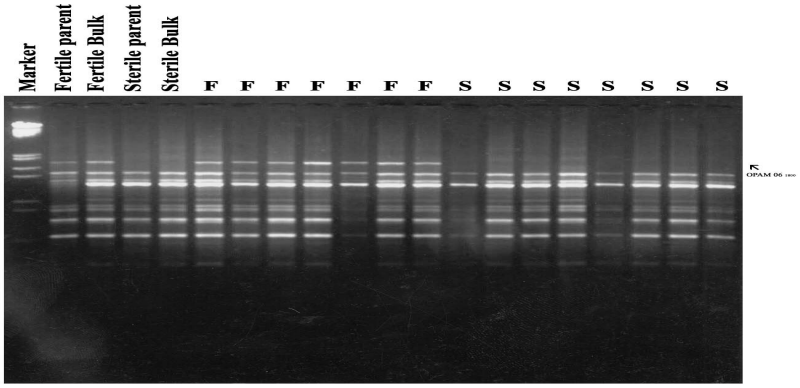

Plate 4. Cosegregation of RAPD marker OPAM 06 $_{1800}$ with fertility restoration gene in F_2 individuals of 234 A x RHA 272

Figure 1. Cosegregation of RAPD marker OPAM 06-1800 with fertility restoration gene in F2 individuals of CMS 234A x RHA 272.

A major problem associated with RAPD technology is the reproducibility of the profiles, which has been the subject of considerable debate among the various investigators. To ameliorate the utility of RAPDs, Sequence Characterized Amplification Regions (SCARs) that have greater reliability than simple RAPDs (Kesseli et al., 1992; Paran and Michelmore, 1993) were developed. Thus, OPAM 06-1800 should be converted to SCAR for increasing reproducibility. This may help in monitoring the fertility restoring gene transfer in sunflower breeding by early screening of the genotypes.

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