IDENTIFICATION OF RAPD MARKER LINKED TO A FERTILITY RESTORER GENE FOR PET-1 CYTOPLASM OF SUNFLOWER (Helianthus annuus L.)

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SUMMARY

Fertility restoration ability of the 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 oligo nucleotide primers were surveyed. The primer OPAM 06₁₈₀₀ was found to produce putative markers, which differentiate parent and bulk from sterile parent and sterile bulk. The co-segregation analysis of the putative marker on the F₂ population confirmed the association of OPAM 06₁₈₀₀ produced by the primer OPAM 06 with the fertility restoration gene. This will help in transfering the fertility restorer gene to the inbreds lacking restoration genes.

Key words: sunflower, fertility restorer gene, RAPD marker

INTRODUCTION

Sunflower 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 program. Modern sunflower breeding began with development of F_1 hybrids after the discovery of cytoplasmic male sterility (Leclercq, 1970) and fertility restorer genes (Kinmann, 1970). The first reliable cytoplasmic male sterile source was isolated by Leclercq (1969) from the interspecific cross *Helianthus petiolaris* Nutt. × *Helianthus annuus* and designated as PET-1 cytoplasm (Serieys, 1987). The restoration of pollen fertility in PET-1 cytoplasm has been reported to be controlled by a single dominant gene (Enns, 1972; Leclercq, 1972; Jan, 2000; Seiler, 2000). In some cases restoration control by two independent complementary dominant genes is also

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reported (Dominguez-Gimenez and Fick, 1975; Horn and Friedt, 1977; Jan *et al.*, 2000).

The development of new fertility restorer by traditional back crossing is costly and time consuming because it requires extensive crossing. Hence, identification of molecular markers closely linked to fertility restoring genes facilitates the breeding of new restorer lines, by reducing the time requirement. Recently, random amplified polymorphic DNA (RAPD) markers have been developed (Wiliams et al., 1990; Welsh and McClelland, 1990). RAPDs are generated by amplification of genomic DNA using a single primer of arbitrary nucleotide sequence to drive the amplification reaction. Several research groups are now using RAPD 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 marker linked to *Rf* genes have been identified in some important crops, such as rape seed (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 marker closely linked to major fertility restoring gene and other fertility related will facilitate the breeding of new restorer lines used in sunflower *cms* system. Here we report on the identification of RAPD markers associated with fertility gene for PET-1 cytoplasm of sunflower using the bulked segregant analysis approach.

MATERIAL AND METHODS

Plant material

Parents of commercial hybrid TCSH 1, *cms* 234 A × RHA 272, were crossed. The F_1 and F_2 generations were subsequently selfed to raise F_3 progeny rows. A mapping populations of 114 F_2 plants were used. The female parent 234 A is sterile whereas the male parent RHA 272 is fertile.

Phenotypic classification of male fertile and sterile plants

The F_2 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 F_2 were planted to establish 50 plants for each F_3 progeny row in Rabi 2001, in order to identify F_2 plants homozygous for dominant fertility restorer allele. Progeny rows of F_3 population with 100% fertile plants were concluded to have come from F_2 plants homozygous for fertility.

Leaf collection and DNA extraction

Leaves were harvested from 114 F_2 plants in the field conditions, freeze-dried and ground to powder. DNA extraction was performed according to the cetyl-trime-thyl-ammonium bromide (CTAB) method (Hoisington *et al.*, 1994).

Constitution of DNA bulk and evaluation of polymorphism

Equal quantities of DNA were bulked from seven homozygous fertile and from eight homozygous sterile F_2 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 114 random primers to identify the polymorphic marker which is present in fertile parent as well as fertile bulk and not in the sterile parent and sterile bulk. The linkage of the polymorphic marker is confirmed using individual segregating population from which the bulks were generated.

RAPD analysis

Genomic DNA was used as template for PCR amplification as described by Williams et al. (1990). A set of 114 arbitrary primers (OPERON Technologies, Inc. California, USA) was used. Amplification reactions were in the volumes of 20 μ l containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% 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 94°C, each cycle consisted of one minute at 94° C, one minute at 36° C and two minutes at 72° C. The 40 cycles were followed by seven minutes of final extension at 72°C. PCR amplified products to electrophoresis on 1.5% agarose gel in $1 \times \text{TBE}$ buffer at 120 V for 4 hours using Hoefer Super Submarine electrophoresis unit (Pharmacia Biotec, USA). The electronic images of ethidium bromide stained gels were captured using Kodak Digital Science DC 120 digital camera (Eastern Kodak Company, Rochester, USA) and the gels were documented using Electrophoresis Documentation and Analysis System (EDAS 12).

No.	Primer series	Name of the primer	Total number of primer
1.	OPA series	OPA 01, OPA 02, OPA 03, OPA 04, OPA 05,	12
		OPA 06, OPA 11, OPA 12, OPA 13, OPA 14,	
		OPA 15, OPA 16	
2.	OPF series	OPF 04, OPF 10, OPF 15, OPF 18	4
3.	OPD series	OPD 10, OPD 20	2
4.	OPS series	OPS 01, OPS 02, OPS 03, OPS 04, OPS 05, OPS 06,	20
		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	
5.	OPT series	OPT 11, OPT 02, OPT 03, OPT 04, OPT 05, OPT 14,	12
		OPT 15, OPT 16, OPT 17, OPT 18, OPT 19, OPT 20	
6.	OPAB series	OPAB 02, OPAB 03, OPAB 04, OPAB 05, OPAB 06,	19
		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	
7.	OPAK series	OPAK 01, OPAK 03, OPAK 04, OPAK 05, OPAK 06,	18
		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	
8.	OPAL series	OPAL 01, OPAL 02, OPAL 03, OPAL 04, OPAL 05,	19
		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	
9.	OPAM series	OPAM 01, OPAM 02, OPAM 03, OPAM 04, OPAM 06,	19
		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	
10.	OPAW series	OPAW 01, OPAW 02, OPAW 03, OPAW 05,	19
		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	

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

RESULTS AND DISCUSSION

Commercial exploitation of cytoplasmic male sterility depends on availability of good restorers. The restorer should posses effective restorer genes for complete restoration of fertility in CMS line. Knowledge on 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 line 234 A with restorer line RHA 272. The F_2 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 single dominant gene. The 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 economic part, exploitation of a *cgms* system is required for the restoration of the *cgms* line. Hence, tagging of fertility restoring gene with molecular marker will help in the screening of genotypes for the presence of fertility restoring genes. It will also help in transfer of fertility restorer gene to the inbreds if it lacks the restoration genes.

RAPD markers have the advantages of cost effectiveness, technical simplicity and non-requirement of sequence information of template DNA (Welsh and McChelland, 1990). In this study, bulked segregant analysis (Michelmore *et al.*, 1991) was followed to identify the markers linked to fertility restoration in the F₂ population of *cms* 234 A × 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 severalfold.



Figure 1: Cosegregation of RAPD marker OPAM 06₁₈₀₀ with fertility restoration gene in F₂ individuals of cms 234 A × RHA 272

A total of 114 random oligonuclotide primers were surveyed for potentional 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 co-segregation analysis of the putative marker on the F_2 population confirmed the association of OPAM 06₁₈₀₀ produced by the primer OPAM 06 with the fertility restoration gene (Figure 1).

The results obtained from this study proved that RAPD analysis is combination with bulk segregant analysis of F_2 population provides a highly efficient strategy to tag the gene of interest *i.e.*, fertility restoration. This was already reported in barley leaf rust resistant gene by Borokova *et al.* (1997), *R1H* gene restoring male fertility in *Beta vulgaris* by Laporte *et al.* (1998), in pepper by Zhang *et al.* (2000), in *Secale cereale* by Miedaner *et al.* (2000).

A major problem associated with RAPD technology is the reproducibility of the profiles and it 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 SCARs for increasing reproducibility. This may help in monitoring the fertility restoring gene transfer in sunflower breeding by early screening of the genotypes.

REFERENCES

- Borner, A., Korzun, V., Polley, A., Malyshev, S. and Melz, G., 1998. Genetics and molecular mapping of male fertility restoration locus (*Rfg 1*) in rye (*Secale cereale* L.). Theor. Appl. Genet. 97: 99-102.
- Damodaran, T. and Hedge, D.M., 2002. Various issues of area and production of principle crops of India, GOI, agricultural statistics at a glance. Oilseed situation: A statistical compendium. ICAR, Directorate of Oilseed Research, India, pp.145.
- Delourme, R., Boucherear, A., Hubert, N., Renard, M. and Landey, B.S., 1994. Identification of RAPD markers linked to a fertility restoring gene for the Ogura raddish cytoplasmic male sterility. Theor. Appl. Genet. 88: 741-748.
- Dominguez-Gimenez, J and Fick, G.N., 1975. Fertility restoration of male sterile cytoplasm in wild sunflowers. Crop Sci. 15: 724-726.
- Enns, H., 1972. New advances in hybrid sunflower. Canada Agriculture 17(4): 11.
- Horn, R. and Friedt, W., 1997. Fertility restoration of new CMS sources in sunflower (Helianthus annuus L.). Plant Breeding 116: 317-322.
- Jan, C.C., 2000. Cytoplasmic male sterility in PI 406647, PI 413024 and PI 413158 and the inheritance of fertility restoration. In: Seiler, G.J. (ed.), Proc. European Cooperative Network on Sunflower, Udine, Italy, Nov. 2-4. 2001, pp. 49-51.
 Jan, C.C., Zhang, T.X., Miller, J.F. and Fick, G.N., 2000. Inheritance of fertility restoration of
- Jan, C.C., Zhang, T.X., Miller, J.F. and Fick, G.N., 2000. Inheritance of fertility restoration of two cytoplasmic male sterility sources of *Helianthus pauciflorus rigidus* Nutt. Crop Sci. November issue, 2002. (In press).
- Kinman, M.L., 1970. New development in the USDA and state experimental station sunflower breeding programmes. In: Proceedings of 4th International Sunflower Conference, Memphis, TN, June 23.25, pp. 181-183.
- Leclercq, P, 1970. Sunflower hybrid using male sterility. Proc. of Fourth Int. Sunflower Conf., Memphis, Tennessee, USA, pp. 123-126.
- Leclercq, P, 1972. La sterile male cytoplasmique de tournesol. I Premieres etudes sur la restauratin de la fertile. Ann. Amelion. Pl. Annales de l'Amelioration de Plantes, 21(1): 45-54.
- Martin, G.B., Williams, J.G.K., Tanksley, S.D., 1991. Rapid identification for markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. Proc. Nat. Acad. Sci., USA, 88: 2336-2340.
- Michelmore, R.W., Paran, I. and Kesseli, R.V., (1991). Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Nat. Acad. Sci., USA, 88:9828-9832.

Penner, G.A., Chong, J., Levesque-Lemay, M., Molnar, S.J., Fedak, G. (1993). Identification of a RAPD marker linked to the oat-stem rust gene Pg3. Theor. Appl. Genet. 85: 702-705.

- Seiler, G.J., 2000. A new cytoplasmic male sterile source from wild *Helianthus annuus* from Texas. FAO progress Report 1999-2000. In: Selier, G.J. (ed.), Proc. of European Cooperative Network on Sunflower. Udine, Italy, Nov. 2-4. 2001, pp. 68-69.
- Serieys, H.A., 1987. Genetic evaluation and use of *Helianthus* wild species and their use in breeding programme. FAO Subnetwork Report, 1984-1986, pp. 1-23.
- Welsh, J., McClelland, M., (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213-7218.
- Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.
- Zhang, G., Bharaj, T.S., Lu, Y., Virmani, S.S. and Huang, N., 1997. Mapping of the Rf-3 nuclear fertility restoring gene for WA cytoplasmic male sterility using RAPD and RFLP marker. Theor. Appl Genet. 94: 27-33.

IDENTIFICACIÓN DEL MARCADOR RAPD VINCULADO CON EL GEN DE RESTAURACIÓN DE FERTILIDAD PARA EL CITOPLASMA PET-1 DE GIRASOL (*Helianthus annuus* L.)

RESUMEN

La capacidad de restauración de fertilidad del restaurador investigado, se ha controlado por parte de un gen dominante. Aplicando el Bulked segregant analysis (BSA) se identificaron los marcadores moleculares vinculados con un gen restaurador importante (*Rf*) utilizando F₂ de la populación del cruzamiento *cms* 234 × RHA 272. Se analizaron en total 144 primers (oligonucleótidos iniciadores) elegidos al azar. Los resultados demostraron que el primer OPAM 06_{1800} había producido marcadores putativos, que hacen la diferenciación de uno de los padres fértil y la muestra de grupo de uno de los padres estéril y de su muestra de grupo. El análisis de cosegregación del marcador putativo en la populación F₂ confirmó el vínculo del primer OPAM 06_{1800} producido por parte del primer OPAm 06, con el gen de restauración de fertilidad investigado. Esto ayudará en la transferencia del gen de restauración de fertilidad en las líneas inbred que no poseen genes de restauración.

IDENTIFICATION DE MARQUEURS RAPD LIÉS AU GÈNE DE RESTAURATION DE FERTILITÉ POUR LE CYTOPLASME PET-1 DE TOURNESOL (Helianthus annuus L.)

RÉSUMÉ

La capacité de restauration de fertilité du restaurateur a été contrôlée par un seul gène dominant. L'analyse "Bulked segregant" (BSA) a été utilisée pour identifier les marqueurs moléculaires liés à un restaurateur important (*Rf*) au moyen d'une population F_2 de *cms* 234 × RHA 272. Un total de 144 "primers" oligo nucléotides ont été analysés. Les résultats ont montré que le "primer" OPAM 06₁₈₀₀ avait produit des marqueurs putatifs qui différenciaient le parent fertile et l'échantillon du groupe du parent stérile du parent stérile et de l'échantillon de son groupe. L'analyse co-ségrégationnelle du marqueur putatif de la population F_2 a confirmé le lien du "primer" OPAM 06₁₈₀₀ produit par le "primer" OPAM 06 avec le gène examiné pour la restauration de fertilité. Ceci aidera lors du transfert du gène restaurateur de fertilité aux lignes inbred qui ne possèdent pas de gène de restauration.