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MAPPING OF THE RESTORER GENE *Rf1* IN SUNFLOWER (*Helianthus annuus* L.)

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SUMMARY

In sunflower, commercial hybrid breeding is based on a single CMS-inducing cytoplasm, the so-called PET1 cytoplasm. The introduction of one dominant, nuclear-encoded restorer gene (*Rf1*) is in most cases sufficient for fertility restoration. Little has been learned so far about the mode of action of the restorer gene *Rf1*. For map-based cloning of the restorer gene *Rf1*, an F_2 population of the cross RHA325 (cms) x HA342 has been used. The χ^2-test confirmed segregation for one dominant gene which corresponds to *Rf1*. For the AFLP analyses 256 *Eco*RI/*Mse*I primer combinations have been used so far. In addition, RAPD analyses were performed using 1,200 decamer primers. Twenty-three primers had polymorphic amplification products, differentiating the bulks, and could therefore be mapped. The hybridization of the marker HP4 against a BAC library resulted in three positive clones. The overlapping end of the smallest clone was used to get a new hybridization against the BAC library.

Key words: ***Helianthus annuus*, cytoplasmic male sterility, restorer gene**

INTRODUCTION

Hybrid breeding leads to higher crop yield by exploiting heterosis effects, i.e. hybrid vigor in specific cross combinations, compared with lines and population varieties. In sunflower, commercial hybrids are based on a single source of cytoplasmic male sterility (CMS), the so-called PET1 cytoplasm. For fertility restoration, the introduction of one dominant, nuclear-encoded restorer gene (*Rf1*) is in most cases sufficient. However, little is known about the mode of action of the gene *Rf1*. Isolation of the gene by map-based cloning will allow to elucidate its role. Saturation with markers of the region, containing the *Rf1* gene, is necessary to obtain such markers which are linked closely enough to the gene to allow the identification of BAC clones, containing the gene of interest or to build up a contig.

MATERIALS AND METHODS

Plant material

The F_2-population RHA325 (CMS) x HA342 segregating for the *Rf1*-gene was used for mapping. Segregation for one dominant gene corresponding to *Rf1* was confirmed by χ_c-test. F_2 plants were identified to be homozygous and heterozygous for the *Rf1*-locus by screening the segregation ratios in the F_3 and F_2BC_1-generations, respectively.

AFLP and RAPD analyses

Bulked-segregant analyses (Michelmore *et al.*, 1991) were carried out applying the AFLP (Vos *et al.*, 1995) and RAPD techniques to identify molecular markers closely linked to the *Rf1*-gene in the F_2 population RHA325 (CMS) x HA342. Bulks of 10 homozygous fertile and 10 homozygous male sterile individuals were screened.

STS development

Markers were excised from agarose gels (1 x TAC) and recovered using a DNA Purification Kit (Amersham, Piscataway NJ, USA). The fragments were ligated in a T/A-vector (pCr2.1, TOPO-TA cloning kit, Invitrogen, San Diego CA, USA) and cloned. Sequences of cloned fragments were used to develop STS markers.

BAC library

The BAC-library was constructed from the restorer line RHA325 using pBeloBAC11 as vector. High molecular weight DNA (HMW-DNA) was obtained by nuclei preparations. High-density BAC filters were prepared with a 5x5 pattern.

Hybridization against the BAC library

Probes were labeled radioactively with α^{32}P-dATP and hybridized for 18 to 36 h at 65°C in hybridization buffer. The filters were washed with 2 x SSC and 0.1% SDS and 0.5 x SSC and 0.1% SDS between 5 to 10 min each time. Afterwards, the filters were exposed for 72 h or longer to Kodak films.

RESULTS AND DISCUSSION

Segregation analyses of fertility restoration

In the F_2 and F_3 populations of the cross RHA325 x HA342 evaluated for segregation of male fertility and sterility, only two phenotypes were observed: male-fertile plants showing normal anthers and producing large amounts of yellow pollen, and male-sterile plants showing very small anthers with no pollen (Table 1.). Segregation ratio of 1 (male fertile, *Rf1Rf1*) : 2 (male fertile, *Rf1rf1*) : 1 (male-sterile, *rf1rf1*)

was confirmed by analysis of F_3 and BC_1 generations in the F_2 population as expected for one restorer gene.

Table 1: Segregation for fertility restoration in the F_2 population of the cross RHA325 (CMS) x HA 342

Cross	Segregation in F_2		χ^2	P
	expected	observed		(df = 2)
RHA325 (CMS) x HA 342	1 : 2 : 1	384 : 785 : 402	0.41	0.8

AFLP and RAPD analyses

Bulked segregant analyses were performed based on AFLP data using 256 *Eco*RI/*Mse*I primer combinations. Forty-nine polymorph primer combinations could be identified, 1 to 4 markers were mapped per primer combination. Five AFLP markers mapped within 1.3 cM genetic distance to the restorer gene.

RAPD analyses were performed using 1,200 decamer primers. Twenty-three primers resulted in polymorphic amplification products differentiating the bulks. Three RAPD marker mapped within 2 cM of the gene of interest (2 in attraction [Y10-750, HP4-450] and 1 in repulsion [H13-337]) (Figure 1) (Horn *et al.*, 2001, 2000b).

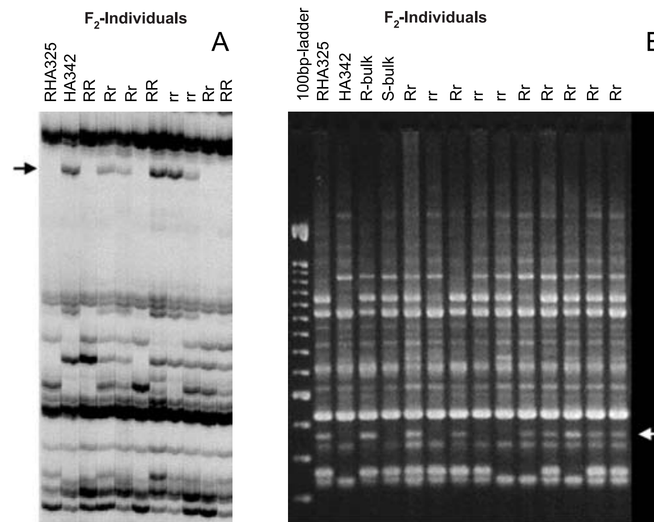

Figure 1: ***A.*** *AFLP analyses using the primer combination E46M60. Segregation in the F_2 population is shown. The marker E46M60-300 is marked by an arrowhead.* ***B.*** *RAPD analyses using the primer OP-HP4 for amplification. Segregation of the marker OP-HP4-450 in the F_2 population is shown. The marker OP-HP4-450 is marked by an arrowhead.*

A linkage map was constructed using JOINMAP. Map distances in centi Morgans (cM) were calculated from recombination frequencies using the Kosambi (1944) function (Figure 2).

Development of STS markers

Two RAPD markers, OP-Y10-750 and OP-HP4-450, were excised from agarose gels, sequenced and successfully converted into the STS markers STSY10-750 and STSHP4-426. The marker STSY10-750 showed a band at 750 bp in the parental line RHA325, the F_1-hybrid, the two R-bulks (fertility restored) and for each individual of the R-bulks, but it was absent in HA342 and the two S-bulks (male-sterile). The marker STSHP4-426 had a polymorph band at 426 bp in the line RHA325 and no band in HA342. In addition, the AFLP-markers E32M60-179 and E41M48-113 were cloned, but the developed primers only resulted in monomorphic amplification patterns (Horn *et al.*, 2000a). However, these cloned markers can be used to screen the BAC library by colony hybridization.

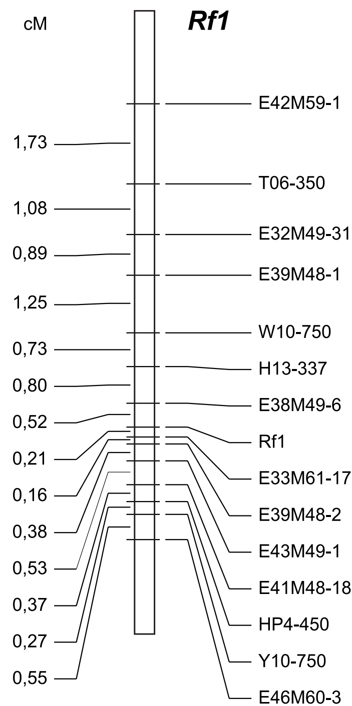

Figure 2: Map of markers linked to the restorer gene Rf1 in sunflower

Marker-assisted selection (MAS) using RAPD markers is effective because a large number of samples can be handled with easy manipulation at the same time. However, there are several problems associated with the reliability and reproducibility of RAPD markers. The use of STS markers developed from RAPD markers is effective in overcoming these problems and improves their utilization for MAS. Co-dominant STS-markers are especially useful because they also allow the identification of heterozygous progeny (Matsui *et al.*, 2001).

DNA fingerprinting of positive BAC clones and contig development

The sunflower library consists of 104,736 clones. Characterizati-on of the BAC clones by *Not*I digest revealed an average insert size of 50 kb with sizes ranging from 30 to 270 kb. Assuming an average size of 50 kb and an estimated haploid genome size of 3,000 Mb of sunflower (Arumuganathan and Earle, 1991), the sunflower BAC library represents about two haploid genome equivalents (Özdemir *et al.*, 2000). The clones were spotted in duplicate on high-density filters (22 cm x 22 cm). Hybridization of the marker HP4 against the BAC-filters identified three positive clones (Figure 3). The positive clones were digested with different restriction enymes, e.g. *Hind*III, *Dra*I, *Bam*HI, *Sma*I. Two clones have the same insert size of 73 kb and the same restriction enzymes pattern. The third clone overlaps with the other two clones and has a size of 24.5 kb. This clone was subcloned into pUC18 using *Bam*HI and *Hind*III fragments to get the ends of the BAC clones. With the

overlapping end a new hybridization against the BAC filters was performed to identify additional clones and to build up a contig around the restorer locus *Rf1*.

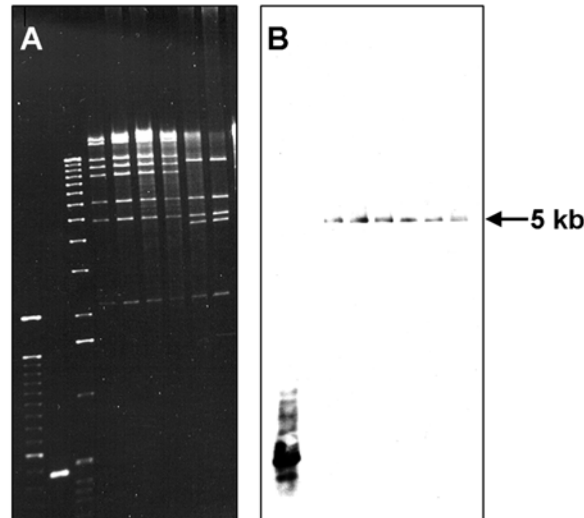

Figure 3: ***A.*** *DNA fingerprinting of positive BAC clones. Each clone is represented by two lanes and was digested with the restriction enzyme SmaI.*
B. *Hybridization with the marker STSHP4-426*

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CREACION DE MAPA DEL GEN RESTAURANTE *Rf1* EN EL GIRASOL (*Helianthus annuus* L.)

RESUMEN

El mejoramiento comercial por hibridación en girasol, está basado en una inducción de citoplasma CMS simple, llamado citoplasma PET1. La introducción de un gen restaurador dominante (*Rf1*) con codificación nuclear, es en la mayoría de los casos suficiente para restaurar la fertilidad. Hasta ahora se conoce poco acerca del modo de acción de este gen restaurador *Rf1*. Para la clonación basada en marcadores del gen restaurador *Rf1* se uso la población F_2 proveniente del cruzamiento de las lineas RHA325 (CMS) x HA342. La prueba χ^2 confirmó la segregación de un gen dominante, el cual corresponde a *Rf1*. Para el análisis de AFLP se usaron 256 combinaciones de primer *EcoR1/MseI.* Para el análisis de RAPD se usaron 1200 decamer primers. Veintitres primers presentaron productos de amplificación polimorficos en los bulks, permitiendo su mapeo. De la hibridación del marcador HP4 con la BAC-genoteca resultaron tres clones positivos. El sobrecruzamiento final del clon más pequeno, se uso para obtener una nueva hibridación con la BAC-genoteca.

CARTOGRAPHIE DU GÈNE *Rf1* RESTAURATEUR DE FERTILITÉ CHEZ LE TOURNESOL (*Helianthus annuus* L.)

RÉSUMÉ

Chez le tournesol, l'amélioration des hybrides commerciaux est basée sue l'induction unique CMS du cytoplasme, ce dernier étant appelé cytoplasme PET1. L'introgression du gène *Rf1* dans le génome est dans la plupart des cas suffisante pour la restauration de la fertilité. Le mode d'action du gène *Rf1* restaurateur de fertilité est peu connu. Afin de réaliser un clonage positionnel de ce gène, une population F_2 dérivée du croisement RHA325 (CMS) x HA342 a été untilisée. Le test χ^2 a permis la confirmation de la ségrégation pour un gène dominant qui correspond á *Rf1*. L'analyse AFLP a été menée à terme au moyen de 256 combinaisons d'amorces *Eco*RI/*Mse*I. Par ailleurs, des analyses RAPD ont été effectuées en utilisant 1200 amorces décamères. Vingt trois amorces ont engendré des produits d'amplification polymorphiques différenciant les bulks. Leur cartographie a été ensuite mise en œuvre. L'hybridation du marqueur HP4 contre la banque d'ADN type BAC a donné trois clones positifs. La terminaison chevauchante du clone le plus court a été utilisée pou l'obtention d'une nouvelle hybridation contre la banque BAC.