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CONSTRUCTION OF A GENETIC MAP AND LOCALIZATION OF MAJOR TRAITS IN SUNFLOWER (*Helianthus annuus* L.)

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SUMMARY

We have developed a segregating mapping population from the cross RHA325(*cms*) × HA342 comprising 183 F_2 individuals. RHA325 is an open American inbred line based on the PET1 cytoplasm while HA342 is a maintainer line for this cytoplasm. The F_2 and derived populations segregate for male fertility vs. sterility, downy mildew (*Plasmopara halstedii*) resistance (Pl_2) vs. susceptibility and oleic vs. linoleic acid content.

A genetic map was developed covering 1751.5 cM with 202 AFLP and 19 SSR markers in 18 linkage groups. Thirteen linkage groups contain one or more SSR markers and are numbered according to Tang *et al*. (2002).

A segregation ratio of 1 (male fertile, *Rf1Rf1*) : 2 (male fertile, *Rf1rf1*) : 1 (male sterile, *rf1rf1*) was observed in the F_2 population as expected for one segregating restorer gene (χ^2=2.83, P=0.24). In F_3, 14 progeny plants of each fertile F_2 individual were evaluated for male fertility to distinguish between F_2 plants being homozygous or heterozygous for the restorer gene. The data obtained from the F_3 progenies were confirmed by segregation analyses in an F_2BC_1 population. The *Rf1* locus was shown to be located on linkage group 13, containing the SSR markers ORS388 and ORS1030.

Using a whole-seedling-immersion test (Gulya *et al*., 1991) applied to F_3 progeny plants we found that the F_2 population RHA325(*cms*) × HA342 segregated for *Plasmopara* reaction at a ratio of 1 (Pl_2Pl_2) :2 (Pl_2pl_2) :1 (pl_2pl_2) ;χ^2=0.83 (P=0.65). The Pl_2 gene was demonstrated to be located on linkage group 8 together with the SSR marker ORS599.

Furthermore, quantitative trait loci for oleic vs. linoleic acid contents (LOD >3) could be localized on linkage group A. Future work will concentrate on marker saturation of the genetic map.

Key words: **cytoplasmic male sterility, genomics, hybrid breeding, molecular markers, mapping, *Plasmopara halstedii*, oleic acid, QTL**

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INTRODUCTION

Sunflower (*Helianthus annuus* L.) is grown around the world mostly as a source of vegetable oil and proteins. The main objective of sunflower breeding is the development of productive F_1 hybrid cultivars with high oil yield.

The importance of molecular markers in sunflower genetic analysis was demonstrated by several research works. The AFLP technique (amplified fragment length polymorphism) is considered an efficient marker system due to its high multiple applicability e.g. genetic mapping, fingerprinting, diversity analysis. Hongtrakul *et al.* (1997) showed that AFLP is a powerful tool for genetic fingerprinting in sunflower. This technique was previously used in the establishment of several genetic maps in crop species, like rice (Mackill *et al.*, 1996), maize (Castiglioni *et al.*, 1999) and more recently sunflower (Flores Berrios *et al.*, 2000, Rachid Al-Chaarani *et al.*, 2001).

Simple sequence repeats (SSR), also called microsatellites, are widely used as molecular markers. Their polymorphism has shown high efficiency and they are used for genetic mapping, population and evolutionary studies, as well as for fingerprinting and pedigree analyses.

The rapid and recent development of molecular marker technology has largely contributed to the establishment of saturated molecular maps which have enabled the mapping of quantitative traits and the dissection and localization of genetic factors underlying quantitative traits, with the final goal of using QTL-marker associations in marker-assisted selection. Many studies have been conducted in crops to identify QTL for important developmental and agronomic traits such as resistance to downy mildew and black stem (Rachid Al-Chaarani *et al.*, 2001).

In this paper, we present a new genetic map of sunflower, constructed with both AFLP and microsatellite markers, based on a population of 183 F_2 plants. QTL for oleic acid vs. linoleic acid were identified.

MATERIALS AND METHODS

Plant material

The F_2 population RHA325(*cms*) × HA342 segregating for the *Rf1-*, Pl_2 gene and oleic acid contents was used for genetic mapping. RHA325 is a restorer line carrying the PET1 cytoplasm; HA342 is a high-oleic maintainer line of the PET1 cytoplasm. F_2, F_3 and F_2BC_1 populations were grown in the field of Gross-Gerau near Frankfurt/Main (Germany). Leaf material was collected from F_2 individuals for DNA analyses and immediately frozen in liquid nitrogen and stored at -20°C.

Isolation of genomic DNA

Genomic DNA was isolated according to Doyle and Doyle (1990). In liquid nitrogen ground powder of 2.5 g leaf material was incubated with 15 ml extraction

buffer (100 mM Tris/HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% $Na_2S_2O_3$) at 65°C for 30 min. After chloroform extraction, the aqueous phase was obtained by centrifugation. The procedure was repeated and the DNA was finally precipitated in the aqueous phase by adding 1 ml ammonium acetate (10 M) and 1 ml sodium acetate (3 M pH 5.5) in addition to two-thirds volume 2-propanol at 4°C. High molecular weight DNA was transferred by a glass hook to a new tube and washed once with wash alcohol (70% ethanol, 10 mM ammonium acetate). DNA that was shortly dried was then solubilized in 1 ml TE (10 mM Tris/HCl pH=8.0, 1 mM EDTA).

AFLP- and SSR-analyses

AFLP analyses using *Eco*RI and *Mse*I primers were performed according to Vos *et al*. (1995). For the selective amplification *Eco*RI primers (500 ng) with three selective nucleotides were labeled using T4 polynucleotide kinase and [γ-^{33}P]-ATP. Labeling was performed for 1h at 37°C in 50 µl kinase buffer (70 mM Tris/HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 1 mM 2-mercaptoethanol). Heating the reaction mixture to 70°C for 10 min inactivated the enzyme. For the selective amplification 5 ng labeled *Eco*RI primer and 30.2 ng *Mse*I primer were used. Alternatively, IRD-labeled *Eco*RI primers from MWG Biotech (Ebersberg, Germany) were used for non-radioactive labeling of the selective amplification products and run on a LICOR (Licor, Bad Homburg).

SSR analyses using the primers from Steve Knapp's group (www.css.orst.edu/knapp-lab/sunflower/genomic_dna_sequence.htm). The PCR reaction needs 25 ng DNA, 2.75 pmol primer (for/rev) and the IRD labeled M13 primer, 1.0 mM dNTPs, 2.0 mM $MgCl_2$, 1% Tween, 1 × Buffer and 0.3 Units Taq, and run on a LICOR.

RESULTS AND DISCUSSION

Genetic map construction

A genetic map comprising 202 AFLP and 19 SSR markers in 18 linkage groups is presented in this paper. The groups range from 7.9 to 172.2 cM in size and have 2-34 markers. The total length of the map is 1645.6 cM which represents at least one maker for every 7.4 cM on average (Table 1). Using inbred lines and AFLP and SSR markers, Mokrani *et al*. (2002) constructed a genetic map of 2539 cM with at least one marker for every 14.9 cM on average. Using recombinant inbred lines and AFLP markers, Flores Berrios *et al*. (2000) constructed a genetic map of 2833.7 cM; updated in Rachid Al-Chaarani *et al*. (2001). Many factors as to the nature of the population studied, the number of individuals and the number of markers, might change the recombination rate and in consequence the distance between the genetic loci. Other genetic maps of sunflower were also developed using different

techniques such as RFLPs (Berry *et al.*, 1995; Gentzbittel *et al.*, 1995; Jan *et al.*, 1998; Gentzbittel *et al.*, 1999) and RAPDs (Rieseberg *et al.*, 1996).

Table 1: Genetic markers assigned to linkage groups

Linkage group	Number of AFLP-markers	Number of SSR-markers	Length (cM)	Average distance (cM)
LG1	16	1	124.2	7.3
LG2	14	1	117.4	7.8
LG3	5	1	38.3	6.4
LG4	11	2	59.0	4.5
LG5	32	2	167.6	
LG6	4	1	59.4	11.9
LG7	10	2	75.0	6.3
LG8	13	1	91.6	6.5
LG9				
LG10				
LG11	13	1	150.2	10.7
LG12	2	1	27.5	9.2
LG13	18	2	172.2	8.61
LG14				
LG15	14	1	134.6	8.9
LG16				
LG17	15	3	129.8	7.2
LG A	15		151.4	10,1
LG B	12		92.8	7.7
LG C	4		32.3	8.1
LG D	2		14.5	7.3
LG E	2		7.9	3.9
S	202	19	1645.6	7.4

Localization of the restorer gene

In the F_2 and F_3 populations of the cross RHA325(*cms*) × HA342 evaluated for the segregation of male fertility versus 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. A segregation ratio of 1 (male fertile, *Rf1Rf1*) : 2 (male fertile, *Rf1rf1*) : 1 (male sterile, *rf1rf1*) was observed in the F_2 population as expected for one restorer gene (χ^2=2.83, P=0.24). In F_3, 14 progeny plants of each fertile F_2 individual were evaluated for male fertility to distinguish between F_2 plants being homozygous or heterozygous for the restorer gene. The data obtained from the F_3 progenies were confirmed by segregation analysis in a F_2BC_1 population. 18 AFLP markers and two SSR markers mapped with the restorer gene in one linkage group. These two SSR markers, ORS388 and ORS1030, were mapped on linkage group 13 by Burke *et al.* (2002) and Tang *et al.* (2002). Fine mapping of the restorer locus has been carried out by

Kusterer *et al*. (2000, 2003) and Horn *et al*. (2002), aiming at the isolation of the restorer gene.

Mapping of *Plasmopara* resistance

Downy mildew of sunflower, incited by *Plasmopara halstedii* (Farl.) Berl. et de Toni., is a major sunflower disease. Using a whole-seedling-immersion test (Gulya *et al.*, 1991) we found that the F_2 population RHA325(*cms*) × HA342 segregated for *Plasmopara* reaction at a ratio of 1 (Pl_2Pl_2) : 2 (Pl_2pl_2) : 1 (pl_2pl_2); χ^2=0.83 (P=0.65). For the Pl_2 gene of cultivated sunflower, which confers resistance to downy mildew races 1 (100), 2 (300), 7 (330), and 9 (703), 14 AFLP markers and the SSR marker ORS599 were mapped together with the Pl_2 on linkage group 8.

Molecular mapping of genes Pl_1, Pl_2, and Pl_6 revealed the co-location of all three *Pl* genes (Bert *et al*., 2001, Bouzidi *et al*., 2002, Mouzeyar *et al.*, 1995, Roeckel-Drevet *et al*., 1996, Vear *et al*., 1997). Vear *et al.* (1997) showed that the Pl_6 locus consists of at least two very closely linked genes. They postulated that the Pl_6 gene is a complex locus, which includes several linked resistance genes. Since the Pl_2 gene and the Pl_6 locus are co-located, Vear *et al*. (1997) concluded that the Pl_2 locus is a smaller part of the complex locus Pl_6. Consequently, they supposed that the Pl_2 locus itself is a cluster of closely linked genes. Brahm *et al*. (2000) developed molecular markers for the Pl_2 gene of cultivated sunflower, which confers resistance to downy mildew races 1, 2, 7 and 9. The markers would facilitate marker assisted selection for downy mildew resistance in sunflower. Bouzidi *et al*. (2002) postulated that the Pl_6 locus contains at least 11 tightly linked genes each giving resistance to different downy mildew races. Cloning and sequence analysis of 13 markers, covering a genetic distance of about 3 cM centered around the Pl_6 locus, indicate that Pl_6 contains conserved genes belonging to the TIR-NBS-LRR class of plant resistance genes.

Characterization of QTL for oil quality

With the phenotypic data from the F_2 plants of (RHA325(*cms*) × HA342) regarding palmitic, stearic, oleic and linoleic acid we used the software PlabQTL (Utz *et al*., 1996) for QTL identification with the CIM procedure. For palmitic acid we found one QTL on linkage group A with a LOD of 3.75. This QTL explains 54.4% of the phenotypic variance. For stearic acid we did not find a QTL with the LOD ≥3.0. For oleic and linoleic acids we found two QTLs located at the same position on linkage group A: one with a LOD of 3.19 (3.31) for oleic (linoleic) acid and the other with LOD 3.79 (3.87). The two QTLs taken together explain 56% of the phenotypic variation (Table 2).

A stearoyl-ACP desaturase losuc (SAD17A), and an oleoyl-PC desaturase locus (OLD7) were found by Pérez-Vich *et al*. (2002) to co-segregate with the previously described genes *Es1* on linkage group1 and *Ol* on linkage group 14 controlling the high stearic and high oleic acid traits, respectively. The authors constructed a link-

age map with AFLP and RFLP markers, and the loci SAD17A and OLD7 were found to underlie the major QTLs affecting the concentrations of stearic and oleic acid, explaining around 80% and 56% of the phenotypic variance of these fatty acids.

Table 2: Map position and phenotypic effect of QTLs detected in a population of F_2 186 plants (RHA325 × HA342)

QTL	Linkage group	Position (cM)*	LOD	R^2 (%)
Palmitic acid	A	88	3.75	54.4
Oleic acid	A	54	3.19	28.4
	A	102	3.79	27.6
Linoleic acid	A	54	3.31	29.3
	A	102	3.87	28.1

* expressed in Kosambi cM from north of the linkage group
R^2: phenotypic variation explained by each QTL
LOD: log10 of the likelihoods odds radio (see Lander & Botstein, 1989)

A minor QTL affecting both stearic and oleic acid levels was identified on LG8. This QTL showed a significant epistatic interaction for oleic acid with the QTL at the OLD7 locus, and was hypothesized to be a modifier of *Ol*. These results provide strong support about the role of fatty acid desaturase genes in determining fatty acid composition in the seed oil of sunflower (Pérez-Vich *et al.*, 2002). The linkage group A in our map may correspond to the linkage group 14 of Pérez-Vich *et al.* (2002). To prove this expectation we are planning to map more SSR markers to this linkage group.

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CONSTRUCCIÓN DEL MAPA GENÉTICO Y UBICACIÓN DE LAS CARACTERÍSTICAS MÁS IMPORTANTES EN GIRASOL (*Helianthus annuus* L.)

RESUMEN

Hemos creado el mapa de segregación para la población del cruzamiento RHA325(*cms*) × HA342 que está compuesta de la planta 183 F_2. RHA325 es una línea consanguínea abierta americana, a base del citoplasma PET1, y HA342 es la línea mantenedora para este citoplasma. F_2 y las poblaciones derivadas, se segregan a la fertilidad / esterilidad masculina, la resistencia (Pl_2) / sensibilidad a tizón (*Plasmopara hastedii*) y el contenido del ácido oleico / linólico.

Se hizo el mapa genético que cubre 1751.5 cM con 202 marcadores AFLP y 19 marcadores SSR en 18 grupos de genes ligados. Trece grupos de genes ligados contienen uno o más marcadores SSR, y están numerados según Tang *et al*. (2002).

En la población F_2, se ha establecido la relación de segregación 1 (fértiles masculinamente, *Rf1Rf1*) : 2 (fértiles masculinamente, *Rf1rf1*) : 1 (estériles masculinamente, *rf1rf1*), lo que se espera para un gen restaurador segregante (χ^2=2.83, P=0.24). En la generación F_3, 14 plantas de la descendencia de cada planta fértil F_2 se evaluaron para la fertilidad masculina con el fin de distinguir las plantas F_2 homocogotas y heterocigotas, para el gen restaurador correspondiente. Los datos obtenidos de la herencia F_3 confirmó también el análisis segregante en la población F_2BC_1. Para el locus *Rf1* se determinó que se encuentra en el grupo de los genes ligados 13, que contiene los marcadores SSR, ORS388 y ORS1030.

Mediante el método de hundimiento de las plántulas enteras (Gulya *et al*. 1991), aplicado en las plantas de la descendencia F_3, hemos determinado que la población de cruzamiento F_2, RHA325(*cms*) × HA342 segrega para la reacción al tizón, en proporción 1 (Pl_2Pl_2) : 2 (Pl_2pl_2) : 1 (pl_2pl_2); χ^2=0.83 (P=0.65). Para el gen Pl_2 está determinado que se encuentra en el grupo de genes ligados ^{1} 8 junto con el marcador SSR, ORS599.

Los locus para las características cuantitativas para el contenido del ácido oleico / linólico (LOD >3) se han ubicado en el grupo de genes ligados A. El futuro trabajo será concentrado en la saturación marcadora del mapa genético.

CONSTRUCTION DE CARTES GÉNÉTIQUES ET LOCALISATION DE TRAITS PERTINENTS DU TOURNESOL (*Helianthus annuus* L.)

RESUME

De nouvelles cartes génétiques de ségrégation pour les populations du croisement RHA325(cms) × HA342 contenant 183 plantes de F_2 ont été créées. La ligne cultivée américaine RHA325 est ouverte, basée au cytoplasme PET1, tandis que la ligne HA342 est le maintien pour ce cytoplasme. La population F_2 et les populations dérivées sont les résultats de ségrégation vers la fertilité mâle vs. stérilité, résistance (Pl_2), sensibilité à la rouille (*Plasmopara hastedii)* et le contenu d'acides oléiques et linoléiques.

Une carte génétique a été faite couvrant 1751,5 de cM avec 202 de AFLP et 19 marqueurs de SSR dans 18 groupes de gènes liés. Treize groupes de gènes liés contiennent un ou plusieurs marqueurs de SSR, numérotés selon Tang et al. (2000). Dans la population F_2 une relation de ségrégation de 1 (mâle fertile, *Rf1Rf1*) : 2 (mâle fertile, *Rf1rf1*) : 1 (mâle stérile, *rf1rf1*) est observée, ce qui est attendu pour un gène restaurateur de ségrégation (χ^2=2,83, P=0,24). Dans la génération F_2 14 plantes des descendants de chaque plante fertile de F_2 sont évaluées pour mâle fertilité afin de distinguer les plantes homozygotes et hétérozygotes de F_2 concernant le gène restaurateur. Les données recueillies de la population de descendants F_3 sont confirmées par l'analyse de ségrégation dans la population F_2BC_1. Pour le locus *Rf1,* il est constaté d'être localisé dans le groupe de gènes liés Nr.13, contenant les marqueurs de SSR ORS388 et ORS1030.

Par la méthode d'immersion totale de semailles (Gulya *et al.*1991) appliquée aux plantes des descendants de F_3 le résultat de ségrégation a démontré une réaction à la rouille d'une relation 1 (Pl_2Pl_2) : 2 (Pl_2pl_2) : 1 (pl_2pl_2) ; χ^2=2,83, (P=0,24) dans la population F_2 du croisement RHA325(cms) × HA342. Pour le gène Pl_2 il est constaté d'être localisé dans le groupe de gènes liés N°8, contenant les marqueurs de SSR et ORS599.

Les locus pour les traits quantitatifs de contenu d'acides oléiques/ linoléiques (LOD>3) sont localisés dans le groupe A de gènes liés. Les recherches futures seront centrées sur la saturation de marqueurs de cartes génétiques.