Mapping a novel fertility restoration gene in sunflower

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ABSTRACT

A novel fertility restoration factor for the PET1 cytoplasm, coming from the public line RHA340, was evaluated together with resistance genes to downy mildew (*Plasmopara halstedii*) *Pl8* and black rust (*Puccinia helianthi*) in a mapping population segregating for the three characters. Linkage between the resistance genes and the fertility restoration was not detected, showing that the restoration factor present in the line RHA340 is different from *Rf1*. Bulk segregant analysis with microsatellite markers allowed to detect markers corresponding to linkage group 7, and this location was confirmed in the mapping population. A map was built showing the order and the distance of these loci.

Key words: bulk segregant – CMS – fertility restoration – mapping – microsatellites – restoration *Rf1*

INTRODUCTION

Hybrid breeding in sunflower is based on a single source of cytoplasmic male sterility (CMS), the so-called PET1 or French cytoplasm, which originates from an interspecific cross between *Helianthus petiolaris* and *Helianthus annuus* (Leclercq, 1969). This cytoplasm is present in all the female, sterile plants. To produce the hybrid seed, the CMS plants are crossed with restorer plants that have the *Rf1* gene, permitting the obtention of fertile plants.

The inbred line RHA340 is a public line that carries the *Pl8* gene (Miller and Gulya, 1991), which was introgressed from *Helianthus argophyllus* using the line HA89 as recurrent parent (Miller and Gulya, 1988). This line is also resistant to black rust and it also has a fertility restoration gene probably introduced during the backcrossing process.

Our objective is to investigate the linkage between the fertility restoration factor and the disease resistance loci; and to establish a linkage map of the region spanning the fertility restoration locus using SSR and Indel markers.

MATERIALS AND METHODS

Plant material

A segregating population was built by crossing the line RHA340 with the line ZENB8, manually emasculated. The latter is a proprietary maintainer line susceptible to downy mildew and black rust. The F_1 plants were sown in a greenhouse, and bagged in order to self-pollinate and obtain F_2 seeds. The F_2 population was sown in the field in Orán, Salta (Argentina). Leaf tissue was collected and reserved for DNA extraction. The F_2 plants were self-pollinated and F_3 seeds were harvested. At the flowering time, pollen was collected and used to pollinate plants of the line ZENA8, the CMS version of the ZENB8 line, with the aim of generating a test cross population. One hundred and seventy individuals from F_2 population were used to produce F_3 families

Pathological tests

Genotyping for both fungal diseases was done by progeny test in groups of 20 seedlings per family. Families with low number of individuals in the evaluations were not considered for the analysis.

Downy mildew evaluations

The whole seed immersion technique described by Mouzeyar et al. (1993) was used for resistance tests using zoosporangia from race 730 (Tourvieille de Labrouhe et al., 2003) maintained in Advanta Semillas, Balcarce, Argentina.

Observations were made 10 days after infection, after the plants had been kept for 24 hs in a saturated atmosphere. Plants were considered susceptible when sporulation in the cotyledons was observed. Plants with low rate of sporulation were transplanted to pots to observe the progress of the disease. All observations were made without knowing the genotype in question.

Black rust evaluation
Two-week-old plants at two pairs of true leaves stage were infected by spraying with a suspension of uredospores (50000 u/ ml). Each plant received an application of approximately 1 ml of inoculum suspension. The uredospores used as inoculum were collected from leaves of plants naturally infected located in a field in Venado Tuerto, Santa Fe, Argentina. The evaluation of this inoculum with differential lines corresponding to the American set showed a pathogenic pattern similar to race 4.

After inoculation with the uredospores suspension, the plants were kept in growing chamber for 24 hs. at 18º C and 100 % humidity to allow the infection to proceed. After this period, the plants were transferred to a greenhouse with 25 º C temperature, and 13 h light photoperiod.

Two weeks after inoculation plants were evaluated for the presence of pustules (uredosores) on the leaves. Plants showing pustules were considered susceptible and plants without signs or symptoms were considered resistant. In all the families, the number of susceptible and resistant individuals was scored and used to classify the families. All observations were made without knowing the genotype in question.

Fertility restoration evaluation
In order to determine the genotype of the F_2 plants from the mapping population, a progeny test was done using the test cross families [(ZenB8/RHA340)F_2/ZenA8] in rows of 20 plants sown in the field in Orán, Salta, Argentina, in June 2003. The families were evaluated at flowering time by counting the number of sterile plants (no pollen) in each of them. The proportion of sterile plants in the F_3 families was used to infer the genetic constitution of the F_2 plant.

SSR genotyping
Leaf tissue was collected from F_2 plants before flowering and dehydrated for preservation until DNA extraction. DNA was extracted from dehydrated leaves using the procedure described by Haymes (1996).

Microsatellites were amplified by PCR using this DNA and primer pairs corresponding to previously mapped molecular markers (Yu et al., 2003; Tang et al., 2002). PCRs were performed as described by Tang et al. (2002).

Amplicons labelled with 6-FAM, HEX and NED were separately amplified, pooled and diluted 50-fold. Samples were prepared for analysis by combining 2 µl of diluted pool, 0.2 µl of Genescan 500 internal lane standard labelled with ROX, and 10 µl of formamide. Electrophoresis was performed in an ABI 3100-avant capillary sequencer with 36 cm capillars and POP4 matrix. Electrophoretograms were analyzed with the software Genemapper 3.0 to assign genotypes following vendor's instructions.

Bulk segregant analysis
To perform the bulk segregant analysis, DNA from plants corresponding to the non-segregant genotypes (100% plants fertile or 100% plants sterile) was quantified and combined in bulks of four plants. The bulk DNA and DNA from the parental lines were amplified with selected primer pairs and the products were analyzed as previously described.

Linkage analysis
Linkage analysis and maps for the linkage group 7 in the mapping population were constructed using Joinmap 3.0 software (Van Ooijen and Voorips, 2001).

RESULTS

Phenotype evaluations
In the entire evaluations susceptible and resistant parents were included as controls, and they always showed 100% and 0 % of diseased plants, respectively.

In all the experiments single dominant genes appear to be controlling the resistances and the fertility restoration as can be observed in Table 1

Table 1. Segregation ratios in the F_2 population regarding downy mildew and black rust resistance following the test of F_3 families.

Trait	Seg.	n (A) Zen B8	n (H)	n (B) RHA340	χ 2	Prob.	Total
Black rust	1:2:1	42	73	40	2.96	0.23	155
Downy mildew	1:2:1	49	74	31	4.4	0.10	154
Rf	1:2:1	42	91	29	4.56	0.10	162

As Table 2 and Table 3 show, black rust and downy mildew resistance are independent of fertility restoration. Independence tests showed no evidence of linkage (χ^2= 0.303, P = 0.999 for downy mildew-restoration and χ^2= 0.97, P = 0.998 for black rust-restoration).

Table 2. Cosegregation between fertility restoration and downy mildew resistance

	Fertility restoration			
Downy mildew resistance	A (sterile)	H (segregant)	B (fertile)	Total
A (susceptible)	11	26	10	47
H (segregant)	14	43	13	70
B (resistant)	13	15	5	33
Total	38	84	28	150

Table 3. Cosegregation between fertility restoration and black rust resistance

	Fertility restoration			
Black rust resistance	A (sterile)	H (segregant)	B (fertile)	Total
A (susceptible)	11	27	8	46
H (segregant)	15	35	13	63
B (resistant)	11	21	7	39
Total	37	83	28	148

This lack of correlation was unexpected because the *Rf1* gene is located in linkage group 13, a few centimorgans away from *Pl8*. (Yu et al., 2003). These results suggest that the restoration factor segregating on this population is not an allele of *Rf1*.

Molecular assays

The genotype of the restoration locus was converted to a codominant score and integrated with molecular data corresponding to 63 loci distributed in all the linkage groups of sunflower genome. The data were analyzed with Joinmap software to detect possible linkages but no markers were grouped with the restoration locus.

In this marker set were included 10 different markers located in linkage group 13. The lack of cosegregation among any of these loci and the fertility restoration shows that this locus is not an allele of *Rf1*, which was mapped in linkage group 13 (Yu et al., 2003) but a different one, located in a different part of the genome.

In order to detect regions of the genome not covered by the selected markers, bulk segregant analysis was conducted using 23 polymorphic markers not included in the original mapping population. Out of all these, only ORS-004, located on linkage group 7, showed linkage disequilibrium, with all the bulks of fertile plants having the allele corresponding to RHA340 and those of the sterile plants the allele of ZenB8. This marker together with IN0182, a proprietary INDEL marker also located in linkage group 7, were analyzed and scored for all the individuals of the population. The linkage analysis showed that the fertility restoration grouped with the two loci with LOD > 9. Table 4 shows the maximum linkage observed for these three loci. The corresponding linkage map was calculated and is shown in Fig. 1.

Table 4: Recombination frequency between *Rf* and selected markers.

Locus1	Locus2	Recombination frequency	LOD
ORS-004	Rf	0.1161	23.19
IN0182	Rf	0.2125	9.10

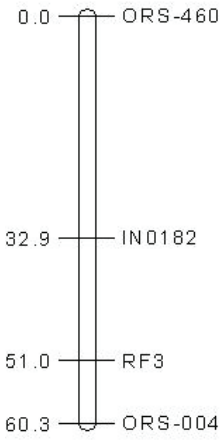

Fig. 1. Map of restoration factor and associated markers.

DISCUSSION

Up to now, two restoration factors have been described in sunflower; *Rf1*, located in linkage group 13 and *Rf2*, which is complementary to *Rf1* in certain genotypes, but is usually fixed in maintainer lines, so it usually does not segregate. Both genes seem to have been originated in *H petiolaris* (Miller and Fick, 1997). The restoration factor present in the line RHA340 is not any of these two, because it is located in linkage group 7 (difference with *Rf1*) and it restores the fertility in PET1 cytoplasm even in the absence of *Rf1* (difference to *Rf2*) being inherited as a simple gene. This novel gene is a fertility restoration that could be traced to the wild *H. argophyllus* used as source of downy mildew resistance during the creation of the line RHA340. We propose the name *Rf3* for this new locus.

Further experiments are being done to include more markers in the map and eventually saturate it. This may allow a cloning of the restoration factor and an explanation its mode of action. Another line of investigation will include the cross of RHA340 with other CMS systems to see if other male sterile cytoplasm can be restored.

This finding could alleviate the genetic bottleneck caused in sunflower due to the exclusive use of *Rf1* to restore PET1 CMS in the production of hybrid cultivars.

The availability of molecular markers linked to this locus facilitates the introgression of this gene in the different lines of breeding programs, to develop new restorer lines.

Summarizing, we studied the linkage of the fertility restoration locus present in the line RHA340 with the resistance genes to downy mildew *PL8* and black rust as well as with molecular markers. The lack of correlation with the resistance genes (linked in repulsion with *Rf1*) and the linkage with markers of linkage group 7 demonstrate that this is a novel restoration gene.

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