MAPPING A RUST RESISTANCE GENE AND THE DOWNY MILDEW *PL*8 GENE IN SUNFLOWER

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Abstract

The resistance genes to downy mildew *Pl*8 and black rust (*Puccinia helianthi*) were evaluated in a mapping population segregating for both characters. Linkage of both genes was detected and the two loci were mapped in the bottom part of linkage group 13, in a region that previously was shown to contain an RGA locus. Microsatellites, Indel and RGA markers closely linked to these two genes were detected. A map showing the order and the distance of these loci was built.

Resumen

Se realizaron experimentos para evaluar el gen *Pl*8 de resistencia a downy mildew y el gen de resistencia a Roya negra provenientes de la línea RHA340 en una población segregante para los dos caracteres. Se trabajó con una población de 170 familias F 2:3 y se analizaron 2 caracteres cualitativos y 13 marcadores moleculares. De los resultados obtenidos se pudo demostrar que los genes de resistencia a ambas enfermedades están localizados en el extremo inferior del cromosoma 13 del mapa público, donde también se localizaron los RGAs, mostrando que en esa zona del genoma existiría un agrupamiento de genes de resistencia.

Introduction

Downy mildew, caused by *Plasmopara halstedii* (Farl.) Berl. and de Toni and black rust, caused by *Puccinia helianthi* Schwein are two of the most important fungal diseases of cultivated sunflower. Single, dominant genes located in chromosomes 8 or 13 generally control the resistance to these two diseases. It has been proposed that the genes *Pl6, Pl*7 and *Pl*8*,* conferring resistance to all the different races of downy mildew are in fact clusters of resistance genes, containing also many copies of other genes or pseudo-genes (Roeckel-Drevet et al., 1996; Gedil et al., 2001, 2001. These genes came from wild *Helianthus* species and have been introgresed by backcrossing into cultivated sunflower. By alignment of maps it is possible to observe that the rust resistance genes reported so far are co-located with the regions containing downy mildew resistance genes (Yu et al, 2003).

The inbred line RHA 340 is a public line that carries the *Pl*8 gene (Miller and Gulya, 1991), which was introgresed from *Helianthus argophyllus* using the line HA 89 as the

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 establish a genetic map of the region spanning the *Pl*8 locus, the rust resistance locus using SSR, RGA and Indel markers.

Materials and Methods

Plant Material. A segregating population was built by crossing the line RHA 340 with the line ZEN B8, manually emasculated. This one is a proprietary elite line susceptible to downy mildew and black rust. The F1 plants were sown in a greenhouse, bagged to self-pollinate and F2 seed was collected. The F2 population was sown in the field in Oran, Salta (Argentina). Leaf tissue was collected and reserved for DNA extraction. The F2 plants were self-pollinated and F3 seeds were harvested. At flowering time, pollen was harvested and used to pollinate plants of the line ZEN A8, the CMS version of the ZEN B8 line, with the aim of generating a test cross population.

Pathological Tests. An F2 population with 170 individuals was used to produce F3 families. Genotyping for both fungal diseases was done by progeny test in groups of 20 seedlings per family. Families with a low number of individuals in the evaluations were not considered for the analysis.

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

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

Downy Mildew Evaluation: Field Assay. The families were evaluated in natural infection conditions sowing the progeny of the test cross (F2 x ZEN A8) in rows of 20 plants without fungicide treatment. Plants were evaluated before flowering for the presence of downy mildew primary systemic infection symptoms (dwarfing, chlorotic and puckered leaves, sporulation on the underside of the leaves). In each row, the total number of plants and the number of diseased plants were scored. The proportion of diseased and resistant plants was used to characterize each family. Previous monitoring results indicated that the race present in the field is race 730.

Black Rust Evaluation. Two-week-old plants at two pairs of true leaf stage were infected by spraying with a suspension of uredospores (50,000/ml). Approximately 1 ml per plant was applied. The uredospores used as inoculum were collected from leaves of naturally infected plants 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 uredospore suspension, the plants were kept in a growth chamber for 24 h at 18C and 100% humidity to allow the infection to proceed. After this period the plants were transferred to a greenhouse with 25C temperature, and 13 h light photoperiod.

Two weeks after inoculation plants were evaluated for the presence of pustules (uredia) in 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.

SSR and RGA Marker Genotyping. DNA was extracted from dehydrated leaves collected prior to flowering using the procedure described by Haymes (1996). Microsatellites were amplified by PCR using DNA and primer pairs corresponding to molecular markers previously described and mapped (Yu et al., 2003, Tang et al., 2002). PCR was performed as described by Tang et al. (2003).

Amplicons labeled with 6FAM, HEX and NED were separately produced, pooled and diluted fifty-fold. Samples were prepared for analysis by combining 2 ul of diluted pool, 0,2 ul of Genescan 500 internal lane standard labeled with ROX and 10 ul of formamide. Electrophoresis was performed in an ABI 3100-*avant* capillary sequencer with 36 cm capillaries and POP4 matrix. Electrophoretograms were analyzed with the software Genescan 2.1 set with filter D. Genotyper 2.0 was used to assign genotypes following vendor instructions.

Resistance gene analogs were amplified as described by Slaubagh et al. (2003) and Radwan et al. (2003). RGA fragments were analyzed by SSCP in an S2 manual sequencer (Gibco, Rockville, Maryland, USA) using MDE matrix (Biowhitaker Molecular Applications, Rockland ME, USA), following vendor instruction. After electrophoresis, the gels were stained with Sybr Gold (Molecular Probes, Eugene, OR, USA) and imaged in a Fluor–S Multiscan (Biorad Laboratories Inc, Hercules, CA, USA).

Linkage and QTL Analysis. Linkage maps for the linkage group 13 in the mapping population were constructed using Joinmap 3.0 software (Van Ooijen and Voorips, 2001).

Simple interval mapping was performed using multiple regression procedure according to the approach described by Haley and Knott (1992) using Plab QTL software (Utz and Melchinger, 1996).

Results

Phenotypic Evaluations. In the entire evaluation, susceptible and resistant parents were included as controls and they always show 100% and 0% diseased plants, respectively.

In all the experiments single dominant genes appear to be controlling the resistance (Table 1). The evaluation of downy mildew resistance in the field and in a chamber showed good agreement, although some families did not match. For further analysis, only families with agreement between both evaluations were selected. Although previous reports indicated that RHA 340 presents type II resistance with cotyledon-limited sporulation, this symptom was never observed and all the plants with sporulation that were monitored for the progress of the disease finally developed all the symptoms of systemic infection.

Table 1. Segregation ratios of the investigated F2 population regarding downy mildew and black rust resistance following the test of F3 families.

Trait	Total	Segregation observed (RR: Rs: ss)	Segregation tested	χ 2	Prob.
Black rust	155	40: 73:42	1:2:1	2,96	0,23
Downy mildew (chamber)	154	31:74:49	1:2:1	4,4	0,10
Downy mildew (field)	147	38:79:30	1:2:1	1,69	0,43
Downy mildew (match)	107	27:54:26	1:2:1	0,11	0,948

As Table 2 shows, the results for downy mildew and black rust are not independent (P=1,02E-22).

Table 2. Co-segregation between black rust and downy mildew resistance.

	DM res.	DM seg.	DM susc.	Total
Rust res.	21	5	0	26
Rust seg.	4	32	0	36
Rust susc.	0	7	23	30
Total	25	44	23	92

Molecular Assays. One Indel and thirty-five microsatellite and markers previously mapped to linkage group 13 were tested for polymorphism between the parental lines. Ten polymorphic markers were observed and selected. These markers were analyzed and scored for all the individuals of the population. Linkage analysis was made with all of them and a map was built to be used as a reference for the next experiment.

RGA Mapping. The RGA markers were tested for polymorphisms between the parental lines of the population. SSCP polymorphisms were found for the markers 1W23, HaNTP3, HaNTP5 and HaNTP6. The markers were scored for the whole population and the data were included in the matrix data. Linkage analysis showed that 1W23, HaNTP3 and HaNTP6 were linked to LG 13, meanwhile HaNTP5 was found linked to LG 15 (data not shown). Figure 1 shows the map of microsatellites, Indels and RGAs obtained for the population.

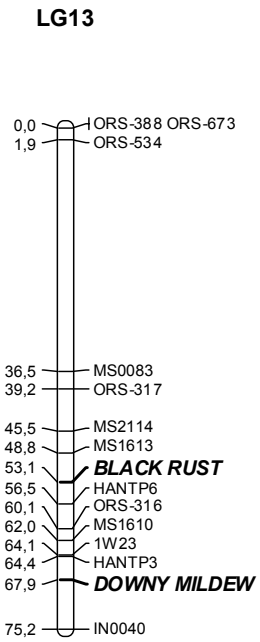

Figure 1. Map of Linkage group 13 showing the location of resistance genes and associated RGAs.

Mapping Resistance Genes as Codominant Markers. The score of the F3 families (Res., Sus., and Segregant) corresponding to the F2 plants was converted to a codominant score (A, B, H) and included in the set of data. Linkage analysis showed strong linkage with microsatellites and RGA markers located in the bottom part of LG 13.

Some inconsistencies were observed when the phenotypic score, converted to codominant score, was evaluated and the location of the genes were mapped, especially the downy mildew resistance gene; this may be an indication of some incorrectly scored families. To be able to map the two genes simultaneously, the mapping was repeated using only the families with a concordant score between the field evaluation and chamber evaluation. The map of the molecular markers and the resistance genes is presented in Figure 1.

QTL Analysis. To reduce the effect of misclassifications in the progeny tests, a different approach was used. The percentage of diseased plants was considered a quantitative score of the resistance of a particular family. Simple interval mapping procedures were used to characterize the resistance to both diseases. In both cases single QTL peaks were detected in the bottom part of linkage group 13. In the resistance to black rust, the QTL peak is located in the interval defined by the markers MS0851 and HANTP6, meanwhile the QTL peak for downy mildew resistance is located in the interval defined by the markers HANTP3, IW23, and IN0040 (Figure 2).

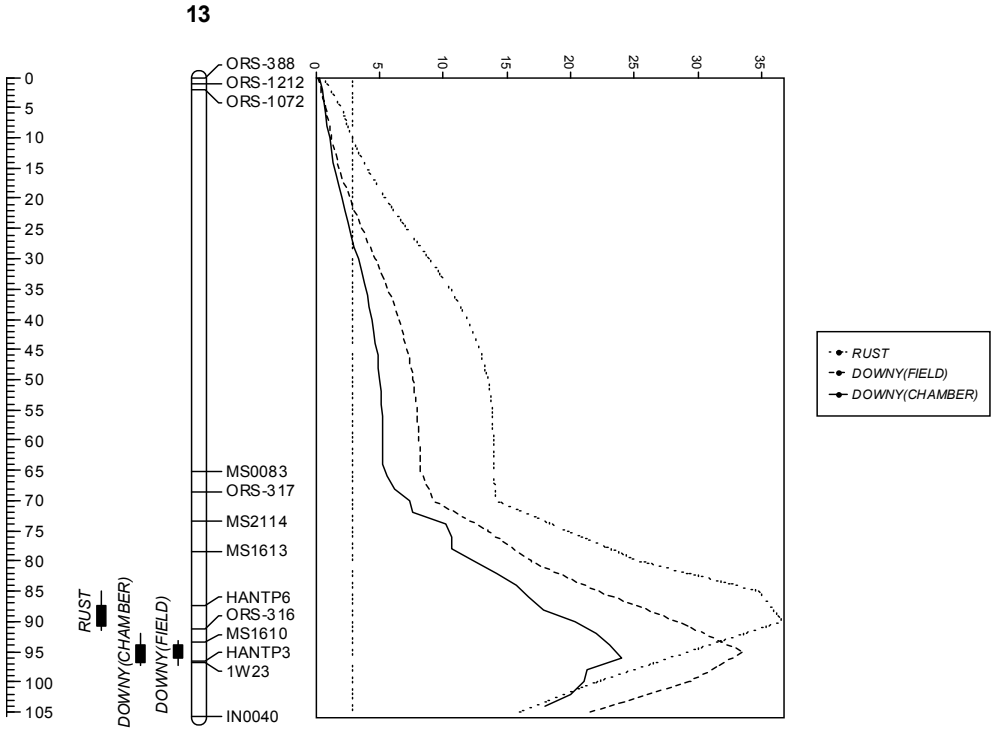

Figure 2. Load score graphic showing the location of QTLs for resistance to downy mildew and black rust. Distance between markers was calculated based on Haldane's function.

Discussion

The two resistance genes evaluated showed strong linkage between them. Vranceanu and Stoenescu (1970) and Fick and Zimmer (1975) reported similar linkage between black rust and downy mildew *Pl*1, but this is the first report of direct evidence of an association between *Pl*8 and a rust resistance gene.

Both resistance genes appear always linked to resistance gene analogs in a region that previously was shown to contain many of these kinds of sequences (Slaubagh, 2003; Gedil, 2001). Some of these RGAs, previously mapped as RFLP probes (Radwan et al., 2003), were remapped as SSCP markers confirming the relation between the resistance gene analogs and the resistant phenotype. The short distance between the genes and the fact that RGAs are distributed in the entire region may indicate that the cluster of resistance genes that include *Pl*8 and *Pl*5 may also include resistance to black rust. The cluster of genes that confer resistance to many races of downy mildew may also be including genes of resistance for other pathogens. These results agree with the mapping of molecular markers associated with rust resistance from other sources that located the genes in the bottom part of LG 13 and the top part of LG 8 (Yu et al., 2003). As was previously noted (Radwan et al., 2003, Slaubagh et al., 2003) these two regions contain duplicated RFLP and SSR loci. The conservation of the order and function of the resistance loci supports the hypothesis that these two regions may be in

fact duplications present in the genome of sunflower, showing similarity not only at the molecular level but also at a functional level.

Keeping in mind that scoring errors produce an increment of map distance, the distance between these two genes as can be seen in this map should be considered a maximum of the distance between both genes. This may explain why the resistance gene loci do not appear in the same interval as the QTL analysis showed, but in both cases they appear in the adjacent interval. Although it was not observed in the controls of the experiments, the cotyledon-limited sporulation, previously described for the line RHA 340 (Mouzeyar et al., 1994), may be one explanation for the classification errors that increase the distance between genes and markers, particularly for the chamber evaluation of downy mildew resistance.

Our results show that it is not surprising that rust resistance was incorporated to the HA 89 line during the backcrossing process by linkage dragging. This means also that it would be possible to breed for downy mildew and black rust resistance with little effort, by the use of molecular markers to prevent crossover between the two resistance loci. The use of the RGA as molecular markers for breeding purposes represents an advance compared with RFLP, although the SSCP is still a complicated technique and further work will be needed to develop more practical types of markers.

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