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Inheritance of Resistance to Downy Mildew (*Plasmopara halstedii*) in Sunflowers

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Abstract

Downy mildew tests on F2 and test cross progenies from crosses of RHA266, RHA274, HA335 or HA338 (race 1) and of HA335 and HA338 (races 1, A and B) gave no segregation for resistance. This suggested that certain resistance genes are at least quite closely linked. Use of RFLP and RAPD markers has shown that at least 3 sources of resistance (*Pl1* from RHA266, *Pl2*(?) from HA61 (resistance to race 1) and *Pl6* (?) from HA335 (resistance to races 1,A and B) are all located in the same area on one linkage group. It may thus be concluded that the downy mildew resistance genes at present used in breeding are not independant as previously reported by many authors, a change probably due to a difference in symptom interpretation. It remains to be determined whether there is a strict gene for gene relationship between sunflower and *Plasmopara halstedii* or whether there are some resistance genes which truly give resistance to more than one downy mildew race.

Introduction

Downy mildew is one of the main diseases causing economic losses in sunflower. It is due to the obligate parasite *Plasmopara halstedii*, for which at least seven different pathotypes have been described according to their reaction on differential sunflower lines (Gulya *et al.*, 1991). Genes giving resistance have been found both in cultivated sunflower (Vranceanu, 1970) and wild *Helianthus* species.

In most cases, these sources of resistance have been reported to have single dominant genes (designated *Pl*) but which give resistance to varying number of downy mildew races (Miller, 1992). Reports of segregation patterns for progenies from crosses between lines giving resistance to the same races always indicated that these genes were independant (for example Vear and Leclercq, 1971 ; Zimmer and Kinman, 1972 ; Miller and Gulya, 1991). Generally, susceptibility was taken as the appearance of zoosporangia on the aerial parts of plants, following seedling tests. However, Vear (1978) and Sackston (1992) have reported that some resistant genotypes, in particular those containing *Pl1* could show sporulation on cotyledons. Mouzeyar *et al.* (1994) observed that, according to genotype, hypersensitive resistance reactions occured in the hypocotyl of resistant, infected seedlings with varying rapidity, so that sporulation could occur on seedlings showing typical resistance reactions.

In France, until 1988, only one race (race 1) was known ; but then two new races were discovered, A in 1988, B in 1989, similar to, but not exactly the same as, American races 3 and 4 (Tourvieille *et al.*, 1991). Studies made by Mouzeyar (1993) of resistance to these new races indicated that, using the criteria of sporulation on leaves as indication of susceptibility, no segregation was observed between lines that had previously been thought to contain independant genes.

This paper reports a summary of this work by classical genetical methods, and the use of molecular biology techniques and the RFLP linkage map developed by Gentzbittel *et al.*(1995) that have been used to check the segregations between *Pl* genes and to try to determine whether a strict gene for gene relation applies for sunflower genotypes and *P. halstedii* races.

Materials and methods

Sunflower genotypes : The lines used are listed in Table 1 with the names of the resistance genes that they have been reported to contain. These names will be used in the results section. All these lines are maintained at I.N.R.A., Clermont-Ferrand by selfing under paper bags. Crosses were made using females with CMS or male-sterilized with gibberellic acid (25 ppm) at 1cm star bud stage, and checked by the vigour of F1. In addition, when possible, tests were made with adequate downy mildew races to confirm F1 hybrids.

Downy Mildew Resistance test : The seed immersion technique described by Mouzeyar *et al.* (1993) was used. Disease reactions were observed two weeks after infection, after 48h saturated humidity. Resistance was defined as absence of sporulation or a slight sporulation on cotyledons only, and susceptibility as fungal sporulation on both cotyledons and true leaves (Vear, 1978).

DNA manipulations : DNA was extracted from leaves collected at flowering for each of the segregating progeny, as described by Gentzbittel *et al.* (1992). For the Bulk Segregant Analyses (BSA) (Michelmore *et al.*, 1991) tests of F3 families made it possible to bulk DNA of 12 homozygous resistant and 12 homozygous susceptible F2 plants from each cross. For RFLP analysis, DNA digestion and Southern hybridization were performed as described by Gentzbittel *et al.* (1992) using the following restriction enzymes : *Bgl II, EcoRI, EcoRV, Hind III* (Amersham). The RFLP probes used had been produced by Gentzbittel *et al.* (1995) and mapped on the linkage map developed by these authors.

Table 1. Inbred sunflower lines used in the studies of downy mildew resistance.

Inbred	Breeder	Resistant to races	Resistance Gene	Author
Suseptible				
GH	I.N.R.A., France			
H52	A.R.S., S.Africa			
Resistant				
RHA266	.S.D.A., U.S.A.	1	*Pl1*	Fick & Zimmer, 1974
RHA274	.S.D.A., U.S.A.	1	*Pl2*	Fick *et al.*, 1975
PAC2	I.N.R.A., France	1	*Pl2*	Vear (unpublished)
HA335	.S.D.A., U.S.A.	1,A,B	*Pl6+?*	Miller & Gulya, 1991
HA338	.S.D.A., U.S.A.	1,A,B	*Pl7+?*	Miller & Gulya, 1991

For RAPD analysis, random decamer primers (Operon Technologies or Bioprobe) were used to identify polymorphic DNA between the two bulks. Amplification reaction and product analyses were made as previously described by Mouzeyar *et al.* (1995).

Results

Downy Mildew resistance segregations :

The results of tests on F2 and test cross (susceptible*F1) progenies are given in Table 2. The absence of segregation between RHA266 and RHA274 indicates that the resistance genes in these two lines, *Pl1* and *Pl2* are not independent. However, it may be noted that if the 20 plants showing cotyledon limited infection were counted as susceptible, the segregation would not be different from 3R : 1S, indicating 2 independant genes giving resistance to race 1.

The progenies from crosses between either HA335 or HA338 and either RHA266 or RHA274, showed no segregation at all when tested with race 1. The tests with the races A and B confirmed that the crosses had been successful, since RHA266 and RHA274 are susceptible and the expected 1R : 1S (test crosses) and 3R : 1S (F2) ratios were observed. The test-cross from (HA338*RHA274) showed segregation, but with an excess of resistant plants which did not agree with either 1R : 1S or 3R : 1S ratios. These results indicate that the genes giving resistance to race 1 in RHA266, RHA274, HA335 and HA338 are not independent.

In addition, the test-cross between HA335 and HA338 gave no segregation to race A, indicating that the genes in these lines, giving resistance to race A, are not independent.

Table 2. Observations of resistance and susceptibility to downy mildew tests of test-cross and F2 progenies from crosses between resistant sunflower lines.

Cross	Progeny	Race	Resistant	Susceptible
RHA266 * RHA274	TC	1	81 (20 CL)	0
HA335 * RHA266	TC	1	84	0
	TC	B	36	28 (1 : 1)
HA335 * RHA274	TC	1	243	0
	TC	A	221	107 (?)
HA338 * RHA266	F2	1	146 (21 CL)	0
	TC	1	47	0
	TC	B	37	43 (1 : 1)
HA338 * RHA274	F2	1	153	0
	F2	B	87	23 (3 : 1)
HA338 * HA335	TC	1	110	0
	TC	A	53	0

TC : Test cross (susceptible * F1)
CL : Cotyledon limited infection
(1:1), (3:1) : results are not significantly different ($p<0.05$) from these hypotheses
(?) : results are significantly different ($p>0.05$) from 1:1 or 3:1.

In order to obtain more detailed information about the localization of these genes, BSA were made using RFLP and RAPD probes. To make the bulks for *Pl1*, F3 families from the cross (GH * RHA266) were tested with race 1. They showed a ratio of 24 homozygous resistant : 76 heterozygous : 35 homozygous susceptible, not significantly different from 1RR : 2RS : 1SS expected for the segregation of a single dominant gene. Bulks from 12 resistant and 12 susceptible F2 plants, followed by individual analyses of all the plants of the progeny, showed that resistance was linked at 5.6 cM to SUN017H3-3, at 7.1cM to SUN124E1-2 and 43.7cM to OPD13(1600), on linkage group 1 (Fig.1). These results are described in detail by Mouzeyar *et al.* (1995).
For *Pl2*, the progeny (GH*PAC2) was used, PAC2 having the same origin of resistance (HA61) as RHA274. Segregation for resistance to race 1 showed 35 homozygous resistance, 57 heterozygous and 19 homozygous susceptible (again not different from 1:2:1). Resistance was linked at 10cM from SUN124E1-2. For *Pl6* resistance to race A, F3 progenies from the cross (H52 * HA335) gave a segregation of 30 homozygous resistant : 84 heterozygous : 45 homozygous susceptible (not different from 1:2:1). The bulks of 12 homozygous resistant and 12 homozygous susceptible, and segregation analyses of all the plants, showed that this gene is located in the same place as Pl1 and Pl2 (Roeckel-Drevet *et al.*, submitted).

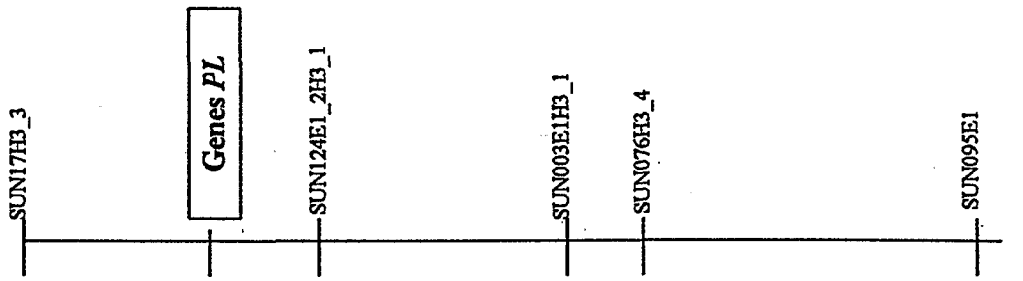

Figure 1. Linkage group 1 of the sunflower RFLP map (Gentzbittel *et al.*, 1995) showing the position of downy mildew resistance genes.

Discussion

All the results, both of downy mildew resistance segregation and linkage to RFLP and RAPD markers indicate that the resistance genes studied, giving resistance to race 1 and to races A and B, are closely linked on the sunflower genome. In the case of resistant genotypes showing cotyledon limited infection, this difference from previous reports is easy to understand since the plants showing cotyledon sporulation would previously have been counted as susceptible. However, in the case of the comparison of HA335 and HA338, there is no evident explaination of the difference from the results of Miller and Gulya (1991), since these lines show no sporulation and their resistance is immediatly effective in the base of the hypocotyl (Mouzeyar *et al.*, 1994). It could be due to the fact that the results presented here use races A and B and not race 4, as used in the first publication.
The excess of resistant plants in the test-cross progeny (HA335*RHA274) tested with race A can be compared with other results involving RHA274 in which excesses of resistant plants have been found (Mouzeyar, 1993).
Linked resistance genes have already been reported in many species, notably for resistance to *Bremia lactucae*, downy mildew of lettuce (Farrara *et al.*, 1987). Comparison with this example

leads to the question of the organisation of sunflower downy mildew resistance genes. Up till recently, each time a new race appeared, the genes found effective against it appeared to give resistance also to all the less virulent races : for example, *Pl2* gives resistance both to race 1 and race 2 (Fick and Zimmer, 1974) and *Pl6, Pl7* and *Pl8*, resistant to race 4, give resistance to all other known races (Miller, 1992). In this situation, since several genes gave resistance to one race, sunflower downy mildew did not appear to follow strictly the gene for gene relationship of Flor (1955).
However, Miller and Gulya (1987) reported that the Russian population Progress might contain more than one resistance gene, each effective against different *P. halstedii* races. Recently, Mouzeyar *et al.* (1994) reported an inbred line, QHP1,developed from a cross with the USDA pool HAR5, that gives resistance, in France, to races 1 and A but not to race B, although the latter is less virulent than race A, other lines being known that are resistant to B but not to A.

If, in agreement with the last two results, the gene for gene relation does apply, it would mean that the resistance genes so far identified are not single genes but clusters. For example, *Pl6* would include *Pl1*, giving resistance to race 1 and *Pl"A"*, giving resistance to race A, in addition to the strict *Pl6*, giving resistance to race 4, and *Pl2* would be a smaller cluster of only *Pl1*, giving resistance to race 1 and *Pl2*, giving resistance to race 2. The present segregation and molecular marking results are in agreement with this hypothesis. The extremely close linkage, apparent from the molecular markers, would explain why the clusters are inherited as single genes, and that, other than QHP1, there do not appear to be publication of genotypes which separate resistance to virulent races from resistance to those that are less virulent. It is true that although enormous numbers of crosses have been made between susceptible, agronomically useful lines and sources of downy mildew resistance, separation of resistance to different races is not a favorable character in breeding, so that any such genotypes may have been immediately discarded.

Studies are in progress in our group, on families from progenies exceeding 100 F2 plants, to determine whether there is some segregation between the resistance to race 1 and that to race A in HA335. It is of considerable practical importance since, so far, *Pl6* could be considered as a "strong" gene, giving resistance to many, perhaps all, races of downy mildew. If, instead, it is a cluster of several genes, with *Pl6* in the strict sense, giving resistance only to race 4, there would seem to be a greater probability that a new, more virulent *P. halstedii* race may occur, to which no member of the existing cluster gives resistance. This would require breeders to find a further resistance gene to add to the cluster.

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References

FARRARA B.F., ILOTT T.W. and R.W. MICHELMORE, 1987: Genetic analysis of factors for resistance to downy mildew (*Bremia lactucae*) in species of lettuce (*Lactuca sativa* L. and *L.serriola*). Plant Pathol., 36, 499-514.

FICK G.N. and D.E. ZIMMER, 1974: RHA271, RHA273 and RHA274, sunflower parental lines for producing downy mildew resistant hybrids. Farm research, 32, 7-9.

FICK G.N., KINMAN M.I. and D.E. ZIMMER, 1978: Registration of RHA273 and RHA274 sunflower parental lines. Crop Sci., 15, p.106.

FLOR H.H., 1955: Host-parasite interaction in flax rust. its gentics and other implications. Phytopathol., 45, 680-685.

GENTZBITTEL L., PERRAULT A. and P. NICOLAS, 1992: Molecular phylogeny of the *Helianthus* genus, based on nuclear restriction fragment length polymorphism (RFLP). Mol. Biol. Evol., 9, 872-892.

GENTZBITTEL L., VEAR F., ZHANG Y.X., BERVILLE A. and P. NICOLAS, 1995: Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.). Theor.Appl.Genet., 90, 1079-1086.

GULYA T.J., SACKSTON W.E., VIRANYI F., MASIREVIC S. and K.Y. RASHID, 1991: New races of the sunflower downy mildew pathogen (*Plasmopara halstedii*) in Europe and North and South America. J. Phytopathol, 132, 303-311.

MICHELMORE R.W., PARAN I. and R.V. KESSELI, 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. Natl. Acad. Sci. USA, 88, 9828-9832.

MILLER J.F., 1992: Update on inheritance of sunflower characteristics. Proc. 13th Int. Sunflower Conf., Pisa, Italy, 905-945.

MILLER J.F. and T.J. GULYA, 1987: Inheritance of resistance to race 3 downy mildew in sunflower. Crop Science, 27, 210-212.

MILLER J.F. and T.J. GULYA, 1991: Inheritance of resistance to race 4 of downy mildew derived from interspecific crosses in sunflower. Crop Science, 31, 40-43.

MOUSEYAR S., 1993: Etude de la résistance du tournesol, *Helianthus annuus* L. au mildiou causé par *Plasmopara halstedii*. Doctoral thesis, Univ Clermont-Ferrand II, France, 99 p.

MOUZEYAR S., TOURVIEILLE de LABROUHE D. and F. VEAR, 1993: Histopathological studies of resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*). J.Phytopathol., 139, 289-297.

MOUZEYAR S., TOURVIEILLE de LABROUHE D. and F. VEAR, 1994a: Effect of host-race combination on resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*). J. Phytopathol., 141, 249-258.

MOUZEYAR S., PHILIPPON J., WALSER P., VEAR F. and D. TOURVIEILLE de LABROUHE, 1994b: Sunflower resistance to French races of downy mildew (*Plasmopara halstedii*). agronomie, 14, 335-336.

MOUZEYAR S., ROECKEL-DREVET P., GENTZBITTEL L., PHILIPPON J., TOURVIEILLE de LABROUHE D., VEAR F. and P. NICOLAS, 1995: RFLP and RAPD mapping of the sunflower *Pl1* locus for resistance to *Plasmopara halstedii* race 1. Theor. Appl. Genet., 91, 733-737.

SACKSTON W.E., 1992: Cotyledon limited infection (CLI) and leaf disk immersion (LDI) inoculation of sunflower by downy mildew (*Plasmopara halstedii*). Proc. 13thInt. Sunflower Conf., Pisa, Italy, 840-848.

TOURVIEILLE de LABROUHE D., MOUZEYAR S., LAFON S. and Y. REGNAULT, 1991: Evolution des races de mildiou (*Plasmopara helianthi*) sur tournesol en France. 3eme Int. Conf. sur les maladies des Plantes, Bordeaux, 77-784.

VEAR F., 1978: Réactions de certains génotypes de tournesol résistants au mildiou (*Plasmopara helianthi*) au test de résistance sur plantules. Ann. Amélior. Plant., 28, 327-332.

VEAR F. and P. LECLERCQ, 1971: Deux nouveaux gènes de résistance au mildiou du tournesol. Ann. Amélior. Plant., 21, 251-255.

VRANCEANU V.I., 1970: Advances in sunflower breeding in Rumania. Proc. 4th Int. Sunflower Conf. Memphis, USA,136-148.

ZIMMER D.E. and M.L. KINMAN, 1972: Downy mildew resistance in cultivated sunflower and its inheritance. Crop Sci., 12, 749-751.