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The genetics of resistance to five races of downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.)

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Abstract These studies were undertaken to determine whether downy mildew resistance genes in sunflower were independent as first reported, or linked as suggested by more recent hypotheses. The segregations for downy mildew reaction of 111 F_3 progenies from a cross between a susceptible line and a line with *Pl2* were used to locate this gene on the sunflower consensus RFLP linkage map. It was shown that *Pl2* was linked to the same RFLP markers on linkage group 1 as *Pl1* and *Pl6*, mapped earlier, and at a very similar distance. The F_3 progenies showed exactly the same segregation patterns when tested with race 1 and race D. One hundred and fifty four progenies from a cross between a susceptible line and HA335, containing *Pl6* (considered as giving resistance to all *Plasmopara halstedii* races), were tested with the five French downy mildew races, 1, A, B, C and D. Two progenies were observed to show segregation for races 1 and D, while appearing homozygous-resistant to races A , B and C. Tests on F_4 progenies confirmed this separation of resistances with fixation of susceptibility to races 1 and D and resistance to races A, B and C. It is concluded that the *Pl6* gene is not a "strong" gene, giving resistance to all downy mildew races, but rather a cluster of genes, each providing resistance to one, or a few, downy mildew races. The genes giving resistance to races 1 and D, on one hand, and to races A, B and C, on the other hand, must be very closely linked, with about 0.6 cM between the two groups.

Key words Linkage · Major gene · Race-specific resistance · RFLP

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Introduction

Downy mildew [*Plasmopara halstedii* (Farl.) Berlese et de Toni] is one of the most widely occurring and important pathogenes of sunflower (Sackston et al. 1990). It is an obligated and specific parasite for which at least ten physiological races have been reported (Gulya et al. 1991). Functionally complete resistance, controlled by major genes, has been selected in cultivated sunflowers (Vranceanu and Stoenescu 1970), or introduced from wild *Helianthus annuus* (Miller and Gulya 1991) or other *Helianthus* spp. (Leclercq et al. 1970; Miller and Gulya 1991).

Reports of genetical studies have concluded that resistance is controlled by single dominant genes denoted as *Pl*. Some genes were reported to give resistance to only one race (Vranceanu and Stoenescu 1970), but others were found to provide resistance to two (Zimmer and Kinman 1972; Miller and Gulya 1987) or more (Vranceanu et al. 1981; Sackston et al. 1990; Miller 1992) races. Miller and Gulya (1987) reported that *Pl5* gave resistance only to race 3, but segregation studies by Vranceanu et al. (1981) led these authors to conclude that this gene provided resistance to races 1, 2 and 3 and to the "Fundulea" race of downy mildew. From these results, it would appear that the *P. halstedii* – sunflower interaction does not strictly adhere to the gene-for-gene concept of Flor (1955), in which each resistance gene gives resistance to only one pathogen

race. Crosses between resistant lines have generally been reported to indicate that resistance genes segregate independently (Vear and Leclercq 1971; Vranceanu et al. 1981; Miller and Gulya 1991). When no segregation occurred in F_2 or test-cross progenies the genes in the two sources of resistance were considered to be the same (Miller and Gulya 1991; Sackston 1992).

However, from the first studies on the races denoted A and B, which appeared in France in 1988 and 1989 (Tourvieille et al. 1991), Mouzeyar et al. (1992) observed no segregation in progenies from crosses between lines containing *Pl1*, *Pl2*, *Pl6* and *Pl7*. This result raised the question of whether these downy mildew resistance genes were not as previously reported, independent, but, on the contrary, quite closely linked. Following the development of a consensus RFLP map for cultivated sunflower (Gentzbittel et al. 1995), *Pl1* (resistance to race 1) and *Pl6* (resistance to race A) were located by Mouzeyar et al. (1995) and Roeckel-Drevet et al. (1996) respectively, using Bulked Segregant Analysis (Michelmore et al. 1991) followed by segregation studies. The two genes were found to be linked to the same RFLP markers at distances which were not significantly different, and in the same interval. This clustering of at least two *Pl* genes in turn led to the question of whether genes such as *Pl6*, giving resistance to all known downy mildew races (Miller 1992), were particularly "strong" genes, or whether they were groups of several, perhaps many, genes, including *Pl1* (which would explain the absence of segregation in the crosses of Mouzeyar et al. 1992).

The present paper reports studies made with the aim of answering these questions. Firstly, the gene known as *Pl2*, reported to give resistance to races 1 and 2 (Zimmer and Kinman 1972), was located on the RFLP map. Secondly, downy mildew reactions of the 154 F_3 progenies used to locate *Pl6* were tested with all the races available in France to determine whether their segregation patterns were exactly the same, or whether recombination between resistances to different races could be detected. The same operation was also applied for progenies containing *Pl2*, using the two French races to which this gene gives resistance.

Materials and methods

Sunflower genotypes

The *Pl2* gene was located and segregation studies made using F_2 and F_3 progenies from the cross GH × PAC2. GH is a completely downy mildew-susceptible line bred by INRA from Romanian material. PAC2 is an INRA resorter line bred from a cross between a susceptible line with male-fertility restoration from *Helianthus petiolaris* and HA61, a USDA line reported to contain *Pl2* (Fick et al. 1978). The segregation studies involving *Pl6* were made on progenies from the cross HA335 × H52. HA335 is a USDA line reported to contain *Pl6* (Miller and Gulya 1991) while H52 is a South African (A.R.S.) line susceptible to all known races of downy mildew.

Downy mildew races

Five downy mildew races were studied, over 3 years, in separate growth chambers and at different periods of the year. Race 1, the European race, was maintained on the completely susceptible open-pollinated variety Peredovik. Races A and B were maintained on the experimental INRA hybrid GH × RHA266 resistant only to race 1, with regular control provided by the INRA inbred lines PMI3 (resistant to race B, susceptible to A) and QHP1 (susceptible to B, resistant to A) (Mouzeyar et al. 1994a). Race A is similar to American race 4, but can be distinguished because the USDA composite DM3 is resistant to A but susceptible to 4. Race B can be distinguished from race 3 because the USDA composite HAR5 and the INRA line QHP1 are both susceptible to B but resistant to 3. The races denoted C and D were first reported in France in 1995 (Roeckel-Drevet et al. 1997). At present, they are indistinguishable from American races 3 and 2 respectively. They are maintained on RHA274 and GH × RHA266 respectively.

Resistance tests

Each of the F_3 and F_4 progenies was subjected to separate tests, using 20–25 seeds for each test. The seed-immersion technique described by Mouzeyar et al. (1993) was used. Disease reactions were observed 2 weeks after infection, following 48 h under a saturated atmosphere. 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 manipulation

DNA was extracted from leaves collected at flowering for each of 111 (GH × PAC2) F_2 plants, as described by Gentzbittel et al. (1995). Two DNA bulks were made from 12 homozygous-resistant and 12 homozygous-susceptible F_2 plants following the method of Michelmore et al. (1991). For RFLP analysis, DNA digestion and Southern hybridization were performed as described by Gentzbittel et al. (1995) using the following restriction enzymes: *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III (Amersham). The RFLP probes used were produced by Gentzbittel et al. (1995).

Linkage analysis

For the unit maps of the GH × PAC2 and HA335 × H52 crosses, MapMaker 3.0 software was used; the maps of the region containing *Pl2* and *Pl6* were constructed with a minimum LOD score of 3.0 and a maximum recombination fraction of 0.45. The order of the loci was computed with the "three point" and "comp" commands, and tested with the "ripple" command. For consensus mapping, the probes S124H3_1 and S097H3 (for GH × RHA266), and S124H3_1 and S039H3 (for HA335 × H52), were employed as anchor loci. MapMaker 3.0 was used to generate a consensus map from all the F_2 plants studied.

Results

Location of *Pl2*

Resistance tests with race 1 were carried out on the F_3 progenies derived from 111 F_2 plants. The genotype of each F_2 plant was deduced from the reaction pattern

of its F_3 progeny. The distribution of the F_2 population was 35 homozygous-resistant, 57 heterozygous-resistant and 19 homozygous-susceptible, which fitted the Mendelian ratio 1RR:2Rr:1rr for the segregation of a single dominant gene. ($\chi^2 = 4.69$, $P > 0.05$).

Twelve homozygous-resistant and 12 homozygous-susceptible F_2 plants were selected for preparation of the two bulks. Two probes that detected polymorphism between the bulks, S124H3_1 and S097H3, were employed for subsequent analysis using the 111 F_2 plants. In the F_2 progeny of GH × PAC2, *Pl2* was found to be linked closely to S124H3_1 on linkage group 1 with 8.3% recombination. The recombination fractions indicate that *Pl2* is situated in the same area as *Pl1* (Mouzeyar et al. 1995) and *Pl6* (Roeckel-Drevet et al. 1996).

Comparison of segregations of F_3 progenies from the cross GH × PAC2

The 111 F_2 progenies tested with race 1 to locate the resistance gene, were also tested with race D. Segregation patterns were strictly comparable, indicating no segregation between the resistances to these two races.

Comparison of segregations of F_3 and F_4 progenies from the cross HA335 × H52

The results of the downy mildew tests on F_3 progenies, with five races present in France, are presented in Table 1. They concern only the progenies for which there was sufficient seed to make all five tests. The numbers of progenies that were homozygous-resistant, segregating, and homozygous-susceptible for each race agrees with a Mendelian segregation of IRR:2Rr:1rr indicating control of resistance by a single dominant gene. All the progenies showed exactly the same behaviour when tested with races A, B and C. However, two progenies that were homozygous-resistant for races A, B and C showed segregation when tested with races 1 and D. Table 2 presents the detailed results of the tests on these progenies.

Table 1 Downy mildew reactions of F_3 progenies from the cross between sunflower lines HA335 and H52 (RR: homozygous-resistant; Rr: segregating-resistant + susceptible; rr: homozygous-susceptible)

No. of progenies	Race 1	Race D	Race C	Race B	Race A
41	RR	RR	RR	RR	RR
84	Rr	Rr	Rr	Rr	Rr
27	rr	rr	rr	rr	rr
2	Rr	Rr	RR	RR	RR

χ^2 test: races 1, D: 41:86:27, $\chi^2 = 4{,}65$ 2 *df* $P > 0.05$; races A, B, C: 43:84:27, $\chi^2 = 4.60$ 2 *df* $P > 0.05$

Table 2 Segregations of the two (HA335 × H52) F_3 families showing differences in reaction to French downy mildew races [the results are the numbers of susceptible (sus.) plants/total number of plants tested]

(Ha335 × H52)B.2.6	race 1 3sus./23 + 2sus./22 race D 8sus./37 race C 0sus./31 race B 0sus./30 race A 0sus./24 + 0sus./20
(Ha355 × H52)B.3.39	race 1 3sus./21 + 5sus./24 race D 10sus./27 race C 0sus./29 race B 0sus./21 race A 0sus./22 + 0sus./23

In order to confirm that this apparent difference in segregation was of genetic origin, samples of these two F_3 progenies were selfed to obtain the F_4 generation. Downy mildew tests of 50 F_4 progenies from each F_3 showed that approximately one-quarter (11 and 15) of the F_4 progenies were homozygous-susceptible to races 1 and D but homozygous-resistant to races A, B and C. These results followed a Mendelian segregation and confirmed those of the F_3. Since the recombination between resistance to races 1 (and D) and A (+ B + C) concerned 2 progenies out of 154, the frequency of recombination was 1.3% (Fig. 1).

Graphical genotypes of the two recombinant individuals, constructed following Young and Tanksley (1988), are presented in Fig. 1. Consensus mapping was used to compute a linkage map of the *Pl* region on linkage group 1 (Fig. 2), based on the analysis of 813 F_2 plants originating from six different F_2 populations and including not only those reported here but also those of Gentzbittle et al. (1995), Mouzeyar et al. (1995) and Roeckel-Drevet et al. (1996).

Discussion

The gene from HA61 giving resistance to race 1 in PAC2, and considered up to now as *Pl2*, was located in the same chromosomal region as *Pl1* and *Pl6*, reported by Roeckel-Drevet et al. (1996). This provides additional evidence that downy mildew resistance genes are grouped, and not independent as previously reported. Further studies are in progress to determine whether downy mildew resistance genes from other sources are also located in the same chromosome region.

Co-location of resistance genes is quite frequent in many different species. According to Vranceanu and Stoenescu (1970) the *Pl1* gene is linked to the gene *R1* responsible for resistance to rust in sunflower. The occurrence of clustering of resistance genes has been reported for *Bremia lactucae* of lettuce. The 13 *Dm*

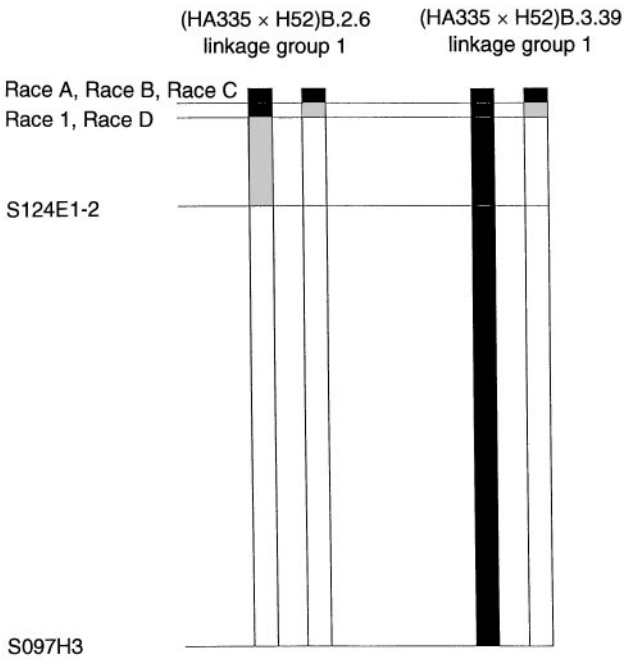

Fig. 1 Graphical genotypes for recombinant plants (HA335 × H52) B.2.6 and (HA335 × H52) B.3.39 illustrating recombination within the *Pl6* locus: *white blocks* indicate that the segment is derived from the susceptible parent (H52); *black blocks* are for segments derived from the resistance parent (HA335), while *stippled blocks* indicate the presence of a cross-over event

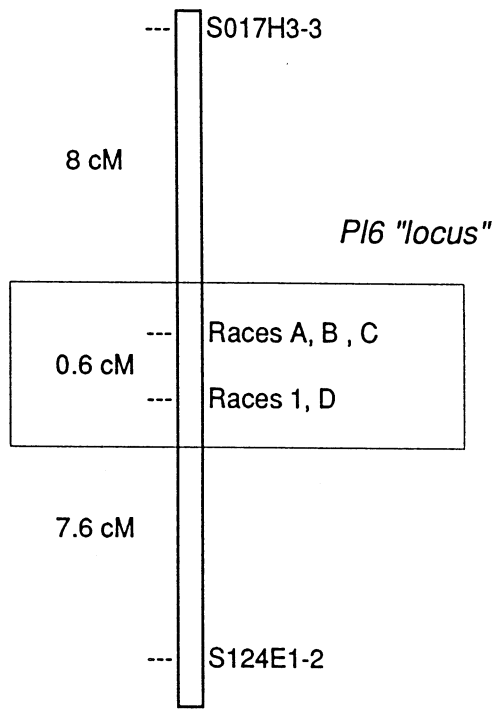

Fig. 2 Part of linkage group 1 of the consensus RFLP map of sunflower showing the position of *Pl* genes

genes responsible for resistance to this species of downy mildew have been mapped in four clusters on the lettuce genome (Farrara et al. 1987). In addition, a clustering of genes giving resistance to different pathogens has been shown in some crops. In tomato, resistance genes *Cf-2* and *Cf-5* to the fungal pathogen *Cladosporium fulvum* are closely linked to the *Mi* locus which confers resistance to root-knot nematodes (Dickinson et al. 1993). In maize, resistance to *Puccinia polysara* (Rpp9) lies 1.6 map units from the *Puccinia sorghi Rp1* locus (Pryor 1987). In *Lactuca serriola*, two genes for resistance to downy mildew were mapped close to existing clusters of resistance genes: the first *R17*, was found in the same cluster as genes for resistance to three pathogenes (Maisonneuve et al. 1994), the second *R18*, was in a cluster with seven *Dm* genes as well as the gene for resistance to root aphid (*Ra*).

The results of downy mildew resistance tests with the progenies containing *Pl6* indicate that from this source it is possible to separate resistance to races 1 and D from resistance to races A, B and C, with about 1.3% recombination. However, in 154 F_3 progenies, resistances to races 1 and D did not segregate; nor did resistances to races A, B and C. Similarly, with the GH × PAC2 progenies, resistances to races 1 and D were not separated in 111 F_3 progenies. Further studies, with larger numbers of progenies will be necessary to determine whether it is possible to separate resistance to each race. At present, it can be concluded that *Pl6* is not one "strong" gene, perhaps with a resistance of the non-race-specific type, but a cluster of several genes, each giving resistance to one, or at most a few, races, as found also in other sunflower genotypes. Resistance to races A, B and C would appear to be closely linked, with resistance to races 1 and D positioned slightly further away (0.6 cM). The gene for resistance to race 1 in the cross GH × PAC2, that was located close to the other *Pl* genes, may, in fact, be *Pl1*, with what is known as *Pl2* being a small cluster of *Pl1* and *Pl2* [resistance to race 2 (or D)].

Clustering of *Pl* genes provides an explanation for many of the unexpected results reported in the past; for example, the lack of segregation between RHA274 (*Pl2*) and Progress (*Pl5*) reported by Miller and Gulya (1987), and between RHA274 and HIR34 (*Pl4* from *H. tuberosus*) reported by Zimmer and Kinman (1972). This means that it is essential to test different sources of resistance with all races to determine if they have different resistance genes, rather than using crossing and test-crossing procedures. However, the present results are in disagreement with reports of segregation indicating independence between genes. In most cases, these differences can probably be explained by different symptom interpretations. The observations leading to conclusions of independent genes considered that the presence of any downy mildew spores on the hypocotyl or cotyledons indicated susceptibility. However, macroscopic observations of plant growth after infection

(Vear 1978), confirmed by microscopic observations of hypersensitive reactions in the hypocotyl (Mouzeyar et al. 1994b), demonstrated that "cotyledon-limited infection" (Gulya et al. 1991), with light sporulation on the cotyledons but not on true leaves, is characteristic of certain resistance genotypes. Thus, certain testcross progenies considered to show segregation for resistance and susceptibility may have been completely resistant but with some cotyledon infection. However, for crosses between lines which do not show this characteristic, the difference in results has yet to be explained. Further work is required since it is possible that downy mildew resistance genes are not all located on the same linkage group.

Similar locations of at least some *Pl* genes could reflect functional relationships between genes, or else a localized mechanism for creating genetic variation. Molecular studies are in progress to identify the genes for resistance involved in the *Pl* locus in order to compare this resistance to sunflower downy mildew with other types of resistance genes.

In conclusion, following these results, it now appears probable that the *P. halstedii*-sunflower interaction does follow the gene-for-gene hypothesis, although complete separation of resistances is necessary for confirmation. Strictly speaking, it would also be necessary to carry out genetic studies on the pathogen, but this is not practicable at present since sexual reproduction cannot be controlled in *P. halstedii*. This conclusion is of considerable practical importance since, if what was called *Pl6* 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 *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. It will be important to study the resistance sources denoted as *Pl7* and *Pl8* (Miller 1992), which also give resistance to all known races, in order to determine whether these are also clusters and, if so, of what size.

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