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P.F. Bert · D. Tourvieille de Labrouhe · J. Philippon
S. Mouzeyar · I. Jouan · P. Nicolas · F. Vear

Identification of a second linkage group carrying genes controlling resistance to downy mildew (*Plasmopara halstedii*) in sunflower (*Helianthus annuus* L.)

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Abstract A sunflower line, XRQ, carrying the gene *Pl5*, which gives resistance to all French downy mildew races shows cotyledon-limited sporulation in seedling immersion tests; consequently, segregations in crosses with other downy mildew resistance sources were tested both by this method and by a secondary infection on leaves. *Pl5* was found to segregate independently of *Pl7* (HA338) but to be closely linked, or allelic, with *Pl8* (RHA340). F_3 and F_4 progenies from a cross with a line containing *Pl2* showed that *Pl5* carries resistance to race 100 which segregates independently of *Pl2*. The *Pl5* gene was mapped on linkage group 6 of the Cartisol RFLP map, linked to two RFLP markers, ten AFLP markers and the restorer gene *Rf1*. Tests with downy mildew race 330 distinguished *Pl5* and *Pl8*, the first being susceptible, the second resistant, whereas both these genes were active against race 304 to which *Pl6* (HA335) and *Pl7* gave susceptibility. It is concluded that *Pl5* and *Pl8* are closely linked on linkage group 6 and form a separate resistance gene group from *Pl6*/*Pl7* on linkage group 1. The origins of these groups of downy mildew resistance genes and their use in breeding are discussed.

Keywords Resistance · Segregation · Mapping · Downy mildew · Sunflower

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P.F. Bert · S. Mouzeyar · I. Jouan · P. Nicolas
U.M.R. "Amélioration et Santé des Plantes",
INRA – Université Blaise Pascal, Campus de Cezeaux, 34,
Ave des Landais, 63177 Aubière, France

D. Tourvieille de Labrouhe · J. Philippon · F. Vear (✉)
U.M.R. "Amélioration et Santé des Plantes",
INRA – Université Blaise Pascal,
Domaine de Crouelle, 234, Ave du Brezet,
63039 Clermont-Ferrand cedex 2, France
e-mail: vear@clermont.inra.fr
Tel.: (33)-(0)-47362-4306, Fax: (33)-(0)-47362-4453

Introduction

Downy mildew (*Plasmopara halstedii*) is one of the most important world wide diseases of sunflower (*Helianthus annuus*). A constant study of new resistance sources is necessary as new physiological races appear regularly (Tourvieille et al. 2000c). To facilitate international co-operation, an international system of race denomination has been adopted since 1998 (Gulya et al. 1998; Tourvielle de Labrouhe et al. 2000b). Resistance was first shown by Vranceanu and Stoenescu (1970) to be controlled by dominant major genes, denoted *Pl*, following a pattern which agrees quite well with the gene-for-gene hypothesis of Flor (1955). First reports suggested that all the known genes were independent, but more recently the genes known as *Pl1*, *Pl2* and *Pl6* were mapped in the same region on linkage group 1 (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996; Vear et al. 1997) of the Cartisol RFLP map (Gentzbittel et al. 1995), with about 0.5 cM between resistances to races 100 and 300 and to races 700, 703 and 710 in the case of *Pl6*. One of the reasons for this change in conclusions may be the recognition (Vear 1978) that plants containing some resistance genes show cotyledon-limited sporulation (CLS) when subjected to the seedling immersion test (Mouzeyar et al. 1993), such that some test-cross progenies, reported originally to show segregation and indicating independent genes, were in fact 100% resistant.

Downy mildew resistance in the INRA inbred line XRQ has been shown to be effective against races 100, 300, 304, 700, 703 and 710, and so is of interest in breeding, especially in France where these races are present. XRQ line was bred from the Russian population Progress, and its resistances to races 710 and 100 were shown not to segregate with those from another Russian population, Novinka (Vear et al. 1998), and to be inherited as a single gene. Following Miller and Gulya (1987) who used the same origins of resistance, this gene was denoted *Pl5*. When subjected to the seedling immersion test, equivalent to primary infections, a large proportion

of plants containing *Pl5* show CLS, making determination of resistance rather difficult. Studies were therefore undertaken to localise it on a molecular map and to search for a closely linked marker which would be useful in breeding programmes. Vear et al. (1998) reported that bulk segregant analysis did not show linkage between this gene and the RFLP markers closely linked with the *Pl6* cluster, and that test-cross progenies from a cross between XRQ and HA335 (*Pl6*) (Miller and Gulya 1991) showed segregation for resistance to race 710. These results suggested that *Pl5* was not located in or near the *Pl6* cluster.

After preliminary studies of downy mildew resistance segregations in progenies from crosses between XRQ and other resistance sources, this paper reports the localisation of this gene on the Cartisol map, using not only RFLPs but also AFLPs, and provides evidence that it is part of a second group of genes giving resistance to all known races of downy mildew, inherited independently from the *Pl6* cluster.

Materials and methods

Sunflower genotypes

The inbred line XRQ was bred by INRA (France) for its downy mildew resistance to all French races of downy mildew from a cross between HA89 and the Russian population Progress (Vear et al. 1998).

For the studies of segregation in test crosses between different sources of downy mildew resistance, the CMS form of XRQ was crossed with USDA lines HA338 (*Pl7*, Miller and Gulya 1991) and RHA340 (*Pl8*, Miller and Gulya 1991). These lines are maintained at INRA, Clermont-Ferrand. The F_1 plants were crossed with two INRA lines (FN and GB) susceptible to all races of downy mildew, to make the test-cross progenies.

For the molecular mapping of *Pl5*, XRQ was crossed with PSC8, an INRA line selected from a population with recurrent selection for capitulum resistance to *Sclerotinia* (Vear et al. 1992) and resistance to downy mildew races 100, 300 and 304 conferred by *Pl2*. Reciprocal crosses were made between the male-fertile forms of the two lines. Eight F_1 plants were selfed by covering the capitula with grease-proof paper bags a few days before flowering to obtain the F_2 generation, which was in turn selfed to obtain the 220 F_3 families. With one exception where there was almost no seed, one plant of each F_3, chosen according to their position in the field, was selfed to obtain the 219 F_4 progenies (as part of the development of recombinant inbred lines).

All the inbred lines tested with different races are maintained at INRA, Clermont-Ferrand. In addition to FN, GB, PSC8, XRQ, HA338 and RHA340, RHA266 (*Pl1*) and HA335 (*Pl6*) were tested.

Downy mildew races

Tests of downy mildew segregations were made in separate growth chambers with races 100, 703 and 710, maintained at INRA, Clermont-Ferrand. Tests on inbred lines with races 700, 300, 304 (Tourvieille de Labrouhe et al. 2000c) and 330 (of American origin, provided by Gulya, USDA, Fargo) were made in P3-level confined growth chambers.

Testing procedures

The usual infection method for resistance tests on germinated seed (primary infection) followed the procedure described by Mouzeyar et al. (1993). Infection of 2-week-old plants (secondary infection) followed the procedure of Tourvieille et al. (2000a). The primary infection test was made alone, or the two tests were made together, with the following procedure:

T: non-infected, germinated seed sown in half trays.
T+12 days: germination of a second seed sample.
T+14 days: preparation of a downy mildew zoosporangia suspension; infection of germinated seed by immersion and of young plants by spraying the terminal bud and first pair of leaves; infected germinated seed sown in the second half of each tray. The trays were maintained in a saturated atmosphere for 24 h.
T+26 days: the trays were maintained in a saturated atmosphere for 48 h to induce sporulation.
T+28 days: observations of both infection series.

Plants showing no sporulation, or only a light sporulation on the cotyledons (CLS) and none on the true leaves, were considered as resistant. For primary infections of test crosses, plants with considerable sporulation on cotyledons and very small true leaves, or which were damped off with no downy mildew sporulation, were considered as indeterminable and were not counted in segregation analyses. Plants with sporulation on true leaves were considered as susceptible. In some cases, for secondary infections it was necessary to use a hand lens to observe sporulation. All observations were made without knowing the genotype in question. Each tray contained susceptible controls for both types of infection and these always showed 100% susceptibility.

For the test crosses, primary infections with races 100 and 710 were made on 100–250 seedlings. Secondary infections with race 710 and both types of test with race 703 were made on 30–80 plants. Primary infection tests were made on 15–20 plants of each of the 220 F_3 and 219 F_4 progenies from the XRQ x PSC8 cross. Race 710 was used for both generations, the F_4 was also tested with race 100. The tests with different races were made on 15–25 seedlings of each inbred line.

RFLP and AFLP analysis

Approximately 5 g of young leaves of XRQ× PSC8 F_2 plants were harvested and ground in liquid nitrogen to obtain a fine powder. Sunflower genomic DNA extraction, digestion by restriction enzymes (*Eco*RI and *Hind*III) and Southern hybridisation were carried out as described by Gentzbittel et al. (1995). The autoradiographs were manually scored. RFLP probes were chosen among those used for the sunflower consensus linkage map (Gentzbittel et al. 1995).

The AFLP protocol developed by Vos et al. (1995) was followed with minor modifications (Bert et al. 1999). PCR products were visualised using the silver-nitrate staining method as described by Tixier et al. (1997). AFLP markers were identified based on the primer-pair combination used and the estimated molecular size. The approximate size of each marker was expressed in nucleotides as estimated in comparison with the mobility of the bands of the 10-base ladder (Sequamark, Research Genetics). Nine primer pairs were selected (E32M49, E32M50, E32M59, E32M60, E32M62, E33M48, E33M49, E33M59 and E35M48) for the generation and screening of AFLP markers.

Data analysis and linkage-group construction

Clearly readable bands were scored as genetic markers. The software package JOINMAP 2.0 (Stam 1993) was used to estimate segregation distortion and determine linkage groups while the software program MAPMAKER 3.0b (Lander et al. 1987) was used to order loci and construct linkage groups. Analyses were performed with a LOD score threshold of 4.0 and a maximum recombination value of 40% (θ=0.40) for grouping and ordering markers. Kosambi's mapping function was applied for the calculation of map distances (Kosambi 1944).

Results

Segregations in primary and secondary infections of test-cross progenies

The results of downy mildew infections on test-cross progenies are presented in Table 1. The first tests (03/99) were difficult to interpret, with plants in an indeterminable category (either damped-off or with the first true leaves which did not grow, according to the growth chamber in which the tests were made). When the second series of tests was made (10/99 or 11/99), there were fewer damping-off problems for the primary infections. It was noted that progenies which showed CLS in primary infections frequently showed yellow spotting of the first leaves, but no sporulation.

The segregation of the test cross from XRQ×HA338 presented an apparent excess of susceptible plants in a first test with race 100, but agreed with a 3 resistant:1 susceptible ratio for race 710. The other results for this cross also agree with the 3 resistant:1 susceptible ratio, so it was concluded that two independent genes each giving resistance to races 100, 710 and 703 were present. In contrast, no segregation appeared in the progeny from the cross between XRQ and RHA340. In the first test there were some indeterminable plants, and in all primary infections at least some plants showed CLS. These results indicate that *Pl5* is independent of *Pl7*, but that it is the same gene or closely linked to *Pl8*.

Segregations in primary infections of (XRQ×PSC8) F_3 and F_4 progenies

In all the tests many plants showed CLS, making the certain identification of some progenies as homozygous resistant (res.), segregating (seg.) or homozygous susceptible (sus.) rather difficult. For race 710, in both F_3 and F_4 generations, there was an excess of progenies judged as heterozygous, and a lack of progenies judged as homozygous susceptible, compared with the theoretical segregation of 1 res.: 2 seg.: 1 sus. in the F_3 and 3 res.: 2 seg.: 3 sus. in the F_4 expected for a single dominant gene. The F_3 generation, used to define the genotype of the F_2 plants on which the molecular analyses were made, showed a segregation of 42 res.: 146 seg.: 32 sus. To agree with the theoretical segregation, 17 progenies of the 220 studied must be considered as wrongly classed as segregating instead of susceptible. For the F_4 (73 res.: 93 seg.: 53 sus.) to agree with the χ^2 for 95% probability, the number of progenies wrongly classed would be 22.

Only the F_4 generation was tested with race 100. The segregation for the reaction of each progeny to the two races is given in Table 2. Sixteen F_4 progenies/219 were

Table 1 Segregation of resistance to downy mildew races 100, 703 and 710 in test-cross progenies from crosses between XRQ (*Pl5*) and HA338 or RHA340 and goodness of fit to a 3 resistant : 1 susceptible ratio

Progeny	Race	Date	Test	Susceptible	Resistant	Total	χ^2 (3:1)	Indeterminable plants excluded from segregations
[(XRQ×HA338)×FN]TC	100	03/99	Primary	53	70	123	21.47 (p<0.01)	14
	710	03/99	Primary	49	117	166	1.81 (p>0.05)	17
	710	11/99	Primary	21	84	105	1.40 (p>0.05)	0
	710	11/99	Secondary	19	55	74	0.01 (p>0.05)	0
	703	10/99	Primary	1	21	22	4.84 (p<0.05)	7
	703	10/99	Secondary	7	31	38	0.82 (p>0.05)	0
[GB×(XRQ×RHA340)]TC	100	03/99	Primary	0	127	127	42.3 (p<0.01)	5
	710	03/99	Primary	0	233	233	77.7 (p<0.01)	17
	710	11/99	Primary	0	113	113	37.6 (p<0.01)	0
	710	11/99	Secondary	0	103	103	34.5 (p<0.01)	0
	703	10/99	Primary	0	38	38	12.7 (p<0.01)	0
	703	10/99	Secondary	0	41	41	13.7 (p<0.01)	0

Table 2 Reactions of F4 progenies from the cross (XRQ× PSC8) using primary infection tests with races 100 and 710

Item	Homozygous resistant race 100	Segregating race 100	Homozygous susceptible race 100	Total
Homozygous resistant race 710	71	2	0	73
Segregating race 710	34	58	1	93
Homozygous susceptible race 710	23	15	15	53
Total	128	75	16	219

Table 3 Reactions of inbred sunflower lines containing different downy mildew resistance genes, to seven downy mildew races. R: resistant; S: susceptible

Inbred line	Resistance gene or cluster	Downy mildew race						
		100	300	304	330	700	703	710
FN	–	S	S	S	S	S	S	S
GB	–	S	S	S	S	S	S	S
RHA266	*Pl1*	R	S	S	S	S	S	S
PSC8	*Pl2*	R	R	R	R	S	S	S
XRQ	*Pl5*	R	R	R	S	R	R	R
HA335	*Pl6*	R	R	S	R	R	R	R
HA338	*Pl7*	R	R	S	R	R	R	R
RHA340	*Pl8*	R	R	R	R	R	R	R

observed as homozygous susceptible. The existence of these susceptible progenies indicates that the two parents of the cross, XRQ and PSC8, do not have the same gene giving resistance to race 100. The ratio 128 res.: 75 seg.: 16 sus. differs from the theoretical F_4 segregation for two genes, 21 res.: 8 seg.:3 sus., because, as for race 710, there is an excess of progenies observed as segregating. If five of the 75 segregating progenies was wrongly judged, the segregation would agree with the theoretical one for two genes. The entirely susceptible category, for which there is little likelihood of error, agrees with the theoretical segregation.

Although the problems of CLS may have caused some progenies to be wrongly judged, the fact that there were no F_4 progenies resistant to race 710 and susceptible to race 100, whereas there were 23 progenies resistant to race 100 and susceptible to race 710, indicates that resistance to race 710 always carries with it resistance to race 100. It can be concluded that a gene giving resistance to race 100 is part of, or closely linked to, the resistance of XRQ to race 710.

Linkage between the *Pl5* locus and DNA markers

Pl5 showed linkage with ten AFLP markers from the total set of AFLP markers generated using the nine primer pairs and with two RFLP markers (S034H3 and S094H3) from the 80 RFLP probes placed throughout the genome. The closest marker was S017H3 at 8 cM. In addition, *Pl5* mapped 15 cM from *Rf1*, a fertility restorer locus. The two RFLP markers and the *Rf1* locus were previously mapped on linkage group 6 of the CARTISOL map (Gentzbittel et al. 1995). This linkage group was 105-cM long with *Pl5* close to one end (Fig. 1).

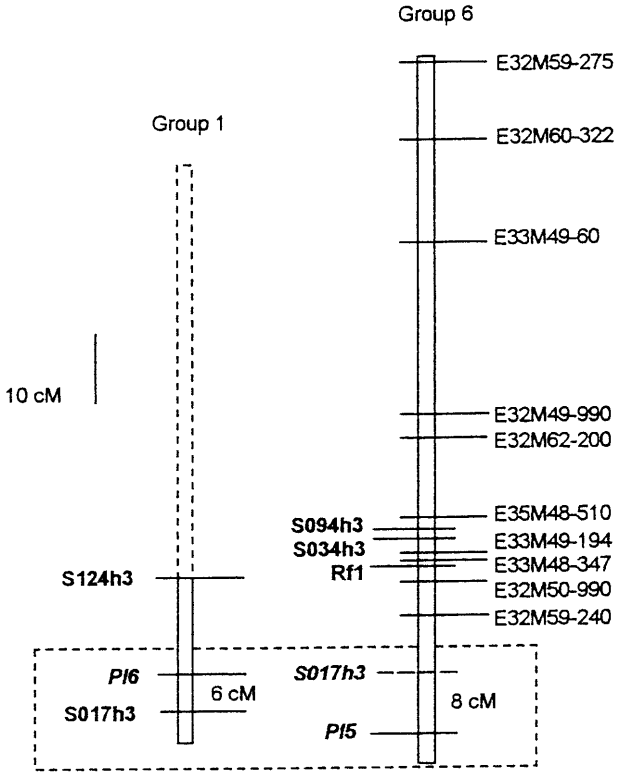

Fig. 1 Genetic map of linkage group 6 with the *Pl5* locus, constructed at a LOD score of 4.0 and a partial genetic map of linkage group 1 showing the *Pl6* region with RFLP markers flanking this locus (Roeckel-Drevet et al. 1996; Gentzbittel et al. 1999). The *Pl5* locus maps 8 cM from the putative position of one S017 marker (Gentzbittel et al. 1999) at the end of group 6, whereas the *Pl6* locus maps 6 cM from another S017 locus at the end of group 1

Distinction of resistance genes according to reaction to downy mildew races

The results of the tests made on a range of downy mildew resistance sources with all the races identified in France and with an American sample of race 330 are presented in Table 3. They indicate that lines carrying *Pl2*, *Pl6*, *Pl7* and *Pl8* are resistant to race 330, whereas *Pl1* and *Pl5* are not active against this race. *Pl5* is thus not identical to *Pl8*. In contrast, tests with race 304 showed that, in this case, *Pl2*, *Pl5* and *Pl8* are effective whereas lines with *Pl1*, *Pl6* and *Pl7* are susceptible. This is further evidence of the difference of the *Pl5*/*Pl8* group compared with *Pl6* and *Pl7*.

Discussion

Working on *Pl5* has highlighted the problem of germinated seed tests which give plants showing cotyledon-limited sporulation, although this phenomenon has never been observed in the field. The tests in different growth

chambers, with different races, also showed the problem of deciding the genotypes of some plants which do not grow normally. Whereas CLS may be genetically determined (Vear 1978), the presence of indeterminable plants probably depends on environmental conditions; for example, when primary and secondary tests were combined there were fewer problems for primary tests, perhaps because of the greater space between the polythene sheeting used to obtain 100% humidity and the compost. To help genotype definition, at least when the numbers of plants are not too large, a secondary infection test, in which the terminal bud of 2-week-old plants was sprayed with a zoosporangia suspension, followed by observation of the first pair of true leaves 2-weeks later, was developed by Tourvieille de Labrouhe et al. (2000a). In this test, the presence or absence of sporulation are indicative of susceptibility and resistance respectively.

Since most of the tests of (XRQ×HA338) test-cross progenies gave normal segregations for two dominant independent genes, there is no reason to hypothesise that genetical segregation of *Pl5* is skewed in primary infection tests of (XRQ×PSC8) F_3 progenies. The lack of agreement with a theoretical segregation in these tests is almost certainly due to difficulties in the classification of some plants. The progenies judged as homozygous resistant or homozygous susceptible are probably correct, but 5–10% of the conclusions concerning those considered as segregating may be erroneous; most often plants with much sporulation but small true leaves are considered as resistant when they are, in fact, susceptible. It would be of interest to submit the F_3 or F_4 families used in the present experiments to the secondary tests.

The problem of interpretation of 5–10% of progenies does not, however, have any great effect on the mapping of *Pl5*. A maximum-likelihood approach was used to estimate recombinant fractions between markers. Lorieux et al. (1995) showed that the estimation of recombinant fractions of codominant markers, such as *Pl5*, was little affected by skewed segregations. Further, the assignation of *Pl5* to linkage group 6 was consistent when LOD threshold values above 4.0 were applied. This study thus indicates that there is a second linkage group (6) carrying downy mildew resistance genes, in addition to linkage group 1 on which the *Pl* genes had been mapped so far.

The existence of genes giving resistances to races 100, 703 and 710 on both of these two linkage groups has been demonstrated. The existence of 15 completely susceptible (XRQ×PSC8) F_4 progenies indicates that resistance to race 100, which is part of, or linked to, *Pl5*, is inherited independently of the same resistance provided by *Pl2* in PSC8 on linkage group 1. Vear et al. (1998) found that the resistance of XRQ to race 710 is inherited independently of *Pl6* (HA335) and the present results indicate that this resistance, and that to race 703, are also independent of *Pl7* (HA338) [which is probably located in the same region as *Pl6* (data not shown)]. In contrast, since *Pl8* (RHA340) showed no segregation when crossed with *Pl5* (XRQ) and tested with races 100, 703 and 710, it may be considered that these two genes are closely linked, that *Pl8* is also situated on linkage group 6, but that the two genes are not the same since their race resistance patterns are not identical.

It was rather unexpected that *Pl5*, which originated from *Helianthus tuberosus*, and *Pl8*, from *Helianthus argophyllus*, should be closely linked, but it may be noted that the genes in the *Pl1*/*Pl2*/*Pl6*/*Pl7* region come from other species: wild *H. annuus* and *Helianthus praecox*. A gene denoted *Pl4* (Vear 1974), also obtained from *H.tuberosus* (Leclercq et al. 1970) and giving resistance to races 100 and 300, did not appear linked to *Pl1*, *Pl2* and *Pl6* (Vear et al. 1998). It will now be of interest to test the markers of *Pl5* on this genotype to determine whether *Pl4* is on linkage group 6.

A further point of difference between the genes on linkage groups 1 and 6 is the appearance of CLS. Although lines with *Pl1* often show CLS, those with *Pl2* only do so quite rarely, and those with *Pl6* or *Pl7* almost never. In contrast, both *Pl5* and *Pl8*, and also *Pl4*, show resistance reactions with CLS very frequently.

The identification of a second linkage group carrying closely linked genes giving resistance to many races of downy mildew is not surprising, since most of the plant resistance genes involved in gene-for-gene interactions are clustered (Michelmore and Meyers 1998). It has not yet been demonstrated that the *Pl5*/*Pl8* region is a cluster of genes with different specificities, but the fact that it carries resistance to at least seven different races, indicates that it is probably a series of closely linked genes. Observation with race 304, to which *Pl2* gives resistance but *Pl6* and *Pl7* give susceptibility, suggests that, in a cluster, there may be many different combinations of individual genes, each giving resistance to one race. *Pl2* could be a small cluster giving resistance to races 100, 300 and 304, included in part of the *Pl6*/*Pl7* region, but with at least the race-304 resistance allele being different between HA335 (*Pl6*) and PSC8 (*Pl2*). Recombination within the region appears to be possible since some lines carrying *Pl2*, backcrossed to introduce the resistance to race 710 of *Pl6* or *Pl7*, show resistance both to races 710 and 304 whereas others have become susceptible to the latter race (data not shown).

Comparison of the molecular maps of linkage groups 1 and 6 as described by Gentzbittel et al. (1999), show that, in both cases, the downy mildew resistance genes are situated in distal positions. In addition, the SUN017 probe detects loci linked at about 5 cM to both resistance gene regions, which suggests that these major resistance loci have arisen by duplication. In an attempt to check whether the *Pl5* locus contains nucleotide-binding site (NBS-LRR) sequences, as was shown for the *Pl6* locus (Gentzbittel et al. 1998), the same RFLP probe was used in this study and failed to detect any polymorphic marker linked to the *Pl5* locus. Thus, this question remains to be answered, and for this purpose NBS-LRR sequences are being used. In the near future, molecular comparison of the *Pl6*/*Pl7* and *Pl5*/*Pl8* regions should provide some information on the origin and the relationships between

different sources of resistances to downy mildew in sunflower.

This study has not provided a close marker which could replace seedling tests in breeding programmes but, since *Pl5* is now mapped on group 6, it will be possible to search for additional AFLP or PCR markers on this group. The results reported here are of importance for breeders since they indicate that it is possible to combine more than one resistance gene in an inbred line, and thus up to four different alleles in a hybrid variety. For example, *Pl2+Pl5* would give resistance to all European races, and these could be combined in a hybrid variety with *Pl6+Pl8*. Such varieties with multigenic resistance should provide protection against the spread of new pathotypes. The identification of molecular markers closely linked to the different resistance genes will be essential to check the presence of more than one gene giving resistance to the same downy mildew races in an inbred line or a hybrid variety.

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