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RFLP and RAPD mapping of the sunflower *Pl1* locus for resistance to *Plasmopara halstedii* race 1

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Abstract The *Pl1* locus in sunflower, *Helianthus annuus* L., conferring resistance to downy mildew, *Plasmopara halstedii,* race 1 has been located in linkage group 1 of the consensus RFLP map of the cultivated sunflower. Bulked segregant analyses were used on 135 plants of an F_2 progeny from a cross between a downy mildew susceptible line, GH, and RHA266, a line carrying *Pl1*. Two RFLP markers and one RAPD marker linked to the *Pl1* locus have been identified. The RFLP markers are located at 5.6 cM and 7.1 cM on either side of *Pl1*. The RAPD marker is situated at 43.7 cM from *Pl1*. The significance and applications of these markers in sunflower breeding are discussed.

Key words *Helianthus annuus* · *Plasmopara halstedii* · Disease resistance · Bulked segregant analysis · Molecular markers

Introduction

Downy mildew of sunflower (*Helianthus annuus* L.) is caused by the obligate parasite *Plasmopara halstedii*. This disease is found in most parts of the world where sunflowers are grown and can cause severe losses in this crop (Sackston 1981). At least seven different races have been described according to their reaction on different sunflower lines (Gulya et al. 1991 a, b).

Resistance is controlled by single dominant genes designated as *Pl* (Vranceanu 1970) and has been found for all known downy mildew races. Some *Pl* genes, such as *Pl1* and *Pl2* were found in cultivated sunflower (Vranceanu 1970; Zimmer and Kinmann 1972) while others were derived from interspecific crosses, such as *Pl4* derived from *H. tuberosus* (Vear 1974), *Pl7* from *H. praecox*, and *Pl8* from *H. argophyllus* (Miller and Gulya 1991). The *Pl1* gene was the first downy mildew resistance gene described in the sunflower (Vranceanu 1970). It confers resistance to race 1, the prevalent race in Europe. In France, resistance to this race is obligatory for the registration of commercial varieties.

Although genetical studies on the inheritance of resistance to downy mildew in sunflower are numerous (Vranceanu 1970; Zimmer and Kinmann 1972; Vranceanu et al. 1981; Miller and Gulya 1991), the relationship between the different *Pl* genes remains unclear. Some *Pl* genes such as *Pl2* and *Pl4* were first described as independent (Vear 1974), but have now been reported not to segregate (Sackston 1992). Some sunflower lines are resistant to more than one race, possibly because they possess more than one *Pl* gene (Sackston et al. 1990). It would therefore be beneficial to locate *Pl* loci on a sunflower linkage map.

Recently, the use of near-isogenic lines (Young et al. 1988) or bulked segregant analysis (Michelmore et al. 1991), together with molecular markers, has accelerated the mapping of many resistance genes in different plant species. Examples are the *Pto* gene in tomato (Martin et al. 1991), the *Dm5/8* gene in lettuce (Michelmore et al. 1991) and the *Are* gene in French bean (Adam-Blondon et al. 1994). RFLP and RAPD markers have already been shown to be useful in studies of polymorphism in the sunflower (Gentzbittel et al. 1994; Teulat et al. 1994).

This paper reports the mapping of the *Pl1* locus for resistance to downy mildew race 1 on the consensus linkage RFLP map of cultivated sunflower (Gentzbittel et al. 1995) using bulked segregant analysis.

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Materials and methods

Sunflower genotypes

An F_2 population of 135 plants was derived from a cross between two sunflower lines, GH and RHA266. GH is susceptible to downy mildew race 1, with no characterised resistance genes. RHA266 contains the resistance gene *Pl1* (Fick and Zimmer 1974) and is resistant to downy mildew race 1. These lines are maintained at INRA by self pollination under paper bags.

Fungal isolate

An isolate of downy mildew race 1 (European race) was used. It is maintained at INRA (Clermont-Ferrand) on HA89, a susceptible sunflower genotype.

Evaluation of disease reaction

The downy mildew resistance genotype of each F_2 plant was determined by testing their F_3 progenies, obtained from selfing under paper bags. For each progeny, at least 20 seedlings were infected and grown as described previously by Mouzeyar et al. (1993). Seedlings were scored as susceptible if fungal sporulation was evident on cotyledons and true leaves, and resistant if no sporulation, or only a slight sporulation, was observed on cotyledons (Mouzeyar et al. 1993, 1994). The corresponding F_2 plants were then classified as homozygous susceptible, homozygous resistant, or heterozygous for *Pl1*.

DNA extraction and bulk preparation

About five fully grown leaves were collected from each of the 135 F_2 plants at flowering. DNA extraction was conducted as described by Gentzbittel et al. (1992). Equal quantities of DNA were bulked from 12 homozygous-resistant and from 12 homozygous-susceptible F_2 plants, according to the method of Michelmore et al. (1991), to give two DNA bulks.

RFLP and RAPD procedures

RFLP analysis

DNA digestion and Southern hybridization were performed as described previously (Gentzbittel et al. 1992) using four restriction enzymes: B*gl*II, *Eco*RI, *Eco*RV and *Hind*III (Amersham). The RFLP probes used were produced and mapped by Gentzbittel et al. (1995).

RAPD analysis

Random decamer primers (kits A to T, Operon Technologies, Calif., USA) were used to identify polymorphic DNA between the two bulks. Amplification was conducted according to Williams et al. (1990) with minor modifications: 40–50 ng DNA were used as template in a 25-µl reaction volume containing 10 mM Tris-HCl pH 9, 200 µM of each dNTP (Pharmacia), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% TritonX100, 0.2% gelatin, 0.2 µM of primer and 1 unit of *Taq* DNA Polymerase (Appligène). Amplification was performed in a 9600 Perkin Elmer Cetus thermal cycler as follows: initial denaturation for 5 min at 91°C, then 40 cycles of 1 min at 91°C, 1 min at 36°C and 2 min at 72°C. The 40 cycles were followed by final extension for 5 min at 72°C. Amplification products were analysed by electrophoresis on 1.4% agarose gels in Tris-acetic acid-EDTA (TAE) buffer and stained with ethidium bromide.

Linkage analysis

Linkage analysis was conducted using the software Mapmaker 3.0 (Lander et al. 1987). Markers were ordered with a minimum LOD score of 3.0 and r_{max}=0.35. Recombination fractions were converted into centiMorgans (cM) by applying the Haldane function. For consensus mapping, the Gmendel package was used (Holloway and Knapp 1993) with the same initial options as for Mapmaker.

Results

Bulked segregant analysis

The downy mildew resistance tests on the (GH×RHA266) F_3 progenies, derived from the 135 F_2 plants, gave 24 homozygous-resistant, 76 segregating and 35 homozygous-susceptible progenies. This segregation fitted the expected ratio for a single segregating gene (1:2:1, χ^2=3.9, $0.10<P<0.25$). Twelve homozygous-resistant and 12 homozygous-susceptible F_2 plants were then selected for preparation of the two bulks. RAPD primers and RFLP probes were screened for polymorphism against the two bulks and the parental lines GH and RHA266. Polymorphic markers were then screened against the 135 F_2 plants including those used for bulk preparation.

RAPD primers. Of the 350 decamer primers tested, 48 (14%) revealed polymorphism between the parental lines but none revealed polymorphism between the two bulks. However, the primer OPD13 amplified a 1600-bp band from the DNA of RHA266 and the resistant bulk, whereas this band was faint in the susceptible bulk and absent from GH (Fig. 1) Thus, this marker was named $OPD13_{1600}$.

RFLP probes. A total of 33 loci distributed on the first seven linkage groups of the RFLP map of cultivated sunflower were tested. Two probes that detected polymorphism between the bulks were identified. SUN017 is a dominant marker which revealed a 2.1-kb band present only in RHA266 and resistant bulk DNA. SUN124 revealed two bands (9 and 7.8 kb) in GH and the susceptible bulk DNA, and a 4.6-kb band in RHA266 and the resistant bulk DNA (Fig. 2).

Mapping of the Pl1 gene

Subsequent segregation analysis using the 135 F_2 plants with the Mapmaker 3.0 programme showed that SUN017, SUN124 and $OPD13_{1600}$ are linked to the *Pl1* locus. SUN017 is 5.6±2.0 cM (LOD=22.1) from the *Pl1* locus whilst SUN124 is situated at 7.1±1.5 cM (LOD=8.4). $OPD13_{1600}$ is 43.7±3 cM (LOD=2.2) from the *Pl1* locus. Analysis using Gmendel software with SUN017 and SUN124 as anchor loci allowed location of the *Pl1* locus on linkage group 1 of the RFLP consensus map of the cultivated sunflower (Fig. 3). (Gentzbittel et al. 1995).

Discussion

The purpose of this study was to locate the downy mildew resistance locus *Pl1* on the sunflower RFLP map. A bulked

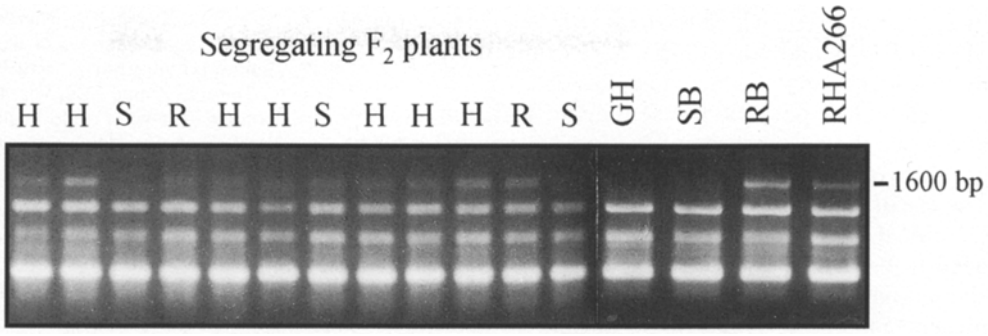

Fig. 1 Amplification of genomic DNA from RHA266 (resistant parent), RB (resistant bulk), SB (susceptible bulk), GH (susceptible parent) and 12 segregating F_2 plants with the OPD13 primer (operon technologies). The 1600-bp band is the $OPD13_{1600}$ product linked to the *Pl1* locus. Downy mildew phenotypes: *R* homozygous resistant, *S* homozygous susceptible and *H* heterozygous resistant

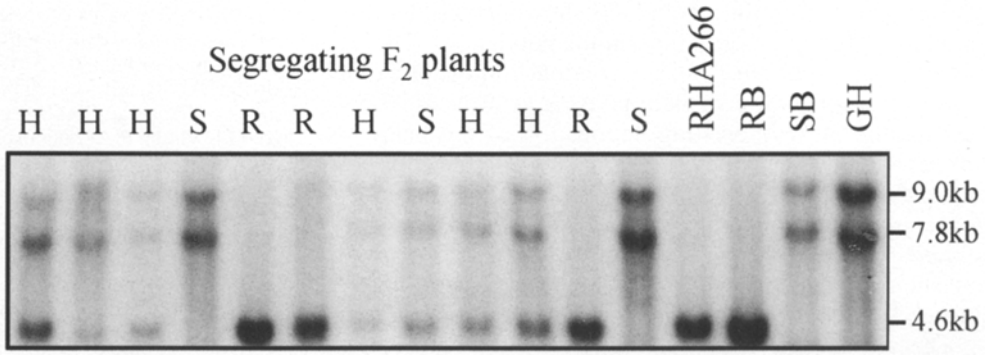

Fig. 2 Autoradiograph showing linkage between the *Pl1* locus and the RFLP locus SUN124. Probe SUN124 was hybridized onto blots of *Eco*RI-digested DNA from the two bulks, the parental lines, and 135 F_2 plants (only 12 F_2 plants are shown). The 9.0-kb and 7.8-kb bands are the GH (susceptible) allele, the 4.6-kb band is the RHA266 (resistant) allele

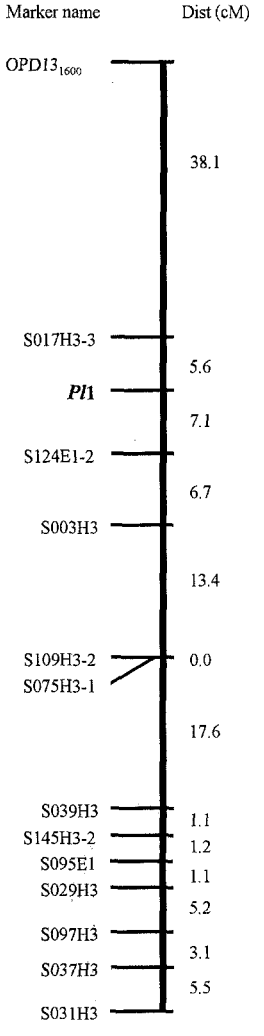

Fig. 3 RFLP map of sunflower linkage group 1 containing the *Pl1* locus for resistance to *P. halstedii* race 1. Map distances in cM are based on the analysis of 135 F_2 plants derived from the cross between the susceptible line GH and the resistant line RHA266. Loci nomenclature is as follows: SXXXYY-Z with SXXX the SUN probe number, YY the restriction enzyme used for mapping (E1 *Eco*RI, H3 *Hin*dIII). For those probes that hybridized to more than one site in the genome, the suffix –Z (–1, –2, –3) indicates each duplicate locus

segregant analysis as described by Michelmore et al. (1991) was used to identify two RFLP markers, one on each side of the *Pl1* locus, and also a linked RAPD marker. Recently, this strategy has also been used for other agronomic characters, such as photosensitive genic male sterility in maize (Zhang et al. 1994). Bulked segregant analysis was found to be useful in the present study, since it was possible to detect a RAPD marker 43.7 cM from the *Pl1* locus (30±5% recombination). Although the susceptible bulk included three recombinant individuals, with the $OPD13_{1600}$ band, it showed only a weak amplification of this band, in comparison with the resistant bulk. This agrees with the report of Michelmore et al. (1991), who considered that segregating markers within a 30% recombination range would be detectable by bulked segregant analysis.

The RFLP probes used in this study were initially selected by Gentzbittel et al. (1994, 1995) and they proved to be useful. In contrast, only 14% of the RAPD primers tested showed polymorphism between GH and RHA266. This result may be explained by the low level of polymorphism which has been found in cultivated sunflower using isozymes (Quillet et al. 1992), RFLP (Gentzbittel et al. 1994) and RAPD markers (Teulat et al. 1994).

Progenies from five crosses involving four inbred lines were used to construct a consensus RFLP map of the cultivated sunflower and SUN017H3-3 and SUN124E1-2 were found to be separated by 52 cM (Gentzbittel et al. 1995). In the present paper, we present a new calculation for this linkage group, in which the number of segregating families was increased (six instead of five crosses and 795 plants compared with 560 plants for the first version of the map). Also, the loci SUN075H3-1 and SUN109H3-2 were found to be identical. This led us to propose a new map of

linkage group 1, on which the locus *Pl1* is located between SUN017H3-3 and SUN124E1-2. The distance between these 2 loci is now estimated at 12 cM.

It should be possible to use the two RFLP markers to accelerate the introduction of *Pl1* into susceptible lines by back-cross procedures, as proposed by Melchinger (1990). The use of these markers, linked to the *Pl1* locus at 5.6 and 7.1 cM, should reduce the number and size of families that need to be tested, since less than 0.5% of plants should show double crossing-over such that the two markers are present and the resistant allele absent. In addition, this could, with a high degree of probability, help to clarify the resistance or susceptibility of plants showing considerable amounts of fungus sporulation on the cotyledons. This is of particular importance since cotyledon-limited infection is very frequent in the presence of the *Pl1* gene (Vear 1978). Perhaps the most interesting application of the markers linked to *Pl1* will be to determine linkage relationships between *Pl* genes, as it has been suggested that certain *Pl* genes are tightly linked (Mouzeyar et al. 1992). Studies with SUN017 and SUN124 , and other linked RFLP markers, should make it possible to determine whether other *Pl* loci are located in the same linkage group as the *Pl1* locus.

The ultimate objective of this work is the molecular cloning of *Pl1* and the analysis of its mode of action. This and the other *Pl* genes induce a hypersensitive-like reaction which is rather unusual in that this occurs, after fungal penetration of the hypocotyl, within the cortical parenchyma (Mouzeyar et al. 1994). Recently, two resistance genes of plant species have been cloned by a map-based cloning strategy: the *Pto* gene for resistance against *Pseudomonas syringae* in tomato (Martin et al. 1993) and *RPS2* in *Arabidopsis thaliana* against the same pathogen carrying the avirulence gene *avrRpt2* (Bent et al. 1994; Mindrinos et al. 1994). In both cases, markers closely linked to the resistance gene were located and these served either to identify YAC clones containing the resistance gene (*Pto*) or for chromosome walking (*RPS2*). In our case, it will be necessary to find other markers more closely linked to *Pl1* than are SUN017H3-3 and SUN124E1-2, in order to initiate such a strategy to clone this gene.

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