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ORIGINAL PAPER

Consensus mapping of major resistance genes and independent QTL for quantitative resistance to sunflower downy mildew

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Abstract Major gene resistance to sunflower downy mildew (*Plasmopara halstedii*) races 304 and 314 was found to segregate independently from the resistance to races 334, 307 and 304 determined by the gene *Pl2,* already positioned on Linkage Group (LG) 8 of sunflower molecular maps. Using a consensus SSR–SNP map constructed from the INEDI RIL population and a new RIL population FU × PAZ2, the positions of *Pl2* and *Pl5* were confirmed and the new gene, denoted *Pl21*, was mapped on LG13, at 8 cM from *Pl5*. The two RIL populations were observed for their quantitative resistance to downy mildew in the field and both indicated the existence of a QTL on LG8 at 20–40 cM from the major resistance gene cluster. In addition, for the INEDI population, a strong QTL on LG10, reported previously, was confirmed and a third QTL was mapped on LG7. A growth chamber test methodology, significantly correlated with field results, also revealed the major QTL on LG10, explaining 65 % of variability. This QTL mapped in the same area as a gene involved in stomatal opening and root growth, which may be suggested as a possible candidate to explain the control of this character. These results indicate that it should be possible to combine major genes and other resistance mechanisms, a strategy that could help to improve durability of sunflower resistance to downy mildew.

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Introduction

Downy mildew, caused by the obligate oomycete parasite *Plasmopara halstedii*, has been known as a disease of sunflower (*Helianthus annuus* L) since before 1950, both in North America (Sackston 1949; Leppik 1962) and Russia (Novotelnova 1960), and since 1966 in France (Louvet and Kermoal 1966), corresponding to development of the sunflower crop. Seedlings are infected mostly through the roots and disease cases damping off, dwarfing and sterility. The discovery of major gene resistance, first reported in 1967 (Goossen and Sackston 1967), played a very important role in the development of sunflower acreages from 1974, particularly in Europe and USA and all modern varieties carry some major resistance genes. This resistance allowed the demonstration of physiological specialisation of the pathogen, into two races, 1 (now 100) in Europe, and 2 (now 300) in North America by Zimmer (1974). Following the identification of new sources of resistance, it has been possible to identify up to 20 races, with different virulence patterns (Gulya, 2007). Some are probably new; others may have pre-existed but could not be precisely identified in the absence of adequate differential lines. Race nomenclature, with 3 digits (e.g., 304, 710), is based

on the reaction of 9 (3 groups of 3) inbred sunflower lines (Gulya et al. 1998), which may need to be increased to 15 (5 groups of 3) (Tourvieille de Labrouhe et al. 2012). Direct race identification of the pathogen is not yet possible; studies have only started recently on effectors of pathogenicity (As-sadi et al. 2011). Reciprocally, sunflower lines originally considered as carrying the same resistance gene or genes now show differing resistance profiles to some of the new races.

Mapping of the genes identified is a first step towards sequence identification and cloning, which could provide information on their diversity and possible efficiency and durability. In sunflower, major genes giving race specific resistance to downy mildew have been mapped since 1995. So far, there appear to be two main clusters, one on LG8 (Mouzeyar et al. 1995; Roeckel-Drevet et al. 1996) and a second on LG13 (Bert et al. 2001; Radwan et al. 2002). On LG8, genes known as *Pl1* (resistance to race 100), *Pl2* (resistance to "3xx" races, Tourvieillle de Labrouhe et al. 2000) and *Pl6/7* (resistance to races "xx0", particularly 703, 710 and 730), have been found (Bouzidi et al. 2002), together with *Pl15,* which appears resistant to all known races (Bertero de Romano et al. 2010). Vear et al. 1997 showed that the *Pl6* cluster on LG8 could be split, with about 0.5 % recombination, giving lines resistant to races 330(US), 703, 710 and 730 but susceptible to race 100 and all the other "3xx" races including 300 (Tourvieille de Labrouhe et al. 2012). Bouzidi et al. 2002 reported that genes of the TIR-NBS-LRR class were present in this cluster.

The other cluster, on LG13 carries *Pl5* (resistance to "30x" or "70x") and *Pl8* (resistance to all known races). Radwan et al. 2002 and Radwan et al. 2011 reported genes in this area to be of the CC-NBS-LRR class. Dussle et al. (2004) mapped *Plarg* on LG1 and Mulpuri et al. (2009) *Pl13*, at the other end of LG1 from *Plarg*. Vear et al. (2012) reported unexpected segregation patterns in tests with race 304 on a series of F3 progenies, suggesting that two genes could be involved in resistance to this race. Although one of them was probably the *Pl2* mapped on a RFLP-SSR map by Bret-Mestries 1996, the position of the other was not known.

Since these major genes are race specific and in recent years, many have been rendered inefficient quite rapidly (Tourvieille de Labrouhe 2003; Gulya, 2007), research has been made into partial, quantitative resistance, which could be more durable. Tourvieille de Labrouhe et al. (2008) reported significant genetic variability in the response of sunflower to infection in the field, and Vear et al. (2008) localised two QTL on LG 8 and LG10 in a RIL population, but the genetic factors underlying these QTL have not so far been identified. Some quantitative resistances have been found to be associated with defeated major resistance genes (Stewart et al. 2003; Poland et al. 2008) and, since many resistance gene candidates (RGC) have been mapped on the different linkage groups (Radwan et al. 2008), this could be the case for sunflower downy mildew. However, the QTL identified would be of most interest if they are different from the major resistance genes and are not race specific.

This paper reports localisation of a newly identified major resistance gene. It also provides an improved map of QTL involved in quantitative resistance in the RIL population already studied (Vear et al. 2008) both in the field and in controlled conditions, as well as in a new RIL population.

Materials and methods

Sunflower genotypes

Resistance to "3xx" races was studied in F3 and F4 progenies from a cross between two INRA restorer lines, PSC8 and 83HR4RM. PSC8 was bred from a recurrent selection population for *Sclerotinia* resistance, with the same resistance profile as PAC2; an INRA line in which *Pl2* was mapped by Bret-Mestries (1996) on LG8, in the same region as *Pl1* and *Pl6*. 83HR4RM was developed from a cross between Russian population VNIIMK 6540 and USDA line RHA274 and then converted to race 710 resistance by introduction of *Pl7* from HA338 by back crossing. Seven F1 plants were selfed to obtain 251 F2 plants which were in turn selfed to give F3 progenies. According to the amount of seed available and the interest of the progeny, these were further selfed to obtain F4 and F5 generations.

Mapping of both major genes and quantitative resistance QTL were carried out on two RIL populations. The INEDI RIL population was obtained by single seed descent (self-pollination to at least F8) from a cross between PSC8 (see above) and XRQ, an INRA line bred from a cross between the USDA line HA89 and a Russian population "Progress". This line carries *Pl5,* mapped by Bert et al. (2001) on the RIL F3 generation which was also used to map *Sclerotinia* resistance and many other agronomic characters. The RIL population is made up of 279 lines, which are maintained and multiplied by self-pollination.

The FU × PAZ2 RIL population was developed from a cross of two other INRA lines: FU, susceptible to all downy mildew races, was bred from a cross between Rumanian and Russian material. PAZ2 came from a cross between a line with *H.petiolaris* restoration and the USDA line HA61 among its ancestors and a population obtained from Zambia. Its downy mildew resistance probably came from HA61. It has the same resistance profile for "3xx" races as 83HR4RM and so was also used to map the gene giving resistance to race 314, to which PSC8 is susceptible. These 180 RIL, obtained by single seed descent

Table 1 Reactions of sunflower inbred lines to eight downy mildew races

	100	300	304	307	314	330	334	710
83HR4RM	R	R	R	S	R	R	S	R
PSC8	R	R	R	R	S	R	R	S
XRQ	R	R	R	R	R	S	S	R
FU	S	S	S	S	S	S	S	S
PAZ2	R	R	R	S	R	R	S	S

(self-pollination to at least F8), were initially developed from F2/F3 progenies to map *Sclerotinia* and phoma resistance (Bert et al. 2004). They are maintained and multiplied by self-pollination.

The reactions of the lines involved in this study to the downy mildew races used are presented in Table 1.

Downy mildew races

The races used for seedling tests were 304, 314, 334, 307, 330(US), 100, 300 and 710, all maintained at INRA, Clermont Ferrand at −80 °C and multiplied when necessary after verification with differential lines. Race 330(US) differs from a Spanish sample of this race because XRQ (the second parent of the INEDI RIL) and VAQ (a new differential proposed by Tourvieille de Labrouhe et al. 2012) are susceptible, whereas they are resistant to 330(SP). In the field, the race naturally present was identified as 710.

Downy mildew resistance tests for major gene analysis

These were made in NS2 or NS3 confined growth chambers, according to whether the race exists in France or is introduced, and for French races, the number of progenies to be tested. Methods were those of Mouzeyar et al. (1993). For each F3 progeny, 20 infected seeds were pricked out. Observations were made 2 weeks later, after 48 h at 100 %RH. Progenies were defined as homozygous resistant (RR), segregating (seg) and homozygous susceptible (SS) according to the absence or presence of sporulation on leaves and cotyledons. Some cotyledon-limited sporulation was observed, especially with race 314, and, in this case, the overall behaviour of the progeny was considered to define the result.

Quantitative downy mildew resistance evaluations

Field

The methodology for these trials was described in detail by Tourvieille de Labrouhe et al. (2008). The RIL were allocated into trials, each of 26 RIL and 4 controls, 2 with good quantitative resistance (PR56, FU) and two highly susceptible (PSU7, GB), with two blocks and 2 row plots of 60 plants per block. To ensure a high level of natural infection, when radicle length was 2–3 cm, 60 mm irrigation was provided by complete cover sprinklers. In 2004, 2005 and 2006, zoosporangia of the local race, 710, were injected into the irrigation water to improve homogeneity of infection over the field. Counts were made 3–4 weeks after sowing (cotyledon stage) of the number of plants emerged and 2–3 weeks later (2–3 pairs of leaves) of the number of healthy plants per plot. Percentage infection was calculated from 100 % healthy plants and ratio of attack on control genotypes made possible comparisons between trials and between years. For the INEDI RIL, only the 106 lines which did not carry *Pl5* (and thus susceptible to race 710) and for which there was sufficient seed, were observed in field trials in 2004 and 2005. For the FU × PAZ2 RIL, 157 lines were observed in the field in 2006 and 169 in 2007.

Growth chamber

Two week-old seedlings were sprayed with a suspension of 10^4 spores/ml of race 710 in an NS2 confined growth chamber (HR 80 %, maintained at 18 °C, photoperiod = 14/10). Two weeks later, symptoms were observed separately on cotyledons (C1), leaves present at infection (L1) and those which appeared after infection (L2), with the following classes: **1**: healthy plants; **2**: bleached (pale green); **3**, **5**, **7**: increasing size of necrotic spots (3: <2 mm, 5: 2–5 mm, 7: >5 mm) but no sporulation; **4**, **6**, **8**: increasing size of necrotic spots (4: <2 mm, 6: 2–5 mm, 8: >5 mm) with sporulation; **9**: dead cotyledons or leaves, **10**, **11**: systemic infection showing chlorosis; and **12**: intense chlorosis giving a mosaic. Using this methodology, 10 plants each of 136 INEDI RIL not carrying *Pl5* and 6 controls, including those used in field trials (PR56, FU, PSU7, GB) were infected each line was characterised by the number of plants in each symptom class.

Identification of candidate gene polymorphism

Helianthus annuus candidate gene sequences were identified based on their homology with *Arabidopsis thaliana* and from sunflower genes previously identified (details in Supplementary Data, File 1, also available at http//www.heliagene.org/Web/public/mapping_downy_mildew_resistance_genes.html). Candidate genes include those involved in putative transcription factors (560), hormone signalling pathways (142), development (18), abiotic stress response (120) and biotic stress response (120). Genomic sequences of these 960 candidate genes were amplified by PCR using Phusion high-Fidelity DNA Polymerase (Finnzymes, Finland) from genomic DNA of 48 inbred lines including

population parents (Supplementary Data, file 2, available on the same web page). PCR products from all genes were pooled respecting stoichiometry and each mix was tagged and sequenced on Illumina GAII by GATC Biotech (Konstanz, Germany). Genomic sequences were assembled from the 76-bp-long single reads produced and 904 candidate gene fragments corresponding to 754 candidate genes were produced. Sequences from 45 genotypes (3 did not produce good results) were aligned using CAP3 (Huang and Madan 1999). SNP were selected using autoSNP (Barker et al. 2003) when at least two genotypes shared the polymorphism (corresponding to a MAF > 5 %). This resulted in the identification of 9,484 SNP corresponding to a density of one SNP every 66 bp in this germplasm. For Illumina BeadXpress and Infinium genotyping, a selection of 207 SNP, each corresponding to one candidate gene, were used for mapping.

Genotyping

RIL DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1990; Porebski et al. 1997). DNA quality was evaluated by NanoDrop spectrophotometry and gel electrophoresis. DNA concentration was quantified using the Quant-iT^{TM} PicoGreen$^{®}$ dsDNA Assay (Invitrogen) on an ABI7900 instrument (Applied Biosystems). DNA concentrations were adjusted to 50 ng/µL for each sample.

The INEDI RIL had previously been genotyped with RFLP and SSR (Vear et al. 2008). In this study, 273 of them were genotyped with (a) public SSR markers, (b) SNP designed on *Helianthus* EST clusters (http://www.heliagene.org) showing close homology with genes from *A.thaliana* considered to be involved in abiotic or biotic stress responses, (c) phenotypic Mendelian traits (recessive branching, male fertility restoration, downy mildew resistance (races 330 and 710), (d) Resistance Gene Candidates (RGC, Radwan et al. 2008), (e) other public EST markers (HT, Lai et al. 2005) and (f) BAC-end derived markers.

The same markers were used to genotype 180 FU × PAZ2 RIL but, as they were initially selected for polymorphism between XRQ and PSC8, only some were polymorphic between FU and PAZ2.

PCR amplifications were carried out on genomic DNA of the four parental genotypes XRQ, PSC8, FU and PAZ2. Polymorphic markers were then amplified on the genomic DNA of all the lines. PCR reactions for SSR markers were carried out in 96- or 384-well plates with a total volume of 10 µl. The reaction mix was 20 ng of template genomic DNA, 1× Taq buffer (Promega), 1.5 mM MgCl2, 75 µM dNTP (Invitrogen), 0.2 µM of forward primer coupled to a 19-base M13 tail in 5′, 0.2 µM reverse primer, 0.2 µM of the 19-base M13 tail primer with 6-FAM, VIC, NED or PET fluorophore (Applied Biosystems) and 0.4U Taq polymerase. For the FU × PAZ2 RIL population, 1× Combinatorial Enhancer Solution (0.54 M betaine; 1.34 % DMSO; 11 µg/ml BSA, 1.34 mM DTT) was added to improve PCR amplification (Ralser et al. 2006).

Amplification reactions were performed by template enrichment with touch down: after an initial 4-min denaturation step at 94 °C, 11 cycles each of 30-s denaturation at 94 °C, 30 s at the annealing temperature which decreased by 1 °C per cycle from 60 to 50 °C, and 45-s elongation at 72 °C. The enrichment test was followed by 25 cycles, each consisting of 30-s denaturation at 94 °C, and 30 s at 50 °C and 45-s elongation at 72 °C. These cycles were followed by a final 5-min elongation step at 72 °C. After the PCR reaction, multi-pooling procedure was used to prepare amplification product electrophoresis. Briefly, diluted PCR labelled with four different fluorescent dyes were pooled. Then a 1 in 20 dilution of PCR amplification was made and 2 µl was added to 7.94 µL HiDiTM Formamide plus 0.06 µl of GeneScan GS500TM LIZ TM (Applied Biosystems). Fluorescently labelled PCR products were subjected to capillary electrophoresis on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Allele sizing was performed using GeneMapper v2.0 software (Applied Biosystems).

For EST and BAC-end derived markers, high-resolution melting (HRM) genotyping was performed by an LC480 instrument (Roche). PCR reactions were performed in a 10-µl final volume containing 20 ng of genomic DNA, 0.15 µM forward and reverse primers, 3 mM MgCl2 and 1× HRM Master mix (Roche). Amplification was performed using a touchdown PCR protocol: after an initial 10-min pre-incubation step at 94 °C, 14 cycles each of 15-s denaturation at 94 °C, 15 s at the annealing temperature which decreased by 0.5 °C per cycle from 64 °C to 58 °C and 20-s elongation at 72 °C. The programme was followed by 46 cycles, each consisting of 15 s at 94 °C, and 15 s at 58 °C and 20-s elongation at 72 °C. PCR products were denatured at 95 °C for 1 min and annealed 50 °C for 1 min. Melting curves were obtained with a ramp from 70 to 95 °C with a increment of 0.02 °C per s, and analyzed using LC480 Gene Scanning software V1.5.0 (Roche), following by normalization, temperature shifting, automated grouping and validation of difference plots.

The RIL were further genotyped in using Illumina BeadXpress or Illumina Infinium assays with the following procedure: an Oligo Pool Assay (OPA) of 384 SNP was defined for a GoldenGate assay with VeraCode on BeadXpress Reader. The GoldenGate assay uses locus- and allele-specific oligos with Cys3/Cys5 labelling to detect SNP at each locus (Shen et al. 2005; Lin et al. 2009). A total of 250 ng of genomic DNA was used for each genotype. Assay was performed according to the manufacturer's protocol, as described by Hyten et al. (2008).

Table 2 Reactions of F3 or RIL progenies in seedling tests (20 plants per progeny) with several downy mildew (DM) races

Progeny	DM race	Resistant	Segregating	Susceptible	X^2
(PSC8 × 83HR4RM) F3	334	56	133	60	1:2:1 1.29 ns
	314	70	115	50	1:2:1 3.51 ns
	304 corrected by 334 + 314 results	108	131	12	7:8:1 2.48 ns
(XRQ × PSC8) RIL	330(US) + 334	131		139	1:1 0.24 ns
	710	124		155	1:1 3.44 ns
(FU × PAZ2) RIL	304	88		91	1:1 0.05 ns

A total of 9,832 SNP were incorporated into a high-density Illumina Infinium custom genotyping array. Hybridizations with a total of 1,236 samples were performed by IntegraGen (Evry, France), according to the instructions provided by the array manufacturer. The SNP data from Illumina BeadXpress plates and Infinium chip were used to obtain genotyping data for both INEDI and FU × PAZ2 populations. The automatic allele calling was made using the Illumina Genecall software with a GeneCall threshold of 0.25. All GenCall data were visually inspected and re-scored if any errors in calling the homozygous or heterozygous clusters were evident. Only homozygous calls were used for this analysis.

Map construction

A consensus map was built from the two genotyping datasets using the CARTHAGENE software (http://www.inra.fr/mia/T/CartaGene/, De Givry et al. 2005) with the following command lines: (a) *dsmergen*, to merge comparable datasets (here, two RIL populations)n sharing orders and distances, in a single consensus data-set, (b) *group 0.4 9*, to define the linkage groups, (c) then *buildfw 3 3 {} 0* to define the framework map for each linkage group, followed by *buildfw 1 1 {…} 0* to include other markers.

QTL detection was performed with MCQTL (Jourjon et al. 2005) with a threshold corresponding to a Type I error rate of 1 % at the genomewide level, as determined after 3,000 replications of the resampling process for each trait. A meta-analysis of the QTL detections obtained in the two RIL populations was carried out in using the software Biomercator (Arcade et al. 2004).

Results

Studies of major resistance gene segregations

Results of downy mildew resistance tests on seedlings are presented in Table 2.

For the cross PSC8 × 83HR4RM, reactions of F3 families to races 314 and 334 agreed with the hypothesis of a single dominant gene in each case: {1 homozygous resistant (RR): 2 segregating (seg): 1 homozygous susceptible (SS)}. There was a slight, non-significant excess of resistant and segregating families for race 314, which is not very aggressive. Some plants had profuse sporulation on cotyledons but no sporulation on true leaves and could be "Type 2" resistant or susceptible (Mouzeyar et al. 1994). A few progenies may have been considered as "RR" or "seg" when they were genetically "seg" or "SS".

Since the two parents of the cross were resistant to race 304, if the same gene was involved, all the F3 progenies would be resistant. In fact, observations of 20 seedlings per progeny showed 139 RR: 100 seg: 12 SS. The twelve homozygous susceptible agree with a Mendelian segregation for two dominant genes, both giving resistance to race 304. Following this hypothesis, there appeared to be an excess of homozygous resistant and a lack of segregating progenies, but this was certainly due to the segregation for two genes in half of the segregating progenies, giving only 1/16 susceptible, not always visible in tests with 20 plants (probability 0.47). Evidence for this came from 23 of the 100 progenies counted as segregating which showed only one susceptible plant. The hypothesis was further confirmed by comparison with reactions to races 314 and 334. There were no cases of susceptibility or segregation to race 304 in progenies, respectively, segregating or resistant to races 314 or 334 so it could be concluded that these two resistances both also gave resistance to race 304. If the progenies segregating for both races 314 and 334 are considered as also segregating for resistance to race 304, the results (108RR: 131seg: 12SS) agree with the theoretical proportions for two dominant independent genes: 7 RR: 8 seg: 1 SS (Table 2).

All the recombinant progenies (resistant to races 304, 314 and 334 or susceptible to all of them), were resistant to races 100, 300 and 330(US). Progenies resistant to race 334 were also resistant to race 307 but susceptible to race 710, whereas those susceptible to races 334 and 307 were resistant to race 710.

The INEDI (XRQxPCS8) RIL were tested in 2000–2004 with race 710 to define those containing *Pl5* and race 330(US), the only one at then available to which XRQ did

not give resistance, to define those containing *Pl2*. A few of these progenies have since been confirmed with race 334. Results with the two races agreed with 1RR: 1SS segregations, for single dominant and independent genes. The slight excess of susceptible lines for race 710 was probably due to the "type II" resistance shown by *Pl5* in XRQ, often with quite profuse sporulation on cotyledons, which could be taken for susceptibility.

The FU × PAZ2 RIL, tested with race 304, also showed a segregation of 1RR: 1SS agreeing with that expected for a single dominant gene. (Table 2).

Quantitative resistance observations

Figure 1 presents the distributions of the reactions of the two RIL populations to attack in the field with re-enforced inoculum of race 710 in 2004, 2005 and 2006 and natural inoculum in 2007. The mean percentage attacks of the four checks in the 5–7 trials concerned each year were the following: 2004: 64.5 %; 2005: 73.5 %; 2006: 48.1 % and 2007: 26.7 %. The mean ratios compared with these check lines were for the INEDI RIL 0.76 in 2004 and 0.88 in 2005, and, for the FU × PAZ2 RIL, 0.74 in 2006 but 0.93 in 2007. The last result, together with the greater range of values, was probably related to the lower mean level and greater irregularity of the natural attack, with no homogenisation of inoculum. For both populations, correlations between years were highly significant: $r = 0.67^{**}$ for INEDI and $r = 0.46^{**}$ for FU × PAZ2.

The qualitative observations concerning reactions of INEDI RIL susceptible to race 710 to infections with this race in a growth chamber were transformed into quantitative scores by a Factorial Correspondence Analysis (FCA), using R (version 2.9.2, function dudi.coa from the ade4 package) (Fig. 2). The first four axes, explaining 40 % of total inertia, were retained for further analysis. To simplify phenotypic description of plant response under such conditions, a resistance index (RI) highly correlated ($r = 0.93^{**}$) with Axis 1 was defined as follows:

RI = ({Nb. of plants presenting necrosis without sporulation on infected leaves} + 1.5*{Nb. of plants with bleaching on infected leaves} + 2*{Nb. of healthy plants})/ Total nb. of plants.

This RI was significantly correlated with field evaluation in both 2004 ($r = 0.47^{**}$) and 2005 ($r = 0.45^{**}$) and showed a bimodal distribution (Fig. 2).

Map construction

Based on the two independent genotyping datasets produced for the INEDI and FU × PAZ2 RIL populations, a consensus genetic map was obtained, spanning 1,886 cM (Haldane) with 451 markers (see details in Supplementary File 3, also available at http://www.heliagene.org/Web/public/mapping_downy_mildew_resistance_genes.html), with an average of 26 markers per linkage group (minimum: 15, maximum: 53).

Major resistance genes

This consensus map shows the locations of *Pl2* (from PSC8) on LG8 and *Pl5* (from XRQ) on LG13, and a second "*Pl2*" gene, which has been denoted *Pl21*, controlling

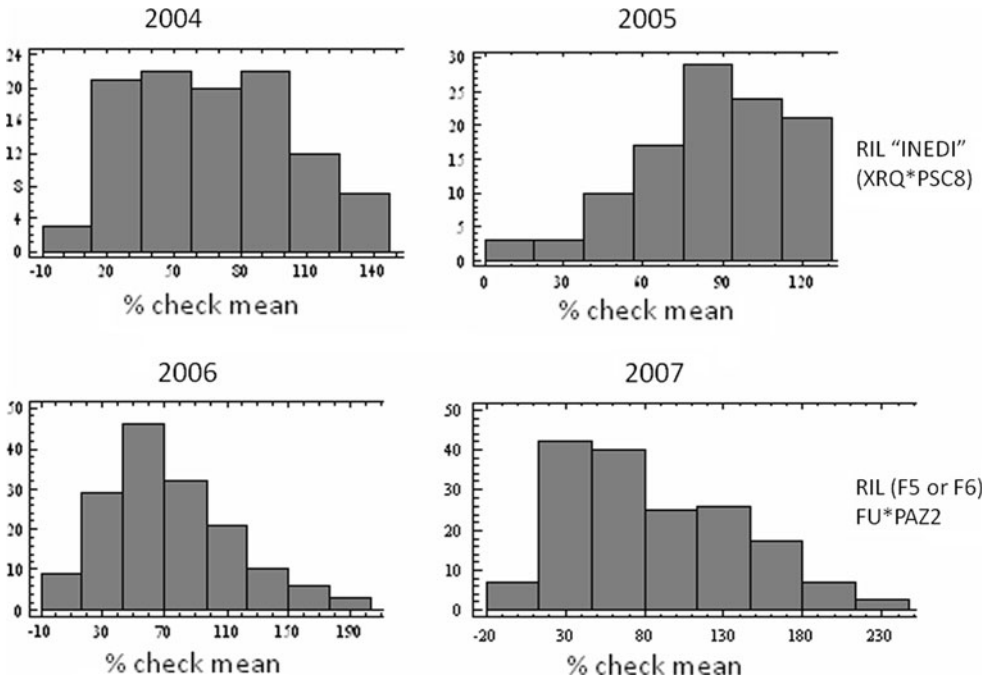

Fig. 1 Distributions of downy mildew attack compared with a mean of 4 check lines for INEDI (XRQx PSC8) (from Vear et al. 2008) and FU × PAZ2 RIL populations

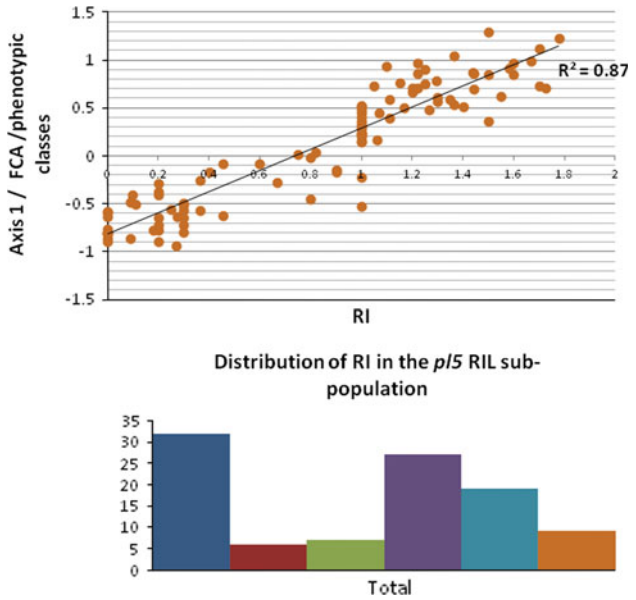

Fig. 2 Quantitative reactions of INEDI RIL not containing *Pl5* to downy mildew race 710 in a growth chamber test: correlation between Axis 1, exhibited by the FCA on qualitative observations, and an empirical Resistance Index (RI) established from the same data, and bimodal distribution of RI for these RIL

resistance to races 304 and 314 in the FU × PAZ2 RIL population (Fig. 3). This gene, carried by the inbred PAZ2, appears to be situated at about 8 cM from *Pl5* on LG13.

QTL involved in quantitative resistance

QTL involved in field expression of quantitative resistance were detected for both RIL populations.

In the INEDI population, in addition to the QTL previously identified (Vear et al. 2008) on LG10 and LG8, a third QTL was mapped on LG7 (Fig. 3). All together, the 3 QTL accounted for 40 % (2005) to 54 % (2004) of the variability, depending on year. The alleles giving best quantitative resistance always came from XRQ. For 2012 growth chamber results, of the four axes taken into account to characterise the profile of response of the INEDI RIL, only Axis 1 allowed identification of a QTL—namely, a very strong QTL on LG10, explaining 65 % of the variability, with a support interval of 0.8 cM (Fig. 3). The RI index gave a QTL at the same position and explaining a similar percentage of the variability. Since it essentially reflects the absence of sporulation in the GC test, it may be concluded that this methodology permits demonstration of a strong genetic factor involved in control of sporulation after early secondary infection. In contrast, as no QTL was detected for the other quantitative axes, this test did not provide a genetic explanation for the other symptoms such as bleaching, drying or "mosaic" phenotypes.

A meta-analysis combining data from field and GC identified two different QTL on LG10 within a 5- to 6-cM interval. However, it should be noted that the first QTL located at position 49.7 cM on LG10 resulted from both field data in 2005 and GC data in 2012. In addition, this QTL was mapped in a region with a particularly dense map. The second possible QTL was detected within a 4-cM interval from the first, which may have affected the detection algorithm. The existence of a single QTL position at 49.7 cM, co-localising with the marker CG0103o1, appears most likely.

In the FU × PAZ2 population, two QTL were detected, the strongest one on LG8, and a smaller one on LG13. This second QTL co-localised with *Pl21*. Therefore, assuming that the allele *Pl21* could play a role in this quantitative response, we discarded the RIL carrying this allele in a second analysis. Using the 90 lines susceptible to race 304, only the QTL located on LG8 was detected. A QTL meta-analysis based on the two RIL populations indicated that their QTL may be different, separated by about 13 cM, but that both are at least 20 cM from the major resistance gene cluster containing *Pl2*.

Discussion

Organisation of major resistance genes in the LG8 and LG13 clusters

The results presented here suggest that two genes give, in particular, resistance to "3xx" downy mildew races, *Pl2* on LG8, controlling resistance to races 304, 307 and 334, present in PSC8, and *Pl21,* located on LG13, giving resistance to races 304 and 314 and present in PAZ2 and 83HR4RM. In contrast, since no segregation was found concerning resistance to races 100, 300 and 330, it seems likely that all the lines had the same gene giving resistance to these races. The existence of two genes had already been suggested in some studies on segregations of F2 progenies with the line RHA274, for races 300 and 330(SP) (Gulya et al. 1991; Molinero-Ruiz et al. 2002), although other results suggested only one gene with race 310 (Molinero-Ruiz et al. 2003). It now appears likely that different results depend on the race used to study segregation.

Figure 4 presents possible organisation of downy mildew resistance genes in the sunflower lines used in the present study, based on the following conclusions and remaining questions:

- *Pl21*, present in 83HR4RM and PAZ2, gives resistance to races 304 and 314, and is situated such that it segregates independently from *Pl2* and from *Pl6/7*. This explains why it has been quite easy to introduce

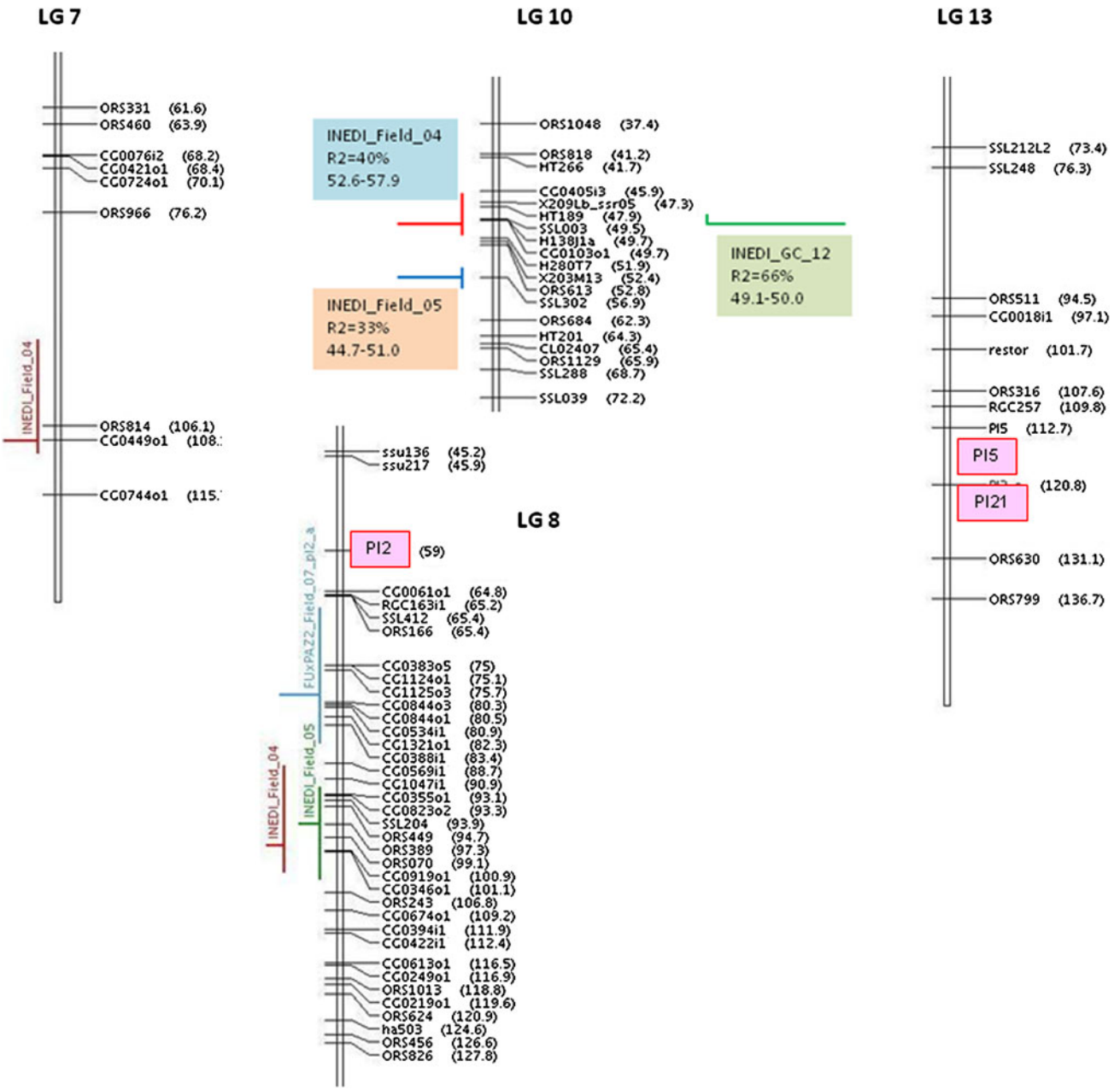

Fig. 3 Linkage Groups (LG) 7, 8, 10 and 13 of a consensus map developed with two RIL populations, INEDI and FU × PAZ2, showing the positions of QTL for quantitative downy mildew resistance and major resistance genes (*Pl*)

resistance to race 710 provided by *Pl6/7* into restorer lines carrying *Pl21*, whereas it was not possible to retain resistance to both races 710 and 304 in "PSC8RM". It should be noted that the original line 83HR4 must contain *Pl2* in addition to *Pl21* since it is resistant to all "3xx" races. It must have lost this gene in recombination when *Pl7* was introduced from HA338, which is susceptible to races 304, 334 and 307.

- Lines resulting from recombination within *Pl6* (Vear et al. 1997), resistant to race 710, but susceptible to races 100 and 300, are also resistant to race 330 (Tourvieille de Labrouhe et al. 2012).
- The PSC8 × 83HR4RM progenies which had neither *Pl2* nor *Pl21* retained their resistance to races 330, 300 and 100, so it is not clear whether the lines 83HR4RM, PSC8 and PAZ2 all carry the same alleles for these resistances on a length of LG8 or whether some of the lines carry them on LG13.
- On LG13, *Pl5,* from XRQ does not give resistance to race 334 or to 330(US), but it should be noted that *Pl5*

Fig. 4 Possible organisation of major resistance gene clusters on LG8 and LG13

gives resistance to race 307 and that *Pl8*, giving type II resistance to all races, is located in the same cluster. Both these genes are thought to come from interspecific hybridisation, so there may not be as much intra-cluster recombination on LG13 as in the cluster on LG8, where most of the genes come from wild *H.annuus*.

The results presented in this paper suggest considerable similarity in the action of the resistance gene clusters on LG8 and LG13. Their remaining difference comes from resistance to races 330(US) and 334 only on LG8 and resistance to races 314, 704 and 714 only on LG13. It has been recently suggested (Radwan et al. 2011) that type I response (no sporulation) could be associated with the expression of TIR-NBS-LRR genes, which have been found (Bouzidi et al. 2002) within the LG8 cluster, while the type II response (sporulation on cotyledons) could be associated with the expression of CC-NBS-LRR genes, which have been found (Radwan et al. 2002) within the LG13 cluster. However, the resistant FU × PAZ2 RIL plants, carrying *Pl21,* showed type I response and lines such as RHA266, carrying *Pl1*, part of the LG8 cluster, show type II response (Vear 1978). In lettuce, another member of the Asteraceae, the cluster of NBS-LRR class major resistance genes to *Bremia lactuca*, another oomycete, has been found very large and highly duplicated, with complex rearrangements. To clarify the structure of the LG8 and LG13 resistance genes clusters as well as resistance responses (type I vs. type II), it will probably be necessary to access the full genomic sequence of these clusters.

Quantitative resistance

For the INEDI RIL population evaluated in the field, Vear et al. (2008) detected only two QTL for quantitative resistance, on LG10 and LG8. With the new map, using the same field data, the QTL on LG10 was positioned with more accuracy and its major effect (32–40 % of variability, depending on year) corroborated. In addition, a very strong QTL (denoted QRM1) was identified at the same position using growth chamber data in 2012. Despite the detection by the MCQTL software of a second QTL in the 2004 experiment, within a 4-cM interval next to the first, the most likely hypothesis appears to be that the same important genetic factor underlying QRM1, strongly co-localising with the marker CG0103o1, was expressed in both the field and the growth chamber. The GC test thus appears valid to measure at least part of the quantitative resistance observable in the field.

With a marker apparently giving a good indication of the QTL position, a start was made to search for candidate genes. Annotation of the marker CG0103, which corresponds to the EST sequence HuCL08172C001 (http://www.heliagene.org/), was found by reciprocal TBlastX to be highly homologous with ATGPA1 (At2G26300). GPA1 is an α-subunit of a heterotrimeric GTP-binding protein, which has been found to be involved in several important biological processes, including the abscissic acid-mediated signalling pathway and stomatal movement. GPA1 also (Zhang et al. 2008) plays a role, in *Arabidopsis*, in the regulation of the PAMP-triggered stomatal response. In the growth chamber resistance evaluation, the leaves of sunflower seedlings were sprayed with zoosporangia suspensions. Allard (1978) observed that primary infection vesicles always occurred close to stomata in young sunflower leaves, so it is quite likely that stomatal openness could be a factor increasing successful infection, as has been shown for *Plasmopara viticola* on grapevine (Allègre et al. 2007). CG0103 may thus be considered as a possible candidate gene, co-localising with the QTL located on LG10, involved in quantitative resistance to downy mildew following early secondary infections on leaves. However, this QTL was also detected in field evaluation, where primary infections do not involve stomatal movement, but penetration of young roots by zoospores. In *Arabidopsis*, GPA1 is involved, through the heterotrimer G-complex, in thigmotropic root growth responses (Pandey and Assmann 2004, Pandey et al. 2008). Expression studies will be necessary to determine whether this candidate is involved in control of primary and secondary downy mildew infections or whether research must concentrate on finding other genes located in the same region. The HuCL08172C001 sequence has been elongated using other unpublished transcriptomic data obtained from the inbred line XRQ (CG0103_fasta.txt, Supplementary data File 4).

Similarly, the QTL on LG7 mapped at the same position as the marker CG0449o2. Again, as for QRM1, it is likely that several genes could be mapped at this locus, but annotation of this candidate, defined from the EST sequence HP021731 (http://www.heliagene.org/HP), was

found by reciprocal TBlastX as the closest homologue of At5G62350. Both HP012731 and At5G62350 are annotated as pectinesterase inhibitors, and HP021731 is also close to At4G12390, also called AtPME1. Interestingly, the pepper pectin methylesterase inhibitor CaPMEI1 has been found to be an antifungal protein involved in basal disease resistance (An et al. 2008), as well as in response to abiotic stresses. All together, this CG0449 may be considered as a first possible candidate controlling the QTL located on LG7. The sequence of HP021731 has been elongated using other unpublished transcriptomic data obtained from the inbred line XRQ (CG0449_fasta.txt, Supplementary data File 5).

Two QTL were identified by meta-analysis, on LG8, depending on the mapping population, separated by about 13 cM. However, it is possible that they are in fact the same, the different positioning being due to software calculations from maps with different precisions. These one or two QTL were located at 20–35 cM from the *Pl2–Pl6* cluster. Several RGC have been mapped on LG8 outside of this major resistance gene cluster (Radwan et al. 2008), and the QTL could be associated with a NBS-LRR class gene not giving complete downy mildew resistance. Stewart et al. (2003) and Poland et al. (2008) showed that modified race-specific resistance genes, such as those belonging to the NBS-LRR family, could be involved in quantitative resistance. In the FU × PAZ2 RIL population, when considering all the RIL including those resistant to race 304, a second QTL was found to map close to the locus *Pl21*on LG13. This could be due to the presence in the field of races other than 710, for example 304, and/or to involvement of *Pl21,* not effective against race 710, in quantitative resistance to this race as suggested above.

All together, a minimum of three QTL have been found to be involved in quantitative resistance of the two RIL populations. One QTL, QRM1, explained such a large part of variability under growth chamber conditions that it is closer to a Mendelian trait than to a QTL. This confirms that refining of phenotypic observations remains a key factor in genetic analysis and also that a "pathosystem" should be not defined only by the host × pathogen description, but also by accurate definition of the environmental conditions in which interactions take place. In the case of the *H. annuus* × *P. halstedii* interaction, the growth chamber test only explains part of field response, but while initially designed to mimic this response in repeatable, controlled conditions, it has made possible the identification of a strong QTL determining part of the overall process.

The question of whether the quantitative resistance identified is non-race-specific has been addressed to a small extent (unpublished data), but, due to Plant Health regulations, it is not possible to compare the responses to different races in the same experiment. Our preliminary results have, nevertheless, indicated that, with races 710 and 304, there is a tendency for the same sunflower genotypes to show less symptoms than others. Downy mildew races have been observed by Sakr et al. (2009) to show considerable differences in aggressiveness and this affects host response, the most aggressive races often producing symptoms which cannot be compared with those produced by those that are less aggressive, masking the impact of genetic factors underlying QTL.

Conclusions

This study has shown that knowledge of the Mendelian genetics of major gene resistance to sunflower downy mildew is not complete. Some of the genes widely used in breeding have still not been mapped and more importantly, their profiles of resistance to races other than the most common are not always known. This paper presents the location of a gene, *Pl21*, widely used for the past 30 years.

This knowledge is important, not only for direct use in breeding, but also to define and exploit quantitative, and perhaps more durable, resistances. This study confirmed the results of Vear et al. (2008) indicating that some genetic factors other than the *Pl* genes are significantly involved in the resistance of sunflower to downy mildew. At least two of these factors, a QTL on LG7, and QRM1, located on LG10, are not located within the major resistance gene clusters (LG8, LG13, LG1), suggesting that the genes underlying these QTL may involve other biological processes than those directly involved in plant × pathogen dialogue and recognition. Despite the lack of sunflower genome sequences, two possible candidate genes which may be involved in a non–*Pl* dependent, quantitative sunflower response to the downy mildew have been identified. This preliminary result should stimulate research on how factors determining abiotic stress responses may also intervene in biotic stresses.

Within the next year, comprehensive genomic data for both *Helianthus annuus* and *Plasmopara halstedii* should become available and these will provide a basis for identification of the molecular factors involved in this plant × pathogen interaction, making it possible to develop strategies giving improved sustainability of sunflower resistance to downy mildew.

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