Theor Appl Genet (2002) 104:490–496 

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# A QTL analysis of sunflower partial resistance to downy mildew (*Plasmopara halstedii*) and black stem (*Phoma macdonaldii*) by the use of recombinant inbred lines (RILs)

Received: 28 February 2001 / Accepted: 14 June 2001

**Abstract** Partial resistance to downy mildew (*Plasmopara halstedii*) and to black stem *(Phoma macdonaldii)* in sunflower were investigated under natural field infection and a controlled growth chamber respectively. Genetic control for resistance to the diseases was determined in recombinant inbred lines (RILs) and their two parents, 'PAC-2' and 'RHA-266.' The experiments were undertaken in a randomized complete block design with two replications, in a field severely infected by downy mildew and in a controlled growth chamber with plants inoculated with an agressive French isolate of *P. macdonaldii*. Each replication consisted of three rows, 4.6-m long, giving 48 plants per RIL or parent in the field and 15 plants in the growth chamber. Genetic variability was observed among the RILs for resistance to both diseases. When 10% of the selected RILs were compared with the mean of the two parents genetic gain was significant for partial resistance to the diseases. Four putative QTLs for resistance to downy mildew on linkage groups 1, 9 and 17 were detected using composite interval mapping. The QTLs explained 54.9% of the total phenotypic variance. Major QTLs (*dmr1–1* and *dmr1–2*) for resistance were found on linkage group 1 with up to 31% of the phenotypic variability explained by two peaks. QTL analysis of resistance to black stem showed seven QTLs on linkage groups 3, 6, 8, 9, 11, 15 and 17. The detected QTLs together explain 92% of the phenotypic variation of the trait. Crosses between RILs contrasted for their resistance to downy mildew and black stem, and exhibiting molecular polymorphism in detected QTLs, will be made in order to focus more-precisely on the genomic region of interest.

Communicated by G. Wenzel

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**Keywords** QTL · Sunflower · Disease resistance · Downy mildew · Black stem · Recombinant inbred lines

## Introduction

Sunflower (*Helianthus annuus* L.) is one of the most-important sources of vegetable oil in the world. Plant improvement implies the ability to create genotypes resistant to different diseases. Downy mildew and black stem caused by *Plasmopara halstedii* and *Phoma macdonaldii*, respectively, are considered as important diseases in sunflower. Downy mildew is reported from every continent and is now epidemic wherever sunflower is cultivated while black stem is present in many European countries, Australia and the USA (Acimovic 1984, 1988). Since 1990 black stem has expanded constantly it is now recognized as one of the most-serious diseases of sunflower in France (Peres and Lefol 1996). Until 1980, the situation with races of *P. halstedii* was very simple, because only two races were known. Since that time, a multitude of races have been identified. In 1980, a third race was identified in USA (Carson 1981) and, in 1984, race 4 was also identified (Gulya et al. 1985). A highly virulent race 5 was reported by Ljubich et al. (1988), restricted also to North America. With intensive sampling and an expanded set of differential genotypes, at least nine races are identified in European countries (Tourvieille et al., 1988; Mouzeyar et al., 1994a,b; Spring et al., 1994; Viranyi and Gulya 1995). There are differences between sunflower genotypes in their susceptibility to downy mildew. The resistance is controlled by single dominant genes designated *Pl* (Mouzeyar et al., 1994b). It has been demonstrated that at least some *Pl* genes are clustered (Mouzeyar et al., 1995; Vear et al., 1997).

Genetic variability for partial resistance to black stem in sunflower has been observed in field conditions (Peres et al., 1994). A study of parental genotypes and their $F_1$ hybrids showed that additive genetic effects, which are important in predicting the progeny performance of

crosses, are significant for some of inbreds lines used in research work (Roustaee et al., 2000a). Additive genetic effects were also reported in sunflower by Deglene et al. (1999) for resistance to *Phomopsis helianthii*, and by Vear et al. (1997) and by Castano et al. (1992) for partial resistance to *Sclerotinia sclerotiorum*.

Identification of chromosome regions with effects on resistance to downy mildew and black stem can increase our understanding of the genetic control of the diseases. Quantitative trait locus (QTL) mapping is a highly effective approach for studying plant disease resistance. With QTL mapping, the role of specific resistance loci and the interaction between resistance genes, plant development and the environment can be analysed (Young 1996). In sunflower, QTLs for *S. sclerotiorum* (Mestries et al., 1998), linkages of molecular markers with resistance genes to rust (Lawson et al., 1998), and candidate genes for downy mildew (Gentzbittel et al., 1998; Vear et al., 2000) have been identified.

The advantage of recombinant inbred lines (RILs) for detecting QTLs have been shown by Austin and Lee (1996). The RILs undergo multiple cycles of meiosis before homozygosity is reached; in consequence, linked genes have a great probability of recombination and their pleiotropic effect can be detected (Burr and Burr 1991). This effect increases the power of testing differences between genotypic classes.

In the field severely infected by the fungus populations, and in climatic conditions with temperature and humidity favourable for infection, a high variability for the number of diseased plants according to genotype is observed. The objectives of present study were thus to contribute to knowledge of the genetic control of resistance in sunflower to downy mildew under natural conditions, as well as to black stem in a controlled growth chamber with seedling inoculations, using recombinant inbred lines (RILs). We also carried out a QTL-mapping analysis to characterize genomic regions involved in resistance to these diseases.

## Materials and methods

### Downy mildew

Recombinant inbred lines (RILs) of *Helianthus annuus* L. were used in this experiment. The RIL population consisted of 77 $F_8$ derived lines developed through single-seed descent (SSD) from a cross between 'PAC-2' and 'RHA-266', which are respectively susceptible and rather resistant to downy mildew in field conditions. The material was kindly provided by INRA (France). Seeds of the 77 above-mentioned RILs and their two parents were planted in a randomized block design with two replications. Each replication consisted of three rows, 4.6-m long, with 50 cm between rows and 30 cm between plants in a row, giving a total number of about 48 plants per plot. Climatic conditions of temperature and moisture, and especially severe soil infection by the pathogen, were favourable for natural contamination of the plants by downy mildew.

Susceptible genotypes were severely infected by *P. halstedii* at a young stage of development. Diseased plants stayed dwarf when compared with resistant individuals. It has already been shown that downy mildew infection culminates in up to a 90% reduction in height and leaf area, and up to one-third fewer leaves (Spring et al., 1991). Five weeks after planting, susceptible plants in each genotype were scored and expressed as a percentage of the total number of plants per plot. This percentage was considered as a measure of susceptibility to *P. halstedii* for each genotype. After flowering, another observation was noted and the results of the first one were confirmed. Mean scores of the percentage of susceptible plants per genotype per replication presented a normal distribution and did not need any transformation.

### Black stem

A population of 83 recombinant inbred lines (RILs) and their two parents, already presented for downy mildew, were used in the experiment. A monopycniospore isolate of *P. macdonaldii* produced from naturally infected plants in the south-west of France was used in this study. The pathological characteristics of this isolate, which is one of the most aggressive isolates of the pathogen, (Roustaee et al. 2000a), were kept constant during the period of the experiment using the method of conservation described by Arabi et al. (1992) for *Drechslera teres*.

The experiment was conducted in a controlled environment chamber at a temperature of 25±1°C (day)/18±1°C (night). The light intensity was 200 µE $m^{-2}$ $S^{-1}$ with a 14-h photoperiod and a relative humidity of 75–80%. The experiment was designed as a randomized complete block with two replications. Each replicate consisted of 15 seedlings of each genotype. Seeds were sterilized for 5 min in a sodium hypochloride solution (6 chlorometric degrees) and washed in sterile distilled water. Fifteen seeds per genotype per replication were planted in plastic containers (40×30×30 cm) filled with moistened vermiculite. Seedlings were irrigated with a nutrient solution (NPK 6-3-6 and micronutrients: Substral, Boulogne Billancourt, France).

Twelve-day old seedlings were inoculated at the junction of the cotyledon petiole and hypocotyl with 20 µl of a pycniospore suspension ($10^6$ pycniospores per ml of water, containing 0.25% of gelatine) using a micropipette. After inoculation, each container was enclosed for 72 h using a special transparent cover (plexiglass) to maintain a near-saturated humidity favourable for fungal inoculation.

Small chlorotic lesions appeared on the surface of the cotyledon petiole 1–2 days after inoculation. Three-days later, they had elongated and transformed into necrotic lesions depending on the reaction of the lines. In severe infection of suceptible lines, the necrotic lesions elongated and then spread down the hypocotyl. Thus, the percentage of surface area in the upper part of the cotyledon petiole occupied by the fungus varied with the susceptibility of the line. Seven days after inoculation, both cotyledon petioles of the seedling were scored according to the percentage of the petiole area exhibiting disease symptoms (necrosis). A rating scale from 1 to 9, based on the percentage of the infected cotyledon petiole area, was used, where: 1=0–5%, 2=6–10%, 3=11–20%, 4=21–30%, 5=31–40%, 6=41–60%, 7=61–80%, 8=81–99%, and 9=100%, with necrosis spreading down the stem. The measures of severity did not need any transformation to normalize the distribution.

### Data analysis and QTL mapping

Analysis of variance was carried out in order to determine the effect of genotype on partial resistance to the diseases. The Newman-Keuls test was used for comparing the means of parents and RILs. The mean of RILs and that of the parents was also compared. The most-resistant RIL (lowest value) was compared with the resistant parent, and genetic gain for 10% of the selected RILs compared to the mean of their parents was determined. Additive environmental variances and narrow-sense heritability were calculated according to Kearsey and Pooni (1996), using least-square estimates of the genetic parameters.

The linkage map used in this study is the completed map described previously (Flores Berrios et al., 2000). In summary, we

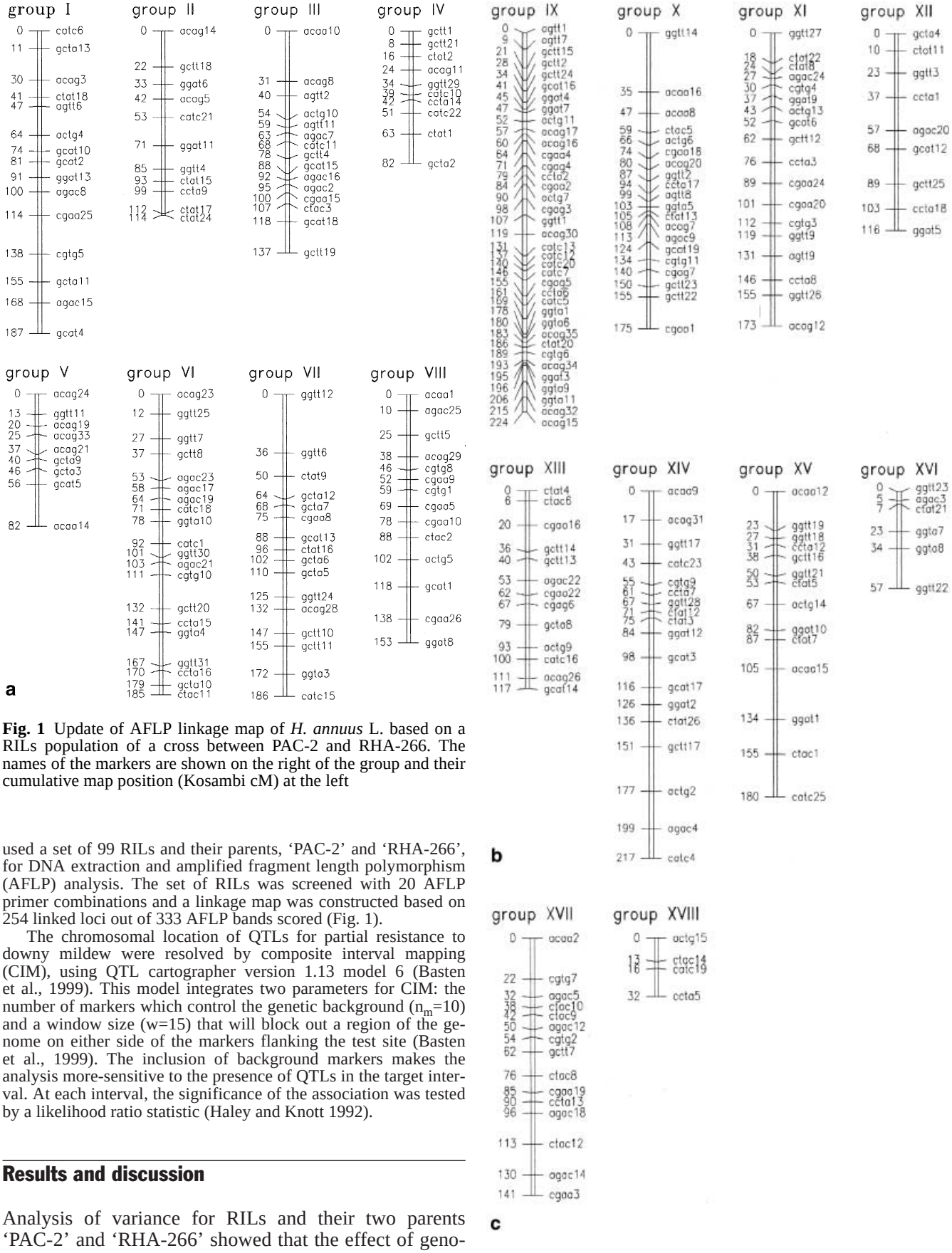


**Fig. 1** Update of AFLP linkage map of *H. annuus* L. based on a RILs population of a cross between PAC-2 and RHA-266. The names of the markers are shown on the right of the group and their cumulative map position (Kosambi cM) at the left

used a set of 99 RILs and their parents, 'PAC-2' and 'RHA-266', for DNA extraction and amplified fragment length polymorphism (AFLP) analysis. The set of RILs was screened with 20 AFLP primer combinations and a linkage map was constructed based on 254 linked loci out of 333 AFLP bands scored (Fig. 1).

The chromosomal location of QTLs for partial resistance to downy mildew were resolved by composite interval mapping (CIM), using QTL cartographer version 1.13 model 6 (Basten et al., 1999). This model integrates two parameters for CIM: the number of markers which control the genetic background ($n_m$=10) and a window size (w=15) that will block out a region of the genome on either side of the markers flanking the test site (Basten et al., 1999). The inclusion of background markers makes the analysis more-sensitive to the presence of QTLs in the target interval. At each interval, the significance of the association was tested by a likelihood ratio statistic (Haley and Knott 1992).

## Results and discussion

Analysis of variance for RILs and their two parents 'PAC-2' and 'RHA-266' showed that the effect of genotype was highly significant for resistance to downy mil-

dew and black stem, whereas no significant effect was observed between the two replications (Table 1). The genetic variability for partial resistance to downy mildew and black stem are presented in Table 2. Parental genotype 'RHA-266' showed a significant higher level of partial resistance to downy mildew when compared with 'PAC-2', whereas the two parents were not significantly different for partial resistance to black stem. The difference between RILs ($\bar{X}$ RILs), and the mean of their parents ($\bar{X}$ P), is not significant for both diseases (Table 2), indicating that the RILs used in this experiment are representative of the entire possible recombinant lines from the cross 'PAC-2×RHA-266.' The best parent ('RHA-266') for downy mildew and 'PAC-2' for black stem, compared with the best RIL, showed a higher level of partial resistance which was statistically significant for black stem. The difference between 10% of RILs (10% S-F8) with the lowest values (more resistance) and the mean of their two parents ($\bar{X}$ P) was significant for both studied diseases (Table 2). This phenomenon, considered as a genetic gain, might be due to the polygenic nature of resistance to the diseases and the accumulation of favourable alleles for resistance in RILs. Narrow-sense heritability (0.81 for downy mildew and 0.58 for black stem) indicates that selection for resistance to both diseases is possible in the progenies of crosses. Genetic variability for resistance to different races of downy mildew has been reported by several researchers (Tourvieille et al., 1988; Mouzeyar et al., 1995; Viranyi and Gulya 1995; Vear et al., 1997). As far as we know the severity of infection by downy mildew in different genotypes under field conditions with soil infected by

**Table 1** Mean squares for downy mildew (DM) and black stem (BS) resistance of sunflower recombinant inbred lines (RILs) and their two parents

| Source of variation | *df*[a] | | Mean square | |
|---|---|---|---|---|
| | DM | BS | DM | BS |
| Total | 157 | 169 | | |
| Genotype | 78 | 84 | 1056.26 *** | 6.696*** |
| Block | 1 | 1 | 25.66[n.s] | 0.297[n.s.] |
| Residual | 78 | 84 | 57.36 | 1.026 |

***=Significant at $P$=0.001; n.s.=not significant
[a] *df*=degrees of freedom

**Table 2** Genetic gain and heritability for downy mildew (DM) and black stem (BS) resistance in recombinant inbred lines (RILs) of sunflower. The values represent the mean of the host reaction rate for susceptibility, expressed in percent for DM, and the scale range, 1 to 9, for BS, from two replications

| Item | Host reaction | |
|---|---|---|
| | DM | BS |
| PAC-2 (P1) | 41.91 | 5.05 |
| RHA-266 (P2) | 12.22 | 6.25 |
| P1–P2 | 29.69* | – 1.20[n.s.] |
| $\bar{X}_P$=(P1+P2)/2 | 27.07 | 5.65 |
| $\bar{X}_{RILs}$[a] | 41.8 | 6.58 |
| $\bar{X}_{RILs}$–$\bar{X}_P$ | 14.73[n.s.] | – 0.93[n.s.] |
| Best RIL (BRIL) | 2.33 | 1.00 |
| GG[c]=BRIL–BP[b] | – 9.89[n.s.] | – 4.05* |
| 10% SF8L[d] | 10.06 | 2.93 |
| GG[e]=10%$SF_8$L–$\bar{X}$ P | –17.01* | – 2.12* |
| $h^2$ | 81.32 | 58.23 |

* Significant at $P$=0.05; ns=not significant at $P$=0.05
[a] $\bar{X}$ RILs, mean of all recombinant inbred lines
[b] BP, best parent ('RHA-266' for downy mildew and 'PAC-2' for black stem)
[c, e] GG, genetic gain when the best RIL or 10% of the selected RILs (10% SF8L) are compared with the best parent ('RHA-266' for downy mildew and 'PAC-2' for black stem)
[d] 10%$SF_8$ L, 10% of the best recombinant $F_8$ lines

**Table 3** Map positions and effect of QTLs detected in recombinant inbred lines (RILs) for downy mildew and black stem resistance

| QTL | Linkage group | Position[a] | LOD | Percent of phenotypic variance explained[b] | Additive effect (a) |
|---|---|---|---|---|---|
| Downy mildew | | | | | |
| *dmr1.1* | I | 21.00 | 3.16 | 20.20 | –12.59 |
| *dmr1.2* | I | 42.83 | 3.94 | 11.10 | – 9.80 |
| *dmr9.1* | IX | 205.82 | 3.70 | 9.30 | 7.42 |
| *dmr17.1* | XVII | 62.21 | 5.12 | 14.30 | 9.26 |
| Black stem | | | | | |
| *bsr3.1* | III | 102 | 3.8 | 6,40 | 0.511 |
| *bsr4.1* | IV | 47 | 5.0 | 13,00 | 0.850 |
| *bsr8.1* | VIII | 133 | 7.6 | 17,30 | 0.912 |
| *bsr9.1* | IX | 89 | 5.6 | 8,90 | 0.581 |
| *bsr11.1* | XI | 73 | 5.7 | 13,50 | 0.901 |
| *bsr15.1* | XV | 179 | 7.5 | 16,60 | 0.986 |
| *bsr17.1* | XVII | 95 | 8.1 | 17,40 | – 0.966 |

[a]=expressed in Kosambi CM, from north of the linkage group
[b]=value determined by QTL Cartographer, Version 1.13 (Basten et al. 1999)

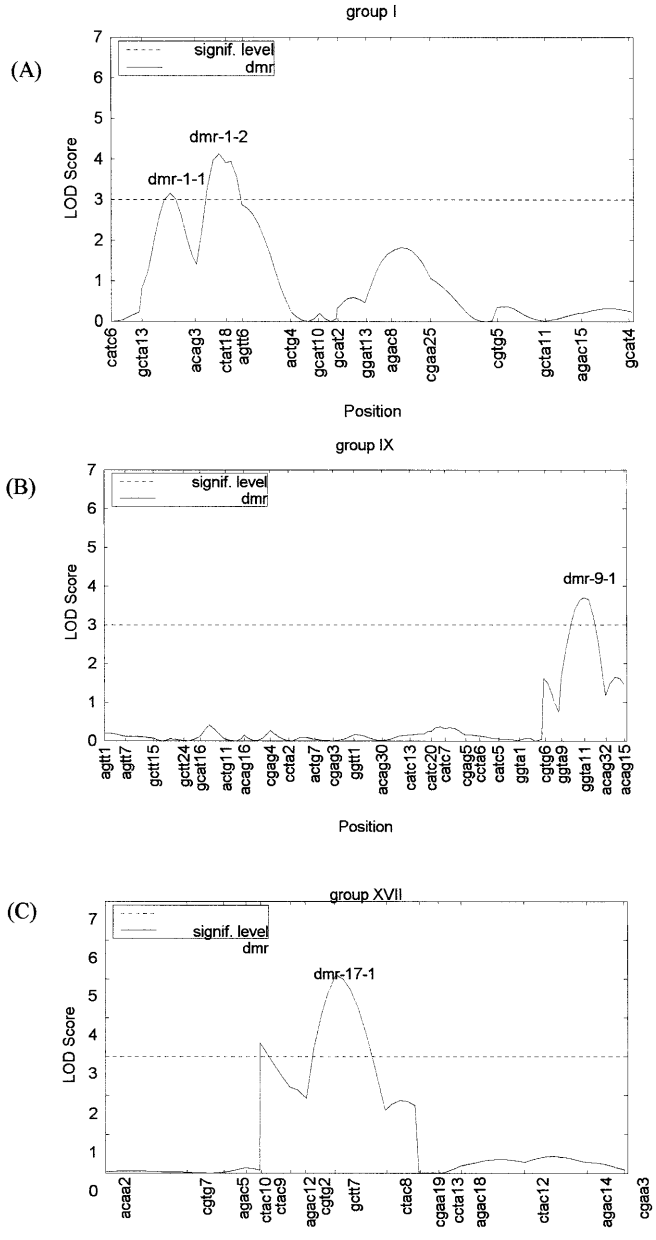


**Fig. 2A–C** Genetic map and LOD-score plots showing the localisations of putative $QTL_S$ associated with resistance to downy mildew (*dmr*) detected by composite interval mapping (CIM) A linkage map of linkage group I. B linkage map of linkage group IX. C linkage map of linkage group XVII

fungus populations is not reported in the literature. Genetic variability and heritability (0.60) for partial resistance to black stem in $F_1$ hybrids and their parents, as well as additive and dominant effects of the genes controlling black stem, were also shown by Roustaee et al. (2000b).

Significant peak values of LOD scores, the position of their peaks, the percentage of phenotypic variance explained and the estimate of QTL effects based on a composite interval-mapping analysis for resistance to downy mildew and black stem are summarized in Table 3. Four QTLs were identified for resistance to downy mildew and seven for resistance to black stem. QTLs were designated as '*dmr*' (downy mildew resistance) and '*bsr*' (black stem resistance) followed by the linkage group and QTL number (Fig. 2). The effects of each QTL are moderate, ranging from 9.30 to 20.20% for both diseases (Table 3). The four detected loci explained 54.9% of the total phenotypic variance of resistance to downy mildew, whereas the seven detected $QTL_S$ for black stem explained 92% of the variance. The transgressive phenotypes observed for both diseases could be explained by the presence of QTLs of opposite sign in each parent (Table 3).

Several researchers have studied the host-pathogen interaction on sunflower downy mildew. From an anatomical standpoint, sunflower genotypes may be resistant by not allowing zoospores to penetrate the roots or by a failure of mechanical/biochemical barriers in the hypocotyl regions. Mouzeyar et al., (1993) showed that the fungal mycelium was apparently blocked by callose and lignin-like materials in the hypocotyls. Two types of resistant-response to downy mildew have been observed in sunflower (Mouzeyar et al., 1994b). The type-I fungus is limited to the root and lower hypocotyls while, with type-II resistance, the fungus colonizes the entire hypocotyls and cotyledons but does not become systemic beyond the cotyledons. Tena and Valbuena (1983) demonstrated that phenylpropanoid biosynthesis is accelerated in mildew-infected plants, typically measured by the increase in phenylalanine ammonia lyase (PAL) activity, and that the resistant hosts respond with elevated PAL levels within 24 h after inoculation. The induction of defense genes, following downy mildew infection in particular auxin-induced genes, was also reported (Mazeyrat et al.,1998). The above-mentioned complex systems for resistance to downy mildew in sunflower are controlled by genes, the expression of which should be modified by the environment. That is why partial resistance is observed in field conditions with the fungus populations.

As far as we know, the description of a putative quantitative component of resistance to downy mildew and to black stem has still not been reported in the literature. In sunflower, QTLs for resistance to *S. sclerotiorum* (Mestries et al., 1998), molecular markers for resistance to rust (Lawson et al., 1998), and candidate genes for downy mildew (Gentzbittel et al., 1998; Vear et al., 2000) have been identified. QTLs for resistance to mildew were also detected in barley (Heun 1992; Backes et al., 1995) and in wheat (Keller et al., 1999). Crosses between RILs contrasted for their resistance to downy mildew or black stem, and exhibiting molecular polymorphism in detected QTLs, could be made in order to focus more-precisely on the genomic region of interest. Additional work is needed to localize the '*dmr*' QTLs with respect to the map position of some *Pl* genes.

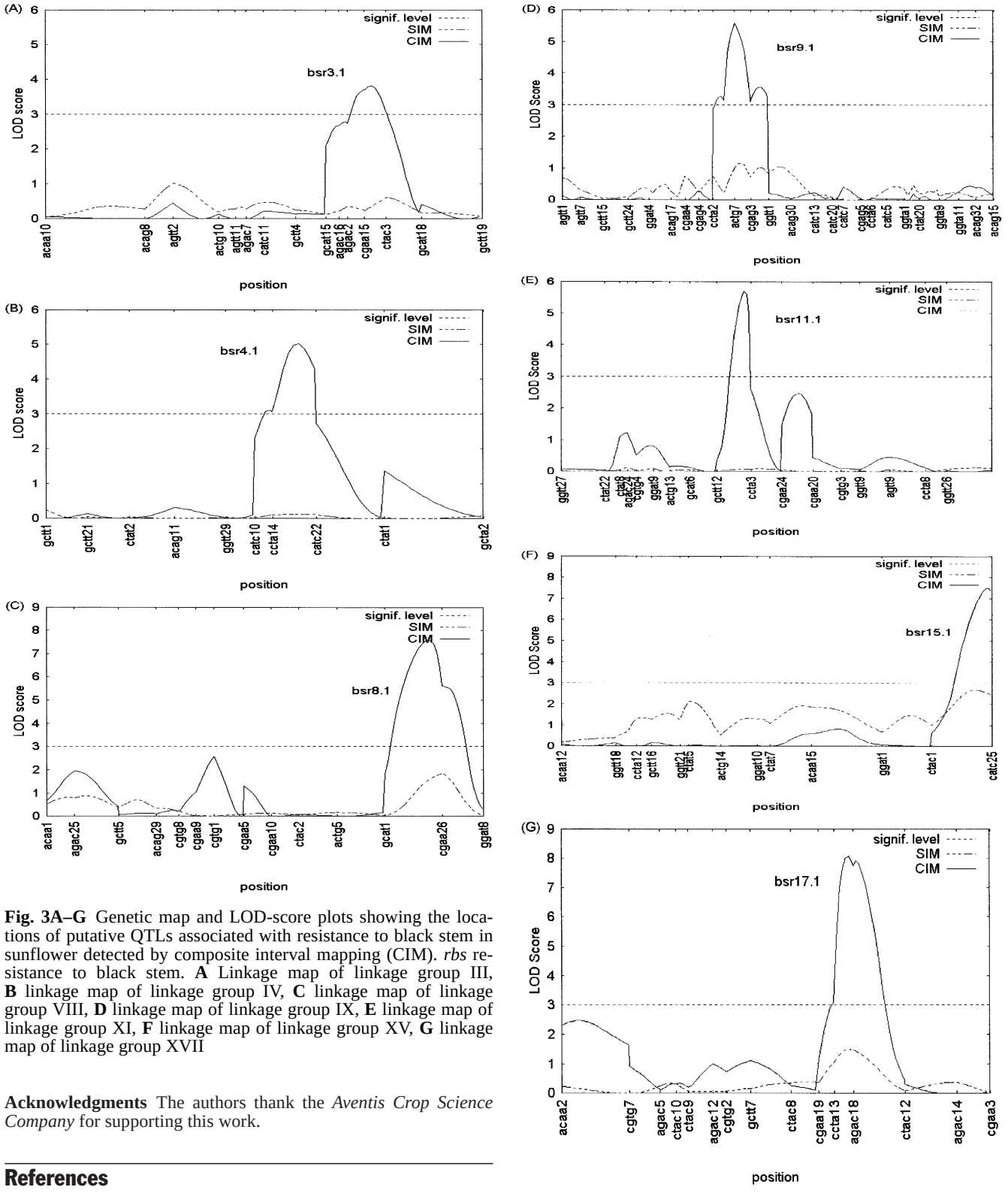


**Fig. 3A–G** Genetic map and LOD-score plots showing the locations of putative QTLs associated with resistance to black stem in sunflower detected by composite interval mapping (CIM). *rbs* resistance to black stem. **A** Linkage map of linkage group III, **B** linkage map of linkage group IV, **C** linkage map of linkage group VIII, **D** linkage map of linkage group IX, **E** linkage map of linkage group XI, **F** linkage map of linkage group XV, **G** linkage map of linkage group XVII

**Acknowledgments** The authors thank the *Aventis Crop Science Company* for supporting this work.

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