Analyses of QTL associated with resistance to *Sclerotinia sclerotiorum* and *Diaporthe helianthi* in sunflower (*Helianthus annuus* L.) using molecular markers

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Summary:

Most traits exhibit continuous variation resulting from the action of multiple genes modified by the environment. Quantitative trait loci (QTL) analysis consists in determining the location and number of genes that condition such quantitative traits and estimating the magnitude of individual gene effect by the use of analytical methods and detailed linkage map. *Sclerotinia sclerotiorum* and *Diaporthe helianthi* are important pathogens of sunflower in France causing white rot and stem canker. Restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) were used to construct two linkage maps of cultivated sunflower based on the progenies of 335 F2 plants from intra specific cross XRQ x PSC8. Genetic factors that condition resistance to leaf and capitulum resistance to *S. sclerotiorum* and to *D. helianthi*, and other agronomic characters are analysed on 220 XRQ x PSC8 F3 families. This QTL analysis will provide new insights on the location and organisation of characterised QTL (eventually common QTL for both pathogen resistances). Genetic mapping of disease resistance genes should help to improve the efficiency of plant breeding and will lead to a better understanding of the molecular basis of resistance.

Résumé :

De nombreux caractères sont le résultat de l'expression de plusieurs gènes, influencée par l'environnement. L'analyse de QTL (Quantitative Trait Loci) consiste à déterminer la localisation et le nombre de locus qui conditionnent le trait quantitatif ainsi que d'estimer la magnitude de l'effet de chacun de ces locus, par le biais de méthodes statistiques et de cartes génétiques détaillées. *Sclerotinia sclerotiorum* et *Diaporthe helianthi* sont les deux principaux champignons nécrotrophes du tournesol en France, causant la pourriture blanche et le phomopsis. Le tournesol présente une résistance de type continue et organe spécifique face à ces deux pathogènes. Le marquage moléculaire de type RFLP (Restriction fragment length polymorphism) et AFLP (amplified fragment length polymorphism) a été employé pour établir une carte génétique afin de caractériser des QTL. Le croisement XRQ x PSC8 permet de réaliser une analyse conjointe de la résistance à *S. sclerotiorum* sur capitule et sur feuille et à *D. helianthi*, ainsi que d'autres caractères d'importance agronomique à travers l'étude de 220 familles F3. Ces analyses permettront donc de caractériser et de comparer les QTL de résistance à ces deux pathogènes entre eux, et de les comparer à ceux déjà obtenus sur d'autres croisements. La localisation de ces QTL sera utile pour la création de nouvelles variétés résistantes et permettra de mieux comprendre la génétique moléculaire de la résistance du tournesol.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary and *Diaporthe helianthi* Munt.-Cvet. *et al*. are two of the most important pathogens of sunflower (*Helianthus annuus* L.) in France, causing white rot and wilt and phomopsis stem canker. Resistance to both pathogens is polygenic and *S. sclerotiorum* poses the additional problem that it can attack all plant organs: roots, stem base, leaves, terminal bud and capitulum. For a given genotype, the level of resistance of each plant part to *S. sclerotiorum* may be quite different and each form of attack on the plant must be considered as a different disease. Analyses of quantitative trait loci (QTL) associated with resistance to extension of *S. sclerotiorum* mycelium on sunflower capitula and leaves using molecular marker have already been made by Mestries *et al.* (1998). Seventy-three RFLP probes were used to construct a genetic map where four QTL loci were demonstrated for leaf resistance and two for capitulum resistance. One of these zones appears to be involved in resistance to both type of *S. sclerotiorum* attack while the others appear specific for resistance of one part of the plant.

Thus, it appeared of interest to identify, in other crosses, QTL for these and other *S. sclerotiorum* resistance characters in order to confirm previous studies, to determine their transportability and possibly to find new QTL. Concerning the analysis of resistance to phomopsis, no QTL analysis has been published so far so it would be of great interest to characterise in the same genotypes QTL for resistances against the two pathogens to improve the efficiency of resistance breeding. In addition, disease resistance cannot be considered alone in breeding: the primary qualities of a variety are its yield, oil content and earliness, so the parental lines of the cross reported here were chosen to show polymorphism for a wide range of agronomic characters.

This paper reports on the tools used, the characters studied and their range of variability in preparation for detailed QTL analyses.

Materials and Methods

Sunflower genotypes:

The two parental inbred lines of the cross were bred by INRA. XRQ was a selection for its downy mildew resistance from a cross between HA89 and the Russian population Progress (Vear *et al*, 1998). PSC8 was a selection from a population with recurrent selection for capitulum resistance to *Sclerotinia* (Vear *et al*, 1992). XRQ, an unbranched PET-1 CMS maintainer shows a medium level of resistance to *Sclerotinia*, but a high level of resistance to phomopsis, high oil content and good seed production. PSC8, a male fertility restorer line exhibits the apical branching gene (b1) phenotype, a high level of *Sclerotinia* resistance, high oil content, but low seed weight per capitulum. The male fertile forms of the two lines were crossed in both directions. The F1 plants were selfed by covering the capitula with grease-proof paper bags a few days before flowering to obtain the F2 generation, which was in turn selfed to obtain the F3 families. No distinction was made according to the direction of the original cross.

Resistance tests:

The *Sclerotina* ascospore test was that described by Tourvieille and Vear (1984), which, after infection of the capitula at the beginning of flowering with an ascospore suspension, measured both percentage attack (on 2 replications of 25 plants) and a latency index, which was the time between infection and symptom appearance compared with the mean delay on two control inbreds infected on the same day. The *Sclerotinia* mycelium tests on leaves and capitula were described by Castaño *et al* (1993). The leaf test gave a lesion length from the mycelium explant infection on the leaf tip, along the main vein. Two replications of 5 plants were infected at one-week intervals. The capitulum test gave a mycelium index which was the lesion area measured three days after infection of the dorsal surface of capitula with mycelium explants, divided by the mean lesion area of a control infected at the same time. Three explants were used to infect each of five capitula in two replications for each F3 family. All the *Sclerotinia* results reported were obtained in 1997. First studies of reaction to phomopsis were made under semi-natural attack (Viguié *et al*, 1999) in 3 locations in 1997 and 1998. In this case the percentage of plants showing stem lesions >5cm were noted on 2 replications of 50 plants in each location. The means results are presented here.

Other field observations:

Flowering date was taken, for each of the 50 plants as the date of ascospore infection. This corresponds to the first day when tubular florets have opened. Symptoms from the ascospore test were observed twice a week. At this time, plants with no *Sclerotinia* symptoms that were completely mature, with the capitulum brown and dry were also noted. This gave a value for maturity date and for the delay from flowering to maturity for each plant that was not attacked by *Sclerotinia*. In addition there was some lodging in these ascospore trials and this was noted (and the plants not counted for percentage success of the ascospore test). Separately, plant height, weight of seeds/capitulum (after drying at 40°C) and seed oil content was measured on 5 plants of each F3 family, not infected with *Sclerotinia*. Oil content was measured on 2g samples from each capitulum by nuclear magnetic resonance (Bruker Mini-Spec 10).

RFLP analysis:

Sixty probes were chosen among those used for the sunflower consensus linkage map (Gentzbittel *et al*., 1995), both for their polymorphism between XRQ and PSC8 lines and their distribution through out the genome. DNA extraction, digestion by restriction enzymes (*Eco* RI and *Hind* III) and Southern hybridisation were carried out as described previously (Gentzbittel *et al*., 1995).

AFLP analysis:

The AFLP protocol developed by Vos *et al.* (1995) was followed with minor modifications as described by 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 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). Clearly readable AFLP bands were scored as dominant genetic markers.

Results

RFLP analysis:

Seventy-three *Eco* RI and 57 *Hind* III probes were chosen among those used for the sunflower consensus linkage map (Gentzbittel *et al*., 1995; Gentzbittel *et al*., 1999), and checked for their polymorphism between XRQ and PSC8 lines and their distribution through out the genome. Twenty-five of the *Eco* RI and 35 of the *Hind* III tested probes were chosen and segregation analysis was performed on the 335 F2 plants. These 60 RFLP probes were scored twice to limit error of scoring.

AFLP marker generation:

An investigation of the reliability of AFLP markers was first performed through a reproducibility test at each step in the AFLP procedure from DNA extraction to final selective amplification. Variation in the banding pattern generated was found to be in the range of 1-2%, (i. e. 1-2 additional or missing amplified DNA fragments over the total number of DNA fragments amplified). As already described by Jones *et al*. (1997), the AFLP technology is very reliable and then suitable for genetic mapping. A total of 64 AFLP primer combinations with the selective bases at the 3'-end of each of the primers were tested on 10 sunflower plants in order to compare fingerprint patterns and determine which ones produced clearly detectable bands and revealed high levels of polymorphism. Of the 64 primer pairs tested, 16 were selected for the generation and screening of DNA markers. Across these 16 primer combinations, the number of well-amplified bands varied between 80 to 120 while the number of polymorphic bands ranged between 14 to 33. From the 335 F2 progenies, 271 markers were scored using the 16 primer combinations. For data scoring, segregation distortion will be checked and highly significantly ($P<0.001$) skewed markers will be removed from further analysis in order to avoid false linkages in map construction.

Analysis of quantitative traits:

Quantitative traits of XRQ x PSC8 F3 families are given in Table 1. All the characters presented showed significant differences between families in preliminary analyses of variance on non-transformed data, except for percentage attack (of *Sclerotinia* or phomopsis) and percentage lodging, when an arc-sin transformation was used.

For the *Sclerotinia* tests results show wide variation, with extremes showing differences of at least 1 to 2 (leaf test) and up to 1 to 6 (% ascospore attack). Comparison with controls showed that for the leaf test, no family was very susceptible, both parents probably carry some favourable alleles. In contrast, for the ascospore test, there were very large variations. The c.v. was higher for % attack, where there are just the two values for each replication, than for the latency index where the results can be a mean of about 40 indices, when there was 80% attack. Although showing wide variation, the mycelium test on capitula was the least precise, with a c.v. of approximately 34%. As for the latency indices, the mean flowering and maturity dates were precise, since they were obtained from up to 50 individual data. In contrast, although showing significant differences between families, percentage

lodging showed a very high c.v., because there were many families without lodging. The phomopsis trials results are very partial, the different results in different locations show the necessity of several trials. For the other agronomic characters, seed weight/capitulum was the most variable, but was less precise than height and oil content.

Table 1.: Analysis of quantitative traits for resistance to *Sclerotinia sclerotiorum* and *Diaporthe helianthi* and for other agronomic characters which varied significantly between F3 families from the cross XRQ x PSC8. Lsd: least significant difference. c.v.: coefficient of variation.

Character	Number of F3 families	Minimum	Maximum	Lsd	c.v.(%)
***Sclerotinia* tests**					
Mycelium/leaf (cm)	220	3.43	7.28	1.54	15.1
Mycelium/capitulum (index)	174	0.15	1.24	0.36	33.8
Ascospore/capitulum (% attack)	220	13.8	84.3	19.2	15.0
Ascospore/capitulum (latency index)	220	0.70	1.37	0.20	9.74
Flowering date (day of year)	220	196	206.5	1.5	0.39
Maturity Date (day of year)	220	239	272	7.7	1.52
Duration flowering-maturity	220	41.7	69.4	7.9	7.2
% lodging	220	0	41.9	17.4	104.3
phomopsis test (% attack)	28	9.0	40.35	14.6	57.5
Seed weight/capitulum (g)	220	0.72	37.2	12.2	69.3
Plant height (cm)	220	125	218	17.2	8.7
Oil content (%)	215	30.2	51.6	17.2	8.7

Discussion

The genetic map of XRQ x PSC8 will soon be completed with approximately 300-350 RFLP and AFLP markers. This map will be then used for characterise and locate QTL for resistance to *S. sclerotiorum* and *D. helianthi,* and the other agronomic characters. The use of

60 polymorphic RFLP probes and about 250 AFLP markers should provide a map with a density similar to the first composite sunflower map published by Gentzbittel *et al*. (1995). It should certainly be possible to determine QTL with good precision. For example, it will be possible to compare the QTL for resistance to *Sclerotinia* mycelium tests with those found by Mestries *et al* (1998) using only 73 RFLP probes.

Of the characters studied, the best polymorphism for disease resistance appears for the *Sclerotinia* ascospore test. This test was also applied on the F3'families (from interpollination of F3 plants) in 1999, to permit studies of year effects. This will give, in addition, a second series of data concerning flowering and maturity. For phomopsis resistance, semi-natural attack trials have been continued, but in addition a test measuring mycelium extension on leaves is being applied to obtain detailed data on certain aspects of phomopsis resistance. Overall, this cross should make it possible to study the genetic control of the main agronomic characters bred in sunflower, and it should thus provide some useful knowledge concerning the interactions between these characters.

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