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Cloning of molecular markers for disease resistance in sunflower, *Helianthus annuus* L.

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Abstract A candidate-gene approach to analyse the resistance of plants to phytopathogenic fungi is presented. The resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*) shows a gene-for-gene interaction (monogenic resistance), whereas resistance to white rot (*Sclerotinia sclerotiorum*) is quantitative, with different levels of resistance for different plant parts. By homology cloning, probes were obtained homologous to some plant resistance genes (nucleotide binding site-like, NBS, genes and serine-threonine protein kinase-like, PK, genes). These clones were used as probes for linkage mapping of the corresponding genes. It was demonstrated that at least three NBS-like loci are located on linkage-group 1, in the region where downy mildew resistance loci have been described. Quantitative trait loci for *S. sclerotiorum* resistance to penetration or extension of the mycelium in different tissues were studied in three crosses. Major QTLs for resistance were found on linkage group 1, with up to 50% of the phenotypic variability explained by peaks at the map position of the PK locus, 25 cM from the downy mildew loci.

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Introduction

Resistance of sunflower to downy mildew (*Plasmopara halstedii*) is controlled by single dominant genes designated *Pl* (Vranceanu 1970) and has been found for all known races of the pathogen. It has been demonstrated recently that at least some *Pl* genes are clustered (Mouzeyar et al. 1995; Vear et al. 1997). A number of plant resistance genes have already been cloned (for review see Bent 1996) These fall into two different classes (Staskawicz et al. 1995): leucine-rich repeat (LRR) genes with or without a nucleotide-binding site (NBS), and genes with a serine/threonine protein kinase domain such as *Pto* in tomato (Martin et al. 1993). *Xa21* in rice (Song et al. 1995) is a gene containing both motifs, i.e. a LRR domain and a kinase domain. A possible hypothesis concerning the *Pl* downy mildew resistance genes of sunflower is that they could be coded either by a LRR or NBS-like gene or a PK-like gene.

The interaction between sunflower and *Sclerotinia sclerotiorum* (the causal agent of white rot and wilt) is a quantitative trait and, for a given genotype, the level of resistance of each plant part may be different. As chemical control is difficult and not economic, it is necessary to develop genetically resistant hybrids. Since the publication of the hypothesis that QTLs could be allelic with major genes governing the same trait, first proposed by Robertson (1985), several coincidental map locations of QTLs and major genes affecting the same trait have been identified (Edwards et al. 1992; Veldboom et al. 1994). The description of the possible role of the *Sh2* gene in starch synthesis in maize (Goldman et al. 1993), or the co-location of carbon metabolism enzyme genes and QTLs for their activities (Causse et al. 1995), tend to provide arguments for a candidate-gene approach to characterize QTLs.

This paper presents a candidate-gene approach, to identify markers both for monogenic and quantitative disease resistance in sunflower.

Materials and methods

Homology cloning and probes

NBS-like probe

For the homology cloning of the NBS-like probe, the *N* gene sequence (accession A54810) and the *L6* gene sequence (accession U27081) were aligned with the CLUSTAL program (Higgins et al. 1992). Two degenerate primers were then generated: forward primer (5′) GGA ATG GGK GGA GTY GGY AAR AC (3′) and reverse primer (5′) ATC ATA ACT TAT TTT KAG (3′). These primers were synthetized by OLIGO-EXPRESS (Paris; France).

Amplification was made on genomic DNA of a downy mildew-resistant line (RHA266) with the following PCR conditions: 94°C (5 min) for denaturation; then 40 cycles of 94°C (30 s) for denaturation; 42°C (30 s) for annealing; 72°C (1 min 30 s) for extension; then a final extension of 72°C (10 min). PCR products were checked on standard agarose gels.

Cloning was carried out in a T/A vector (pCR2.1, InVitrogen, Netherlands) following the manufacturer's instructions. After sequencing, the resulting sequence of the probe was analysed for homology with data banks using the BLAST program (Altschul et al. 1990).

PK-like probe

The primers used for homology cloning of a PK-like probe were derived from S-receptor kinase-like degenerate oligonucleotides (a kind gift of Dr. M. Cock and Prof. C. Dumas, ENS de Lyon, France). The primers used were (5′) GGI GGI TTY GGI ATH GTI TWY AAR GG (3′) as a forward primer and (5′) AAI ATI CKI GCC ATI CCR AAR TC (3′) as reverse primer. The amplification conditions and cloning were as described for the NBS-like probe, except that the annealing temperature was 55°C.

Plant material and disease evaluations

Sunflower genotypes

The F_2 populations employed for linkage mapping and QTL analysis are described in Table 1. Two of the crosses used involved a common parent (PAC1), known to exhibit a good level of resistance to the penetration of *S. sclerotiorum* ascospores. SD is a line with a good level of resistance to the extension of the fungus in the tissues, while CP73 is a very susceptible genotype. Thus, the S1 cross involved two inbred lines with good levels of resistance, while the S3 cross was between a highly susceptible line and a highly resistant line. The S4 cross involved a susceptible line (GH) and a highly resistant line (PAC2). For each cross, about 140 F_3 or F_4 families were tested in the field.

S. sclerotiorum resistance evaluations

The F_3 or F_4 families were tested in 1994 (SD × PAC1) and 1995 (CP73 × PAC1), from North to South of France, in five locations. The F_3 and F_4 families of the GH × PAC2 cross were tested in 1994 and 1995 in only one location.

A Percentage attack and a latency index: ascospores test on capitula (Tourvieille and Vear 1984) were made with two replications of 20–25 plants at Clermont-Ferrand and one other replication in each of the four other locations. The percentage attack is considered to estimate the resistance to the fungal penetration, while the latency index (delay in symptom appearance) is considered to be a measurement of resistance to the extension of the fungus within the tissues.

The method used to determine the rate of mycelium extension on leaves was as described by Castano et al. (1992). After measurements, the infected leaves were removed in order to maintain normal plant development. For the mycelium test on the capitulum the method employed was that of Vear and Guillaumin (1977), at physiological maturity. These two tests were each made on two replications of five plants at Clermont-Ferrand. Correlations between these four different criteria were computed following standard methods.

Resistance to downy mildew analyses involved only the S3 (GH × PAC2) cross and were previously described by Vear et al. (1997). The 111 F_2 progenies from this cross were assessed for resistance both to race 1 and race D of *P. halstedii*.

Linkage mapping and QTL analysis

DNA preparation, Southern blotting and hybridization were carried out by standard techniques. The MAPMAKER/EXP program (Lander et al. 1987) was used to compute the linkage maps of each cross (minimum LOD score 3.0, maximum recombinant fraction 0.40). For linkage-group 1, the consensus linkage map between the three crosses used was constructed using MAPMAKER/EXP, by combining the data files of each cross. The overall method for linkage mapping, including nomenclature of the loci, follows that of Gentzbittel et al. (1995).

QTL analysis was made by each of the three following methods: one-way ANOVA, the interval-mapping method (MAPMAKER/QTL, Lincoln et al. 1992) or marker regression method (Kearsey and Hynes 1994), with a significance level of 0.001 or a LOD score of 3.0. When necessary, data were subjected to

Table 1 Segregating populations used for NBS-R3 and PK-like probe mapping and QTL analysis for resistance to *S. sclerotiorum*

Cross	Genotypes	Characteristics	Number of F_2 plants studied
S1	SD × PAC1	SD: resistant to fungal extension PAC1: resistant to fungal penetration	139
S3	CP73 × PAC1	CP73: susceptible PAC1: resistant to fungal penetration	280
S4	GH × PAC2	GH: susceptible PAC2: resistant	161

transformation, using either log10 or arcsin of the square-root, in order to improve normalization and the equality of variances. The proportion of the phenotypic variance explained by segregation of a significant marker was determined by the R^2 value. Usually, the mean value of each resistance trait for the overall trials in each cross was chosen for QTL analysis, after testing the homogeneity of the trait in each location. When a probe was not polymorphic in a cross, its QTL effect was estimated using the nearest flanking marker of the interval where the probe is assigned (interval-mapping method) or by using the whole linkage-group information (marker-regression method).

Results

Homology cloning and mapping

NBS-like probe

By restricting the multiple sequence alignments to the *N* gene (Whitham et al. 1994) and the *L6* gene (Lawrence et al. 1995), it was possible to design degenerate primers to two conserved domains of these genes. After the PCR reaction on the genomic DNA (T = 42°C) of a resistant line (RHA266), the product obtained was about 650 bp long. Cloning and sequencing of the NBS-R3 product (GenBank accession U96642) revealed a significant homology with the *N* (Whitham et al. 1994) and *L6* (Lawrence et al. 1995) genes at the deduced amino-acid level (Fig. 1).

Mapping of the NBS-R3 probe was carried out in the three crosses employed (Table 1). Autoradiographs (Fig. 2) revealed a rather complex pattern, suggesting a multigenic family. It was possible to map one locus in each cross used (139, 280 and 161 F_2 individuals, respectively). Each NBS-R3 locus (NBSR3-E1, NBSR3-E5_1 and NBSR3-E5_2) co-segregated with loci assigned to linkage-group 1 of the sunflower: the S124E1H3-2, S145H3-2 and S003H3 loci. The consensus map of linkage-group 1 with the three crosses used is presented in Fig. 3 A. A total of 580 individuals were genotyped, with ten loci revealed. A set of three anchor loci were mapped in each cross. Linkage-group 1 covers 90 Kosambi cM, with the NBS-R3 loci located towards the upper end. No evidence was obtained to show whether the three NBS-R3 loci, each mapped in one cross, were, in fact, the same locus.

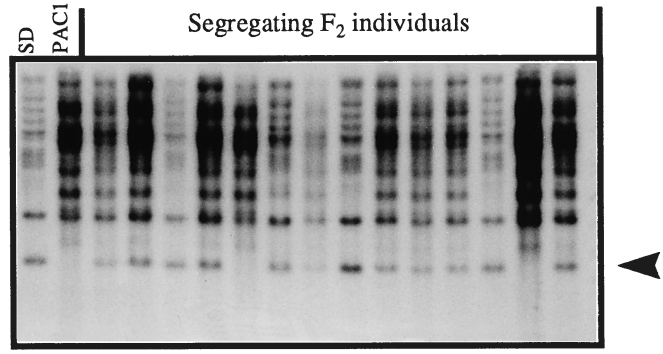

Fig. 2 Example of the NBS-R3 probe segregation autoradiogram in the S1 cross. The *first two lanes* are parental DNAs (SD and PAC1 respectively), digested with *Eco*RV. DNAs from F_2 individuals (part of a segregating population) follow. The *arrow* labels the segregating allele at the locus NBSR3-E5_1 on linkage-group 1

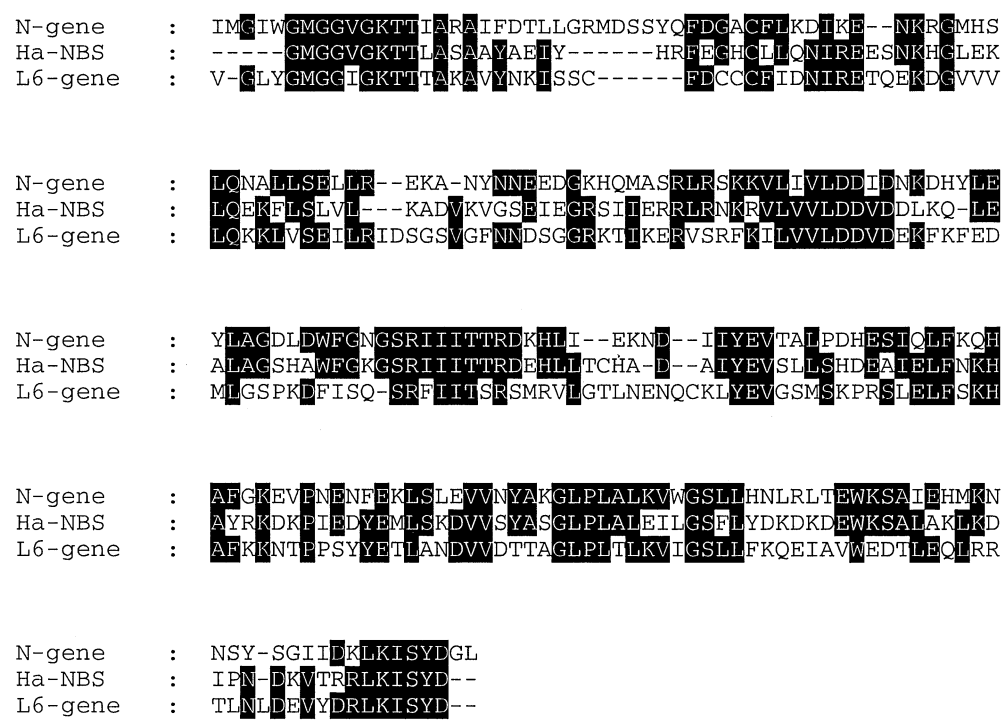

Fig. 1 Partial alignment of the deduced amino-acid sequences of the sunflower NBS-R3 probe (*Ha-nbs*) with that of the disease resistance genes of tobacco *N* (Whitham et al. 1994) and flax *L6* (Lawrence et al. 1995), using the CLUSTAL method. *Black shaded letters* indicate identical residues

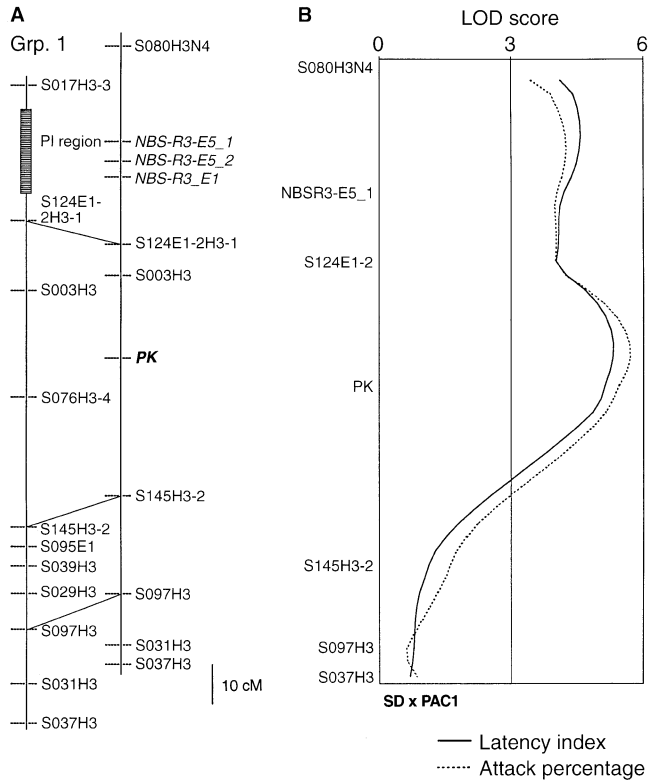

Fig. 3 **A** *Right*: consensus mapping of NBS-R3 loci and the PK locus on linkage-group 1, in the three crosses studied. *Left*: for comparison; the data are from Gentzbittel et al. (1995) for the map of linkage-group 1, and from Mouzeyar et al. (1995) and Vear et al. (1997) for the mapping of *Pl* loci for downy mildew resistance. Anchor loci are indicated with *lines*. **B** Example of a scan showing QTLs associated with *S. sclerotinia* resistance for latency index, and percentage attack (F_3 and F_4 generations) in the SD × PAC1 cross

PK-like probe

After the PCR reaction on genomic DNA at an annealing temperature of 55°C, the product obtained was about 300-bp long. Cloning and sequencing of this product (GenBank accession U96643) revealed homologies exclusively with serine/threonine protein kinases at the deduced amino-acid level. Homology was found with the *Atreclkin* gene (Hervé et al. 1996) and the *Atlec* gene (Swarup et al. 1996). Both genes originated from *Arabidopsis thaliana* and contain a kinase domain and an extracellular lectin-like domain. Partial alignment of the sunflower PK-like probe and the kinase domains of these genes is shown Fig. 4. The PK-like probe also shows significant homology with the disease resistance gene *Pto* in tomato (Martin et al. 1993) and with the kinase domain of the *Xa21* gene in rice (Song et al. 1995) (Fig. 5).

The PK-like probe was mapped in the S1 and S4 crosses and revealed a very simple pattern (Fig. 6), suggesting a low-copy number sequence. The PK-like locus co-segregated, in the same way as the NBS-R3 loci, with loci assigned to linkage-group 1: i.e. the S124E1H3-2, S145H3-2 and S003H3 loci. The consensus map of linkage-group 1 for the PK locus is shown in Fig. 3 A.

QTL analysis of *S. sclerotiorum* resistance

In order to obtain information about the overall level of *S. sclerotiorum* resistance, QTLs controlling the reactions to four different tests were mapped. There was a significant correlation between the percentage attack and the latency index ($r = 0.76$). QTLs were analysed for each location unit-trial, as well as on the means of all the trials.

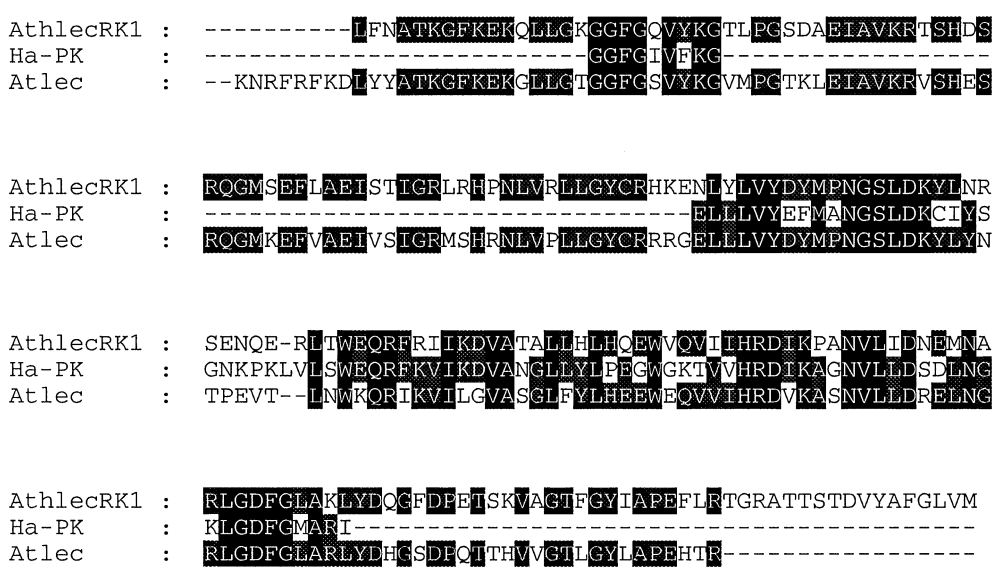

Fig. 4 Partial alignment of the deduced amino-acid sequence of sunflower PK probe (*Ha-PK*) with the kinase domain of *A. thaliana* receptor kinase genes containing a lectin domain, *AthlecRK1* (Herve et al. 1996) and *Atlec* (Swarup et al. 1996). The alignment was produced by using the CLUSTAL method. *Black shaded letters* indicate identical residues

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Pto-gene   :   --------RDGAKVALKRRTP-----------ESSQGIEEFETEIETLSFCR
Ha-PK      :   ---------GGFGIVFKGEL-------------------------------
Xa21-gene  :   FAPTNLLGSGSFGSVYKGKLNIQDHVAVKVLKLENPKALKSFTAECEALRNMR

Pto-gene   :   HPHLVSLIGFCDE-----RNEMILIYKYMENGNLKRHLYGSD---LPTMSMSW
Ha-PK      :   ----------------------LLVYEFMANGSLDKCIYSGN---KPKLVLSW
Xa21-gene  :   HRNLVKIVTICSSIDNRGNDFKAIVYDFMPNGSLEDWIHPETNDQADQRHLNL

Pto-gene   :   EQRLEICIGAARGLHYLHTR---AIIHRDVKSINILLDENFVPKITDFGISKK
Ha-PK      :   EQRFKVIKDVANGLLYLPEGWGKTVVHRDIKAGNVLLDSDLNGKLGDFGMARI
Xa21-gene  :   HRRVTILLDVACALDYLHRHGPEPVVHCDIKSSNVLLDSDMVAHVGDFGLARI

Pto-gene   :   GT--------------
Ha-PK      :   ----------------
Xa21-gene  :   LVDGTSLIQQSTSSMG
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Fig. 5 Partial alignment of the deduced amino-acid sequence of the sunflower PK probe (*Ha-PK*) with the kinase domain of resistance genes *Pto* (Martin et al. 1993) and *Xa21* (Song et al. 1995). The alignment was produced by using the CLUSTAL method. *Black shaded letters* indicate identical residues

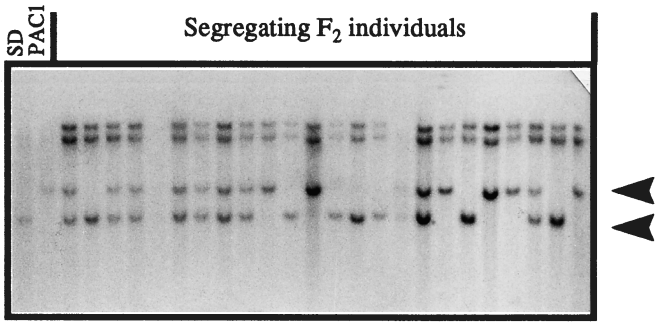

Fig. 6 Example of the PK probe segregation-autoradiogram in the S1 cross. The *first two lanes* are parental DNAs (SD and PAC1 respectively), digested with *Hind*III. DNAs from F_2 individuals (part of a segregating population) follow. *Arrows* label the segregating alleles at the PK locus, on linkage-group 1

The QTL analyses of sunflower resistance to *S. sclerotiorum* are presented in Table 2. Only the mean value of each trait over the five locations of each cross is presented. The major QTLs found for the percentage attack and the latency index covered the region surrounding the PK-like locus (Fig. 2 B), this locus being almost at the peak position in the S1 cross (LOD score = 5.10 and LOD score = 5.28 for percentage attack and latency index respectively). For the S3 cross, the PK locus effect was estimated using the S003H3–S145H3-1 interval for interval mapping, or by using the whole linkage-group information for marker-regression mapping. This QTL explained more than 50% of the phenotypic variance in the S3 cross, but only 16% in the S1 cross. A positive association of the PK locus with the QTL for either latency index or percentage attack was found not only on the mean value of the trait in each cross, but also in three of the five locations in the S1 cross (LOD score ranging from 3.03 to 7.12, data not shown), and in all the five locations for the S3 cross (LOD score ranging from 3.51 to 8.66). The PK locus is associated with a QTL for the mycelium index on leaves in the S4 cross (LOD score ranging from 3.75 in the F_3 generation to 8.48 in the F_4 generation). This QTL also explained about 50% of the phenotypic variance in the S4 cross. Additionally, the NBS-R3 loci showed significant associations with the QTLs for latency index, percentage attack and mycelium index on leaves , but the part of the phenotypic variance explained was smaller than that related to the PK locus (Table 2). It can be suggested that this significant association is a consequence of the genetic linkage between the NBS-R3 and PK loci on linkage group 1.

Discussion

Co-localisation of the NBS-like probe and the *Pl* loci

The hypothesis on which this study was based was that LRR-NBS-like genes or PK-like genes could be involved in the expression of the resistance of sunflower to downy mildew. It may be suggested that the NBS-R3 probe is homologous to the *N* and *L6* genes. Alignment of the NBS-R3 probe with the *N* gene revealed that about 660 bp in 5′ and 2100 bp in 3′ are missing on the corresponding cDNA. 5′RACE-PCR and 3′RACE-PCR experiments are currently in progress to obtain the full-length cDNA of the NBS-R3 gene.

This study has shown that both NBS-like and PK-like probes are located in the vicinity of the *Pl* region on linkage-group 1. Autoradiographs suggest that the NBS-R3 probe is a repeated sequence, of which all the polymorphic bands are located on linkage-group 1. Consensus mapping of this linkage group (Fig. 3 A), based on the three crosses used (580 F_2 individuals),

Table 2 Effects of the PK-like and NBS-like probes on the *S. sclerotiorum* resistance traits in sunflowers. In brackets: estimated effect at the position of the locus, in the case where the probe was non-polymorphic in the cross studied. nd: the entire linkage group exhibits no polymorphism in the cross studied

Probe	Linkage group	Percentage attack						Latency index						Mycelium on leaves						Mycelium on capitulum					
		S1[a]		S3[b]		S4[c]		S1		S3		S4		S1		S3		S4		S1		S3		S4	
		LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2	LOD	R^2
NBS-R3	1	4.00	14.3	5.03	23.5	0.06	0.2	4.03	13.6	4.66	29.7	0.14	0.5	0.36	1.5	0.31	1.9	3.34	9.7	1.05	3.9	0.07	0.4	0.39	2.7
PK-like	1	5.10	15.7	[6.67]	59.6	1.80	8.9	5.28	16.4	[6.87]	52.0	1.66	6.4	0.47	1.9	[0.24]	1.3	8.48	49.9	1.38	5.4	[0.05]	0.5	0.06	0.2

[a] S1: SD × PAC1 cross
[b] S3: CP73 × PAC1 cross
[c] S4: GH × PAC2 cross

revealed that the NBS-R3 loci map in the region where the *Pl* loci have been assigned (Mouzeyar et al. 1995; Vear et al. 1997). Thus, the NBS-R3 gene could be a candidate for resistance to downy mildew in sunflower. The same approach was used in recent studies to map candidate disease-resistance genes in soybean (Kanazin et al. 1996; Yu et al. 1996) and in potato (Leister et al. 1996). Further studies are in progress to define the genetics of the *Pl* locus in sunflower, in order to determine whether each *Pl* gene (specific to a given race of downy mildew) corresponds to one NBS-R3 locus.

Major QTL effect of the PK-like locus

It may be suggested that the PK-like fragment is related to the Serine/Threonine protein kinase family, since significant homologies with the *Pto* gene (Martin et al. 1993) and with the kinase domain of the *Xa21* gene in rice (Song et al. 1995) are exhibited by this fragment. Homologies to protein kinases containing a putative lectin domain (Herve et al. 1996; Swarup et al. 1996) are also found for the PK-like probe. The cloning of the complete gene corresponding to the PK-like fragment is under investigation and should give indications concerning its structure.

The co-location of QTLs with major genes affecting the same trait has been well documented, in particular for traits governing the morphology of the plant (Veldboom et al. 1994; Mitchellolds 1996). In barley, the co-location of a QTL for disease resistance at the adult plant stage and a monogenic resistance locus at the seedling stage has been also reported for spot blotch resistance (Steffenson et al. 1996), indicating a potential relationship between monogenic and quantitative resistances.

Marker-assisted selection

The ultimate objective of this work is to clone the functional complete genes corresponding to the NBS-R3 and PK-like probes and to test whether they can be employed for complementation experiments. Nevertheless, in the short term, these probes could be useful as molecular markers for the introgression of resistance both to *S. sclerotiorum* and *P. halstedii* into susceptible sunflower lines.

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