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Identification of non-TIR-NBS-LRR markers linked to the *Pl5*/*Pl8* locus for resistance to downy mildew in sunflower

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Abstract The resistance of sunflower, *Helianthus annuus* L., to downy mildew, caused by *Plasmopara halstedii*, is conferred by major genes denoted by *Pl*. Using degenerate and specific primers, 16 different resistance gene analogs (RGAs) have been cloned and sequenced. Sequence comparison and Southern-blot analysis distinguished six classes of RGA. Two of these classes correspond to TIR-NBS-LRR sequences while the remaining four classes correspond to the non-TIR-NBS-LRR type of resistance genes. The genetic mapping of these RGAs on two segregating F2 populations showed that the non-TIR-NBS-LRR RGAs are clustered and linked to the *Pl5*/*Pl8* locus for resistance to downy mildew in sunflower. These and other results indicate that different *Pl* loci conferring resistance to the same pathogen races may contain different sequences.

Keywords Disease resistance · *Helianthus annuus* · *Plasmopara halstedii* · Non-Toll-Interleukin-1 Receptor (non-TIR) · Nucleotide Binding Site (NBS) · Leucine-Rich Repeats (LRR)

Introduction

Downy mildew, caused by *Plasmopara halstedii* (Farl.) Berl. & de Toni, is one of the main diseases causing economic losses in cultivated sunflower (*Helianthus annuus* L.). The resistance to this disease was first shown by Vranceanu and Stoenescu (1970) to be controlled by dominant major genes, denoted *Pl*, following a pattern which agrees quite well with the gene-for-gene hypothesis of Flor (1955). So far, ten *Pl* genes have been described from both cultivated sunflower (Vranceanu and Stoenescu 1970) and wild *Helianthus* species (Miller and Gulya 1991; Vear et al. 2000).

Vear et al. (1997) showed that the *Pl6* locus from wild *H. annuus* (Miller and Gulya 1991) could be split into at least two genetically distinct regions, one giving resistance to races 100 and 300, and a second giving resistance to races 700, 703 and 710. Further evidence for the grouping of resistance genes came from the mapping of the *Pl6* gene by Roeckel-Drevet et al. (1996) in the same area on the RFLP map (Gentzbittel et al. 1999) as *Pl1* (Mouzeyar et al. 1995) and *Pl2* (Vear et al. 1997). A second region carrying downy mildew resistance genes was reported by Bert et al. (2001), who mapped *Pl5* (from the Russian population Progress) on linkage group 6 and found that its resistances to races 100, 703 and 710 did not segregate with those controlled by *Pl8* (from *Helianthus argophyllus*, Miller and Gulya 1991).

In recent years, many different plant disease-resistant genes (R-genes) have been cloned and sequenced, greatly advancing our understanding of molecular genetic mechanisms underlying disease resistance in plants (Michelmore 1995a, b; Staskawicz et al. 1995; Bent 1996; Hammond-Kosack and Jones 1997; Parker and Coleman 1997; Martin 1999; Ellis et al. 2000).

To isolate R-gene analogs of different crop plants, well-conserved regions of the NBS domains have been used to design degenerate primers that amplify resistance gene analogs (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Feuillet et al. 1997; Aarts et al. 1998; Collins et al. 1998; Gentzbittel et al. 1998; Ohmori et al. 1998; Shen et al. 1998; Speulman et al. 1998). Two groups of NBS-LRR (nucleotide binding site-leucine-rich repeats) resistance genes have been described in plants. The first group, TIR-NBS-LRR, includes *L*6 from flax (Lawrence et al. 1995), *N* from tobacco (Whitham et al. 1994) and *RPP*5 from *Arabidopsis thaliana* (Parker et al. 1996).

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Genes belonging to this group code N-terminal domains with Toll/interleukin-1 receptor (TIR) homology and are absent from monocotyledons (Meyers et al. 1999). The second group, non-TIR-NBS-LRR, includes *RPM*1 (Grant et al. 1995) and *RPS*2 (Bent et al. 1994) from *A. thaliana*. Genes belonging to this group lack the TIR domain and are present in both monocotyledons and dicotyledons (Meyers et al. 1999).

The NBS sequence of R genes are characterised by the presence of up to seven conserved domains including the P-loop, Kinase-2 and GLPL motifs (Meyers et al. 1999). The presence of these conserved domains has facilitated the cloning of RGAs from diverse species by PCR, using degenerate oligonucleotide primers. Identification of RGAs should help to generate markers for map-based cloning of resistance genes.

In sunflower, Gentzbittel et al. (1998) used degenerate primers designed from the conserved NBS domains of *N* from tobacco (Whitham et al. 1994), *RPS2* from *A. thaliana* (Mindrinos et al. 1994) and *L*6 from flax (Lawrence et al. 1995). The resulting amplification products were shown to be members of a multigene family. One clone was sequenced and mapped close to *Pl6*. Sequence analysis of this RGA showed considerable homology with the nucleotide-binding domains of previously cloned resistance genes in other species. Bouzidi et al. (2002) found 13 STSs linked to the *Pl6* locus on linkage group 1 all belonging to the TIR-NBS-LRR subclass and clustered within about 3 cM which contain genes giving resistance to downy mildew races 100, 300, 700, 703 and 710. The same markers failed to detect other resistance loci on other linkage groups.

Gedil et al. (2001a) used degenerate oligonucleotide primers targeted to conserved NBS DNA sequence motifs to amplify RGA fragments from sunflower genomic DNA. PCR products were cloned, sequenced and assigned to 11 groups. RFLP analysis mapped six RGA loci to three linkage groups. Four of these RGAs were non-TIR-NBS-LRR and the rest were TIR-NBS-LRR. In the same study, one TIR-NBS-LRR RGA (Ha-4w2) was found linked to the *Pl1* gene giving resistance to *P. halstedii* race 100. However, the other RGAs cloned, especially those belonging to the non TIR-NBS-LRR subclass, were not tested for linkage with other loci giving resistance to *P. halstedii*. Therefore, it was interesting to test whether RGA markers of the non-TIR-NBS-LRR subclass of plant resistance genes could be linked to *Pl* loci which segregate independently of *Pl6*.

For this purpose, degenerate and specific primers were used to clone several non-TIR-NBS-LRR RGAs. Four of these were selected and used as RFLP probes for genetic mapping of two segregating F2 populations carrying *Pl5* and *Pl8*.

Materials and methods

Sunflower genotypes

The two origins of downy mildew resistance that were studied were INRA lines YSQ and QIR8. YSQ was developed from a cross between a line resistant to race 710, provided by Dalgren and Co. (USA), and a susceptible INRA line (DV). Previous results (Vear et al. 2000) had shown that its resistance to races 100, 703 and 710 did not segregate with the resistance gene denoted *Pl5*, in the line XRQ, mapped on linkage group 6 by Bert et al. (2001). Since YSQ, like XRQ, is resistant to all French downy mildew races and to a Spanish isolate of race 330, but susceptible to an American isolate of this race, it was considered that the two lines probably contained the same resistance gene *Pl5*. QIR8 was bred by INRA from a cross between an INRA line containing *Pl2* (PIR2) and RHA340, a USDA line containing *Pl8* (Miller and Gulya 1991) and resistant to all known downy mildew races. As for YSQ, the resistance of RHA340 to races 100, 703 and 710 was found not to segregate with *Pl5* in XRQ (Vear et al. 2000). To check the position of resistance genes *Pl5* and *Pl8*, and to find RGAs linked with them, crosses were made between two INRA downy mildew susceptible lines, OC and CAY, and YSQ and QIR8, respectively. The F1 plants were selfed to obtain about 200 F2 plants in each case, from which DNA was isolated. These plants were, in turn, selfed to obtain F3 families which were tested to determine the resistance genotype of the F2 plants.

Downy mildew races

Tests of downy mildew segregations were made with race 710 for the F3 progenies of OC × YSQ and CAY × QIR8. In addition, to confirm the results of these tests for CAY × QIR8, since segregation between resistance to races 703 and 710 has never been observed (Vear et al. 1997, 2000; Bert et al. 2001), the progenies difficult to classify were subjected to tests with race 703, in a separate growth chamber.

Resistance tests

The downy mildew resistance genotype of each F2 plant was determined by testing their F3 progenies. For each progeny, at least 20 seedlings were infected and grown as described previously by Mouzeyar et al. (1993). Seedlings were scored as susceptible if fungal sporulation was evident on cotyledons and true leaves, and resistant if no sporulation was observed on true leaves (Mouzeyar et al. 1993, 1994). Plants with sporulation on cotyledons (Type II resistance) were considered as resistant, but progenies with many such plants were re-tested to confirm classification as resistant, susceptible or segregating. The corresponding F2 plants were then classified as homozygous susceptible, homozygous resistant, or heterozygous.

DNA manipulations

Young leaf tissue from the F2 plants was collected and freeze-dried. DNA was isolated using the CTAB method, as described by Saghai Maroof et al. (1984). Equal quantities of DNA were bulked from 12 homozygous-resistant and from 12 homozygous-susceptible F2 plants, according to the method of Michelmore et al. (1991), to give the two DNA bulks of each cross.

Oligonucleotide primers and PCR amplification

One degenerate oligonucleotide primer pair (Leister et al. 1996; Yu et al. 1996) and four specific oligonucleotide primer pairs were used in this study. The specific primers were designed using the sunflower RGAs sequences (Genbank Accession number

Table 1 Sequences of forward and reverse primers amplifying RGAs linked to *Pl5* and *Pl8* Degenerate IUB code: I (inosine); R (A or G); D (G, A or T); Y(C or T)

Primer pair	Forward primer sequences	Reverse primer sequences
HaNTP1	5'GGIGGIGTIGGIAAIACIAC3'	5'YCTAGTTGTRAYDATDAYYYTRC3'
HaNTP2	5'GGIGGIGTIGGIAAIACIAC3'	5'TTCCCAGTCGTCATAGTTTTCA3'
HaNTP3	5'GAATATTGTATAACGATACACGAG3'	5'TTCCAGTAGCCCTAGAATGAAATG3'
HaNTP4	5'GACATTTATATAACGACGCACAAG3'	5'TTCCAGGAGCCCCCAAATGAAATG3'
HaNTP5	5'GACATTTATATAACGACGCACAAG3	5'TTCCATGGGCACATGAACGAAATG3'
HaNTP6	5'GACTATTGTACAATGAAAAGCAAG3'	5'TTCCAGGAGCACATGCATGAAATG3'

AF272766, AF272767, AF272768 and AF272769) described by Gedil et al. (2001a). The sequences of all primer pairs are presented in Table 1. The PCR amplification with degenerate primers was carried out with 100 ng of sunflower DNA from the parents and the two bulks of the OC × YSQ cross. In the presence of 0.2 mM of each dNTP, 1 U (1 μl) of *Taq* DNA polymerase (Advantage 2, Clontech, France), 1 × *Taq* polymerase buffer [40 mM Tricine-KOH pH 8.7, 15 mM KOAc and 3.5 mM $Mg(Oac)_2$] and 1 μM of each primer, PCR was carried out in a 2400 Perkin-Elmer thermocycler under the following conditions: initial denaturation at 95 °C for 3 min, 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s. The PCR specific primer amplifications were carried out with 50 ng of DNA in the presence of 0.2 mM of each dNTP, 0.4 U (0.4 μl) of *Taq* DNA polymerase (Advantage 2, Clontech), 1 × *Taq* polymerase buffer and 0.5 μM of each primer. Following initial denaturaton at 95 °C for 3 min, 35 PCR cycles of 94 °C for 10 s, 58 °C for 30 s and 72 °c for 1 min 30 s were performed. PCR products were separated using standard TAE agarose-gel electrophoresis.

RGA cloning and sequence analysis

The PCR products were cloned into the pGEM-T Easy vector (Promega). A total of 96 colonies were sequenced on both strands using the Dye-Terminator method (Genome Express, France). The BLASTX program (Altschul et al. 1997) was used for homology search against the data banks. The sequences were aligned using the CLUSTALX software with default options (Thompson et al. 1997) and the resulting alignments were shaded using the GENEDOC software (Nicholas et al. 1997).

RFLP analysis and genetic mapping

DNA digestion and Southern hybridisation were performed as described previously (Gentzbittel et al. 1999) using four restriction enzymes: *Eco*RI, *Eco*RV, *Hin*dIII and *Bgl*II. The RGA probes were prepared by digesting 10 μg of the corresponding plasmids with 100 U of the restriction enzyme *Eco*RI. The digestion products were separated by standard agarose electrophoresis. The complete inserts were purified from the gel and used in subsequent experiments. The different RGAs were tested for polymorphism and those which produced polymorphic bands on the bulks were scored on 150 F2 plants of each cross. Two genetic maps were constructed for linkage group 6, comprising *Pl5* and *Pl8* polymorphic RGA loci, and three RFLP markers from the map of Gentzbittel et al. (1999). Linkage analysis was made with the software Mapmaker 3.0 (Lander et al. 1987). Markers were ordered with a LOD value threshold of 3.0 and a maximum recombination fraction of 50.

Results

Resistance tests and bulked segregant analysis

Both progenies showed Type II resistance, with plants showing sporulation on cotyledons. For the cross OC × YSQ, 209 F3 families were first tested with race 710, then 44 that were difficult to classify were re-tested with the same race, and the conclusion was modified for seven families. The segregation for OC × YSQ was concluded to be 64 homozygous resistant (RR): 92 heterozygous (Rr): 53 homozygous susceptible (rr). This agrees with the hypothesis of a single dominant gene (χ^2: 4.22, $P > 0.05$), although with a slight excess of plants considered as homozygous resistant. In the case of the cross CAY × QIR8, when tested with race 710, the F3 progenies were observed as a segregation of 43 RR: 73 Rr: 19 rr. This is significantly different from a 1:2:1 segregation (X^2: 9.43). Since resistances to races 703 and 710 have co-segregated without exception in all studies in our laboratory (Vear et al. 1997, 2000; Bert et al. 2001), to reduce possible error from difficult observations of reaction to race 710, the 72 progenies, which showed heavy sporulation on cotyledons with this race, were re-tested with race 703 in a different growth chamber. In this case there was less sporulation on cotyledons and it was easier to distinguish resistant and susceptible plants. Six progenies were observed as Rr instead of RR, eight as rr instead of Rr and three as rr instead of RR, giving a final ratio of 34 RR: 71 Rr: 30 rr (χ^2: 0.60). These conclusions were used for the genotypes of F2 plants for mapping.

Mapping of loci *Pl5* and *Pl8*

The *Pl5* locus showed linkage with two RFLP markers, S094H3 and S069H3, located on linkage group 6 of the RFLP map of Gentzbittel et al. (1999). The first was dominant, revealing a band only in the susceptible bulk and the susceptible parent OC, whereas the RFLP marker S069H3 was co-dominant, producing fragments polymorphic between the two bulks and the two parents. Mapping analysis using 150 F2 plants and the two probes revealed that the *Pl5* locus mapped 18.4 cM and 20.4 cM from S094H3 and S069H3, respectively. Similarly the *Pl8* locus in QIR8 showed linkage (21.1 cM) with the RFLP marker $SO17H3_6$, also previously mapped on group 6, which produced fragments polymorphic between the two

bulks and the two parents. Thus, it was confirmed that *Pl5* and *Pl8* are located on linkage group 6 and in the same area (see Fig. 3)

Cloning and sequence analysis of sunflower RGAs

Using DNAs from the parents and the two bulks of the OC × YSQ cross, PCR amplifications were obtained with the different pairs of primers. The length of the amplification products were the same for the parental and the bulked DNAs. The degenerate primer pair HaNTP1 produced an approximately 300-bp band. When we used the combination between the degenerate and specific primers, HaNTP2, the approximate 250- and the approximate 300-bp bands were obtained. The specific primer pairs HaNTP3, HaNTP4 and HaNTP5 produced an approximate 250-bp band, and the specific primer pair HaNTP6 gave an amplification product at about 270 bp. The PCR products were excised, purified from the gel and cloned into the pGEM-T Easy vector. Fourty eight clones (24 clones from the resistant parent and 24 clones from the susceptible one), amplified using the degenerate primer pair HaNTP1, were chosen randomly and sequenced. From the other primer-pair experiments, four clones from each parent were chosen randomly and sequenced. A total of 96 clones were sequenced on both strands. Sequencing results demonstrated that there were PCR products of different size even with the same primer pair (Table 2).

Table 2 RFLP profile classification of 16 RGAs amplified from OC and YSQ DNA with degenerate and specific NBS primers

Class[a]	RGA	Primers	Length (bp)	Parent[b]	Polymorphism[c]	NBS-LRR subfamily	Accession Number
A	Ha-NTIR3	HaNTP5	277	Resistant	Yes	Non-TIR	AF528539
	Ha-NTIR4	HaNTP5	277	Susceptible	Yes	Non-TIR	AF528540
	Ha-NTIR9	HaNTP1	308	Susceptible	Yes	Non-TIR	AF528541
	Ha-NTIR16	HaNTP1	308	Resistant	Yes	Non-TIR	AF528542
	Ha-NTIR12	HaNTP5	269	Resistant	Yes	Non-TIR	AF528543
B	Ha-NTIR5	HaNTP3	277	Susceptible	Yes	Non-TIR	AF528544
	Ha-NTIR6	HaNTP3	277	Resistant	Yes	Non-TIR	AF528545
	Ha-NTIR10	HaNTP2	284	Resistant	Yes	Non-TIR	AF528546
	Ha-NTIR11	HaNTP2	248	Resistant	Yes	Non-TIR	AF528547
	Ha-NTIR15	HaNTP2	248	Susceptible	Yes	Non-TIR	AF528548
C	Ha-NTIR7	HaNTP4	278	Resistant	Yes	Non-TIR	AF528549
	Ha-NTIR8	HaNTP4	278	Susceptible	Yes	Non-TIR	AF528550
D	Ha-NTIR2	HaNTP6	277	Resistant	Yes	Non-TIR	AF528551
	Ha-NTIR1	HaNTP6	277	Susceptible	Yes	Non-TIR	AF528552
E	Ha-TIR13	HaNTP1	308	Susceptible	Yes	TIR	AF528553
F	Ha-TIR14	HaNTP1	305	Resistant	No	TIR	AF528554

[a] Based on RFLP analysis and sequence comparison
[b] The parent from which the fragment was cloned
[c] Polymorphism between parents in the crosses OC × YSQ and CAY × QIR8

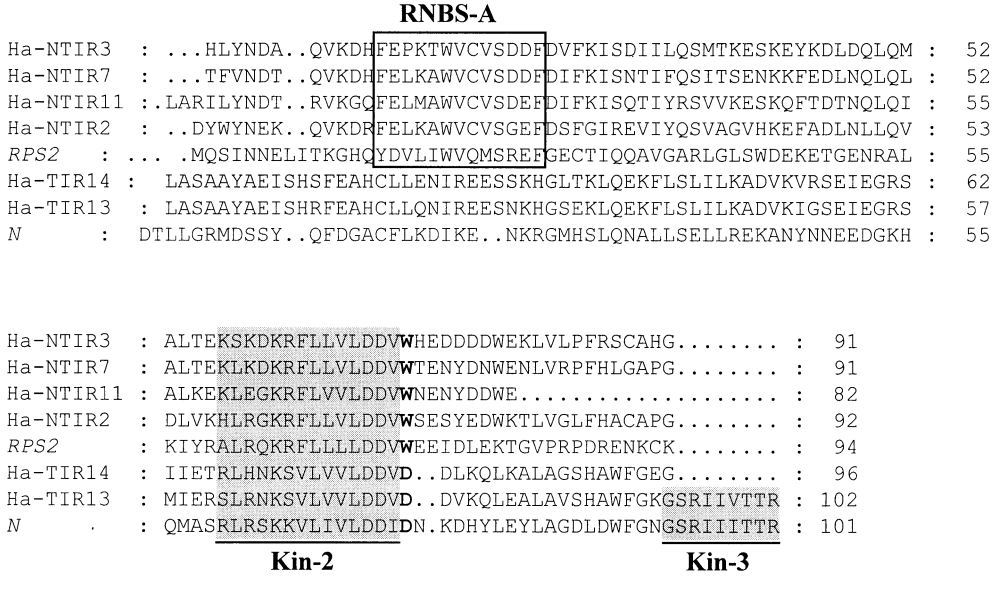

Fig. 1 Partial alignment of deduced amino-acid sequences of four non-TIR-NBS-LRR, two TIR-NBS-LRR RGAs and of two R-genes, tobacco *N* (accession number, U15605) and *A. thaliana RPS2* (accession number, U14158). The Computer program CLUSTALX was used in alignment analysis. Alignments were *shaded* using the Genedoc software. The Kin-2 domains and Kin-3 are *underlined*. The RNBS-A (FDLx-AWVCVSQxF) motif is *boxed*

A total of 52 clones out of 96 shared homology with genes from the NBS-LRR class of plant resistance genes. In contrast, 2 out of 48 clones amplified by degenerate primers showed homology with hypothetical proteins from *A. thaliana*, and 42 clones showed no significant homology with any known gene in the databases. Thus, these 44 clones were considered as not related to disease resistance genes and excluded from further analysis.

As expected, homology search showed that all the clones obtained by specific primers showed homology with genes from the NBS-LRR class of plant resistance genes, and with the clones of Gedil et al. (2001a) from which the primers were derived. For each primer pair combination, the eight clones sequenced (four clones originating from each parent) were aligned, and this showed that some of these clones were identical. After eliminating this redundancy, the 52 clones were found to correspond to 16 unique sequences. In order to group these RGAs, they were used as RFLP probes and those which had identical RFLP profiles were then arranged in the same class, giving six classes denoted A to F (Table 2). Of the 16 RGAs, 14 belonged to the non-TIR-NBS-LRR subfamily that contains the *RPS*2 gene from *A. thaliana* (Mindrinos et al. 1994), and two RGAs belonged to the TIR-NBS-LRR subfamily of resistant genes that contains the *N* gene from tobacco (Whitham et al. 1994). One representative RGA from each class was selected for sequence comparison and alignment. The amino-acid alignment between four non-TIR-NBS-LRR and two TIR-NBS-LRR sequences is shown in Fig. 1. Some of the motifs characteristic of the TIR-NBS-LRR or non-TIR-NBS-LRR subclasses are indicated according to Meyers et al. (1999).

Within each class of RGA, the pair-wise identity at the amino-acid level varied between 85 and 98% within class A, between 50 and 85% within class B, about 92% within class C and about 87% within class D. In contrast, classes E and F contained only one clone each.

When the subfamilies were compared, the identity between the two RGAs (TIR13 and TIR14) belonging to the TIR-NBS-LRR subfamily was about 72%, while the identity between the four RGAs of the non-TIR-NBS-LRR subfamily ranged from 47 to 68%. The mean percent identity between the two subclasses was weak and ranged from 15 to 17% (Table 3).

Similarity between these sequences and those obtained by Gedil et al. (2001a) ranged from 42% (between Ha-TIR14 and Ha-IW22) to 96% (between Ha-NTIR3 and Ha-IB39).

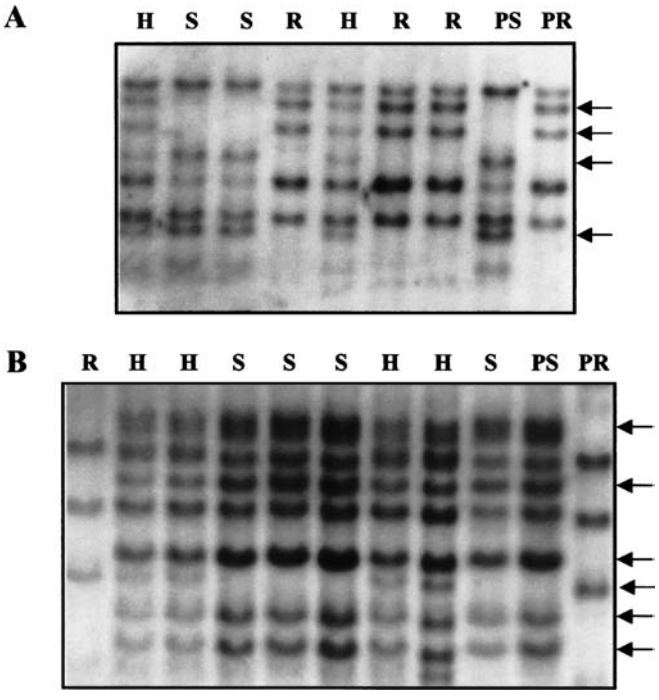

Fig. 2 Autoradiographs showing the linkage between the *Pl5* locus (**A**), *Pl8* locus (**B**) and the RGA (Ha-NTIR11) which was used as a RFLP probe. Downy mildew phenotypes: *R* homozygous resistant, *S* homozygous susceptible and *H* heterozygous. The polymorphic bands between the parent and the two bulks of each cross are indicated by the *arrows*

Linkage between sunflower RGAs and the *Pl5*/*Pl8* cluster

Of the 16 RGAs cloned and sequenced, one, TIR14, did not show any polymorphic profile in either cross using different restriction enzymes. This RGA was excluded from further linkage analysis. The RGA TIR13, which belongs to the TIR-NBS-LRR subclass, showed polymorphic bands in both crosses, but none of these bands was linked to *Pl5* or *Pl8*.

Four RGAs (Ha-NTIR7, Ha-NTIR11, Ha-NTIR2 and Ha-NTIR3), representative of the 14 clones of the non-TIR-NBS-LRR subfamily, were used to pursue the linkage study with the *Pl5*/*Pl8* locus. Southern blots showed multiple bands of varying intensity with these probes. Some of these bands were polymorphic between the two bulks of each cross. An example of a profile obtained with the Ha-NTIR11 clone is shown in Fig. 2. It may be noted that for a given RGA probe, all the

Table 3 Percent amino-acid sequence identities of four non-TIR-NBS-LRR, two TIR-NBS-LRR RGAs and of two R-genes (*N* from tobacco, Whitham et al. 1994, and *RPS2* from *A. thaliana*, Mindrinos et al. 1994). Values were calculated using the CLUSTALX program

Item	Ha-TIR14	Ha-TIR13	*N*	*RPS2*	Ha-NTIR2	Ha-NTIR11	Ha-NTIR7
Ha-TIR13	72						
N	30	39					
RPS2	10	12	12				
Ha-NTIR2	16	16	13	22			
Ha-NTIR11	15	17	12	20	47		
Ha-NTIR7	15	16	14	23	56	57	
Ha-NTIR3	15	16	14	22	52	52	68

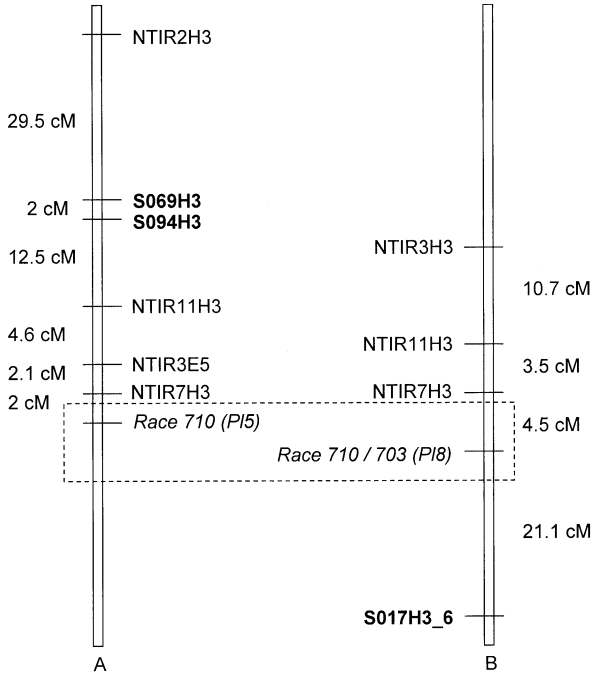

Fig. 3 Genetic map of sunflower linkage group 6 showing the relative positions of downy mildew resistance loci *Pl5* (**A**), *Pl8* (**B**), RGA (Ha-NTIR7H3, accession number AF528549; Ha-NTIR3E5, accession number AF528539; Ha-NTIR11H3, accession number AF528547; Ha-NTIR3H3, accession number AF528539 and Ha-NTIR2H3, accession number AF528551) and RFLP markers (S069H3, S094H3 and $S017H3_6$). RFLP markers are shown *in bold*. Genetic distances were calculated using the Kosambi map function and are shown in centimorgans (cM). The suffixes E5 and H3 indicate the restriction enzymes *Eco*RV and *Hin*dIII respectively

polymorphic bands were found absolutely linked and thus were considered as unique loci.

All the non-TIR-NBS-LRR RGAs were found linked to *Pl5* and *Pl8*, and the RFLP markers S094H3, S069H3 and $S017H3_6$ on linkage group 6 of the map of Gentzbittel et al. (1999). The map of the regions containing *Pl5* and *Pl8* is shown in Fig. 3A and B. The closest markers to *Pl5* were Ha-NTIR7H3 and Ha-NTIR3E5 at 2 cM and 4.1 cM, respectively; the marker Ha-NTIR2H3 mapped 52 cM from *Pl5* (Fig. 3A). The closest markers to *Pl8* were Ha-NTIR7H3 and Ha-NTIR11H3 at 4.5 cM and 8 cM, respectively; the Ha-NTIR3H3 marker mapped at 18.7 cM from *Pl8* (Fig. 3B).

Discussion

Mapping of *Pl*5 and *Pl*8 on linkage group 6

Bert et al. (2001) reported that *Pl*5, present in the inbred line XRQ, was linked to ten AFLP markers and two RFLP markers, S094H3 and S034H3, on linkage group 6 of the map of Gentzbittel et al. (1999). *Pl5* was found to be situated at 36 cM from the first RFLP and 38 cM from the second (Bert et al. 2002). It was 27 cM from *Rf1*, the restoration gene. In this study, a different RFLP marker, S069H3, reported by Gentzbittel et al. (1999) to be 1 or 2 cM from S034H3 and S094H3, confirmed that the *Pl5* gene in the line YSQ was also situated on linkage group 6, in the same area. The difference in positioning, about 15 cM, is probably due to the different segregating populations used for mapping, but the difficulties in determining downy mildew resistance genotypes in the presence of Type II resistance could also contribute to the difference.

Bert et al. (2001) reported that the resistance controlled by *Pl8* did not segregate with that of *Pl5*. The results presented here demonstrate that *Pl5* and *Pl8* are linked, and may form a major cluster for resistance to all known races of *P. halstedii* on linkage group 6. The linkage of *Pl8* to $S017H3_6$ confirms the position of the locus close to this RFLP marker suggested by Bert et al. (2001). According to their position relative to the marker Ha-NTIR7H3, resistance to races 703 and 710 controlled by *Pl5* and *Pl8* may be separated by about 2 cM. This distance indicates the possible complexity of the locus. The clustering of the resistance genes has been reported for many plant species such as the *Cf4*/*Cf9* cluster in tomato (Parniske et al. 1999) and *Dm3* in lettuce (Michelmore 2000). In sunflower, a major cluster for resistance to downy mildew had been described on linkage group 1 (Bouzidi et al. 2002), with genes also conferring resistance to all known races of downy mildew, so the two clusters appear to be functionally similar and it was important to determine whether they have different structures.

Linkage of non-TIR-NBS-LRR RGAs to the *Pl5*/*Pl8* cluster

Since the initial cloning of some plant resistance genes, several research groups have demonstrated that PCR-amplification-conserved disease-resistance motifs can be used to identify disease-resistance loci (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Hayes and Saghai Maroof 2000). In sunflower, a TIR-NBS-LRR RGA was localised within the Pl6 locus by Gentzbittel et al. (1998) using degenerate primers. In a similar study, Gedil et al. (2001a) used two pairs of degenerate oligonucleotide primers and obtained different RGAs from sunflower. They reported that three markers (HR-1W23, HR-1B39 and HR-1W41) were clustered on one end of linkage group 13 of the map of Gedil et al. (2001b). All of the NBS clones that detected these loci were of the non-TIR-NBS-LRR subfamily and shared 56–69% amino-acid identity. They also found that two TIR-NBS-LRR markers segregated independent of other RGA loci (HR-4W2 and HR-1W22) that were mapped on linkage groups 8 and 15 of the map of Gedil et al. (2001b). Thus it appears that there are both TIR-NBS-LRR and non-TIR-NBS-LRR sequences in sunflower and that they may be distributed in different regions of the sunflower genome. Bouzidi et al. (2002) reported that the

Pl6 locus on linkage group 1 of the map of Gentzbittel et al. (1999) appears to contain only TIR-NBS-LRR RGAs. None of these markers detected other resistance loci on other linkage groups.

In a recent study Yu et al. (2002) concluded that, by mapping phenotypic marker loci such as downy mildew resistance (*Pl* genes) and fertility restoration (*Rf1*) on different RFLP maps made up of different anonymous RFLP probes, linkage groups 1 and 6 of the map of Gentzbittel et al. (1999) correspond respectively to linkage groups 8 and 13 of the map of Yu et al. (2002). In addition, Yu et al. (2002) mapped SCAR markers that were previously linked to the rust resistance genes *R*1 and *R*adv (Lawson et al. 1998) on linkage groups 8 and 13, respectively, of the map of Yu et al. (2002). As stated by these authors, the two linkage groups contain duplicated clusters for resistance both to *P. halstedii* and *Puccinia graminis*. It will be interesting to verify for each linkage group whether these clusters are overlapping or that they are tightly linked.

In this study, RGAs of the non-TIR-NBS-LRR subclass were cloned using either degenerate or specific primers. As expected, the clones obtained were similar to those of Gedil et al. (2001a) but not identical. The percentage identity between the clones described by Gedil et al. (2001a) and those obtained in the present work varied between 42% (between Ha-TIR14, this work, and Ha-IW22, Gedil et al. 2001a) and 96% (between Ha-NTI3, this work, and Ha-IW41, Gedil et al. 2001a). These variations in sequence comparison may indicate that either the different sequences are part of different genes and/or that it is due to sequence variation between sunflower lines. The identification of these RGAs was based on the conservation of some motifs or amino-acid characteristics of the non-TIR-NBS-LRR subfamily (Meyers et al. 1999). TIR and non-TIR-NBS-LRR sequences are distinguishable by amino-acid motifs internal to the NBS domains while some of the motifs are present in both subclasses such as P-loop or GLPL motifs. Sequence comparison of the RGAs obtained in this study, and partial sequences from *RPS2* (Mindrinos et al. 1994) and *N* (Whitham et al. 1994) genes, showed the presence of the motif RNBS-A (FDLx-AWVCVSQxF) which is characteristic of the non-TIR-NBS-LRR sequences (Meyers et al. 1999). The presence of a tryptophan residue (W) as the final amino acid in motif Kinase-2 in some sequences, and an aspartic acid (D) in others, can be used to distinguish the two subclasses (Meyers et al. 1999; Penuela et al. 2002). The last authors exploited the existence of conserved motifs characteristic of each subclass to clone specifically non-TIR-NBS-LRR RGAs in soybean. In the grapevine genome, Donald et al. (2002) reported that analysis of the Kinase motifs predicted that 19 out of the 22 RGA clones, obtained using this PCR strategy, were of the non-TIR subfamily.

The present results showed that the *Pl5* and *Pl8* regions on linkage group 6 of the map of Gentzbittel et al. (1999) appear linked to the RGAs of the non-TIR-NBS-LRR subfamily (Ha-NTIR7H3, Ha-NTIR11H3, Ha-NTIR3E5, Ha-NTIR2H3 and Ha-NTIR3H3). In contrast, one marker (Ha-TIR13H3) of the TIR-NBS-LRR subfamily segregated independently of the other RGAs of the non-TIR-NBS-LRR subfamily, and *Pl5* and *Pl8*. These results and those of Bouzidi et al. (2002) indicate that in sunflower there are at least two regions controlling resistance to the same races of *P. halstedii* and that these regions may contain different types of NBS-LRR sequences. However, further RGAs of both classes should be cloned and mapped to test this hypothesis. If the *Pl1*/*Pl2*/*Pl6* cluster on linkage group 1 and the *Pl5*/*Pl8* cluster on linkage group 6 contain different classes of NBS-LRR RGAs, conferring resistance to the same races of *P. halstedii*, then the markers developed here and those of Gedil et al. (2001a) and Bouzidi et al. (2002) will be useful in marker-assisted selection to accumulate different *Pl* genes in the same sunflower variety, which may enhance the durability of field resistance.

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