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ORIGINAL PAPER

Candidate genes for quantitative resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.)

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Abstract Partial resistance to *Mycosphaerella pinodes* in pea is quantitatively inherited. Genomic regions involved in resistance (QTLs) have been previously identified in the pea genome, but the molecular basis of the resistance is still unknown. The objective of this study was to map resistance gene analogs (RGA) and defense-related (DR) genes in the JI296 × DP RIL population that has been used for mapping QTLs for resistance to *M. pinodes*, and identify co-localizations between candidate genes and QTLs. Using degenerate oligonucleotide primers designed on the conserved motifs P-loop and GLPL of cloned resistance genes, we isolated and cloned 16 NBS-LRR sequences, corresponding to five distinct classes of RGAs. Specific second-generation primers were designed for each class. RGAs from two classes were located on the linkage group (LG) VII. Another set of PCR-based markers was designed for four RGA sequences previously isolated in pea and 12 previously cloned DR gene sequences available in databases. Out of the 16 sequences studied, the two RGAs RGA-G3A and RGA2.97 were located on LG VII, *PsPRP4A* was located on LG II, *Peachi21, PsMnSOD, DRR230-b* and *PsDof1* were mapped on LG III and *peaßglu* and *DRR49a* were located on LG VI. Two co-localizations between candidate genes and QTLs for resistance to *M. pinodes* were observed on LG III, between the putative transcription factor *PsDof1* and the QTL *mpIII-1* and between the pea defensin *DRR230-b* gene and the QTL *mpIII-4.* Another co-localization was observed on LG VII between a cluster of RGAs and the QTL *mpVII-1*. The three co-localizations appear to be located in chromosomal regions containing other disease resistance or DR genes, suggesting an important role of these genomic regions in defense responses against pathogens in pea.

Keywords Ascochyta blight · Disease resistance · Resistance gene analogs (RGAs) · Defense-related genes · Molecular mapping · QTL

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Introduction

Ascochyta blight, caused by *Mycosphaerella pinodes* (Berk. and Blox.), is the most important foliar disease of field pea (*Pisum sativum* L.) worldwide. Breeding pea varieties with resistance to *M. pinodes* is difficult due to the availability of only partial levels of resistance (Ali-Khan et al. 1973; Ali et al. 1978; Bretag 1989, 1991; Xue et al. 1996; Kraft et al. 1998; Xue and Warkentin 2001; Prioul et al. 2003) inherited as a complex polygenic trait (Zlamal 1984; Wroth 1999). In the

last 3 years, QTL mapping studies have attempted to dissect the genetic basis of quantitative resistance to ascochyta blight in pea. Timmerman-Vaughan et al. (2002, 2004) identified numerous QTLs for resistance to field epidemics located on the seven pea linkage groups. Six QTLs common to two populations were identified on linkage groups II, III, IV, V and VII (Timmerman et al. 2004). However QTL specificity for pathogens of the ascochyta complex (*Mycosphaerella pinodes, Ascochyta pisi* and *Phoma medicaginis)* was not specified. Tar'an et al. (2003) reported three QTLs for resistance to *Mycosphaerella* blight under field conditions. In a previous study, we identified six and ten QTLs for resistance to *M. pinodes* at the seedling (controlled conditions) and at the adult plant stages (field conditions) respectively, four QTLs being common to both plant stages (Prioul et al. 2004). These QTL mapping studies have considerably increased our knowledge on the genetic architecture of pea partial resistance to *M. pinodes*, but the biological functions of the resistance factors underlying these QTLs still remain unknown. Such information on the biochemical mechanisms underlying resistance will be helpful to improve the efficiency of MAS based construction of resistant genotypes and provide a more efficient and durable resistance to *M. pinodes* in pea.

The candidate gene approach has been successfully used in plant genetics for QTL characterization, by testing associations among QTLs and genes potentially involved in the biochemical pathways leading to trait expression. Co-localizations between resistance QTLs and resistance genes (*R* genes), resistance-gene analogs (RGAs) or defense response genes (DR genes) were reported in a variety of plant species, leading to the hypothesis of a possible involvement of these genes in the effects of some resistance QTLs (for review, see Pflieger et al. 2001a).

In pea, *R* genes involved in resistance against fungi (Dirlewanger et al. 1994; Timmerman et al. 1994; Coyne et al. 2000), viruses (Gritton and Hagedorn 1980; Marx et al. 1985; Provvidenti and Hampton 1991; Timmerman et al. 1993; Dirlewanger et al. 1994), or bacteria (Hunter et al. 1998, 2001), as well as defense-related cloned sequences (Gilpin et al. 1997; Weeden et al. 1998, 1999) and NBS-LRR RGAs (Timmerman-Vaughan et al. 2000) were located on published pea maps. Up to now, only one major resistance gene has been cloned in pea, conferring resistance to a pathotype of PSbMV (Gao et al. 2004). Comparative mapping studies have also shown that three genomic regions containing RGAs also included QTLs for resistance to Ascochyta blight (Timmerman-Vaughan et al. 2002). Physiological and biochemical studies of the pea–*M. pinodes* interaction reported that *M. pinodes* elicitor (Shiraishi et al. 1978a, 1992; Matsubara and Kuroda 1987) induces many defense responses in pea, such as accumulation of pisatin, a major phytoalexin in pea (Shiraishi et al. 1978b), activation of the genes encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (for review, see Yamada et al. 1996), activation of PR proteins (β-1,3-glucanase, chitinases) (Yoshioka et al. 1992), generation of superoxide anion (Kiba et al. 1997), enhancement of ATPase activity (Kiba et al. 1995, 1996, 1997), activation of the polyphosphoinositide metabolism (Toyoda et al. 1992, 1993, 1998). However, the mechanisms by which *M. pinodes* elicitor recognition, activation of signal transduction pathways leading to defense responses and expression of the QTLs of partial resistance are connected are still not well understood.

The aim of the present study was to map candidate genes for resistance on the JI296 × DP genetic linkage map and compare their genomic localizations with QTLs for resistance to *M. pinodes* previously identified in the RIL population (Prioul et al. 2004). Candidate genes were (1) previously cloned pea DR genes chosen according to their potential role in the *M. pinodes*/pea interaction or in disease resistance mechanisms, (2) RGAs previously isolated in pea by Timmerman-Vaughan et al. (2000) and likely to map in similar genomic regions as QTLs identified in the JI296 × DP population, and (3) RGAs cloned in the present study. In this paper, we report the development and mapping of a set of PCR-based DR and RGA markers and discuss the genomic co-localizations between these candidate genes and QTLs for *M. pinodes* resistance in pea.

Materials and methods

Plant material

Two homozygous pea genotypes were used as sources of total genomic DNA for PCR amplification: DP and JI296 (i.e. cv. 'Chemin Long'), partially resistant and susceptible to *M. pinodes*, respectively (Prioul et al. 2003, 2004). Genomic DNA was extracted from dried young leaves using the CTAB method of Doyle and Doyle (1990).

A 135 $F_{2:6}$ recombinant inbred line (RIL) population derived from the cross JI296 × DP, previously described by Prioul et al. (2004), was used for mapping studies.

Isolation, cloning and sequencing of RGA sequences

Degenerate RGA-consensus primers were designed on the conserved motifs P-loop and GLPL (Meyers

et al. 1999). Primer pairs were designated (1) RGAino, with forward sequence 5′-GGI GGI GTI GGI AAI ACI AC-3′ (Leister et al. 1996) and reverse sequence 5′-IAG IGC IAG IGG IA(A/G) ICC-3′ (Fourmann et al. 2001), and (2) RGAdeg with forward sequence 5′-GGT GGG GTT GGG AA(A/G) AC(A/T/C/G) AC-3′ (Fourmann et al. 2001) and reverse sequence 5′-CAA CGC TAG TGG (A/T/C/G)A(A/G) (A/T/C/G)CC-3′ (Fourmann et al. 2001).

PCR amplifications were performed in 50 µl containing 1.5 mM MgCl2, 0.2 mM of each dNTP, 1.5 µM of each primer, 200 ng of genomic DNA and 1.5U of Taq polymerase (Eurobio). PCR conditions were 5 min at 94°C; 1 min at 94°C, 1 min at the annealing temperature (Table 2) and 2 min at 72°C for 30 cycles; and 5 min at 72°C.

PCR products were purified and concentrated on centrifugal Filter Devices Microcon (Amicon process), cloned into pGEM-T vector system (Promega, Madison, Wis.) and used to electroporate DH10B cells (Gibco BRL). Clones containing inserts were screened by PCR using universal M13 primers. A total of 707 putative recombinant colonies were obtained, including 291 and 363 clones isolated from DP and JI296, respectively, using RGAino primers (clones individually named as *IDx or IJx*, where x is the number of the clone), and 5 and 48 clones obtained from DP and JI296, respectively, using RGAdeg primers (clones individually named as *DDx or DJx*).

To select non-redundant clones before sequencing, aliquots containing 15 µl of the PCR product from each clone were digested with the restriction enzymes *HaeIII* (Appligene) and either *AluI* (Appligene) or *RsaI* (Gibco BRL), following the manufacturer instructions. The restriction fragments were separated on a 5% agarose gel (1X TAE buffer, 150 V, 3 h30 migration). A total of 130 non redundant clones were retained for sequencing. All sequencing reactions were performed by Genome Express laboratory (Montreuil, France).

PCR amplification of RGAs and DR candidate genes

Three classes of candidate genes were tested for mapping: RGAs cloned in the present study, 4 RGAs previously cloned in pea by Timmerman-Vaughan et al. (2000), and 12 pea DR genes chosen in the GenBank database. The putative biological functions of cloned DR genes and RGAs are detailed in Table 1. Sequence-specific primers were designed using the OLIGO 6.0 software (http://www.oligo.net/). Primer specifications for DR genes and RGAs are indicated in Tables 2 and 3, respectively.

Amplifications were performed in 25 µl using 50 ng of genomic DNA of each parental line DP or JI296, 1.5–2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 1 µM of each primer, 1U Taq polymerase (Eurobio). PCR conditions were 5 min at 94°C; 1 min at 94°C, 1 min at the annealing temperature (Tables 2 and 3) and 2 min at 72°C for 30 cycles; and 5 min at 72°C. PCR products were separated either on a 1.4% agarose gel (1X TAE buffer, 110 V, 1 h30 migration) or on a 5% non-denaturing acrylamide gel (1X TBE buffer, 220 V, 15–20 h migration). DNA from bands of interest was removed from the gel and reamplified according to Brunel et al. (1999) with slightly different conditions: 5 min at 94°C; 1 min at 94°C, 45 sec at the annealing temperature, and 1 min at 72°C for 25 cycles; and 5 min at 72°C. These products were then directly sequenced to control for specific amplification of the expected sequence.

Sequence analysis

RGA sequences amplified from DP and JI296 were analyzed using the GCG software package (version 10.2, Genetics Computer Group, Madison). RGA clones presenting less than five different bases were considered as the same initial sequence, as reported by Fourmann et al. (2001). RGA multiple sequence alignments were performed using the CLUSTALW option of the GCG Wisconsin package with default parameters.

For DR genes and RGAs isolated by Timmerman-Vaughan et al. (2000), sequences amplified from DP and JI296 were validated for their homology with the chosen candidate gene sequence by performing similarity searches against sequences deposited in the non-redundant sequence databases (NCBI), using BlastN and BlastX algorithms (Altschul et al. 1997). To identify useful polymorphism for further marker development, pairwise comparisons of sequences and editing of restriction maps were performed using the PILEUP and MAP options of the GCG package, respectively.

Genetic mapping

Specific candidate gene markers showing polymorphism between JI296 and DP were genotyped on the RIL population. For each segregating marker, a chi-square analysis was used to test for deviations from the expected (1:1) segregation ratio. The MAPMAKER/EXP software (version 3.1) (Lincoln et al. 1992) was used to map the candidate genes on the JI296 × DP genetic map previously described by Prioul et al. (2004), using the "try" command to assign molecular markers to linkage group (minimum LOD score of 2.0; maximum recombinant fraction of 0.30), and the

Table 1 Pea DR and RGA sequences previously published used for the candidate gene approach

Candidate	Genbank accession no.	Function	Sequence origin	Reference
DR genes				
PEAPAL1	D10002	Phenylalanine ammonia-lyase	DNA	Yamada et al. (1992)
PEAPAL2	D10003	Phenylalanine ammonia-lyase	DNA	Yamada et al. (1992)
Pschitin	X63899	Basic class1 A1-chitinase	mRNA	Vad et al. (1993)
Peachi21	L37876	Basic class I endo-chitinase (Chi2)	DNA	Chang et al. (1995)
Peaßglu	L02212	Beta-1,3 glucanase	DNA	Chang et al. (1993)
Hmm6	U69554	(+) 6a-hydroxymaackiain 3-*O*-methyltransferase	mRNA	Wu et al. (1997)
DRR49a	U31669	Disease resistance response protein PR10	DNA	Culley et al. (1995a)
DRR206-d	U11716	Disease resistance response protein 206-d (DRR206-d)	DNA	Culley et al. (1995b)
DRR230-b	L01579	Disease resistance response protein 39 (DRR230-b)	mRNA	Chiang and Hadwiger (1991)
PsDof1	AB026297	Elicitor-responsive Dof protein	mRNA	Seki et al. (2002)
PsPRP4A	AF137351	Pathogenesis-related protein 4a (PRP4A)	mRNA	Savenstrand et al. (2000)
PsMnSOD	U30841	Manganese superoxide dismutase precursor (MnSOD)	DNA	Wong-Vega et al. (1991)
RGAs				
RGA-G3A	AF123703	Putative NBS-LRR type disease resistance protein (RGA-G3A)	DNA	Timmerman-Vaughan et al. (2000)
RGA1.1	AF123695	Putative NBS-LRR type disease resistance protein (RGA1.1)	DNA	Timmerman-Vaughan et al. (2000)
RGA2.65	AF123699	Putative NBS-LRR type disease resistance protein (RGA2.65)	DNA	Timmerman-Vaughan et al. (2000)
RGA2.97	AF123701	Putative NBS-LRR type disease resistance protein (RGA2.97)	DNA	Timmerman-Vaughan et al. (2000)

"ripple" command to test final orders (LOD threshold of 2.0). Three additionnal molecular markers were added to the JI296 × DP genetic map increasing the number of common markers with other published pea genetic maps: *sL01* (Frew et al. 2002) was mapped on LG VI, *sP2P5* (Timmerman-Vaughan et al. 2002) on LG II, and *sY16* (Timmerman-Vaughan et al. 2002) on the distal part of LG Vb.

Statistical analyses

Statistical analyses for candidate gene-phenotype association were performed using the SAS version 6.12 package (SAS Institute, Cary, NC, USA) and the QTL CARTOGRAPHER Windows v1.30 software (Basten et al. 1994, 2001). Markers that co-localized with QTLs for ascochyta blight resistance were checked by one-way ANOVA, Kruskal–Wallis non-parametric test and composite interval mapping (CIM) analysis, as described in Prioul et al. (2004).

Results

RGA isolation

Out of the 130 sequenced clones, 64 showed high similarities with RGA sequences when compared with GenBank accessions (BlastX *e*-values varying from e^{-84} to e^{-46}). The remaining 66 clones were not analyzed further since they corresponded to PCR products that (1) were generated by one of the primer alone, suggesting the existence of two opposite complementary sequences on DNA allowing PCR amplification, (2) showed similarities with retrotransposon sequences, myosins or integrases, or (3) did not show any similarities with any sequences in the GenBank database. Out of the 64 retained RGA sequences, 61 were obtained with RGAino primer pair and 3 with RGAdeg primer pair. Five sequences were not analyzed further due to the poor quality of their electrophoregrams. Considering a likely Taq polymerase (Eurobio) error frequency of 1×10^{-4} in base incorporation, the remaining 59 clone sequences were divided into 16 groups, sequences within each group being considered as identical. For each parental line, a reference clone was chosen as the most probable sequence for each group. The 16 reference sequences were conserved for further analysis, corresponding to insert sizes varying from 451 bp (incomplete sequence) to 516 bp.

Figure 1 presents a multiple alignment and classification of the 16 RGA putative amino acid sequences. The conserved domains P-loop (upper primer), RNBS-A-TIR, RNBS-A-nonTIR, kinase 2, RNBS-B, RNBS-C and GLPL (lower primer) previously defined by Meyers et al. (1999) were found in the sequences.

Table 2 Primer specifications, optimized PCR conditions and generated polymorphism between DP and JI296 for DR genes

Candidate gene	Upper primer (5′→3′)[a]	Lower primer (5′→3′)[a]	Tm[b] (°C)	Fragment size (bp)	Polymorphism[c] DP/JI296
PEAPAL1	CTG GGT GAT GGA GAG TAT GAA	TTG CTG TGT GTG GTA GTG TAT G	63	300	100% amino acid identity
	TGG AAA CAG TAG CAG CAG CC[d]	TTG CTG TGT GTG GTA GTG TAT G	60	1000	Size
PEAPAL2	TAT CAT AGC CAC TCA ACA CCA A	CTG ACA GCT CCA CCT TCA CAC	50	640	100% amino acid identity
	GGT CCG TCT TGG TGG TGA	TTG CTG TGT GTG GTA GTG TAT G	60	450	100% amino acid identity
	GGT CCG TCT TGG TGG TGA	AAG AAA TTG GAA GAG GAG CA[d]	50	1950	*Dde, HaeIII*
Pschitin	CTT GGC TCA AAC TTC TCA	GGC TTG ATG GCT TGT TTG C	60	1000 (intron)	100% amino acid identity
Peachi21	CTT TCC CCA ACT TCG CCA ATA A	TAG TCG AGA ATG AAA TGG TCT GAG A	55	876	100% amino acid identity
	GTC TCA AAA TAT GAG CAA	AGC GTC GTA GGT GTA AAA	51	350	*TspRI*
Peaβglu	AAA CAA CCT ACC ACC AGC AAA	AAA CAA CAA CAT TCA CCC AAC C	55	686	*Tfi1*
Hmm6	GGA GAT ATG TTC AAG TCT GT	AGC AGT TCT TTC TAA TGT ACT C	53	624 (intron)	*MnlI*
DRR49a	ATT GTT GAA GGM AAC GGT GG	AGC CTT GAA AAG ACCATC MCC	58	350	*SspI*
DRR206-d	GCT TTA AGT TCA GCA ATT CC	AAA GAA CTT GAT ATA AAC ACC	58	470	100% amino acid identity
DRR230-b	ATG GAG AAG AAA TCA MTA GC	GTG CGC TTT GTT YYT RCA GTG	54	950 (intron)	*SspI, VspI*
PsDof1	AAG CCT CAG CCG GAA CAA GC	TCC ATT TCC AAG GAA TAA ACC	55	410	*TaqI*
PsPRP4A	ACA TAC AAT AAC TAC AAC CC	TCT CAC TGT TAC CTG AGC TCC	55	320	+/−
PsMnSOD	GCT CTC GAA CAG CTT CAC GAT	CTT TGG TGG TTC ACC ACC TCC	60	750	*Sse91*

[a] *M* A/C, *Y* C/T, *R* A/G

[b] Annealing temperature

[c] Polymorphism generated for mapping assay: restriction enzymes for CAPS markers

+/− Presence/absence

Size size polymorphism

[d] Primers defined by Weeden et al. (1999)

Fifteen sequences had the RNBS-A-TIR domain and were divided into four classes based on the presence of specific motifs in the RNBS-A-TIR domain and along the sequence: class I consisted of three sequences isolated from both parental lines, class II contained nine fragments isolated from both parental lines, class III was composed of two fragments isolated from JI296 and class IV was composed of the unique JI296 sequence *IJA3*. *DJ37* is the only sequence to possess the RNBS-A-nonTIR domain (class V). Except for class I, RGAino and RGAdeg primer pairs led to the identification of distinct RGA classes. No high similarities were found between pea RGA sequences and known *R*-genes, except with the tobacco *N* gene (genbank accession U15605) conferring resistance to the Tobacco Mosaic Virus or the tomato *I2* gene (genbank accession AF118127), conferring resistance to *Fusarium oxysporum*. Amino acid identities of 45%, 38%, 34% and 36% were found between the tobacco *N* gene and reference sequences of classes I, II, III and IV, respectively. Class V *DJ37* presented 44% amino acid identity with the tomato *I2* gene. Highest homologies (up to 97% of amino acid identity) were found between pea RGA sequences identified in this study and published sequences putatively involved in plant disease resistance, isolated from *Pisum sativum* (*RGA-G3A*, *RGA2.159*) and closely related legume species such as *Cicer arietinum* (*CP3*), *Cajanus cajun* (*PP10*) or *Medicago sativa* (*AF230841*). High amino acid identity percentages were found within the same pea RGA class but varied from 23.9% to 70.4% between classes (Table 4). The relationships within the 16 pea RGA

Table 3 Specific primer pairs defined on RGAs, PCR conditions and generated polymorphism between DP and JI296

RGA class	Primer pair	Upper primer (5′→3′)	Lower primer (5′→3′)	Tm[a] (°C)	Fragment size (bp)	Representative clones[b]	Polymorphism[c] DP/JI296
Second-generation primers							
I	RGA1a	TTG CCA AAG CCA TTT ATA	GCC CCC AGC ATA AGC AAT	54	479	***ID257***, *ID4, DJ18*	None
I	RGA1b	GGG AGC ATG TTT GAG GGA A	GCC CCC AGC ATA AGC AAT	54	453	***ID257***, *ID4, DJ18*	None
II	RGA2	GCT ACT GCT TTG TAT GCT A	TCT TTT GTC AAG TAT TAG TAA	54	449	***IJB174***, *ID144, ID181, ID268, ID68, ID193, ID280, ID261, IJB91*	*HpyF44III*
III	RGA3	AGA CGA CTC TTG CTA CTG	GAG AGG AAC TTG GTA AAC	51	374	***IJA27***, *IJB187*	+/−
IV	RGA4	GAA GAC GAC TCT TGC TAT	AAA AGT GGA ACT TGG TAA	46	375	***IJA3***	None
V	RGA5	TTG GAA ACA ACA CTT GCT GA	GTT TTC GTT GCC ACC ATA A	46	447	***DJ37***	None
Allele-specific primers							
II	IJB174	GAC GAC TCT TGC TAC TGC	ATG AAG TAG ATG AGG TAT	54	355	*IJB174*	+/−
II	IJB91	GAC GAC TCT TGC TAC TGC	TGA GGG AGA CGG ATC GGG	51	295	*IJB91*	+/−
Cloned Pea RGAs (Timmerman-Vaughan et al. 2000)							
	RGA-G3A	GTA TGC TAG AAT CTC TAA TCA	TGT CAA GTA TAT GTA ACC ACT C	53	433		*BpiI, HpyF44III, MaeI, BseNI*
	RGA1.1	ATT TGT GTA TCC GAG GAT TTC	CTG CCA ATT TCT TCT AGG TTT	54	419		*NlaIII*
	RGA2.65	TTG CGA AAG CCA TCT ACA AT	TGC CAA CTA AAT AGC TCA AT	54	403		size
	RGA2.97	TTA GGA ACG AGG GAG CAT AG	ATT CTT TAG CCA TTT CAC GC	48	445		*NlaIII, Tru9I*

[a] Annealing temperature

[b] Clones chosen as the consensus sequence are indicated in *bold*

[c] Polymorphism generated for mapping assay: restriction enzymes for CAPS markers

+/− Presence/absence

reference sequences identified in this study and Genbank accessions are illustrated in Fig. 1.

RGA mapping

Second-generation specific primer pairs designed for classes I, IV and V of isolated RGAs (primer pairs RGA1a, RGA1b, RGA4 and RGA5) amplified PCR products related to the corresponding groups, but no polymorphism useful for mapping could be found between DP and JI296. The primer pair RGA2 gave an electrophoresis profile with two bands on DP and JI296, both sharing high homology with the class II sequences. When comparing sequences obtained between the two parental lines, seven and two nucleic acid differences were found between the two lower bands and the two upper bands, respectively. A CAPS marker was developed (Table 3) and one of the RGA2 loci (corresponding to the lower band) was mapped on linkage group VII (Fig. 2). Two allele-specific primers, designed in regions of the class II *IJB174* and *IJB91* sequences, generated sequences showing polymorphism between the two parental genotypes (orphan bands in the JI296 parental line) and were mapped on linkage group VII, close to the RGA2 locus (Fig. 2). The primer pair RGA3 amplified a single band in DP and two bands in JI296. After having checked that all three PCR products were related to the class III, the polymorphic RGA3 locus was mapped on linkage group VII in the vicinity of the class II RGA *IJB174* and *IJB91* (Fig. 2). For each of the four RGA markers added to the genetic map on LGVII, a high segregation distortion ($P < 0.0001$) was observed favoring the DP allele (Fig. 2), as for most of the neighboring molecular markers in this genomic region.

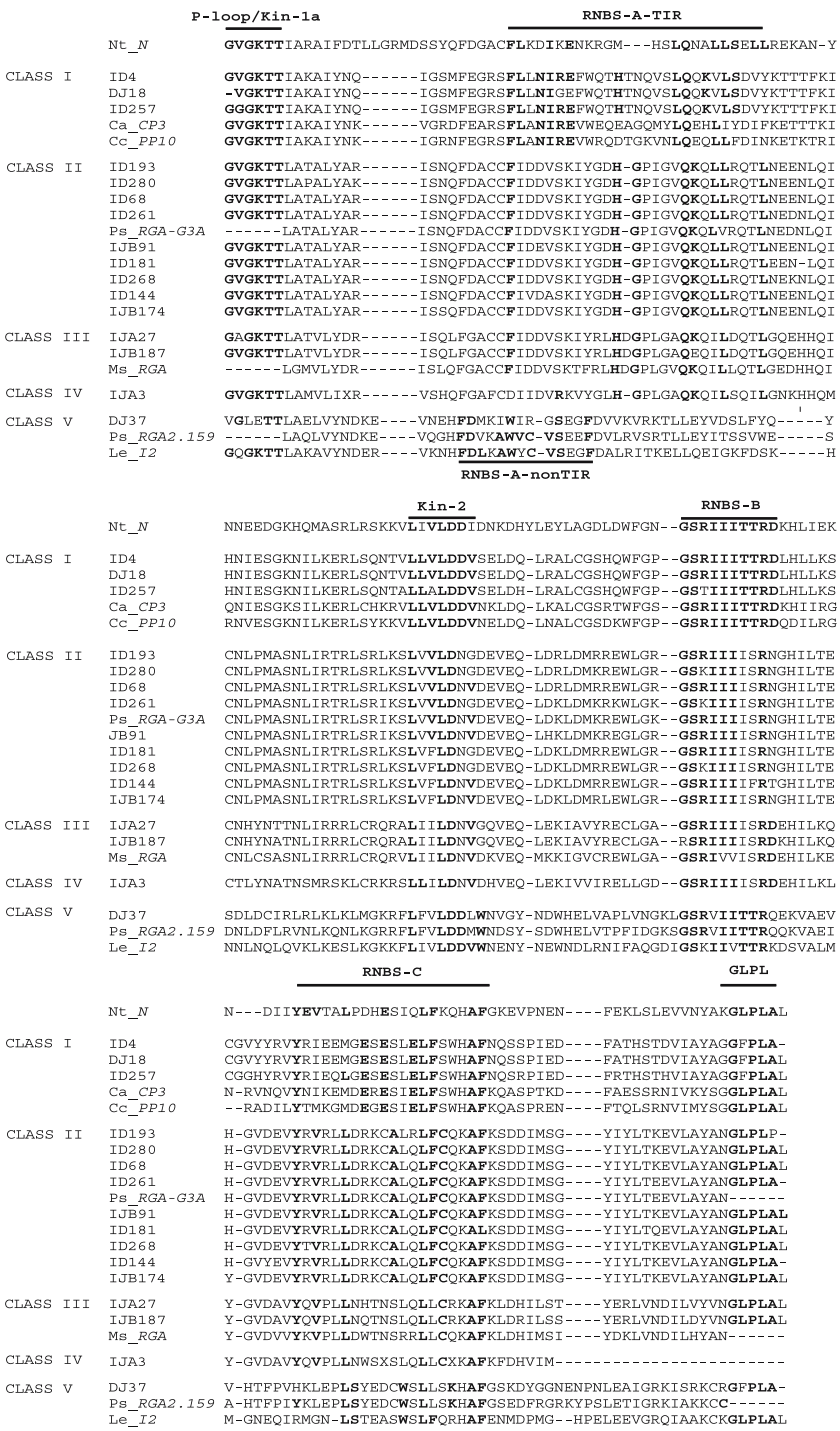

Fig. 1 Alignment of the predicted amino acid sequences of the five classes of RGA sequences isolated from pea. Sequences of *Nicotiana tabacum N* (U15605), *Lycopersicum esculentum I2* (AF118127), *Cajanus cajun PP10* (AF186633), *Cicer arietinum CP3* (AF186625), *Medicago sativa* (AF230841), *Pisum sativum RGA-G3A* (AF123703) and *RGA2.159* (AF123702) are included in this analysis. The conserved motifs defined by Meyers et al. (1999) are indicated in *bold*

The specific primers designed on the four RGAs previously isolated in pea by Timmerman-Vaughan et al. (2000) successfully amplified the candidate sequences and generated polymorphism between the two parental lines. *RGA-G3A* and *RGA2.97* were mapped on LG VII, in the vicinity of the four RGA markers developed in this study (Fig. 2). *RGA1.1* was assigned to LG III but its accurate location still remained unclear. *RGA2.65* was not mapped further due to difficulties in following the marker in the segregating population.

Genomic amplification and mapping of DR candidate genes

Out of the primer pairs defined for the 12 DR candidate genes, two (from *Pschitin* and *DRR206-d* sequences) successfully amplified target genomic

Table 4 Amino acid identities (%) between the five classes of pea RGA sequences

	Class I	Class II	Class III	Class IV	Class V
Class I	94.5				
Class II	33.6	95.3			
Class III	29.9	60.2	94.7		
Class IV	29.4	55.2	70.4	–	
Class V	25.3	29.8	31.0	23.9	–

sequences but did not reveal any polymorphism between parental lines (Table 2). These primer pairs were not used further. For the ten other DR genes, polymorphism between DP and JI296 was revealed and, except for *PEAPAL1* and *PsPRP4A*, was generated by digestion with restriction enzymes. The CAPS markers are listed in Table 2. PCR amplifications from genomic DNA revealed the presence of introns for *Pschitin*, *Hmm6* and *DRR230-b* sequences.

Out of the 12 candidate genes tested in the present study, 8 were mapped on the JI296 × DP genetic map (Fig. 2). *PsPRP4A* was located on LG II; *Peachi21, PsMnSOD, DRR230-b* and *PsDof1* were mapped at different positions on LG III; *peaβglu* and *DRR49a* were mapped in a same genomic region on LG VI, and *Hmm6* was located on LG VII in the vicinity of the *RGA2.97* locus. The genes *Hmm6, PsMnSOD* and *PsDof1* showed significant deviation from the expected 1:1 ratio (Fig. 2). *PEAPAL1 and PEAPAL2* were not mapped due to difficulties in following these markers in the segregating population.

Genomic co-localizations between candidate genes and QTLs for resistance to *M. pinodes*

CIM analysis (data not shown), one-way ANOVA ($P \leq 5 \times 10^{-3}$) and the Kruskall–Wallis non-parametric test ($P \leq 5 \times 10^{-3}$), performed on different ascochyta blight resistance scoring criteria previously described in Prioul et al (2004), confirmed the co-segregations between *PsDof1* and the QTL *mpIII-1*, *DRR230-b* and the QTL *mpIII-4*, and between *IJB91*, *IJB174*, *RGA2* and *RGA-G3A* and the QTL *mpVII-1* (Table 5, Fig. 2).

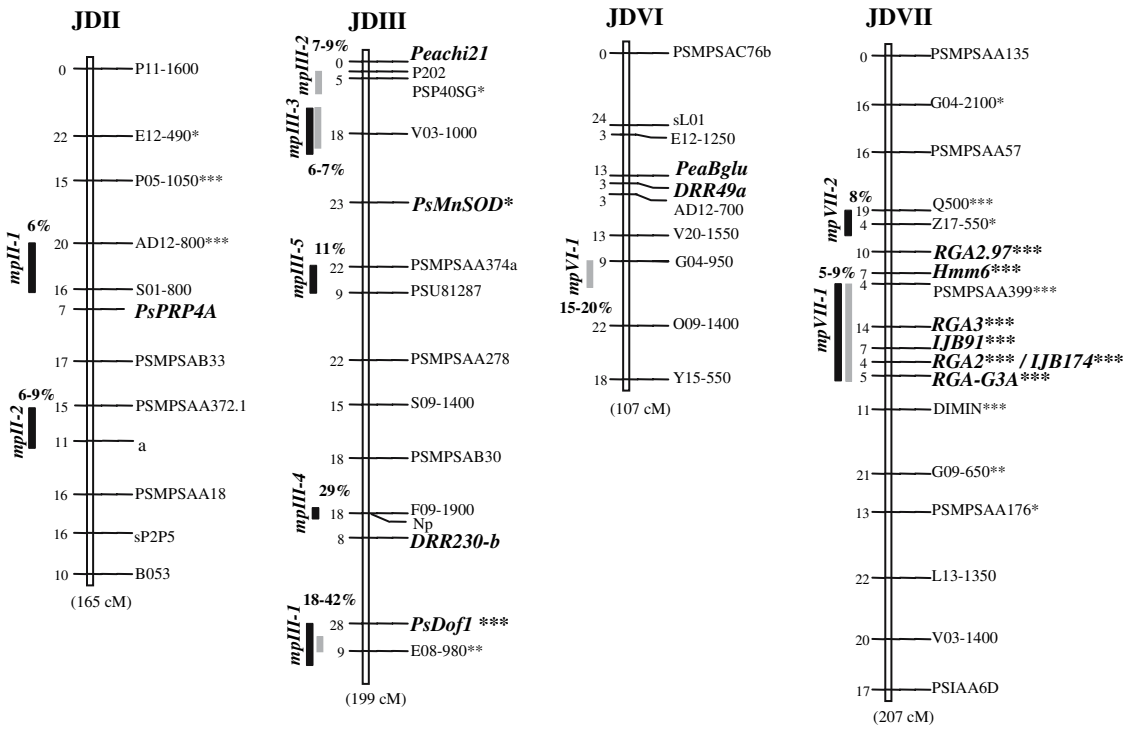

Fig. 2 Localization of 14 candidate gene markers on the JI296 × DP genetic map, including 8 DR gene markers and 6 RGAs (4 from this study and 2 previously isolated by Timmerman-Vaughan et al. 2000). Candidate genes are displayed in bold italic. The size of each linkage group is indicated in Kosambi centiMorgans (cM) below each group. Markers with segregation distortion at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ are indicated by *, ** and ***, respectively. QTLs previously detected in the JI296 × DP RIL population (Prioul et al. 2004) are indicated by *vertical bars* to the left of each linkage group. *Shaded bars* represent QTLs identified at the seedling stage, under controlled conditions. *Black bars* represent QTLs identified at the adult stage, under field conditions. The names of the QTLs are reported in adjacent position. The percent number above each QTL is the range of percentage of phenotypic variation (R^2) individually explained by the QTL, depending on different resistance criteria used in Prioul et al. (2004). The length of the bar represents a 1-LOD support interval from the peak LOD of the QTL

Table 5 Significant associations ($P \leq 5.10^{-3}$) between candidate genes and the different disease scoring criteria used in the mapping QTL study for resistance to *M. pinodes* in the JI296 × DP RIL population, under both controlled and field conditions (Prioul et al. 2004). Probabilities were obtained using one-way ANOVA/Kruskal–Wallis non-parametric test

Candidate gene	Linkage group	Controlled conditions		Field conditions			
		AUDPC stipules	AUDPC stems	AUDPC stipules	AUDPC stems	%HS4	%HT3
DRR230-b	III	ns/ns	ns/ns	ns/ns	<0.0001/<0.0001	ns/ns	ns/ns
PsDof1	III	<0.0001/<0.0001	<0.0001/<0.0001	<0.0001/<0.0001	0.0003/0.0004	<0.0001/<0.0001	<0.0001/<0.0001
IJB91	VII	0.0049/ns	ns/ns	ns/ns	ns/ns	ns/ns	ns/ns
IJB174	VII	0.001/0.0016	ns/ns	ns/ns	ns/ns	ns/ns	ns/ns
RGA2	VII	0.0002/0.0002	0.0012/0.0025	ns/ns	ns/ns	ns/ns	ns/ns
RGA-G3A	VII	<0.0001/<0.0001	0.0008/0.0029	ns/ns	ns/ns	ns/ns	ns/ns

ns Not significant, *AUDPC* Area under the disease progress curve, *%HS4* proportion of stipule height injured ratios until the score 4, *%HT3* proportion of stem height injured ratios until the score 3

Discussion

Using a PCR-based candidate gene approach, we isolated and mapped several RGAs and DR genes on the JI296 × DP genetic map, some of them occurring in genomic regions containing QTLs for resistance to *M. pinodes* on LGs III and VII.

Development and mapping of candidate RGA and DR gene markers

RGAs isolated in this work, using degenerate oligonucleotide primers based on NBS-LRR type of cloned *R* genes, fell into 5 distinct classes, with classes I, II and IV corresponding to new RGA types in comparison with the 9 pea RGAs previously identified by Timmerman-Vaughan et al (2000). Discrepancies between the two studies may be due either to the use of different pea genotypes or the possibility that some RGA sequences may be absent in some lines (Collins et al. 1998), or to the set of degenerate primers used and the fact that slight differences in primer sequences and/or primer combinations could lead to the amplification of different RGA fragments (Leister et al. 1996; Aarts et al. 1998). The close relatedeness of these RGAs with RGAs isolated from other closely related legumes corroborates the existence of legume specific families of NBS-LRR *R* genes (Meyers et al. 1999; Zhu et al. 2002). As previously reported in legumes (Yu et al. 1996; Zhu et al. 2002; Bertioli et al. 2003), an unbalanced ratio of TIR-NBS LRR and non-TIR NBS LRR sequences (15/1) was also observed in this study. Such a ratio may reflect either a bias during the PCR towards TIR sequences unrevealing differences in the P-loop and GLPL motifs between TIR and non-TIR NBS sequences, as hypothesized for the *Arachis* genome (Bertioli et al. 2003) and/or the presence of a smaller number of nonTIR-NBS than TIR sequences in the pea genome. Using a non-TIR-NBS specific primer should allow to test between both hypothesis. There are potentially much more RGAs to detect in pea. Indeed, 147 NBS-LRR RGAs have been identified in the model legume *M. truncatula*, mostly organized in clusters on the genome, several of these clusters being syntenic between *M. truncatula* and pea (Zhu et al. 2002). Although more RGAs should be mapped on the pea genome, our results and the ones of Timmerman-Vaughan et al. (2000) suggest the existence of a RGA cluster on LG VII including 5 RGAs distributed over 41 cM (Fig. 2). By comparative mapping with *M. truncatula*, this cluster appears to be syntenic to an extended cluster of at least 30 TIR NBS-LRR RGAs spanning the majority of the LG VI in *M. truncatula* (Zhu et al. 2002; NSF Plant Genome Project 2002). Syntenic blocks of *R* gene loci were also previously identified between LG I of pea and LG V of *M. truncatula* (Zhu et al. 2002). Consequently, NBS-LRR RGAs isolated and mapped in *M. truncatula* could be useful tools for identification of more RGAs in pea.

In the present study, we also designed PCR-based markers for 4 NBS-LRR sequences previously isolated in pea. The RGAs *RGA-G3A* and *RGA2.97* were unambiguously mapped on the JI296 × DP genetic map, occuring at similar genomic locations as in the JI1794 x Slow population (Timmerman-Vaughan et al. 2000). Using the PCR-based approach on pea DR sequences available in public databases, we successfully mapped 8 DR genes on the JI296 × DP genetic map. Three of these genes were not mapped previously on published molecular linkage maps, namely *PsDof1*, *Hmm6* and *PsPRP4A*. Our mapping results are in agreement with previously reported map locations found for *DRR49a* (corresponding to *pi49*), *Peaβglu* (corresponding to *β-1,3-glucanase)* and *PsMnSOD* (corresponding to *Sodmt*) (Gilpin et al. 1997; Weeden et al. 1998, 1999), but also clarify the locations of *Peachi21* and *DRR230*. Indeed, we confirmed the linkage between *Peachi21* and the *P202* marker (a 5 cM

interval; data not shown), as previously described in Gilpin et al. (1997), and located *Peachi21* on LG III. We also unambiguously assigned *DRR230-b* (corresponding to *pi39*) to LG III, whereas it was previously located on LG III/IV by Gilpin et al. (1997). Because of the absence of polymorphism between the parental lines or difficulties to follow the markers in the segregating population, *Pschitin*, *PEAPAL1* and *PEAPAL2* were not mapped on the JI296 × DP map, whereas these genes were previously mapped on other linkage maps (Gilpin et al. 1997; Weeden et al. 1999).

Except for *PsMnSOD, PEAPAL1 and PEAPAL2* for which PCR-based STS markers have been developped by Weeden et al. (1999), remaining DR genes and the 4 RGAs previously reported on linkage maps were located by other groups using RFLP probes. Based on PCR, our markers require a lower technical complexity for sample preparation and marker detection than RFLP, are less time-consuming, and can easily be transfered to any laboratory. The major problems we found with RGA and DR marker development were either the low rate of mutations in the coding sequence of the genes (i.e., *PEAPAL1*, *PEAPAL2*), thus limiting the usefulness of the CAPS strategy, or the inability to exploit mutations by digestion with restriction enzymes or allele-specific design with the 3′ end of the primer located on the mutation (Ugozzoli and Wallace 1991). The current development of SNP genotyping technologies should provide us new tools to take better advantage of these mutations and develop new types of markers.

Co-localizations between candidate genes and QTL for resistance to *M. pinodes*

A first co-localization was identified on LG III between the *PsDof1* gene, encoding a putative transcription factor that may bind to DNA through a Dof (DNA-binding with one finger) domain (Seki et al. 2002), and the QTL *mpIII-1*, a major QTL explaining 18–42% of the total phenotypic variation at different growth-stages and in various environmental conditions (Prioul et al. 2004). As *PsDof1* was initially isolated from a cDNA library constructed from *M. pinodes* elicitor-treated pea epicotyls (Seki et al. 2002), it has been suggested to be involved in the elicitor-induced activation of elicitor-responsive genes (Seki et al. 2003). Thus, *PsDof1* represents a good candidate for the QTL *mpIII-1*.

A second genomic co-localization was observed on LG III between *DRR230-b*, a member of the pea defensin gene family, and the QTL *mpIII-4* which explained 29% of the stem resistance in the field (Prioul et al. 2004). *DRR230-b* cDNA was first isolated from pea pods in response to infection by the fungal pathogen *Fusarium solani* (Chiang and Hadwiger 1991). More recently, Lai et al. (2002) reported the induction of two related defensin genes (*DRR230-a* and *DRR230-c*) in response to different fungal pathogens, including *Ascochyta pinodes* (teleomorph *Mycosphaerella pinodes*), and bacterial pathogens (compatible, incompatible and non-host strains). It has been hypothesized that pea defensins may play a role in the general plant defense responses, as suggested by their accumulation in response to wounding, ozone exposure and pathogen infection (Sävenstrand et al. 2000; Lai et al. 2002), and would act as general inducible determinants of disease resistance (Lai et al. 2002). Although further QTL analysis studies across environments should be performed to confirm particularly the involvement of the QTL *mpIII-4* in pea partial resistance to *M. pinodes* (Prioul et al. 2004), *DRR230-b* can be considered as a putative candidate for this QTL.

The third genomic co-localization was found on linkage group VII between a cluster of RGA sequences (*RGA2*, *RGA3*, *IJB174*, *IJB91*, *RGA-G3A*) and *mpVII-1*, a minor-effect QTL identified from seedling and adult stage condition scorings (Prioul et al. 2004). In their previous study, Timmerman-Vaughan et al. (2002) also reported a co-localization on LG VII between *RGA-G3A* and a QTL for resistance to field epidemics of ascochyta blight. In our work, statistical analyses showed a specific association between the 4 RGAs belonging to class II (i.e., *RGA-G3A*, *RGA2, IJB174* and *IJB91)* and the *M. pinodes* disease severity AUDPC scored at the seedling stage (Table 5), suggesting a stage-specific expression of these genetic factors. In our previous QTL mapping study, we used two distinct methodologies to assess partial resistance to *M. pinodes*: under controlled conditions, we focused on seedling resistance to infection only, scoring disease severity progress during 20 days after inoculation with *M. pinodes;* whereas under field conditions, we focused on the overall response of the adult plant to *M. pinodes* infection, including both resistance to infection and resistance to fungus progress upward on the plant. As the QTL *mpVII-1* was detected in both conditions and given the global assessment methodology used in field trials, we can hypothesize an involvement of these genetic factors in the very early steps of adult-plant resistance. Although further experiments are still needed to establish a functional link between these RGAs and the expression of resistance, the four mapped RGAs could be considered as valuable candidate genes for the QTL *mpVII-1*.

When comparing our results with previous reports about the mapping of disease resistance genes in pea,

we identified two chromosomal regions of the pea genome that might possess a hot spot of genes with a putative role in pea disease resistance. The distal part of linkage group III, where we located two QTLs for resistance to *M. pinodes* (Prioul et al. 2004) and the DR genes *DRR230-b* and *PsDof1*, was also previously reported to carry the *Rmp4* gene, involved in stem resistance to *M. pinodes* in pea seedlings and linked to *Np* (Clulow et al. 1991), the *Fw* gene, conferring resistance to *Fusarium oxysporum* f. sp. *pisi* race 1 (Dirlewanger et al. 1994; Weeden et al. 1998), the QTL *Asc3.1* for resistance to Ascochyta blight (Timmerman-Vaughan et al. 2002) and two RGAs, *RGA1.1* and *RGA2.65* (Timmerman-Vaughan et al. 2000). The chromosomal region of LG VII spanning from *Q500* to *DIMIN* (covering ~ 45 cM) carries QTLs for resistance to Ascochyta blight (Timmerman-Vaughan et al. 2002; Prioul et al. 2004), the *Hmm6* gene (this study), encoding an enzyme involved in the terminal step for synthesis of pisatin and isolated in pea tissues after infection with the bacteria *Nectria haematococca* (Wu et al. 1997), RGAs (this work; Timmerman-Vaughan et al. 2002), the *Ppi2* gene for resistance to *Pseudomonas syringae* pv. *pisi* (Hunter et al. 2001). Probably the further use in pea mapping of bridge markers such as microsatellites allowing to align genetic maps from different crosses (Loridon et al. 2005) will help in confirming map to map co-localisations between candidate genes and identified resistance genes or QTLs to various pathogens.

The DR genes assayed in the present study only represent a small subset of the genes potentially involved the *M. pinodes*/pea interaction. Moreover, only a small number of RGAs has been mapped on the JI296 × DP map, and we cannot exclude the hypothesis that *R* genes that do not encode NBS-LRR genes may also be involved in resistance to *M. pinodes*. We also know that the methodology used did not enable us to test and check all the alleles of a gene family. Further analyses based on the choice of additional candidate genes and the use of complementary methodologies, including methods aiming at evaluating gene transcription after inoculation with *M. pinodes* (i.e., SSH, micro-arrays...), are under investigation and should allow to identify new candidate genes for resistance QTLs.

Conclusion

RGA and DR markers developed in this study (1) successfully amplify potentially functional genes, (2) as far as RGAs are concerned, may be closely linked to disease *R* genes or QTL for resistance to various pathogens, and (3) are PCR-based, and can therefore be easily transferred to any laboratory and are of good value for a further use in marker-assisted selection (MAS). Moreover, all the candidate gene markers developed in the present study could be potentially useful in identifying the molecular factors underlying resistance to other pea diseases.

Using the same RIL population for both the candidate gene approach and the QTL mapping study, we reported three co-localizations between candidate genes and QTLs for resistance to *M. pinodes*, which suggest that quantitative resistance to *M. pinodes* could be explained by an association of genes acting at different levels in the *M. pinodes*/pea interaction. Our results corroborate previous findings in other plant species where the molecular basis for quantitative resistance were reported to be involved in recognition (Kanazin et al. 1996; Pflieger et al. 1999), transcriptional regulation (Trognitz et al. 2002) or plant defense (Faris et al. 1999; Geffroy et al. 2000; Pflieger et al. 2001b, Trognitz et al. 2002). Nevertheless, further experiments are required to (1) confirm the three co-localizations between the candidate genes and the resistance QTLs, (2) discard co-segregations that may have occured by chance only (Pflieger et al. 2001a), (3) validate the implication of the candidate gene(s) in the phenotypic variation.

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References

Aarts MGM, Lintel Hekkert BT, Holub EB, Beynon JL, Stiekema WJ, Pereira A (1998) Identification of R-gene homologous DNA fragments genetically linked to disease resistance loci in *Arabidopsis thaliana*. Mol Plant Microbe Interact 11:215–258

Ali SM, Nitschke LF, Dubé AJ, Krause MR, Cameron B (1978) Selection of pea lines for resistance to pathotypes of *Ascochyta pinodes*, *A. pisi* and *Phoma medicaginis* var. *pinodella*. Aust J Agric Res 29:841–849

Ali-Khan ST, Zimmer RC, Kenaschuk EO (1973) Reaction of pea introductions to ascochyta foot rot and powdery mildew. Can Plant Dis Surv 53:155–156

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Basten CJ, Weir BS, Zeng ZB (1994) ZMAP-a QTL cartographer. In: Proceedings of the 5th World congress on genetics applied to livestock production. Guelph, Ontario, pp 65–66

Basten CJ, Weir BS, Zeng ZB (2001) QTL CARTOGRAPHER, version 1.15. Department of Statistics, North Carolina State University, Raleigh, N.C

Bertioli DJ, Leal-Bertioli SCM, Lion MB, Santos VL, Pappas Jr G, Cannon SB, Guimarães PM (2003) A large scale analysis of resistance gene homologues in *Arachis*. Mol Genet Genomics 270:34–45

Bretag TW (1989) Resistance of pea cultivars to ascochyta blight caused by *Mycosphaerella pinodes*, *Phoma medicaginis* and *Ascochyta pisi*. Ann Appl Biol 114(Suppl):156–157

Bretag TW (1991) Epidemiology and control of ascochyta blight of field peas. PHD thesis, La Trobe University. Victoria, Australia

Brunel D, Froger N, Pelletier G (1999) Development of amplified consensus genetic markers (ACGM) in *Brassica napus* from *Arabidopsis thaliana* sequences of known biological function. Genome 42:387–402

Chang MM, Culley DE, Hadwiger LA (1993) Nucleotide sequence of a pea (*Pisum sativum* L.) β-1,3-glucanase gene. Plant Physiol 101:1121–1122

Chang MM, Horovitz D, Culley D, Hadwiger LA (1995) Molecular cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. Plant Mol Biol 28:105–111

Chiang CC, Hadwiger LA (1991) The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. Mol Plant Microbe Interact 4:324–331

Clulow SA, Matthews P, Lewis BG (1991) Genetical analysis of resistance to *Mycosphaerella pinodes* in pea seedlings. Euphytica 58:183–189

Collins NC, Webb CA, Seah S, Ellis JG, Hulbert SH, Pryor A (1998) The isolation and mapping of disease resistance gene analogs. Mol Plant Microbe Interact 10:968–978

Coyne CJ, Inglis DA, Whitehead SJ, McClendon MT, Muehlbauer FJ (2000) Chromosomal location of *Fwf*, the Fusarium wilt race 5 resistance gene in *Pisum sativum*. Pisum Genet 32:20–22

Culley DE, Brown S, Parsons MA, Hadwiger LA, Fristensky B (1995a) Cloning and sequencing of disease-resistance response gene DRR49a (Ypr10.PS.1; pI49) from *Pisum sativum* (Accession No. U31669) (PGR95–070). Plant Physiol 109:722

Culley DE, Horovitz D, Hadwiger LA (1995b) Molecular characterization of disease-resistance response gene DRR206-d from *Pisum sativum* (L.). Plant Physiol 107:301–302

Dirlewanger E, Isaac PG, Ranade S, Belajouza M, Cousin R, de Vienne D (1994) Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theor Appl Genet 88:17–27

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98:219–225

Fourmann M, Charlot F, Froger N, Delourme R, Brunel D (2001) Expression, mapping, and genetic variability of *Brassica napus* disease resistance gene analogues. Genome 44:1083–1099

Frew TJ, Russell AC, Timmerman-Vaughan GM (2002) Sequence tagged site markers linked to the *sbm1* gene for resistance to pea seedborne mosaic virus in pea. Plant Breed 121:512–516

Gao Z, Johansen E, Eyers S, Thomas CL, Ellis THN, Maule AJ (2004) The potyvirus recessive resistance gene, *sbm1*, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. Plant J 40:376–385

Geffroy V, Sévignac M, De Oliveira JCF, Fouilloux G, Skroch P, Thoquet P, Gepts P, Langin T, Dron M (2000) Inheritance of partial resistance against *Colletotrichum lindemuthianum* in *Phaseolus vulgaris* and co-localization of quantitative trait loci with genes involved in specific resistance. Mol Plant Microbe Interact 3:287–296

Gilpin BJ, McCallum JA, Frew TJ, Timmerman-Vaughan GM (1997) A linkage map of pea (*Pisum sativum* L.) genome containing cloned sequences of known function and expressed sequence tags (ESTs). Theor Appl Genet 95:1289–1299

Gritton ET, Hagedorn DJ (1980) Linkage of the *En* and *st* genes in peas. Pisum Newsl 12:26–27

Hunter PJ, Ellis N, Taylor JD (1998) Mapping genes for resistance to *Pseudomonas syringae* pv. *pisi* in *Pisum sativum*. http://www.bspp.org.uk/icpp98/3.4/29.html

Hunter PJ, Ellis N, Taylor JD (2001) Association of dominant loci for resistance to *Pseudomonas syringae* pv. *pisi* with linkage groups II, VI and VII of *Pisum sativum*. Theor Appl Genet 103:129–135

Kanazin V, Frederick Marek L, Shoemaker RC (1996) Resistance gene analogs are conserved and clustered in soybean. Proc Natl Acad Sci USA 93:11746–11750

Kiba A, Toyoda K, Yamada T, Ichinose Y, Shiraishi T (1995) Specific inhibition of cell wall-bound ATPase by fungal suppressor from *Mycosphaerella pinodes*. Plant Cell Physiol 36:809–817

Kiba A, Toyoda K, Ichinose Y, Yamada T, Shiraishi T (1996) Specific response of partially purified cell wall-bound ATPase to fungal suppressor. Plant Cell Physiol 37:207–214

Kiba A, Miyake C, Toyoda K, Ichinose Y, Yamada T, Shiraishi T (1997) Superoxide generation in extracts from isolated plant cell walls is regulated by fungal signal molecules. Phytopathology 87:846–852

Kraft JM, Dunne B, Goulden D, Armstrong S (1998) A search for resistance in peas to *Mycosphaerella pinodes*. Plant Dis 82:251–253

Lai FM, DeLong C, Mei K, Wignes T, Fobert PR (2002) Analysis of the DRR230 family of pea defensins: gene expression pattern and evidence of broad host-range antifungal activity. Plant Sci 163:855–864

Leister D, Ballvora A, Salamini F, Gebhardt C (1996) A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. Nature Genet 14:421–429

Lincoln M, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP ver. 3.0. Technical report, 3rd edn. Whitehead Institute, Cambridge, Mass

Loridon K, McPhee K, Morin J, Dubreuil P, Pilet-Nayel ML, Aubert G, Rameau C, Baranger A, Coyne C, Lejeune-Hènaut I, Burstin J (2005) Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theor Appl Genet 111(6):1022–1031

Marx GA, Weeden NF, Provvidenti R (1985) Linkage relationships among markers in chromosome 3 and *En*, a gene conferring virus resistance. Pisum Newsl 17:57–60

Matsubara M, Kuroda H (1987) The structure and physiological activity of a glycoprotein secreted from conidia of *Mycosphaerella pinodes*. II. Chem Pharm Bull (Tokyo) 35:249–255

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

NSF Plant Genome Project (2002) Towards the complete gene inventory and function of the *Medicago truncatula* genome. In: National Science Foundation Plant Genome Program

Report for DBI-0110203. http://www.medicago.ucdavis.edu/Medicago/pdffile/2002MtNSF_Report.pdf
Pflieger S, Lefebvre V, Caranta C, Blattes A, Goffinet B, Palloix A (1999) Disease resistance gene analogs as candidates for QTLs involved in pepper/pathogen interactions. Genome 42:1100–1110
Pflieger S, Lefebvre V, Causse M (2001a) The candidate gene approach in plant genetics: a review. Mol Breed 7:275–291
Pflieger S, Palloix A, Caranta C, Blattes A, Lefebvre V (2001b) Defense response genes co-localize with quantitative disease resistance loci in pepper. Theor Appl Genet 103:920–929
Prioul S, Onfroy C, Tivoli B, Baranger A (2003) Controlled environment assessment of partial resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.) seedlings. Euphytica 131:121–130
Prioul S, Frankewitz A, Deniot G, Morin G, Baranger A (2004) Mapping of quantitative trait loci for partial resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.), at the seedling and adult plant stages. Theor Appl Genet 108:1322–1334
Provvidenti R, Hampton RO (1991) Chromosomal distribution of genes for resistance to seven potyviruses in *Pisum sativum*. Pisum Genet 23:26–28
Sävenstrand H, Brosché M, Ängehagen M, Strid A (2000) Molecular markers for ozone stress isolated by suppression substractive hybridization: specificity of gene expression and identification of a novel stress-regulated gene. Plant Cell Environ 23:689–700
Seki H, Nakamura N, Marutani M, Okabe T, Sanematsu S, Inagaki Y, Toyoda K, Shiraishi T, Yamada T, Ichinose Y (2002) Molecular cloning of cDNA for a novel pea Dof protein, PsDof1, and its DNA-binding activity to the promoter of *PsDof1* gene. Plant Biotechnol 19:251–260
Seki H, Marutani M, Inagaki Y, Yoyoda K, Shiraishi T, Ichinose Y (2003) Possible involvement of AAAG motif and PsDof1 in elicitor-induced gene expression in pea. Sci Fac Agr Okayama Univ 92:21–26 (http://www.lib.okayama-u.ac.jp/www/aggaku/pdf/92_021_026.pdf)
Shiraishi T, Oku H, Yamashita M, Ouchi S (1978a) Elicitor and suppressor of pisatin induction in spore germination fluid of pea pathogen, *Mycosphaerella pinodes*. Ann Phytopathol Soc Jpn 44:659–665
Shiraishi T, Oku H, Tsuji Y, Ouchi S (1978b) Inhibitory effect of pisatin on infection process of *Mycosphaerella pinodes* on pea. Ann Phytopathol Soc Jpn 44:641–645
Shiraishi T, Saitoh K, Mo Kim H, Kato T, Tahara M, Oku H, Yamada T, Ichinose Y (1992) Two suppressors, supprescins A and B, secreted by a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol 33:663–667
Tar'an B, Warkentin T, Somers DJ, Miranda D, Vandenberg A, Blade S, Woods S, Bing D, DeKoeyer D, Penner G (2003) Quantitative trait loci for lodging resistance, plant height and partial resistance to Mycosphaerella blight in field pea (*Pisum sativum* L.). Theor Appl Genet 107:1482–1491
Timmerman GM, Frew TJ, Miller AL, Weeden NF, Jermyn WA (1993) Linkage mapping of *sbm-1*, a gene conferring resistance to pea seed-borne mosaic virus, using molecular markers in *Pisum sativum*. Theor Appl Genet 85:609–615
Timmerman GM, Frew TJ, Weeden NF, Miller AL, Goulden DS (1994) Linkage analysis of *er-1*, a recessive *Pisum sativum* gene for resistance to powdery mildew fungus (*Erysiphe pisi* D.C.). Theor Appl Genet 88:1050–1055
Timmerman-Vaughan GM, Frew TJ, Weeden NF (2000) Characterization and linkage mapping of R-gene analogous DNA sequences in pea (*Pisum sativum* L.). Theor Appl Genet 101:241–247
Timmerman-Vaughan GM, Frew TJ, Russel AC, Khan T, Butler R, Gilpin M, Murray S, Falloon K (2002) QTL mapping of partial resistance to field epidemics of Ascochyta blight of pea. Crop Sci 42:2100–2111
Timmerman-Vaughan GM, Frew TJ, Butler R, Murray S, Gilpin M, Falloon K, Johnston P, Lakeman MB, Russell A, Khan T (2004) Validation of quantitative trait loci for Ascochyta blight resistance in pea (*Pisum sativum* L.), using populations from two crosses. Theor Appl Genet 109:1620–1631
Toyoda K, Shiraishi T, Yoshioka H, Yamada T, Ichinose Y, Oku H (1992) Regulation of polyphosphoinositide metabolism in pea plasma membranes by elicitor and suppressor from a pea pathogen, *Mycosphaerella pinodes*. Plant Cell Physiol 33:445–452
Toyoda K, Shiraishi T, Yamada T, Ichinose Y, Oku H (1993) Rapid changes in polyphosphoinositide metabolism in pea in response to fungal signals. Plant Cell Physiol 34:729–735
Toyoda K, Koyama M, Mizukoshi R, Ichinose Y, Yamada T, Shiraishi T (1998) Phosphorylation of phosphatidylinositols and production of lysophospholipid in pea plasma membrane are coordinately regulated by elicitor and suppressor from *Mycosphaerella pinodes*. Sci Rep Fac Agr Okayama Univ 87:109–116
Trognitz F, Manosalva P, Gysin R, Niño-Liu D, Simon R, del Rosario Herrera M, Trognitz B, Ghislain M, Nelson R (2002) Plant defense genes associated with quantitative resistance to potato late blight in *Solanum phureja* x Dihaploid *S. tuberosum* hybrids. Mol Plant Microbe Interact 15:587–597
Ugozzoli L, Wallace RB (1991) Allele-specific polymerase chain reaction. Methods Enzymol 2:42–48
Vad K, de Neergaard E, Madriz-Ordenana K, Mikkelsen JD, Collinge DB (1993) Accumulation of defense-related transcripts and cloning of a chitinase mRNA from pea leaves (*Pisum sativum* L.) inoculated with *Ascochyta pisi* Lib. Plant Sci 92:69–79
Weeden NF, Ellis THN, Timmerman-Vaughan GM, Swiecicki WK, Rozov SM, Berdnikov VA (1998) A consensus linkage map for *Pisum sativum*. Pisum Genet 30:1–4
Weeden NF, Tonguc M, Boone WE (1999) Mapping coding sequences in pea by PCR. Pisum Genet 31:30–32
Wong-Vega L, Burke JJ, Allen AH (1991) Isolation and sequence analysis of a cDNA that encodes pea manganese superoxide dismutase. Plant Mol Biol 17:1271–1274
Wroth JM (1999) Evidence suggests that *Mycosphaerella pinodes* infection of *Pisum sativum* is inherited as a quantitative trait. Euphytica 107:193–204
Wu Q, Preisig CL, VanEtten HD (1997) Isolation of the cDNAs encoding (+)6a-hydroxymaackiain 3-*O*-methyltransferase, the terminal step for the synthesis of the phytoalexin pisatin in *Pisum sativum*. Plant Mol Biol 35:551–560
Xue AG, Warkentin TD, Greeniaus MT, Zimmer RC (1996) Genotypic variability in seedborne infection of field pea by *Mycosphaerella pinodes* and its relation to foliar disease severity. Can J Plant Pathol 18:370–374
Xue AG, Warkentin TD (2001) Partial resistance to *Mycosphaerella pinodes* in field pea. Can J Plant Sci 81:535–540
Yamada T, Tanaka Y, Sriprasertsak P, Kato H, Hashimoto T, Kawamata S, Ichinose Y, Kato H, Shiraishi T, Oku H (1992) Phenylalanine ammonia-lyase genes from *Pisum sativum*: structure, organ-specific expression and regulation by fungal elicitor and suppressor. Plant Cell Physiol 33:715–725
Yamada T, Shiraishi T, Ichinose Y, Kato H, Seki H, Murakami Y (1996) Regulation of genes for phenylpropanoid synthesis in pea elicitor and suppressor. In: Mills D, Kunoh H, Keen NT, Mayama S (eds) Molecular aspects of phatogenicity and

resistance: requirement for signal transduction. American Phytopathological Society, St Paul, pp 151–162

Yoshioka H, Shiraishi T, Nasu K, Yamada T, Ichinose Y, Oku H (1992) Suppression of activation of chitinase and ß-1,3-glucanase in pea epicotyls by orthovanadate and suppressor from *Mycosphaerella pinodes*. Ann Phytopathol Soc Jpn 58:405–410

Yu YG, Buss GR, Saghai Maroof MA (1996) Isolation of a superfamily of candidate disease-resistance genes in soybean based on a conserved nucleotide-binding site. Proc Natl Acad Sci USA 93:11751–11756

Zhu H, Cannon SB, Young ND, Cook DR (2002) Phylogeny and genomic organization of the TIR and Non-TIR NBS-LRR resistance genes family in *Medicago truncatula*. Mol Plant Microbe Interact 15:529–539

Zlamal P (1984) Genetics of horizontal resistance to anthracnose in peas. Sbornik UVTIZ. Genet Slechteni 20:191–192