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ORIGINAL PAPER

Identification of quantitative trait loci controlling partial clubroot resistance in new mapping populations of *Arabidopsis thaliana*

Mélanie Jubault · Christine Lariagon · Matthieu Simon · Régine Delourme · Maria J. Manzanares-Dauleux

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Abstract To date, mechanisms of partial quantitative resistance, under polygenic control, remain poorly understood, studies of the molecular basis of disease resistance have mainly focused on qualitative variation under oligogenic control. However, oligogenic conferred resistance is rapidly overcome by the pathogen and knowledge of the relationship between qualitative and quantitative resistance is necessary to develop durably resistant cultivars. In this study, we exploited the *Arabidopsis thaliana-Plasmodiophora brassicae* pathosystem to decipher the genetic architecture determining partial resistance. This soil-borne pathogen causes clubroot, one of the economically most important diseases of *Brassica* crops in the world. A quantitative trait locus (QTL) approach was carried out using two segregating populations (F_2 and recombinant inbred lines) from crosses between the partially resistant accession Burren and the susceptible accession Columbia. Four additive QTLs (one moderate and three minor) controlling partial resistance to clubroot were identified, all the resistance alleles being derived from the partially resistant parent. In addition, four epistatic regions, which have no additive effect on resistance, were also found to be involved in partial resistance. An examination of candidate genes suggested that a potentially diverse array of mechanisms is related to the different QTLs. By fine-mapping and cloning these regions, the mechanisms involved in partial resistance will be identified.

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M. Jubault · C. Lariagon · R. Delourme ·
M. J. Manzanares-Dauleux (✉)
Amélioration des Plantes et Biotechnologies Végétales,
UMR118 INRA-Agrocampus Rennes-Université de Rennes 1,
BP35327, 35653 Le Rheu Cedex, France
e-mail: maria.manzanares@rennes.inra.fr

M. Simon
Station de Génétique et Amélioration des Plantes,
Centre de Versailles, INRA, 78026 Versailles, France

Introduction

Plants, like animals, are able to launch successful defense responses against invading micro-organisms. In order to limit pathogen growth, plants have evolved a sophisticated, multilayered system of passive and active defense mechanisms, leading to partial resistance (a compatible interaction) or to complete resistance (an incompatible interaction). Complete resistance is the most studied of these defense systems, it usually relies on molecules that specifically recognize, either directly or indirectly (known as the 'Guard' hypothesis), a particular pathogen or strain of a given pathogen. These molecules are encoded by *Resistance (R)* genes, and each R protein initiates a defense response in the presence of a pathogen-derived elicitor protein that is termed the Avirulence (*Avr*) determinant (Hammond-Kosack and Parker 2003). The genetic relationship between R and Avr proteins is commonly known as gene-for-gene resistance (Flor 1971).

A great number of disease resistances, however, do not fit the gene-for-gene system, these include partial resistance of quantitative nature controlled by multiple genes. Despite the prevalence of quantitative variations in resistance levels in natural populations and crop plants (Young 1996), its molecular basis remain currently unknown. Indeed, it is not clear whether the genetic pathways that mediate quantitative and qualitative variations in resistance are the same or involve different genes. On the one hand,

QTLs and *R* genes have frequently been observed to co-localize (Gebhardt and Valkonen 2001; Kover et al. 2005; Perchepied et al. 2006; Wisser et al. 2005), suggesting that quantitative resistance could result from the action of weak *R* gene alleles and qualitative resistance from particularly strong alleles. Mutations in the rice *Xa21* and the tomato *I2* R-genes, involved in qualitative resistance to different kinds of pathogens, resulted in partial resistance phenotypes (Ori et al. 1997; Wang et al. 1998) and confirmed the previous hypothesis. On the other hand, genes involved in defense responses (such as the production of antimicrobial compounds, cell wall strengthening, callose formation, lignification, the oxidative burst) (Gebhardt and Valkonen 2001; Ramalingam et al. 2003; Trognitz et al. 2002; Wisser et al. 2005) or genes encoding metabolic enzymes (Taler et al. 2004) could also be conferring quantitative resistance.

Clubroot, caused by the obligate biotroph protist *Plasmodiophora brassicae* Woron., is one of the economically most important diseases of *Brassica* crops in the world. This soil-borne pathogen causes the hypertrophy (abnormal cell enlargement) and hyperplasia (uncontrolled cell division) of infected roots into characteristic clubs. These obstruct nutrient and water transport, stunt the growth of the plant and consequently reduce crop yield and quality. Since the pathogen survives as resting spores for a long period (up to 15 years) in the soil, control of the disease by agricultural practices and/or chemical treatments is difficult and/or expensive. Thus, the development of resistant cultivars is currently the most efficient way to control clubroot in all *Brassica* crops. Both qualitative and quantitative clubroot resistances were identified in different *Brassicaceae* species including the three most commonly cultivated species: *Brassica napus*, *Brassica rapa* and *Brassica oleracea* (Hirai 2006). However, the type of clubroot resistance introduced into commercial cultivars is usually monogenic or oligogenic and rapidly overcome. Successful strategies for breeding clubroot resistant cultivars will depend on the relationship between the different types of resistance (race-specific or race non-specific, qualitative or quantitative) and the impact of their association on size and genetic composition of pathogen populations. Thus, knowledge of clubroot resistance gene functions and associated mechanisms is required for the development of durable host-plant resistance. However, although numerous studies on the genetic control of clubroot resistance in *Brassicas* have been carried out (Hirai 2006), clubroot resistance genes or QTLs have not been isolated and their potential function remains currently unidentified.

The wild *Brassicaceae Arabidopsis thaliana* is also a host for clubroot (Koch et al. 1991). This model plant provides several advantages for cloning and characterizing plant disease resistance genes. Indeed, the multitude of publicly available molecular tools, including the complete genome sequence for Columbia (Arabidopsis Genome Initiative 2000) and partial genome sequences for numerous other accessions (Nordborg et al. 2005), means that the cloning of disease resistance genes can progress more quickly in Arabidopsis than in other plant species. Furthermore, *P. brassicae* does not present host specificity in *Brassicaceae* (i.e. the same isolate can infect different species). Consequently, the pathosystem *P. brassicae – A. thaliana* appears to be a good model for the analysis of the molecular mechanisms involved in *Brassicaceae* clubroot resistance. Knowledge acquired on the model plant could then be rapidly integrated and transferred to cultivated crops. Up to now, research on clubroot using Arabidopsis as a model host system was mainly focused on the potential involvement of several metabolic pathways in the pathogenesis of the disease, such as hormonal regulation by auxin (Grsic et al. 1999; Ludwig-Muller et al. 1999; Neuhaus et al. 2000) or cytokinins (Devos et al. 2006; Siemens et al. 2006) and trehalose synthesis (Brodmann et al. 2002). With the exception of the identification of *RPB1*, a gene located on chromosome 1 involved in complete clubroot resistance (Fuchs and Sacristán 1996), very little information is currently available on the genetic control of clubroot resistance in Arabidopsis.

The observation of accessions of worldwide origin has revealed that there is natural variation in the responses of *A. thaliana* to clubroot infection (Alix et al. 2007; Fuchs and Sacristán 1996; Siemens et al. 2002). Alix et al. (2007) identified the accession Burren (Bur-0) as partially resistant to the *P. brassicae* isolate eH. This finding makes it possible to use segregating populations to genetically dissect quantitative trait loci (QTLs) controlling these defense mechanisms and gain insight into the molecular basis of quantitative partial resistance and its possible relationship to qualitative resistance. This type of strategy has led to the identification of genetic factors required for Arabidopsis resistance or susceptibility to other bacterial and fungal pathogens (Denby et al. 2004; Kover et al. 2005; Kover and Cheverud 2007; Llorente et al. 2005; Perchepied et al. 2006).

Consequently, to investigate the genetic basis of partial clubroot resistance in *A. thaliana*, a F_2 population was generated by crossing the susceptible accession Columbia (Col-0) and the partially resistant accession Bur-0. A recombinant inbred line (RIL) population produced from crosses between the same parents (Bur-0 × Col-0) was also available at INRA Versailles. Using these two populations, we identified several QTLs (both additive and epistatic) conferring partial clubroot resistance in *A. thaliana*. The complete genomic sequence of Arabidopsis was then scanned for putative candidate genes underlying the QTLs and their role in partial resistance is discussed.

Materials and methods

Plant materials

F_2 population

An F_2 population, consisting of 264 lines, was generated by crossing the clubroot susceptible accession Col-0 (N60000) (female parent) and the partially resistant accession Bur-0 (N1028) (male parent). Parent seeds were obtained from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/). For clubroot-resistance analysis, F_3 seeds were obtained from the self-pollination of each F_2 plant. Young leaves of each F_2 plant were freeze dried and stored at −80°C for DNA isolation using the CTAB method (Doyle and Doyle 1990).

RIL population

The Bur-0 × Col-0 RIL population was generated at INRA Versailles (http://dbsgap.versailles.inra.fr/vnat/Fichier_collection/Rech_RIL_pop.php). The original 347 recombinant inbred lines were produced from a cross between the Bur-0 (172AV) and Col-0 (186AV) accessions; both parental lines were derived by Single Seed Descent from accessions N1028 and N1092 respectively, from the Nottingham Arabidopsis Stock Centre. Lines were taken through to the F_6 generation using the Single Seed Descent method without selection. Then one plant per line was selected for selfing to obtain F_7 seeds, which were used as a bulk for genotyping (equivalent to genotyping the F_6 plants). Two hundred and twenty two of the 347 available Bur-0 × Col-0 RILs were used in our QTL mapping: 164 RILs which constituted the core-population (this sub-set grouped the lines with the most recombined genotypes with a balanced representation of parental alleles, lines with missing data points were removed) and 58 other RILs which were randomly selected.

Pathogen

The selection isolate 'eH' (Fähling et al. 2003), used in this study, belongs to the most virulent *P. brassicae* pathotype P1 (Somé et al. 1996). It was kindly provided by J. Siemens (University of Dresden, Germany).

Tests for clubroot resistance

One hundred and fifty-two of the 264 available Col-0 × Bur-0 F_3 families were evaluated for their resistance against isolate eH. Twenty-four plants per F_3 family and 24 plants per parental line [Bur-0 (N1028) and Col-0 (N60000)] were evaluated in a randomized block design with two blocks.

Two hundred and twenty two of the 347 available RILs were tested in three distinct tests, with common lines in each test. The 164 RILs which constituted the core-population were evaluated in the two first clubroot tests. Twenty-nine lines were common between these two tests. A third test was performed on 58 other RILs randomly selected in the overall population. Seventeen core-population lines previously tested in the two first tests were also added as control. For each RIL test, 24 plants per line (RILs together with the corresponding parental lines [Bur-0 (172AV) and Col-0 (186AV)] were evaluated in a randomized block design with two blocks.

All resistance tests were performed as described by Alix et al. (2007). Arabidopsis seeds were placed on wet blotting paper in Petri dishes at 4°C for 2 days to synchronize germination, then seeds were individually sown in 4 cm-diameter pots containing a (2/3 compost, 1/3 vermiculite) mix sterilized by autoclaving. Arabidopsis plants were grown under controlled environmental conditions (16 h light at 22°C and 8 h dark at 19°C) and inoculated 7 days after germination [stage 1.04 (Boyes et al. 2001)]. The inoculum was prepared according to Manzanares-Dauleux et al. (2000a) and inoculation was performed by applying 1 ml of resting spore suspension (10^7 spores ml^{-1}) to the crown of each seedling. Arabidopsis plants were evaluated for clubroot resistance 3 weeks after inoculation [from stage 3.90 to 6.50 (Boyes et al. 2001)], and symptoms were recorded using the scale previously described for *B. oleracea* (Manzanares-Dauleux et al. 2000b): 0, no visible swelling; 1, very slight swelling usually confined to lateral roots; 2, moderate swelling on lateral roots and taproot; 2+, severe clubs on all roots, but some roots remain and 3, no root left, only one big gall. A disease index (DI) was calculated as described by Manzanares-Dauleux et al. (2000b): DI = (n1(25 + n2(50 + n2+(75 + n3(100)/N, where 'ni' is the number of plants in the symptom class 'i' and N is the total number of plants tested; a line displaying a DI of zero is completely resistant and does not develop any clubroot symptoms while a line with a DI of 100 is highly susceptible.

Genetic maps

F_2 population

Molecular markers A total of 24 simple sequence length polymorphisms (SSLP), 21 cleaved amplified polymorphisms (CAPS) (Konieczny and Ausubel 1993) and derived cleaved amplified polymorphisms (dCAPs) (Neff et al. 1998), and 15 Indel markers were used for map construction. SSLP markers were obtained from Bell and

Ecker (1994) and from the Arabidopsis database (TAIR: http://www.arabidopsis.org). Indel, CAPS and dCAPS markers were specifically designed for this study using small insertions/deletions or mutations identified between Col-0 and Bur-0 (Nordborg et al. 2005) (Table 1). Primer3 software and the dCAPS Finder 2.0 program (Neff et al. 2002) were used for designing primers and choosing restriction enzymes. Each marker name consists of the chromosome number followed by its physical position in kb. Indel, CAPS and dCAPS markers were amplified by PCR in 10 µl of reaction mixture containing 10 ng of genomic DNA, 1X *Green GoTaq*™ *Reaction Buffer* (Promega), 0.5 U of *GoTaq*™ *DNA Polymerase* (Promega), 2.5 mM of $MgCl_2$ (Promega), 2.5 mM of each dNTP and 5 pmol of each primer. Thermal cycling conditions comprised denaturation at 94°C for 30 s, followed by 35 cycles

Table 1 Informative CAPS and Indel markers between Col-0 and Bur-0 generated for this study

Marker[a]	Type	Enzyme	Primers	
			Forward primer	Reverse primer
At1_00029	dCAPS	*Taq*I	TTGAAGATGGGGAGATGGAG	GCTTCAACAAGAAGACCTTTCTC
At1_03041	Indel		TTCGTGTTACATGCCCTCTG	CCTCCTCCGTTGAGAACAAG
At1_05380	CAPS	*Tru*9I	ACTGGAGTTCGTGGGTGAAC	GCCATCTAAATCACCGGAAA
At1_07197	Indel		CAAATCCCCTCACAAAGAGG	GCATCCTTCTCTCCTTACGA
At1_08169	CAPS	*Rsa*I	ATGGACGGTGAGGATCGTAG	TTCATGGATCGCGACAAATA
At1_09973	Indel		ACGTTTTCATCGTACTTGTAGTTGTAG	TGAAAATTACGCCAAGGAAGA
At1_11487	Indel		TACGTTGGTTTACGCCAAAT	GACGCCGGCCAGAAAGTA
At1_18155	Indel		ACTAGTTGTGCTAAGGTAAAGAATAAAGA	TTCTGTCAGTTGATTCCTTTGG
At1_22569	Indel		AGCAGTGTACCAGACTATATCTGTCG	AGACTACCCAACGACCATCG
At1_24694	Indel		GTGGAAGGATCGAATCATCA	ATGAAAGAAACATTTATTTGTTAGGA
At1_26992	CAPS	*Taq*I	TGTTAAACAGACCCGGTACAAA	CCTCATTAACCTCTCCTTCAACA
At2_06280	Indel		TTTGGGGGTTTTGCTTAATG	TTGTTGGCCATGTTTAAAAGC
At3_07842	Indel		CGTCGCACCAAAACAAATTA	TCTTTGTTACGAAAGTTGTGAGC
At3_10849	Indel		CCAAATTGTGTCATTCAACAAAA	GGAAAACGAGAAAGAGATTTGG
At3_15129	Indel		TGCCCTTGATCGAGTATTTG	GGAGGGAAGCAGCAGAGTAA
At4_11984	CAPS	*Hae*III	GATCAGGAGGAAACCGACAA	CTCGAACCTTCCGTGAAGAC
At4_13078	CAPS	*Hae*III	GGAAAGAGCCACAAATCCAA	CAAGCATATGGAGCTGGTCA
At4_14176	CAPS	*Mbo*I	TGTCCTGGCTCCTGAAGTTT	CGAGCATCTAAGTCAGCCATC
At4_15329	CAPS	*Vsp*I	GGGTTTCTTCAGTAAGAGTTTTTAGA	TGTGGGTTTGTCGTAGATTGA
At4_16337	CAPS	*Dde*I	AATGCTTTTGGCGAGTATGG	CGGTACCAAAAGGAGAAAAGA
At4_16636	Indel		TGGTTGATCTCAAGCATTGG	AATCTTGCCGAAAAGGGTTT
At5_02192	Indel		CAGCCAAGATAAAGGTCATGG	CGTTATTGCACCCTTTGCTT
At5_03126	CAPS	*Dde*I	TCGAAGGCGAGAATAACATTG	CATCAGCCCAAACCCTTAAA
At5_04010	CAPS	*Hae*III	AGGTTTTGCGCAATTCAATC	TGAGGAAGCATCAAACAGAAAA
At5_05425	CAPS	*Hinf*I	AACCGAAACTACACCTGCAAA	AAATGGGCTTTCTTTTGTGG
At5_06221	CAPS	*Nla*III	ACCCAGTTGAACCAACCACT	TTGCTACAATGGCCTCACAC
At5_07004	CAPS	*Cac8*I	CTTACCGCTTCTCCAACCAA	GTTTCACACTTAGCTTTGTCGTT
At5_09447	CAPS	*Hha*I	GAAAAGCTTTCAGGCGACAG	TCCTACACGGGTTTCCACAT
At5_12698	CAPS	*Apo*I	TGAACTGCAGATTTTGAAATGG	TTCAGTGTTGGAAAATCGTGA
At5_15065	CAPS	*Alu*I	TCAGTTGCTCGTTCCTCTTG	TTAGGTTGGATGCCCACAGT
At5_17569	Indel		GGCCTTTATCAAACCGGTAA	CCTTACCACGAATCTTTTCCA
At5_18987	Indel		TTGATGACTTTGGTGGAGCA	GCCATGGCTGAAGAAACTGT
At5_20318	CAPS	*Nco*I	TTGTCAAGCTTTGGGTTCCT	TCATATTTGTAACCTGTCATACAGAAC
At5_20915	dCAPS	*Hha*I	ATGTGTAAGAGACAAGAAGTAGTAAAGC	AAGAGCAAGTTCACGCATAAG
At5_23115	dCAPS	*Ssp*I	CGGACGTTGATACGAAAGC	GCCAATCAAATATAAAATATCTCCAATA
At5_24996	CAPS	*Hpa*II	ATTTGAGAAATCGGCGTGAG	CAACGAGGGTAACGTTTCGT

[a] Each marker name consists of its chromosome number and physical position in kb

of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR or digestion products were run on 3% agarose gels.

Map construction For map construction, all molecular markers showing polymorphism between Col-0 and Bur-0 were then used for segregation analysis on the 152 F_2 plants. Segregation of each marker in the F_2 population was analyzed by a chi-square test for deviation from the expected segregation ratio (a 1:2:1 (codominant marker) or 3:1 (dominant marker) ratio). Marker linkage analysis was carried out using MAPMAKER/EXP 3.0 (Lander et al. 1987). The two point analysis command "group" (LOD = 6, maximum distance between markers = 40 cM) was first used to define which linkage groups the molecular markers belong to. Marker order was established with the "compare" command. All genetic distances are expressed in centimorgan values calculated by the Kosambi function (Kosambi 1944).

RIL population The linkage map was created using 85 single nucleotide polymorphisms (SNP) genotyped with the SNPlex technology (Applied Biosystems) and two microsatellites markers. Each marker name consists of the chromosome number followed by its physical position in kb. Segregation of each marker in the RIL population was analyzed by a chi-square test for deviation from the expected 1:1 segregation. The genetic map was established using MAPMAKER/EXP 3.0 with the Kosambi mapping function.

Statistical methods

General

The data obtained from each resistance test were statistically analyzed using a generalized linear model [(PROC GLM of Statistical Analysis System (SAS) software (SAS Institute Inc., 2000)]. Broad-sense heritability (h^2) was estimated from an ANOVA with the following formula $h^2 = \sigma_g^2/\left[\sigma_g^2 + (\sigma_e^2/n)\right]$ where σ_g^2 is the genetic variance, σ_e^2 is the environmental variance and n is the number of replicates per line.

QTL analysis

The genotypic and phenotypic data sets were imported into the computer program QTL CARTOGRAPHER version 2.5 (Wang et al. 2007). Composite interval mapping (CIM) was used, which takes background genetic variation into account, for more accurate QTL detection and characterization. To conduct CIM, Model 6 of QTL CARTOGRAPHER was used specifying a SF2 population and a RI1 population for the F_2 and RIL populations, respectively, and scanning the genome every 2 cM. Five markers, selected by a forward-backward stepwise regression analysis, were used as cofactors in the CIM procedure with a 10 cM window size. This procedure estimated the log-likehood (LOD) score, additive effect, and percentage of phenotypic variance every 2 cM along each chromosome. Empirical threshold levels, which confirmed the existence of a QTL at the genome-wise significance level of 0.05, were obtained by analyzing 1000 permutations of the data, according to the method of Churchill and Doerge (1994). QTL locations were determined as the positions with a peak LOD score exceeding the threshold significance level (3.3 and 2.4 for the F_2 and RIL populations respectively). One LOD support interval was established as approximately 95% QTL confidence interval (van Ooijen 1992).

In addition to additive effects, pairwise epistatic interactions were searched for in the RIL population using a two-way ANOVA model with an interaction component between pairs of markers. The significant level threshold used to evaluate the significance of epistatic effects was 1.34×10^{-3}, expecting five false positives. The overall R^2 was estimated with a full ANOVA including all additive and digenic epistatic effects.

Results

Identification of QTLs controlling partial clubroot resistance in the F_2 population

Map construction

Each F_2 individual was genotyped with a total of 60 markers, including 24 SSLPs, 21 CAPS/dCAPS and 15 Indel markers (Table 1). The distribution of Col-0 and Bur-0 alleles was as expected [a 1:2:1 (codominant marker) or 3:1 (dominant marker) ratio] with the exception of the At1_22569 and At1_24694 markers at the bottom of the chromosome 1. These segregation data were used to obtain the linkage map shown in Fig. 1. The 60 markers were assigned to five linkage groups with a total length of 422 cM and an average spacing of 7 cM. The longest distance between markers was 19.3 cM, between CIW5 and DET1.2 on the top of the chromosome 4. The genetic length of each linkage group was comparable to the lengths reported for other mapping populations (Alonso-Blanco et al. 1998; Lister and Dean 1993; Loudet et al. 2002) and all markers were located as expected according to their physical position on the Col-0 genomic sequence.

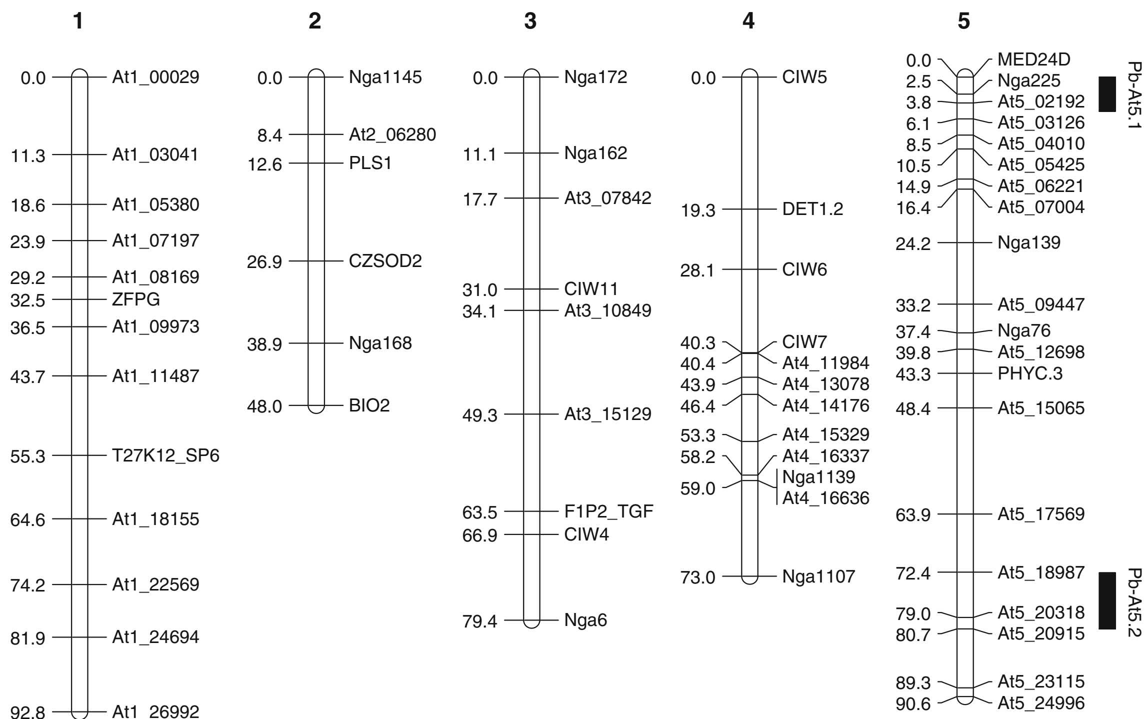

Fig. 1 Arabidopsis QTLs controlling partial resistance to *Plasmodiophora brassicae* in the F_2 Col-0 × Bur-0 population. Each marker name consists of its chromosome number and physical position in kb. The bar length is equal to the one-LOD likelihood confidence interval

Statistical analysis

To determine the genomic regions involved in resistance, we performed a full QTL analysis in the F_2 population obtained from the cross between the susceptible Col-0 and the partially resistant Bur-0. Plants from 152 lines in the Col-0 × Bur-0 F_2 population, as well as the parental accessions, were evaluated for their response to inoculation by *P. brassicae*. Analysis of variance revealed significant phenotypic variation among lines ($p = 0.0009$) and no significant differences between blocks ($p = 0.95$). Heritability was $h^2 = 0.40$. The Bur-0 and Col-0 parental lines showed a mean DI of 82 and 99 respectively. Analysis of resistance in the F_2 population showed a continuous distribution pattern, suggesting a quantitative and polygenic control of the partial resistance (Fig. 2). The trait expressed high transgressive segregation with a minimum DI of 60. This suggests that the Col-0 parental accession carries favorable alleles for clubroot resistance.

QTL analysis

Quantitative data were used to map QTLs responsible for clubroot resistance, and two QTLs located on chromosome 5 were identified (Fig. 1). The first QTL, designated as *Pb-At5.1* (for ***P****lasmodiophora* ***b****rassicae* ***A****rabidopsis* ***t****haliana)*, was near the Nga225 marker at the top of chromosome 5 (LOD = 3.4) with a 5 cM confidence interval. The second QTL, designed *Pb-At5.2*, was mapped to the bottom of chromosome 5 (LOD = 4.6), near the At5_20318 marker, with a confidence interval of 8 cM (Table 2). Resistance alleles for both clubroot resistance loci were derived from the partially resistant parent Bur-0. *Pb-At5.1* accounted for 9.2% of the total variation and *Pb-At5.2* for 12.9%. The percentage of the total phenotypic variation (R^2) explained by the two QTLs was 27.2%. Substitution of *Pb-At5.2* susceptibility alleles for resistance alleles had the greatest impact on the DI score (i.e. 1.7 DI points explained) (Table 2).

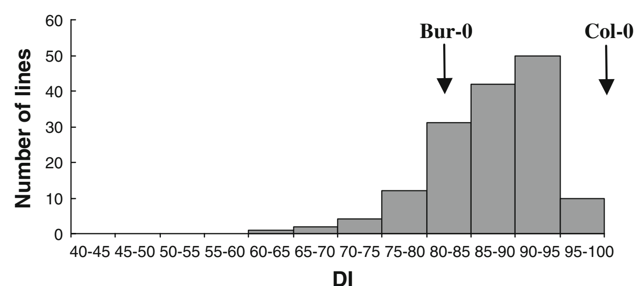

Fig. 2 Frequency distribution of the adjusted mean DI of the 152 Col-0 × Bur-0 F_2 population. The arrows depict the mean values of the parental lines

Table 2 Summary of significant QTLs controlling clubroot resistance identified in the Bur-0 × Col-0 and the Col-0 × Bur-0 progenies

Progeny	QTL name	Chr.	Position (cM)[a]	Marker[b]	Confidence interval (cM)	LOD score	Additive effect[c]	R^2 (%)[d]
F_2 Col-0 × Bur-0	Pb-At5.1	5	2.6	Nga225	0–5	3.4	+0.8	9.2
	Pb-At5.2	5	79.0	At5_20318	72.5–80.7	4.6	+1.7	12.9
RIL Bur-0 × Col-0	Pb-At5.1	5	11.3	c5_05319	8.0–14.5	2.4	+1.8	3.6
	Pb-At5.2	5	62.9	c5_19316	59.3–63.5	14.9	+4.4	22.9
	Pb-At1	1	12.4	c1_05593	9.5–16.3	3.7	+2.1	5.0
	Pb-At4	4	43.1	c4_14819	36.6–48.8	4.2	+2.3	6.2

[a] Position from the first marker of the chromosome

[b] Marker nearest the peak LOD score

[c] The additive effect indicates the effect on DI of Col-0 allele in comparison to Bur-0 allele

[d] Percentage of phenotypic variation explained by QTL

Identification of QTLs controlling partial clubroot resistance in the RIL population

To validate and clarify the location of QTLs first detected in the Col-0 × Bur-0 F_2 population, we performed a full quantitative trait analysis in the RIL population derived from the cross between partially resistant Bur-0 and susceptible Col-0.

Map construction

The 222 Bur-0 × Col-0 RILs were used to build a genetic map with 87 markers. No significant deviation from the expected 1:1 distribution of Col-0 and Bur-0 alleles was detected in the core-population. However, segregation distortion was observed in several genomic regions in the genetic map built with data from all 222 RILs (data not shown). The total length of the genetic map is 363 cM. The average distance between two adjacent markers is 4.4 cM and the maximal distance between two consecutive markers is 11.7 cM.

Statistical analysis

The 222 RILs were evaluated in three distinct tests with respectively 29 and 19 common lines. ANOVA performed on the results of each test revealed significant phenotypic variations among lines ($p < 0.0001$, $p = 0.0009$ and $p = 0.0083$ respectively) and no significant differences between blocks. Heritabilities were $h^2 = 0.70$, $h^2 = 0.49$ and $h^2 = 0.43$. Parents and common lines showed a similar behavior in the three clubroot tests. As ANOVA performed on the common lines did not reveal a significant test effect ($p = 0.79$), subsequent QTL analyses were realized on mean values. The Bur-0 and Col-0 parental lines showed a mean DI of 59 and 84 respectively. Analysis of the resistance in the RIL population also showed a continuous distribution pattern (Fig. 3). The mean DI for the 222 RILs Bur-0 × Col-0 was approximately equal to the mid-parent value (77), and the trait expressed high transgressive segregation in both directions with a minimum DI of 43 and a maximum DI of 96.

QTL analysis

QTL mapping performed on the RIL population revealed four significant QTLs governing partial resistance to clubroot on chromosomes 1, 4 and 5 (Fig. 4). The first QTL was at the top of chromosome 5 (LOD = 2.5), near the c5_05319 marker with a confidence interval of 7 cM. The second QTL was mapped to the bottom of chromosome 5 (LOD = 14.9), near the c5_19316 marker, with a 4.2 cM confidence interval. The physical locations of these two QTLs are similar to the respective positions of *Pb-At5.1* and *Pb-At5.2* QTLs which were previously identified in the F_2 population. Two additional QTL were specifically detected in the RIL population. The first QTL, *Pb-At1*, was detected at the top of chromosome 1 (LOD = 3.7), near the c1_05593 marker, with a 7 cM confidence interval. The second one, *Pb-At4*, was found at the bottom of chromosome 4 (LOD = 4.2) near the c4_14819 marker. It was detected with a confidence interval of 12 cM. The four QTLs *Pb-At5.1*, *Pb-At5.2*, *Pb-At1* and *Pb-At4* accounted for 3.6, 22.9, 5.0 and 6.2% of the total variation, respectively (Table 2) and together they

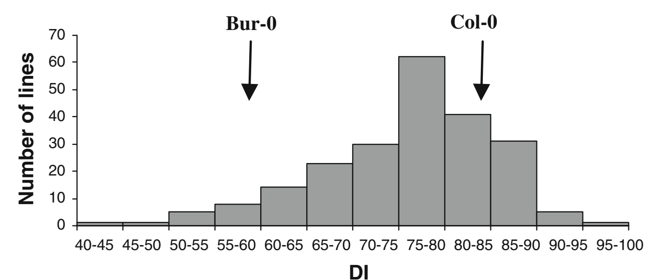

Fig. 3 Frequency distribution of the mean DI of the 222 RILs Bur-0 × Col-0. The arrows depict the mean values of the parental lines

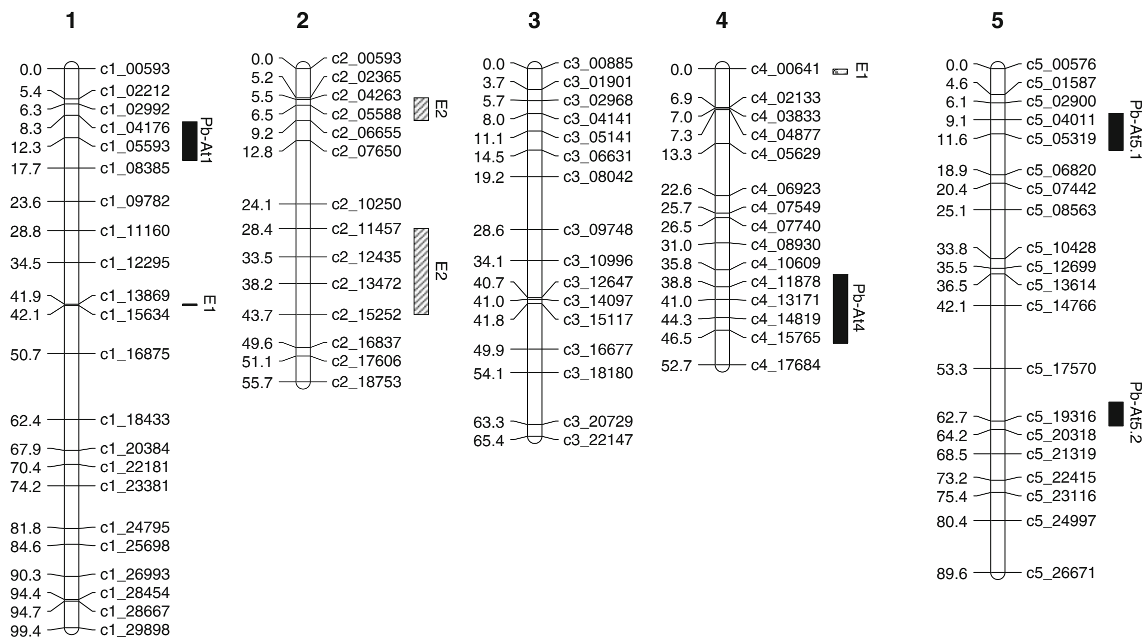

Fig. 4 Arabidopsis additive and epistatic QTLs controlling partial resistance to *P. brassicae* in the Bur-0 × Col-0 RIL population. Each marker name consists of its chromosome number and physical position in kb. Additive QTLs detected in the RIL population are indicated by black-colored bars. The bar length is equal to the one-LOD likelihood confidence interval. Significant epistatic interactions detected by a complete pair-wise search are shown by pairs of hatched bars

explained 33.8% of the total phenotypic variation. The additive allele effect of these four QTLs was in the same direction, alleles from Bur-0 increased the resistance compared with Col-0 alleles. Substitution of *Pb-At5.2* susceptibility alleles for resistance alleles had the greatest impact on the DI score (i.e. 4.4 DI points explained) compared to the three other *Pb-At* QTLs (Table 2).

We also searched for pairwise epistatic interactions between all the 87 markers in the RIL population. On the total number of 3 741 pairwise tests performed, 17 interactive effects were found to be significant ($P<1.34 \times 10^{-3}$). These 17 interactive marker-to-marker effects corresponded to eight different genomic regions. We only retained four of these regions since they showed several markers in interaction. These four genomic regions independently accounted for up to 11.4% of the phenotypic variance (Table 3). The interactions were found between regions that had no significant additive effect on resistance (Fig. 4). Additive effect loci and interactions were combined in complex ANOVA models to determine the overall R^2 corresponding to the total part of variance explained by the set of different QTLs detected (additive and epistatic QTLs) (Table 4). The phenotypic variances explained by only additive QTLs were 33.8%. In the complex model including the additive and the epistatic QTLs, the overall R^2 values were 42.5%.

Discussion

The present study is the first genetic analysis of partial clubroot resistance in *A. thaliana*. We demonstrated that

Table 3 The most significant interactions between genetic markers determined using two-way ANOVA ($p < 1.34 \times 10^{-3}$) in the RIL population

Epistatic regions	Markers in interaction[a]	p	R^2	Phenotypic DI RIL means with alleles[b]			
				BB	BC	CB	CC
1	c1_15634 × c4_00641	3.82×10^{-4}	11.4	**69.8**	78.8	77.9	77.8
2	c2_05588 × c2_12435	1.46×10^{-4}	8.1	77.1	**73.0**	73.1	79.4

[a] We considered QTLs identified by the marker loci that displayed the highest R^2 value

[b] Combination of two alleles from Bur-0 (BB), two alleles from Col-0 (CC), the allele from Bur-0 for the first marker and the Col-0 allele for the second marker (BC) and conversely (CB). Note: bold-type font indicates the most resistant class

Table 4 Full ANOVA model including all additive and digenic epistatic QTLs controlling partial clubroot resistance in the Bur-0 × Col-0 RIL population. Bold-type font indicates significant QTLs (α=0.05)

QTLs	Markers[a]	p	R^2
Pb-At5.1	c5_05319	**0.012**	3.6
Pb-At5.2	c5_19316	**<0.0001**	22.6
Pb-At1	c1_05593	**0.02**	2.4
Pb-At4	c4_14819	**0.0073**	3.1
c1_15634 × c4_00641		**0.0088**	11.4
c2_05588 × c2_12435		**0.0059**	8.1
Complete model			42.5

[a] We considered QTLs identified by the marker nearest the peak LOD score

partial clubroot resistance in the Arabidopsis accession Bur-0 is inherited in a polygenic fashion. Four additive QTLs (one moderate-effect locus *Pb-At5.2* and three minor-effect loci, *Pb-At5.1*, *Pb-At1* and *Pb-At4*) controlling partial resistance to clubroot were identified. These resistance alleles were all derived from the partially resistant parent Bur-0. In addition, four epistatic regions, which have no additive effect on resistance, were also found to be involved in partial resistance, independently accounting for up to 11.4% of the phenotypic variance.

Among the additive QTLs, the *Pb-At5.2* QTL with a moderate effect and the minor-effect *Pb-At5.1* QTL associated with partial clubroot resistance were detected in both the Col-0 × Bur-0 F_2 and Bur-0 × Col-0 RIL populations. Thus, the QTLs *Pb-At5.1* and *Pb-At5.2* were reliable and consistent in both genetic backgrounds. The two other QTLs, *Pb-At1* and *Pb-At4*, were detected only in the Bur-0 × Col-0 RIL population. This may have resulted, on the one hand, from the homogeneous behavior of RILs which allows more accurate evaluation of quantitative traits and on the other hand, from the larger size of the RIL population (152 F_2 lines compared to 222 lines in the RIL population) which facilitates the detection of small-effect QTLs (Holland 2007; Mackay 2001).

Clubroot resistance in *Brassicaceae*

Both qualitative and quantitative clubroot resistances were already identified in different *Brassicaceae* species. In *A. thaliana*, Fuchs and Sacristán (1996) studied a monogenic inherited resistance to *P. brassicae* isolate 'e' in the accession Tsu-0 and mapped the clubroot resistance gene, designated *RPB1*, roughly between 11 465 kb and 11 538 kb on the top of chromosome 1 (Arbeiter et al. 2002). However, this *RPB1* position does not correspond to either the additive QTL, *Pb-At1*, or the epistatic QTL on chromosome 1 detected in the present study.

Genetic studies of clubroot resistance in *B. oleracea* and *B. napus* showed the polygenic nature of the trait (Landry et al. 1992; Manzanares-Dauleux et al. 2000a, 2003; Moriguchi et al. 1999; Rocherieux et al. 2004). These studies demonstrated that quantitative resistance to clubroot could be controlled by both broad-spectrum and isolate-specific as well as small- to very strong-effect QTLs (explaining up to 98.1% of the phenotypic variation). In *B. oleracea*, a study was carried out on quantitative resistance carried by the kale, C10, to five *P. brassicae* isolates (Rocherieux et al. 2004) including the isolate (eH) used in the present study. Two moderate-effect QTLs (each one explaining 21% of the phenotypic variation) and three minor-effect QTLs (explaining 4–6% of the phenotypic variation) were detected with the eH isolate, explaining 70% of the total phenotypic variation. The genetic architecture of quantitative clubroot resistance against the eH isolate in *B. oleracea* and *A. thaliana* seems to be similar with the coexistence of several moderate-effect QTLs (each one explaining about 20–25% of the phenotypic variation) and minor-effect QTLs (each one explaining about 4–9% of the phenotypic variation). In *B. oleracea*, the two major QTLs, *Pb-Bo1* and *Pb-Bo2* are broad-spectrum, the former conferring total or partial resistance depending on the clubroot isolate. In a similar manner, the major clubroot resistance gene *Pb-Bn2*, which is efficient against the eH isolate in *B. napus*, co-localizes with a moderate-effect QTL which confers resistance to another isolate (Manzanares-Dauleux et al. 2003). The Bur-0 accession appears to be also partially resistant to another *P. brassicae* isolate (Ms6) (Alix et al. 2007). Thus, an interesting future line of study will be to identify the QTLs controlling Bur-0 partial resistance against this other clubroot isolate, in order to determine whether the *Pb-At* QTLs, and in particular the moderate-effect *Pb-At5.2*, confer specific resistance to eH, or whether these represent some form of broad-spectrum resistance factors. Furthermore, in addition to additive QTLs, epistasis appears to significantly contribute to clubroot resistance in *Brassicaceae*. Indeed, in both *A. thaliana* and *B. napus* (Manzanares-Dauleux et al. 2000a), epistatic interactions were found to have an important effect on resistance, since they accounted for up to 11.4 and 20.0% of the phenotypic variance respectively. The syntenic relationships between clubroot resistance loci in these three species remain currently unknown.

In *B. rapa*, at least four independent major clubroot resistance genes (*Crr1*, *Crr2*, *Crr3*, *CRb*) and one weak QTL (*Crr4*) were identified and mapped (Hirai 2006). The region adjacent to *Crr3* shows synteny to the top of the long arm of *A. thaliana* chromosome 3 (Saito et al. 2006). The region containing the *Crr1*, *Crr2* and *CRb* clubroot resistance genes was also found to align with a central

section of the long arm of *A. thaliana* chromosome 4 (Suwabe et al. 2006), a region which also corresponds to the minor-effect QTL *Pb-At4*, detected in the present study. Even if the *Pb-At4* confidence interval is rather large, our results, obtained with a different *P. brassicae* isolate, confirm the existence of a functional clubroot resistance gene in this region.

Candidate loci for Arabidopsis clubroot resistance QTLs

Comparisons between the genetic data available on clubroot resistance loci in the *Brassicaceae* indicate that QTLs controlling partial clubroot resistance may correspond to allelic variants of qualitative resistance genes, which lead to intermediate phenotypes. However, whereas this hypothesis has been frequently proposed for several pathosystems (Gebhardt and Valkonen 2001; Kover and Cheverud 2007; Perchepied et al. 2006; Pflieger et al. 1999), no pathogen resistance QTL has yet been cloned and the molecular nature of such genes remains hypothetical. Thus, it was interesting to investigate whether putative disease resistance genes or other candidate genes are found within the genomic regions identified as QTLs in the present study.

Several hypothetical *R*-genes (NBS-LRR) are located within the confidence interval defined for the *Pb-Ats* QTLs, making them potential good candidate genes. The moderate-effect QTL *Pb-At5.2* co-localize thus with several clusters of resistance genes. Some of these *R* genes have already been associated with qualitative resistance against pathogens, such as *TTR1*, which confers resistance to tobacco ringspot nepovirus (Lee et al. 1996), *RPS4*, which confers specific resistance to *Pseudomonas syringae* pv. Tomato (Gassmann et al. 1999), and *RRS1*, which confers resistance to *Ralstonia solanacearum* (Deslandes et al. 2003). Moreover, two other resistance QTLs, *PRP-Ps4* to *Pseudomonas syringae* (Perchepied et al. 2006) and *QRP3* to *Plectosphaerella cucumerina* (Llorente et al. 2005), were also previously detected in this region. Besides, the minor-effect QTL, *Pb-At4*, also co-localizes with 3 NB-LRR genes among which *RPS2*, confers specific resistance to *Pseudomonas syringae* pv. Tomato (Bent et al. 1994). Partial resistance QTLs could also correspond to genes involved in defense responses. Genes involved in the production of antimicrobial compounds, in cell wall strengthening, and in the oxidative burst are found subjacent to the *Pb-At* QTLs. Finally, a last class of genes appears also to be good candidates for the partial clubroot resistance QTLs. Taking into account the nature of clubroot symptoms e.g. abnormal cell enlargement and uncontrolled cell division, genes underlying *Pb-Ats* QTLs involved in auxin response, cell-expansion or root architecture could also be involved in partial clubroot resistance.

In summary, several genes with known or predicted roles in pathogen response or in clubs formation occur within the confidence intervals of the *Pb-Ats* QTLs. However, the confidence intervals found for these QTLs involve rather large genomic regions and it is also possible that a locus with a different function and structure underlies the observed *P. brassicae* resistance QTLs. Consequently, any positional candidate gene at this point should be regarded only as hypothetical. Further fine mapping on these four regions is ongoing to narrow down the most appropriate gene for these QTLs. Cloning the underlying genes will provide a basis to identify the mechanisms implicated in partial resistance and the relationship between quantitative and qualitative resistance. Furthermore, further comparative studies on *Brassica* using information obtained in Arabidopsis, will improve our understanding of the evolution of clubroot resistance in the *Brassicaceae* and should yield valuable data for optimizing the breeding strategies for clubroot resistant *Brassica* crops.

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