Theor Appl Genet (2003) 108:168–174
DOI 10.1007/s00122-003-1408-8

ORIGINAL PAPER

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Identification of QTLs related to cocoa resistance to three species of *Phytophthora*

Received: 15 March 2003 / Accepted: 15 May 2003 / Published online: 13 September 2003

Abstract This study aimed to compare the genetic control of cacao resistance to three species of *Phytophthora*: *Phytophthora palmivora*, *Phytophthora megakarya* and *Phytophthora capsici.* The study was conducted on 151 hybrid progenies created in Côte d'Ivoire and grown in a green-house in Montpellier. *Phytophthora* resistance was screened by leaf-test inoculation with two different strains per species. Selection of the best individuals for resistance to *P. palmivora* at a 10% selection rate, would lead to a genetic progress of 47% in the disease evaluation for this species and a genetic progress of 42% and 21% for the two other species. A genetic map with a total length of 682 cM was built with 213 markers, 190 AFLPs and 23 microsatellites. QTLs were identified using composite interval mapping. QTLs were found located in six genomic regions. One of these was detected with five strains belonging to the three *Phytophthora* species. Two other regions were detected with two or three strains of two different species. Three additional QTLs were detected for only one species of *Phytophthora*. Each QTL explained between 8 to 12% of the phenotypic variation. For each strain, between 11.5% to 27.5% of the total phenotypic variation could be explained by the QTLs identified. The identification of multiple QTLs involved in resistance to *Phytophthora* offers the possibility to improve durability of resistance in cocoa by a possible cumulation of many different resistance genes located in different chromosome regions using marker-aided selection.

Communicated by H.F. Linskens

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Introduction

Black pod caused by several species of *Phytophthora* is one of the most-important diseases affecting cacao (*Theobroma cacao* L.) and is responsible for important yield losses. A detailed study of the taxonomy of these *Phytophthora* species was published by Brasier and Griffin (1979). *Phytophthora palmivora* (Butl.) Butler (1919) is found throughout the cacao growing zone, whereas *Phytophthora capsici* Leonian (1922) emond. Tsao and Alizadeh (1988) only occurs in Latin America and *Phytophthora megakarya* Brasier and Griffin (1979) only exists in Central and West Africa. *P. megakarya* is the species that causes most damage (up to 80% of pod losses). It is currently spreading in Africa and has now reached the East of Côte d'Ivoire, the first producing country. Like many other agronomic traits, resistance to *Phytophthora* exhibits a continuum of phenotypic variations in the species *Theobroma cacao* suggesting the implication of several genes in resistance to *Phytophthora*; but, among all the germplasm analysed, no tree has ever been found to be completely resistant in the field. A polygenic control of resistance has already been suggested (Blaha and Lotodé 1976; Enriquez and Soria 1996) and field resistance seems to be additive (Tan and Tan 1990; Cilas et al. 1998). Artificial inoculation tests were developed to enable an early evaluation of resistance in cocoa. Blaha and Lotodé (1976) classified a hundred clones according to their level of resistance using an artificial inoculation test. Recently a new method based on inoculation made on leaves was developed by Nyassé (1997), and a significant positive correlation between leaf-test data and pod-rot rate in the field was observed. The resistance trait heritability and the validity of leaf inoculation test were studied for *P. megakarya* in a diallel crossing scheme in Cameroon (Ndoumbé et al. 2001; Nyassé et al. 2002). These results suggested that the leaf

test could be used as an early predictor of cacao resistance in the field. Leaf tests have already been used for quantitative traits analyses for resistance on cacao (Flament et al. 2001) and sugar beet (Setiawan et al. 2000). Molecular mapping of the cacao genome was initiated by Lanaud et al. (1995) and a high-density genetic map was recently constructed (Risterucci et al. 2000) using RFLPs associated with microsatellites and AFLP markers. The high multiplex ratio achievable with AFLPs offered a great advantage for the analysis of a large number of loci with a good distribution of markers along the genome, and enabled dense linkage maps to be developed more rapidly for quantitative-trait analysis such as disease resistance (Nandi et al. 1997; Meyer et al. 1998; Ghislain et al. 2001).

The objective of this study was to use a QTL approach to have a more-precise understanding of the genetic control of the resistance to three species of *Phytophthora*: *P. palmivora*, *P. megakarya* and *P. capsici.* It is important to know whether a common locus of resistance usable as a priority for marker-assisted selection can be located on the genome, and whether selection for resistance to one of the *Phytophthora* species could increase the level of resistance to the other species.

Materials and methods

Plant material

The study was conducted on a progeny created in Côte d'Ivoire and derived from a cross between (SCA6×H) and IFC1. The female parent (SCA6×H) is a hybrid individual derived from the cross between Scavina 6 (SCA6), an Upper Amazon Forastero clone known for its high level of resistance to pod rot and for its productivity, and H, a Trinitario. The male parent, IFC1, is a highly homozygous Lower Amazon Forastero clone (Amelonado type) cultivated in West Africa and susceptible to pod rot. Seeds (151) were sent to Montpellier and sown in the greenhouse

Evaluation of resistance

The level of resistance was evaluated by leaf-disc inoculation with three *Phytophthora* species and two strains for each species with a different level of pathogenicity: strains 517 (medium level of pathogenicity) and Tri (high level) for *P. palmivora*, strains NS 269 (high level) and 309 (medium level) for *P. megakarya*, strains TRI3 (high level) and MHU76.2 (medium level) for *P. capsici*. Healthy 2-month-old leaves were collected within a period of 2 h early in the morning. Leaves were labelled and placed in an ice box containing humidified foam rubber, to maintain 100% relative humidity. Leaf discs (2 cm) were cut with a cork borer. The discs were placed in plastic trays (302 discs per tray) with the underside of the leaf upwards and humidified. After completing an inoculation tray, discs were slightly humidified by spraying with very fine droplets of sterile distilled water. The trays were then closed.

Inoculation was carried out the following morning. Ten microliters of a suspension containing 2×10^5 zoospores/ml were put on each leaf disc. Four series of inoculations were performed for *P. palmivora* and *P. megakarya* and two series for *P. capsici.* Each experiment consisted of ten trays, each containing two discs of each genotype; one disc was inoculated with each strain in each tray.

Symptom severity was scored 4 and 6 days after inoculation. The scale was based on increasing size of the chlorotic/necrotic area with, 0: no symptoms; 1: penetration points, 2: network of points; 3: weblike patch; 4: mottled patch; and 5: true patch (necrosis).

Statistical analysis

Statistical analyses were carried out with STATISTICA 5.1 software. After checking that the frequency distributions fitted a normal law by using a Shapiro and Wilk test, analyses of variance were carried out with the variables: "score at 4 days" and "score at 6 days". As the result obtained with those two variables, or with their difference (generally small), were similar, only the results obtained for the variable "score at 6 days" were used for the rest of the analysis. A two-way analysis of variance was carried out to estimate the effect of interaction between species and individuals. Then, analyses per species were used to estimate the strain effect and the effect of interaction between strains and genotype. The individuals of the progeny were classified according to their level of resistance for each species and were compared by the Spearman rank test. Genetic progress was estimated from a selection of the best 10% individuals. The progress was the ratio between the phenotypic-value average of the selected individuals and the average of the whole progeny.

Genetic mapping

DNA extraction

DNA extraction was performed from fresh leaves according to Risterucci et al. (2000).

Molecular markers

AFLPs (Vos et al. 1995) were revealed with the Gibco BRL AFLP analysis system II as recommended by the supplier. Due to the high level of homozygosity of the IFC1 clone, only the map of the parent (Sca6×H) was etablished. AFLP markers segregating in the progeny were selected showing a pattern of absence of the band in the IFC1 (homozygous) and the presence of band the in (Sca6×H) (heterozygous). Genotyping in the same time as the Sca6 clone, it was possible to detect some alleles specific to Sca6 or to H. Some microsatellites (Tautz and Rentz 1984) were also used to establish the map and identify the chromosomes in reference to the map established by Risterucci et al. (2000).

The primer *Eco*R1+2 for AFLP and one primer for microsatellites were end-labelled with γ-[^{33}P]-ATP. After adding 20 µl of loading buffer (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol), the mixes were denatured at 92°C for 3 min and 3 µl of each sample were loaded onto a 5% polyacrylamide gel with 7.5 M urea and electrophoresed in 0.5% TBE buffer at 55 W for 1 h 40 min. The gel was dried for 30 min at 80°C and exposed overnight to X-ray film (Fuji RX).

Linkage analysis

Linkage analyses were performed using the JOIN MAP program, version 3.0 (Van Ooijen and Voorrips 2001). The segregation of 213 markers was studied and a LOD score of 4.0 was used to identify the linkage groups. The Kosambi mapping function was used to convert recombination frequencies into map distances.

QTL mapping

QTL analysis was performed using composite interval-mapping analysis (Zeng 1994) using MapQTL version 2.0 (Van Ooijen et al. 2000) and QTL Cartographer version 1.16 (Basten et al. 2002). A forward-backward stepwise regression was performed to choose co-

Table 1 Statistical parameter of the distribution of "score at 6-days" observed for each species of *Phytophthora* on the progeny (Sca6×H)×C1 and the clone Sca6. SD = standard deviation. W = Shapiro and Wilk normality test, ** significant deviation at $P<0.001$

Variable	No.	Mean	Median	Min	Max	S.D.	W	Sca6 mean
P. palmivora	151	1.382	1.375	0.50	2.56	0.39	0.98**	1.03
P. megakarya	151	0.909	0.825	0.27	2.37	0.35	0.92	1.22
P. capsici	151	1.023	0.900	0.00	2.80	0.54	0.95**	1.17

factors before performing QTL detection. A 1,000 permutation test was performed with QTL Cartographer to estimate the appropriate significance threshold for analysis. A LOD threshold of 2.4 corresponding to a genome-wise significance level of 0.10 was chosen.

Results

Genetic map

Fifteen AFLP primer combinations were used for genotyping the progeny, generating a total of 1,238 unambigous bands. Of these only clearly scorable fragments were analysed. Six to 22 polymorphic fragments in a size range of 70 to 500 bases were observed by primer combination. In addition 23 microsatellites selected from the reference map (Risterucci et al. 2000) could be mapped and used for linkage-group identification. Each segregating marker was first tested with a χ^2 test for goodness-of-fit to the expected 1:1 Mendelian segregation ratio. Skewed segregation was detected for 12 loci (5.6% of the total), with ten significant at $P=0.05$ and two at $P=0.01$. The loci affected by these skewed segregations were located on linkage group 6 between markers AF12/6 and AF 8/12, and on linkage group 2 between markers AF9/12 and AF 12/18. A total of 213 loci were assigned to ten linkage groups that putatively corresponded to the ten gametic chromosomes of *T. cacao*. These loci corresponded to 190 AFLPs and 23 microsatellites, with 146 alleles identified as provided by Scavina6 and 67 alleles as provided by the Trinitario genotype H. The total length of the map of (Sca6×H) was 682 cM with individual linkage groups varying between 32 cM and 96 cM. The average distance between markers was 3.2 cM. Only three gaps were larger than 20 cM, the largest gap being 26 cM. Such distribution and density make this map a useful framework for quantitave trait loci identification.

Analysis of resistance values

A statistical analysis of the results of inoculation tests showed that the distributions were close to normality, except for the one concerning *P. megakarya,* for which high scores were less frequent (especially with 309). The general statistics for scores at 6 days are given in Table 1. A comparison of the means of the resistance value of the progeny with that of the clone SCA6, which is known to be highly resistant, revealed a slight improvement of the progeny resistance for *P. megakarya* and *P. capsici*, but for *P. palmivora*; the mean level of resistance in the progeny is lower than for SCA6. The results of the analyses of variance for scores at 6 days showed that there was a significant difference in the level of resistance between the individuals of the progeny, with respect to the three species of *Phytophthora* (Table 2). There was, however, a significant interaction between *Phytophthora* species and the plant. The analysis per species of *Phytophthora* tested, revealed a significant difference between the strains of the same species, with no interaction between strain and plant for the same species. The classification of means by the Newman and Keuls test led to the identification of groups of individuals with different mean resistance levels (18 groups). Spearman rank coefficients of correlation comparing the classifications of individuals for each of the three species, revealed that the results of the tests were positively correlated. The weakest correlation, however, was found between *P. palmivora* and *P. megakarya*. By choosing the individuals most-resistant to *P. palmivora* and applying a 10% selection rate, the level of resistance of these individuals was significantly improved for the two other species; however, this gain was lower for resistance to *P. megakarya* due to the low level of pathogenicity of the 309 strain on this progeny (Table 3).

Table 2 Analysis of variance of the trait "score at 6-days", *df* = degree of freedom, MS = mean square,** significant at 1%

Source	*df*	MS	*F*
Species	2	894	454.8**
Series (species)	1	2,085	2,120.1**
Tray (series)	8	217	27.6**
Strain (species)	3	12	4.4**
Genotype	150	736	4.9**
Species × genotype	281	773	2.8**

A comparison of the 30 most-resistant individuals selected for each species revealed that six trees on average were common, i.e. 20% of the resistant trees. With random-sampling that would have been 5%.

QTL detection

QTLs were sought for each isolate of *P. palmivora*, *P. megakarya* and *P. capsici.* The composite interval mapping-method was tested with two softwares, MapQTL and the QTL Cartographer. The differences between the

Table 3 List of trees (genotype number) selected at a rate of 10% (sorting on *P. palmivora* scores) and scores obtained at 6 days for the two other species *P. megakarya* and *P. capsici*; genetic progress = ratio between average of selected individuals and the average of the progeny

Genotype	*P. palmivora*	*P. megakarya*	*P. capsici*
86	0.50	0.70	0.30
59	0.60	1.33	0.90
98	0.63	0.63	0.30
44	0.69	0.58	0.70
78	0.70	0.83	0.40
17	0.73	0.45	0.30
66	0.73	0.70	0.40
7	0.73	0.53	0.64
92	0.77	0.60	0.60
87	0.83	0.83	0.90
24	0.83	0.60	0.90
16	0.85	0.73	0.90
40	0.85	0.90	0.50
123	0.88	0.70	0.50
Mean	0.73	0.72	0.59
Std. deviation	0.11	0.21	0.24
Genetic progress	47%	21%	42%

two softwares consisted in the method of choice of the cofactors required for the analysis. MapQTL allows chosing the cofactors empirically, whereas QTL Cartographer integrates a stepwise regression analysis to choose the cofactors and determine their relevance and order of importance, but their number can be reduced if necessary. Only results obtained with the QTL Cartographer are shown. These QTLs were found significant with the MapQTL as well.

In total, 13 QTLs were identified. These QTLs were located in six chromosome regions named q*Psp*-1-1, q*Psp*-1-2, q*Psp*-3, q*Psp*-5, q*Psp*-6 and q*Psp*-7, respectively, on linkage groups 1, 3, 5, 6 and 7. QTL Cartographer results are summarized on Table 4 and Fig. 1. Three regions (q*Psp*-5, q*Psp*-6 and q*Psp*-1-2) gather QTLs identified for 2 or 3 species of *Phytophthora*. The highest proportion of phenotypic variation was observed on q*Psp*-5 (R^2=12.4%) for a test with strain TRI3 of *P capsici*. QTLs were identified on this region at a significant threshold for five different strains belonging to the three species of *Phytophthora* (but not for strain 309). The percentage of phenotypic variation explained by each QTL was between 7.5% and 12.4%. Two other regions gathered QTLs detected with two *Phytophthora* species, q*Psp*6 for *P. palmivora* (strains 517 and Tri) and for *P. capsici* (strain MHU 76-2), and q*Psp*-1-2 for *P. palmivora* (strain 517) and for *P. capsici* (strain MHU 76-2). Three other regions, q*Psp*-1-1, q*Psp*-3 and q*Psp*-7 showed QTLs detected only in one species and one strain. For each QTL, the origin (Sca6 or H) of both markers in coupling at each side of the favorable QTL allele were identified. For each QTL, both markers has the same origin (Sca6 or H). This situation allow us identify the probable origin of favorable resistance alleles. Favorable alleles were probably brought by Scavina 6 an Upper Amazon Forastero clone for two QTLs q*Psp*-6 and q*Psp*-3. For the four other QTLs, the favorable alleles were probably brought by the Trinitario genotype H.

Discussion

This study reports the first analysis of genetic control of cocoa resistance simultaneously to three different species of *Phytophthora*. Substantial variability in the level of resistance was detected within the progeny for the three *Phytophthora* species. Data analysis showed that there is little interaction between species and genotype, essentially due to strain 309, and that selection for resistance to a single species (e.g. *P. palmivora*) would provide genetic gains for improving resistance to the other species. For *P. palmivora,* a selection of the best individuals would lead to a genetic gain of 47% for this species, and to 42% for *P. capsici* and 21% for *P. megakarya*. Given the correlation existing between the values of resistance to the three species, breeding cocoa trees resistant to *P. palmivora*, the most widespread species, would also lead to genetic gains with respect to the other species. These results are in agreement with the QTL analyses. Indeed

Table 4 Identification of QTLs for leaf test resistance to *Phytophthora* using composite interval mapping (* favorable allele provided by the Sca6 genotype). Pos: genetic distance in cM from the left marker

Species	Strain	Chromosome region	Interval position	Linkage group	Pos	LOD	Effect	R2 (%)
P. megakarya	NS269	q*Psp*-1-1	AF 10/4–AF 3/4	1	1.1	4.06	−0.36	10.0
		q*Psp*-5	AF 6/14–AF 7/10	5	1.9	3.64	−0.27	9.5
		q*Psp*-7	AF 4/14–AF 5/7	7	0.0	3.12	0.36	8.0
P. megakarya	309	q*Psp*-3	AF 8/20*–AF 12/2*	3	0.4	3.26	−0.25	11.5
P. palmivora	517	q*Psp*-1-2	AF 14/8–AF 10/7	1	0.2	3.18	−0.28	8.0
		q*Psp*-5	AF 6/14–AF 7/10	5	8.9	3.89	−0.26	10.1
		q*Psp*-6	AF 9/5*–AF 9/9*	6	6.9	3.33	0.25	9.1
P. palmivora	Tri	q*Psp*-5	AF 6/14–AF 7/10	5	5.9	2.93	−0.27	7.5
		q*Psp*-6	AF 9/5*–AF 9/9*	6	9.5	3.40	0.24	8.6
P. capsici	MHU76–2	q*Psp*-1-2	AF 14/8–AF 10/7	1	2.2	3.06	−0.47	8.3
		q*Psp*-5	AF 6/14–AF 7/10	5	0.1	3.72	−0.39	10.3
		q*Psp*-6	AF 9/5*–AF 9/9*	6	3.9	3.86	0.27	11.0
P. capsici	TRI 3	q*Psp*-5	AF 6/14–AF 7/10	5	5.9	3.03	−0.41	12.4

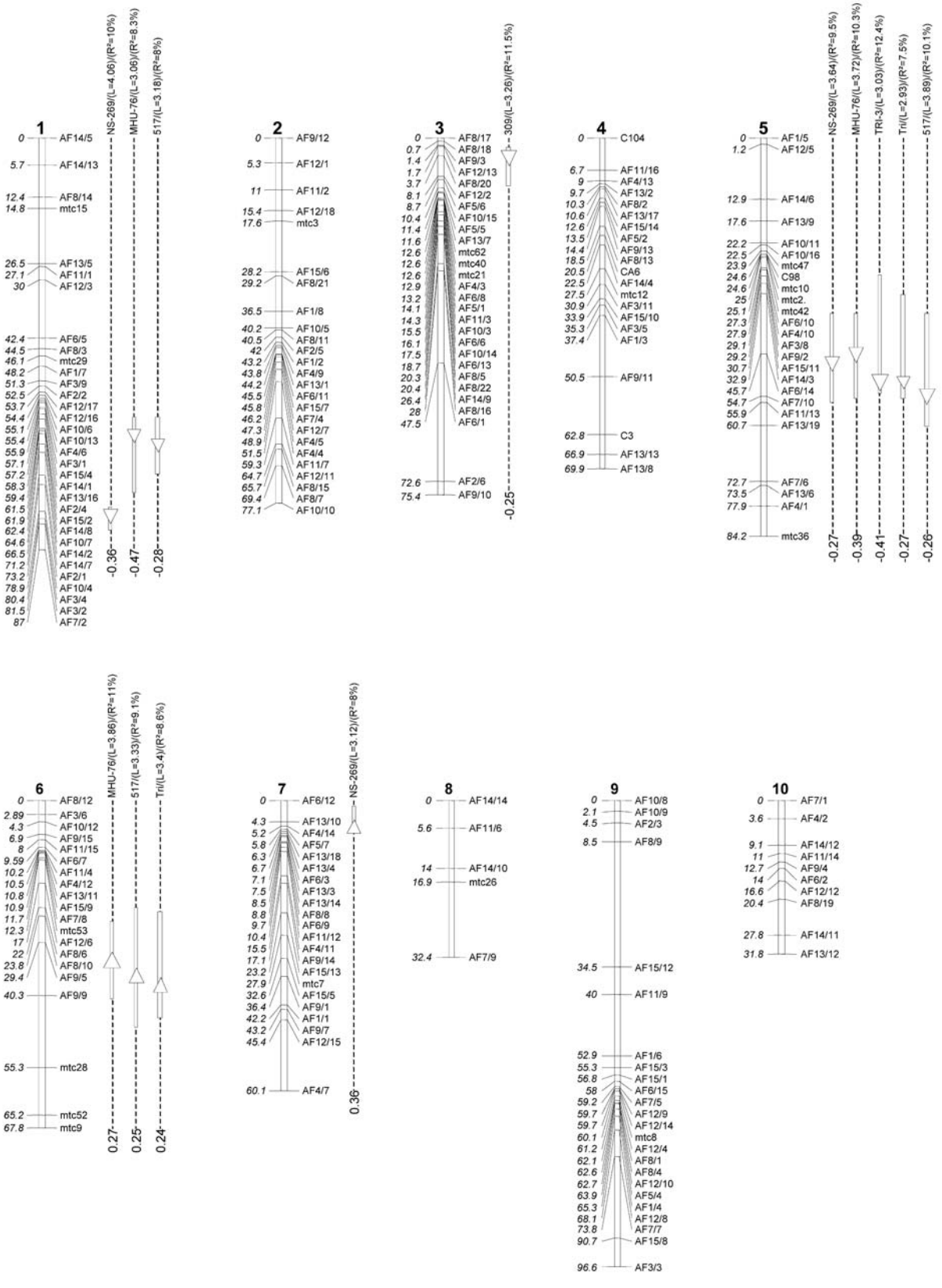

Fig. 1 Molecular marker linkage map of *T. cacao* L. for the cross (SCA6×H)×IFC1. Loci are listed to the right and recombination distances (cM) to the left of each linkage group. Locations of QTLs for leaf test resistance to *Phytophthora* are indicated by *bars* to the right of the linkage groups, strains (NS269, 309, 517, Tri, MHU 76–2, TRI3), L = LOD score and R2% are indicated to the top of each bar, effects are indicated to the bottom of each bar

QTLs for resistance respectively to three and two species were detected in common regions q*Psp*5 and q*Psp*6. Favorable common alleles of resistance to two or three of the species of *Phytophthora* were detected in Sca6 as well in the Trinitario H clones, which belong to two different genetic groups. These results are particularly interesting in the present situation of the progression of *P. megakarya* which is responsible for the largest yield losses in Africa, even though *P. palmivora* is currently dominant in Côte d'Ivoire. Indeed when selecting for resistance to *P. palmivora*, it should be possible to increase the resistance of clones to *P. megakarya*. It could be particularly worthwhile transferring these QTLs into an elite clone through a marker-assisted selection scheme. Other QTLs located on chromosomes 1, 3 and 7 seemed to be more specific to one species of *Phytophthora.*

Some QTLs, identified in this study, were located in the same chromosome regions than QTLs related to *Phytophthora* identified in other studies. That was the case for q*Psp*-3 found for strain 309 of *P. megakarya* on linkage group 3 (even if the global level of pathogenicity of this strain was low). In the same region, near the mTc 21 microsatellite marker, a QTL was found by Flament et al. (2001) with a leaf test performed to study the cocoa resistance to *P. palmivora* of another Forastero clone T60/887 located in Cote d'Ivoire. A study of QTLs related to resistance to *P. palmivora* evaluated by pod tests in a progeny located in Costa Rica by Crouzillat et al. (2000) led also to the identification of some QTLs in the same regions, one on linkage group 7 and one on linkage group 5 (corresponding to LG 1 and LG 9 respectively on the Crouzillat map). In spite of a larger population size in our study, the LOD threshold and phenotypic variance explained by each QTL were not very high. These results due to the large number of genes involved in resistance are comparable to those found in other plants for resistance to *Phytophthora* diseases. Leonard-Schippers et al. (1994) used a leaf test to estimate the resistance of potato to *P. infestans* and found 11 QTLs involved in the resistance; Lefebvre and Palloix (1996) found 13 QTLs of resistance to *P. capsici* in pepper with a test applied to stems and roots.

Genes conferring resistance to the major classes of plant pathogens have been cloned in several species (reviewed in Hammond-Kosack and Jones 1997). In a review made on the organisation of genes controlling disease resistance in the potato genome, Gebhardt and Valkonen (2001) underligned several co-localisations between QTLs and R genes in the potato function-map for resistance. Particularly on chromosome 5 where a cluster of genes for resistance to fungi, virus, insects and nematodes was found in the same region, this also detected the largest effect on quantitative resistance to *P. infestans* (Leonard-Schippers et al. 1994). Pflieger et al. (2001) found similar co-localization between defence-response genes and quantitative disease resistance loci to *P. capsici* in pepper. A polymerase chain reaction based-strategy for isolating putative resistance genes using degenerate PCR primers was initiated on cocoa by Lanaud et al. (2001) and Kuhn et al. (2001). A co-localisation was also observed by Lanaud et al. (2001) between defence genes and QTLs detected in this study on the q*Psp*-5 region, and related to resistance to the three *Phytophthora* species.

The history of race-specific resistance to several species, especially of *P. infestans*, indicates that new races can evolve rapidly enough to render the race-specific resistance ineffective. Because the potential exists for new races to arise that will overcome race-specific resistance, the trend in breeding for resistance is to search for and develop general resistance that is not as vulnerable to attack by new races. In most cases, general resistance is controlled by multigenes, and the actual resistance is rate-limiting and not immunity. The identification of multiple QTLs involved in resistance to *Phytophthora* offers the possibility to improve the durability of resistance in cocoa by a possible cumulation of many different resistance genes located in different chromosome regions using marker-aided selection. The utility of a mapping approach involving the main traits of interest is also to identify the linkage between favorable and unfavorable loci of each trait of interest, and to more-effectively estimate and manage the number of plants needed to select the worthwhile combinations. The marker alleles used for the introgression survey on MAS can be also used for characterization of unrelated gerplasm and finding new sources of resistance.

Acknowledgements We are grateful to Dr. Brigitte Courtois for critical reading of this manuscript, we also thank CAOBISCO (Association of the chocolate biscuit and confectionery industries of the EEC) for their financial participation in these studies. The experiments comply with the current laws of France in which the experiments were performed.

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