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Quantitative trait loci analysis of citrus leprosis resistance in an interspecific backcross family of (*Citrus reticulata* Blanco × *C. sinensis* L. Osbeck) × *C. sinensis* L. Osb

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Abstract Leprosis, caused by citrus leprosis virus (CiLV) and transmitted by the tenuipalpid mite *Brevipalpus phoenicis*, is one of the most important viruses of citrus in the Americas. Sweet oranges (*Citrus sinensis* L. Osb.) are highly susceptible to CiLV, while mandarins (*C. reticulata* Blanco) and some of their hybrids have higher tolerance or resistance to this disease. The mechanisms involved in the resistance and its inheritance are still largely unknown. To study the quantitative trait loci (QTL; quantitative trait loci) associated with the resistance to CiLV, progeny analyses were established with 143 hybrid individuals of 'Pêra' sweet orange (*C. sinensis* L. Osb.) and 'Murcott' tangor (*C. reticulata* Blanco × *C. sinensis* L. Osb.) from controlled crossings. Disease assessment of the hybrid individuals was conducted by infesting the plants with viruliferous mites in the field. The experiment consisted of a randomized completely block design with ten replicates. The evaluated phenotypic traits were incidence and severity of the disease on leaves and branches, for a period of 3 years. The MapQTL^{TM} v.4.0 software was used for the identification and location of possible QTL associated with resistance to CiLV on a genetic map obtained from 260 AFLP and 5 RAPD markers. Only consistent QTLs from different phenotypic traits and years of evaluation, with the critical LOD scores to determine the presence or absence of each QTL calculated through the random permutation test, were considered. A QTL was observed and had a significant effect on the phenotypic variation, ranging from 79.4 to 84% depending on which trait (incidence or severity) was assessed. This suggests that few genes are involved in the genetic resistance of citrus to CiLV.

Keywords Citrus leprosis virus · Disease resistance · Tangor · Sweet orange

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Introduction

Among the viral diseases that affect citrus, leprosis, caused by citrus leprosis virus (CiLV), is considered

one of the most important due to the severe symptoms it can induce in its hosts and due to the high costs of acaricides used to control the mite vector, *Brevipalpus phoenicis* (Acari: Tenuipalpidae). In fact, the presence of the vector in citrus orchards is pivotal to the epidemiology of the disease, since CiLV does not invade its host systemically, remaining only in the typical chlorotic and necrotic lesions it induces in citrus leaves, branches, and fruits (Bassanezi and Laranjeira 2007). These symptoms, associated with severe fruit and leaf drop, can lead to a significant reduction in the production and quality of the fruits (Rodrigues et al. 2003).

In Brazil, this disease was first reported and characterized in 1931 (Bitancourt 1955). There are previous reports of leprosis in Florida, USA; however, the disease has not been found in that country for decades (Childers et al. 2003a). Currently, leprosis is restricted to several countries in South and Central Americas and has reached the south of Mexico (Bastianel et al. 2006a).

Three species of *Brevipalpus*, which are cosmopolitan and polyphagous mites found on more than 900 host plants (Childers et al. 2003b), are reported as vectors of CiLV. In Brazil, *B. phoenicis* has been associated with leprosis since the beginning of the 1960s (Mussumeci and Rosseti 1963), while *B. californicus* and *B. obovatus* were considered vectors of the pathogen in the United States and Argentina, respectively (Knorr 1968; Vergani 1945).

There are two types of CiLV, an extremely rare nuclear form of the virus that accumulates in the nucleus of infected cells and produces intranuclear viroplasm and virions (CiLV-N), and the prevalent cytoplasmic form (CiLV-C) responsible for more than 99% of all of the leprosis reports in the world (Kitajima et al. 1974; Locali et al. 2003; Rodrigues et al. 2003; Bastianel et al. 2006a). Because of its morphology and its presence in the cytoplasm or in the nucleus of infected cells, CiLV has been considered a tentative member of the *Rhabdoviridae* family; however, recent data on its genome completely exclude it from this family and suggest that CiLV be the type member of the new genus *Cilevirus* (Locali-Fabris et al. 2006).

There is very little information on the genetic resistance of the *Citrus* genus to CiLV. All the sweet orange cultivars are known as highly susceptible to leprosis (Rodrigues et al. 2000). Resistance to CiLV-C has been found in little mandarins and some of its hybrids (Rodrigues et al. 2003; Bastianel et al. 2006a; Bastianel et al. 2008). However, for a thorough resistance/inheritance study, a great number of plants in a segregant population for this trait is necessary.

Molecular markers have been widely used in citrus breeding to identify zygotic hybrid individuals produced from controlled crossings and for genetic mapping, enabling the study of several traits of agronomical importance, such as disease and pest resistance (Cristofani et al. 1999; Ling et al. 2000; Asíns et al. 2004; Bernet et al. 2005; Siviero et al. 2006; Chen et al. 2008).

In this study, we report the localization of quantitative trait loci (QTLs) in a linkage map using AFLP (amplified fragment length polymorphisms) and RAPD (random amplified polymorphism DNA) markers developed from 143 hybrid individuals obtained from crossings between a resistant and a susceptible parent, 'Murcott' tangor (*C. reticulata* L. Blanco × *C. sinensis* L. Osb.) and 'Pêra' sweet orange (*C. sinensis* L. Osb.), respectively. These plants were established in the field, infested with CiLV-C-viruliferous mites, and evaluated for the incidence and severity of leprosis for 2 or 3 years.

Materials and methods

Plant material

A controlled crossing between a 'Murcott' tangor (female parent) and a 'Pêra' sweet orange (male parent) was conducted in the spring of 1997, and the selection of individual zygotic plants was conducted with RAPD and SSR molecular markers based on standard procedures (Litt and Luty 1989; Willians et al. 1990) with modifications described by Machado et al. (1996) and Oliveira et al. (2000), respectively. Almost 2,000 young plants were used to select a total of 312 hybrids by morphologic, RAPD and SSR markers (Oliveira et al. 2000, 2002). Of them, 143 plants were chosen randomly to compose the progeny for the mapping study. They were then grafted onto Rangpur lime (*C. limonia* L. Osb.) and evaluated in the field with the clones from the parental plants for 3 years under a randomized completely block design with ten replicates each for a total of 1,450 plants.

The experiments were conducted at the Centro APTA Citros/IAC, Cordeirópolis, SP, Brazil.

Virus inoculation

A non-viruliferous population of *B. phoenicis* was reared on sweet orange fruits according to standard procedures (Bastianel et al. 2006b). For viral acquisition, the individuals were transferred and kept for 48 h on leaves of sweet orange exhibiting initial symptoms of leprosis. Afterwards, viruliferous mites were transferred to the experimental plants maintained in the field at a rate of 30 mites per plant by attaching a symptomatic leaf with mites to the plants. All plants were infested approximately 6 months after grafting.

Phenotypic assessment

Disease incidence (percentage of leaves with lesions) and severity were evaluated after 1 and 3 years of viruliferous mites' infestation. Two scales were used to express severity, as described in Bastianel et al. (2006b). A diagrammatic scale of scores expressed in percentage of the foliar area affected with leprosis (0.39, 2, 6, 16.5 and 39.5%; Rodrigues et al. 2002), and a descriptive scale, based on Rodrigues (2000), with scores from zero to five (0 = no lesions; 1 = few lesions restricted to a particular region of the plant; two = lesions in more than one plant tissue and/or distributed in more than one sector of the plant; three = abundant lesions throughout the plant; four = abundant lesions throughout the plant, and leaf and/or fruit drop; and five = four plus branches die back). Assessments with the descriptive scale were performed yearly, for 3 years period.

AFLP and RAPD markers

The reactions for AFLP markers were prepared using the AFLP Plant Mapping kit (Applied Biosystems), according to instructions from the manufacturer for species whose genome size is between 500 and 6,000 Mb, with primers labeled with fluorophores specific for the ABI Prism 377 Automatic Sequencer, with few modifications (Oliveira et al. 2007). RAPD markers were obtained based on Machado et al. (1996), who used random decamer primers (Operon). The chi-square test [χ^2 ($P \leq 0.05$, DF = 1 or 3)] was used to test the Mendelian segregation hypothesis of 1:1 and 3:1 for each of the AFLP and RAPD markers. Only those markers that did not show a skewed segregation ($P > 0.05$) were used to construct the linkage maps.

Linkage map and QTL localization

The genetic map was obtained using the JoinMap software v.3.0 (Van Ooijen and Voorrips 2001) through the simultaneous analysis of markers that had 1:1 and 3:1 Mendelian segregations. The 3:1 type segregation markers were used as a linkage bridge to generate the integration of 1:1 markers for both parents. The map was obtained with LOD $\geq$ 3.0 and a recombination frequency (Max. θ) $\leq$ 0.40. The Kosambi function (Kosambi 1944) was used to convert the recombination fractions into centiMorgans (cM).

Seven phenotypic data, that is, four evaluations done for 2 years (for incidence and severity using a diagrammatic scale) and three evaluations done for 3 years (severity using descriptive scale), were used for the QTL Mapping through the MapQTL^{TM} v.4.0 software (Van Ooijen et al. 2002). Each variable obtained for the response to the disease was used separately to localize the genomic regions associated with the resistance to CiLV-C. Those reproducible QTLs found over different years of evaluation with significant LOD scores were considered consistent for the analysis.

The non-parametric test Kruskal–Wallis methodology and the Interval Mapping (IM) were initially used for all molecular markers and for the mean of each trait according to the MapQTL^{TM} computer program (Van Ooijen et al. 2002). Multiple QTL Mapping (MQM) was applied, where the co-factors were chosen based on the selection mode (the automated cofactor selection package) and the percentage of explanation values of the markers flanking the QTLs detected by IM and MQM mapping. The critical LOD score value to determine the presence or absence of each QTL was calculated through the random permutation test (1,000 repetitions) (Churchill and Dodge 1994). Both strategies were performed with the MapQTL^{TM} v.4.0 software.

Results

Response of the hybrids individuals to leprosis in the field

No typical leprosis symptoms were observed in 'Murcott' tangor 3 years post-infestation with viruliferous mites. On the other hand, all 'Pêra' sweet orange showed typical leprosis symptoms, of which more than 50% died due to the high incidence and severity of the disease. Fifteen hybrids were resistant to leprosis and remained asymptomatic throughout the experiment, even under high CiLV-C inoculum pressure in the field. One hundred and twenty-eight hybrid individuals presented different levels of symptoms. RT-PCR assays detected CiLV-C in the samples of mites, including those collected in 'Murcott' tangor. A study of the phenotypic response of the hybrid individuals to leprosis in the field and the phenotypic distribution for traits related to the disease was previously presented (Bastianel et al. 2006b).

Linkage map construction

The segregation analysis of the markers in the progeny revealed 89 AFLP markers and 16 heterozygous RAPD for the 'Murcott' tangor, 122 AFLP and 11 heterozygous RAPD for 'Pêra' sweet orange with a 1:1 Mendelian segregation in the population, and 205 AFLP and six heterozygous RAPD for both parents, which segregated as markers at the 3:1 ratio in the population. The linkage analysis conducted through JoinMap software detected 265 linked markers distributed in ten LGs, with 57 heterozygous loci for 'Pêra' sweet orange, 46 heterozygous loci for the 'Murcott' tangor, and 162 heterozygous loci for both parents, with 44.96, 57.3 and 73.5% of the total mapped markers, respectively (Fig. 1). Only linkage groups with more than three markers were considered for the map generated.

QTLs mapping

The Krustal–wallis test assessed the hypothesis of association of each of the polymorphic markers and the mean of the phenotypic traits obtained. More than one marker was found linked to all phenotypic traits and they were localized on the linkage groups III and VII of the genetic map (Table 1).

The analysis through the IM, MQM, and Krustal–Wallis methods confirmed the presence of two QTLs in the interval closest to one of the markers detected for all of the phenotypic traits on linkage groups III and VII. However, the permutation test (1,000 repetitions) detected a critical LOD score that confirms the presence of one reproducible QTL (over the 3 years and with the evaluated phenotypic traits) only in the group III. This should be considered a major QTL, which explains 79.4–84% of trait variance, depending on which trait is measured (Table 2). The identified QTL was localized closest to the M-CAA/E-ACT92 AFLP marker, which was derived from the resistant 'Murcott' tangor parent (Fig. 2).

Discussion

The breeding program and the study of disease resistance of citrus have shown considerable progress since the 1990s due to the association of biotechnology tools with conventional breeding (Cristofani et al. 1999; Ling et al. 2000; Asíns et al. 2004; Bernet et al. 2005; Chen et al. 2008; Rao et al. 2008). Unquestionably, this association has been essential to understand the genetic mechanisms involved in the resistance to complex pathosystems such as citrus leprosis. Despite the importance of leprosis for the citrus industry in the Americas, very little is known about heritability of resistance to CiLV in such host. Few studies that have been carried out to date with the objective to assess citrus resistance to CiLV (Bastianel et al. 2006a, 2008). The large majority of these reports the behavior of different varieties in the field under natural conditions of mite infestation, and only few of them compare levels of severity. One of the challenges in the studies of inheritance of disease resistance is related to the standardization of the phenotypic evaluation. Leprosis symptomatology shows a progressive evolution with typical lesions on leaves, branches, and fruits, which require a special evaluation system so that temporal and spatial conditions do not affect the results (Bastianel et al. 2006b).

Therefore, to ensure such conditions, more than one characteristic was assessed to indicate the incidence and severity of the disease at different periods post-infestation with viruliferous mites (percentage of the leaves with symptoms, diagrammatic and descriptive scales for severity).

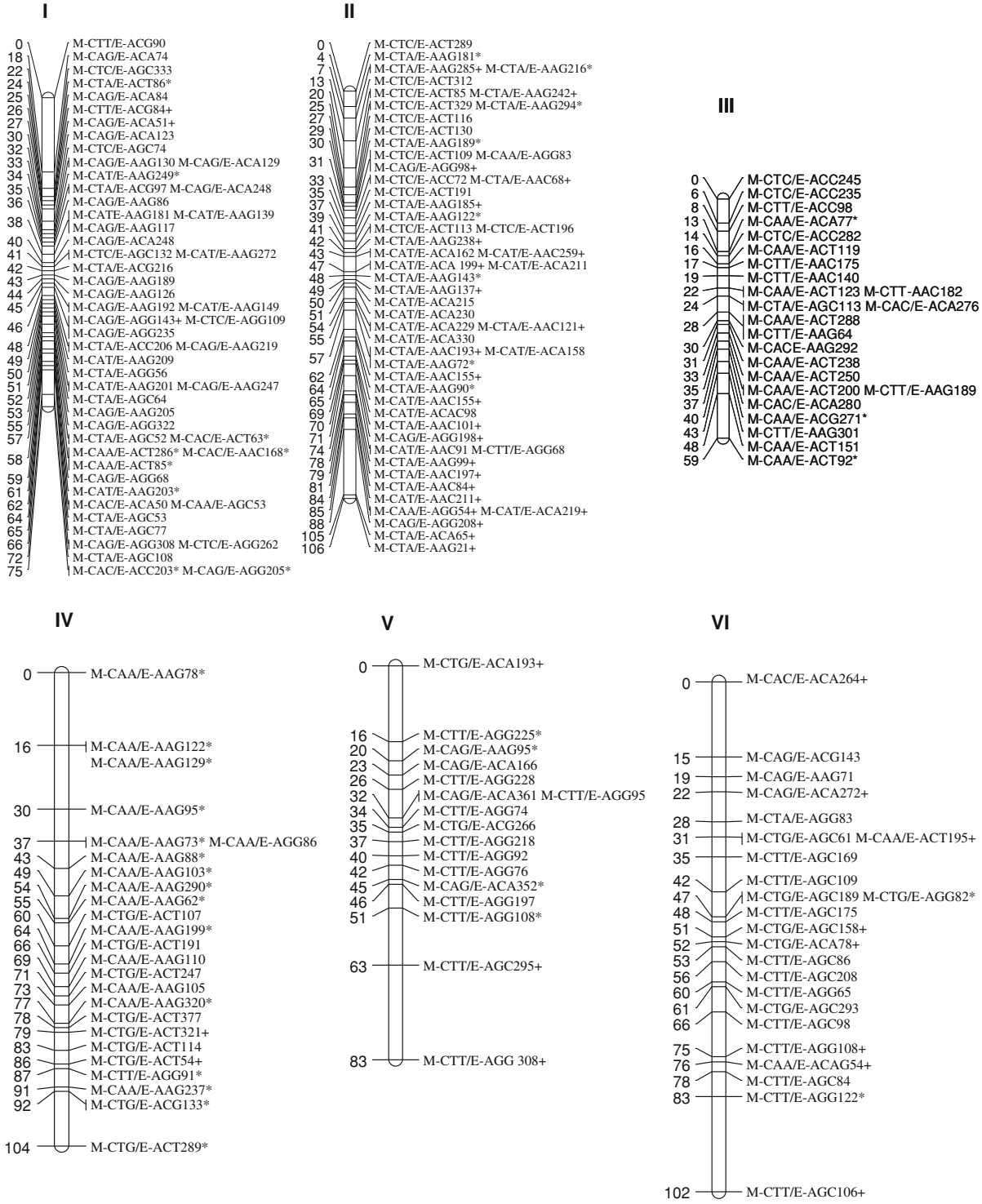

Fig. 1 Genetic map constructed with 265 AFLP and RAPD markers in a population of 143 hybrid individuals from crossing between 'Murcott' tangor and 'Pêra' sweet orange. On the *right* of the linkage groups are all markers that showed co-segregation; on the *left*, the distance in centiMorgans (cM) for each pair of markers. "+" 'Pêra' sweet orange markers (1:1), "*" 'Murcott' tangor markers (1:1)

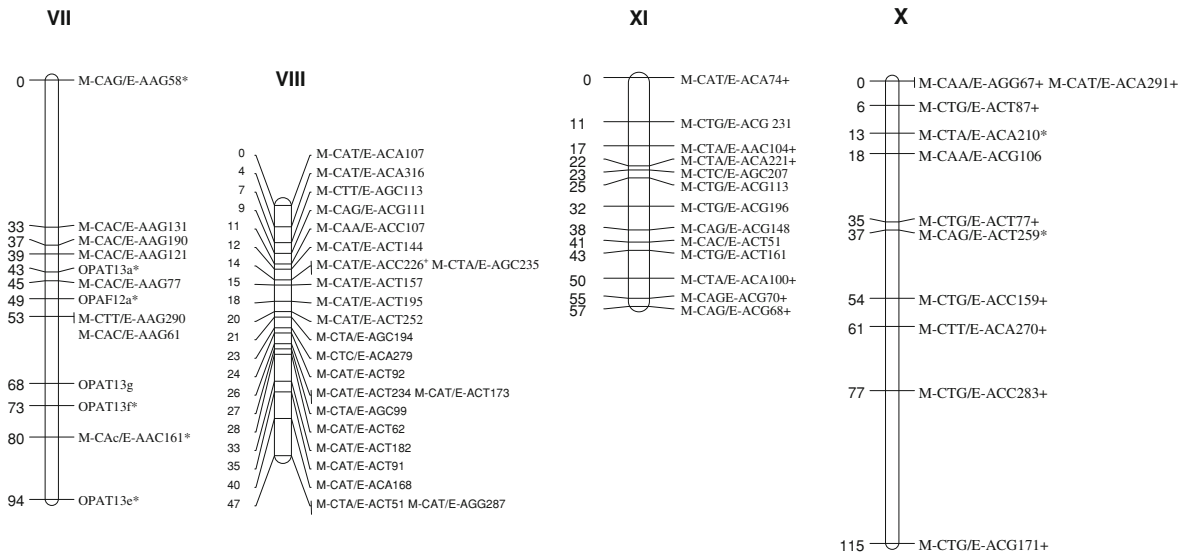

Fig. 1 continued

Table 1 The most significant AFLP and RAPD markers established through the Krustal–Wallis analysis associated with the phenotypic characteristics studied

Characteristic (year of the evaluation)	Marker (linkage group III)	Marker (linkage group VII)
Percentage of leaves with lesions (year 1)	M-CAA/E-ACT151*	OPAT13a**
	M-CAA/E-ACG271**	OPAF12a**
	M-CTC/E-ACC245*	OPAT13f**
Diagrammatic scale (year 1)	M-CTT/E-ACC245**	OPAT13a***
	M-CAA/E-ACT151**	OPAF12a**
Descriptive scale (year 1)	M-CTC/E-AAC175*	OPAT13a***
	M-CTC/E-AAC182*	OPAF12a**
	M-CAA/E-ACT151**	OPAT13f**
		M-CAC/E-AGG58*
Descriptive scale (year 2)	M-CTT/E-AAG64*	OPAT13a**
	M-CTC/E-AAC182*	OPAF12a*
	M-CAA/E-ACT151*	OPAT13f*
		M-CAC/E-AGG58*
		M-CAC/E-AAC161*
Descriptive scale (year 3)	M-CAA/E-ACT151*	OPAT13a**
	M-CAA/E-ACG271*	OPAF12a**
	M-CTC/E-ACC245*	OPAT13f**
Percentage of leaves with lesions (year 3)	M-CTC/E-ACC245**	OPAT13a***
	M-CAA/E-ACT151**	OPAF12a**
Diagrammatic scale (year 3)	M-CTC/E-AAC175*	OPAT13a***
	M-CTC/E-AAC182*	OPAF12a**
	M-CAA/E-ACT151*	OPAT13f**
		M-CAC/E-AGG58*

* Level of significance 0.1

** Level of significance 0.05

*** Level of significance 0.01

Table 2 Details of the major quantitative traits loci (QTLs) identified for resistance to citrus leprosis virus, detected on group III with indication of map position (over two LOD support intervals), maximal LOD scores observed in the 3 years of investigation and percentage of explained variance

Characteristic (year of the evaluation)	Interval (cM)	LOD	Critical LOD	Variance	% Expl.
Percentage of leaves with lesions (year 1)	47.7–59.3	14.74	12.9	0.27	83.0
Diagrammatic scale (year 1)	47.7–59.3	11.49	9.2	94.25	79.4
Descriptive scale (year 1)	47.7–59.3	11.67	9.9	0.67	84.0
Descriptive scale (year 2)	47.7–59.3	11.93	11.0	0.13	79.8
Descriptive scale (year 3)	47.7–59.3	14.74	12.6	0.27	83.0
Percentage of leaves with lesions (year 3)	47.7–59.3	11.49	9.1	94.25	79.4
Diagrammatic scale (year 3)	47.7–59.3	11.67	10.1	0.67	84.0

In a previous report, we demonstrated that incidence, descriptive and diagrammatic scale measurements together showed high correlation with resistance to leprosis, allowing the successful identification of resistant hybrids after challenge with CiLV-C (Bastianel et al. 2006b). High disease incidence leads to a greater disease severity, which indicates that the response of the plant genotype is associated to the primary common events of the infection by the virus. Based on the descriptive and diagrammatic scales, it was possible to correlate higher percentage of symptomatic leaves with the occurrence of symptoms in branches and with higher disease severity. However, there was no correlation between the number of mites and the occurrence of leprosis symptoms in the field, which suggests that the genotypes evaluated did not show a differential response to the colonization of the vector (Bastianel et al. 2006b). Therefore, as suggested by Rodrigues (2000), different mechanisms of resistance to the virus and to the colonization of the mite might be involved in the leprosis pathosystem.

A recent study showed that CiLV-C infection induced immediate and subsequent changes in gene expression by the host and that the infection has the potential to give advance signaling of the imminent infection (Freitas-Astúa et al. 2007). The fact that CiLV-C causes only chlorotic and necrotic local lesions in susceptible hosts and never invades them systemically (Marques et al. 2007), has raised questions regarding whether or not the symptoms could be a variation of the hypersensitive response (HR) observed in viral-incompatible interactions. However, Freitas-Astúa et al. (2007) suggested that the two responses are very different at the molecular level and hence, the manifestation of leprosis symptoms should not be considered as a HR. Other genetics mechanisms seem to be involved in the response to citrus leprosis, a very complex pathosystem.

In this manner, the construction of saturated genetic maps is an important step in the understanding of genetic inheritance of the resistance against diseases and it has been an important tool to help programs of citrus breeding. The challenges of the conventional breeding of this group of woody perennial plants are enormous and lead to the use of new tools for the study of variability and inheritance of the resistance to biotic and abiotic factors. Thus, linkage maps have been constructed specially to identify regions associated to QTLs. However, so far, most of the maps produced for citrus have been originated from RFLP and RAPD markers. Recently, the development of microsatellites based on EST libraries brought new perspectives for the genetic research, including studies of genetic variation, gene tagging and evolution, and mapping and analysis of QTL (Chen et al. 2006; Palmieri et al. 2007; Chen et al. 2008; Rao et al. 2008).

This study was undertaken to construct genetic maps for 'Murcott' tangor and 'Pêra' sweet orange, with AFLP and RAPD markers evaluated in an F_1 population originated from this crossing. Our results showed that AFLP markers can be used to generate DNA fingerprinting and to construct linkage maps for citrus. AFLP markers are considered more efficient than other classes of markers such as RFLP and RAPD (Powell et al. 1996) and have been used even more to construct maps of different species (Saliba-Colombani et al. 2000).

The high number of loci found segregating in a 3:1 ratio (73.5%) in the progeny indicates a high genetic similarity among the parents. In fact, 'Murcott'

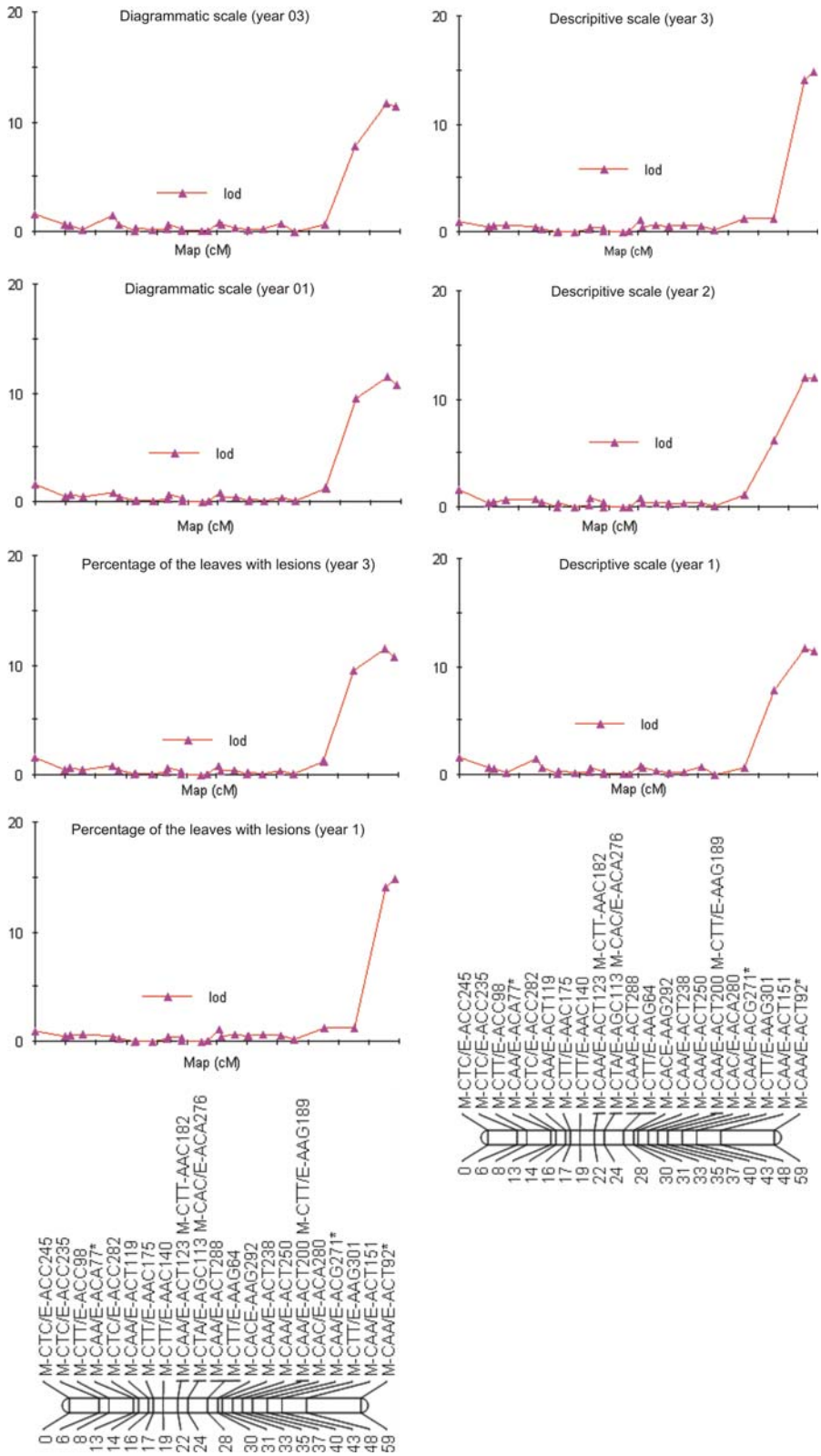

Fig. 2 QTL linked to the inheritance of the resistance to citrus leprosis virus in the linkage group III detected close to the 'Murcott' tangor (M-CAA/E-ACT92) marker for the seven phenotypic traits measured. On the *right* of the linkage groups are all markers that showed co-segregation; on the *left*, the distance in centiMorgans (cM) for each pair of markers

tangor is a hybrid of *C. reticulata* and *C. sinensis* (Araújo et al. 2003). Therefore, the hybrid population of this study could be considered from a backcross. On the other hand, the taxonomical complexity found in citrus suggests that *C. sinensis* is, also, an ancestral hybrid from *C. reticulata* (Barret and Rhodes 1976; Araújo et al. 2003). This fact supports the probability of a degree of relationship between the parental progenitors used in this study.

Using the Joinmap software, the integrated map was produced through the analysis of 443 AFLP and RAPD markers, with 3 segregation patterns found (lm × ll, nn × np, and hk × hk). In this case, heterozygous loci for both parents that showed a 3:1 segregation ratio were used as "bridges" to integrate markers. All linkage groups (LG) of the citrus map contain markers common to 'Pêra' sweet orange and 'Murcott' tangor, which results in the integration of the parental maps. The heterozygous markers in both parents showed uniform distribution in the LG of the integrated maps (Fig. 1). The linkage analysis found 265 markers linked to the LG in the integrated map. Non-linked markers are frequent in citrus mapping. Luro et al. (1994), by using isoenzymatic markers, RFLP and RAPD, for 53 citrus hybrids, found approximately 13% markers not linked to any linkage group, while Cai et al. (1994), using RFLP and RAPD markers for *C. grandis* and *Poncirus trifoliata* hybrids, were able to find 28.9% not-linked markers, similarly to what was found in the present study.

According to Garcia et al. (2006), an integrated map comprising different types of molecular markers (RFLPs, RAPDs, SSRs, and AFLPs) should bring several advantages such as increasing saturation, extending the characterization of polymorphic variation throughout the entire genome. In addition, the location of QTLs is facilitated if an integrated map is available (Maliepaard et al. 1998). However, in heterozygous parents, for each segregating locus, it is possible to find different numbers of segregating alleles or markers. This situation complicates linkage analysis and mapping once parental linkage phases of marker pairs are unknown a priori, which makes the detection of recombination events difficult (Maliepaard et al. 1997; Wu and Ma 2002).

According to Maliepaard et al. (1998), the integrated map combines markers segregating in one or the other parent with those segregating in both parents. For marker pairs heterozygous in both parents, the combined recombination frequency estimate is an average over the recombination frequencies in the male and the female meioses. This combined estimate may differ from the single-parent estimates and thereby cause a changed marker order in the integrated map in comparison with the single-parent map. Since differences in the estimated distances of both maps may reflect real differences in the recombination frequencies of both parents, these can best be presented separately. Both parental maps can be used separately to investigate QTLs segregating from a single parent. However, if QTLs may be present in both parents and for studying the different allelic combinations at QTLs, it is better to use the integrated map with an all-marker mapping approach (Maliepaard and Van Ooijen 1994; Maliepaard et al. 1998).

In the present study, the results obtained through the MQM suggest a QTL with a high LOD score value (LOD > 9.1). This explains 79.4–84% of disease severity variance (Table 2; Fig. 2). This QTL was found at the same region of the linkage group III for all the phenotypic traits related to the disease (percentage of leaves with lesions, descriptive and diagrammatic scales of severity) studied and in the 3 years of evaluation, thus indicating a consistent QTL. The fact that the same QTL was detected for all studied phenotypic traits suggests that any one of them can be used in studies of this nature. However, despite the fact that diagrammatic scales are more often used to assess disease in field experiments, in the particular case of leprosis, the descriptive scale can be easier to use in field evaluations of a great number of plants. In addition, it has proved to be efficient in estimating disease intensity, since it takes in consideration the presence of lesions in all aerial tissues of the plants.

We discussed in a previous analyses that all phenotypic traits presented values for the coefficient of clonal repeatability close to those found for qualitative characters, for the evaluations carried out 1 year after inoculation (Bastianel et al. 2006b). Moreover, the analyses from the frequency of average values for all traits showed distinct phenotypic classes in their distributions, which is commonly observed for phenotypic traits controlled by one or few genes.

Traits of high heritability have a simple genetic control and they are probably controlled by a lower number of genes. Thus, for these characteristics, there is a greater probability of detecting the QTLs. Our results suggested that a gene of a great effect might

be involved in the leprosis resistance in the 'Murcott' tangor. However, it cannot exclude the possibility of other genes with lower effect to be involved in the resistance heritability as well.

In citrus, genetic mechanisms of mono- and oligogenic inheritance have been reported for resistance to other pathogens such as the nematode *Tylenchulus semipenetrans* (Ling et al. 2000) and the *Citrus tristeza virus* (Cristofani et al. 1999). For citrus leprosis, the mechanisms for genetic inheritance were recently studied. Here, we report the first analysis of QTLs for this complex pathosystem, and further studies are in progress to completely elucidate the genetic mechanisms involved in the disease. A network with the citrus hybrid populations was established in two field trials in traditional areas of citrus cultivation in the state of São Paulo, where leprosis is endemic. Those plants will be assessed phenotypically using the descriptive scale of notes for validation of the QTL found in the present study and concomitantly for other agronomic traits of interest, such as fruit productivity and quality.

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