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Genetic analysis of citrus leafminer susceptibility

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Abstract Damage caused by the citrus leafminer (CLM), *Phyllocnistis citrella*, is highly dependent on the citrus flushing pattern. Chemical control is only required in young trees, both in nurseries and in newly established orchards. However, this situation is completely different in countries where the causal agent of citrus canker, the bacterium *Xanthomonas axonopodis* pv. *citri* exists. CLM infestation results in a higher incidence of citrus canker infection. Among preventive control strategies that provide environmentally sound and sustainable solutions, resistant or tolerant varieties remain the most economical means of insect control. The objective of the present study is to genetically analyse the resistance/susceptibility to CLM and two other traits that might be related, the deciduous behaviour and leaf area of the tree, in a progeny of citradias derived from the cross between two species with different CLM susceptibility—*C. aurantium* L. and *Poncirus trifoliata*—using linkage maps of each parent that include several resistance gene analogues. We detected two antibiosis and six antixenosis putative quantitative trait loci (QTLs) in a random sample of forty-two of those citradias. An important antibiosis QTL ($R^2 = 18.8–26.7\%$) affecting both percentage of infested leaves and number of pupal casts per leaf has been detected in *P. trifoliata* linkage group Pa7, which is in agreement with the CLM antibiotic character shown by this species, and independent from any segregating QTL involved in its deciduous behaviour. The maximum value for the Kruskal-Wallis statistic of the other putative antibiosis QTL coincides with marker S2-AS4_800 in sour orange linkage map. Given that the sequence of this marker is highly similar to several nucleotide binding site-leucine-rich repeat (NBS-LRR)-type resistance genes, it might be considered as a candidate gene for insect resistance in citrus.

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Introduction

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), is a pest native to southern Asia that colonized leafminer-free citrus growing areas in both the Mediterranean Basin and the Americas during the last decade of the 20th century (Urbaneja et al. 2001). As *P. citrella* oviposits on very young citrus leaves (5–45 mm long; Garrido and Gascòn 1995), its damage is highly dependent on the presence of young shoots and, consequently, on citrus flushing patterns. Larvae bore a serpentine mine under the leaf cuticle and feed on the epidermal cell layer of still-developing leaves (Garrido et al. 1998). *P. citrella* has four larval instars. The fourth-instar larvae spin a cocoon, which is usually protected by folding the leaf edge, in which pupation takes place (Garrido et al. 1998). Affected leaves dry and break easily, and may fall prematurely. Although damage is conspicuous, leaf-area losses are not considered to be important in adult bearing trees (García Marí et al. 2002), and chemical control is only required in young trees, both in nurseries and in newly established orchards. However, this situation is completely different in countries where the causal agent of citrus canker, the bacterium *Xanthomonas axonopodis* pv. *citri* exists (EPPO 2003a). Mines caused by *P. citrella* provide abundant wounding on new flushes which dramatically increases the amount of leaf mesophyll tissues exposed to *X. axonopodis* pv. *citri* spores. This scenario results in a higher incidence of citrus canker infection (Sohi and Sandhu 1968; Sinha et al. 1972). The effect is further magnified because the

mines become naturally infected at much lower inoculum concentrations than via the stomata route (Goto 1992), the natural entry of this bacterium in citrus (EPPO 2003b). The interaction between CLM infestation and citrus canker has become immediately apparent in locations where the disease existed prior to the introduction of the CLM (Gottwald et al. 2002). Wounds caused by the CLM remain susceptible for 7–14 days compared to only 24 h for wounds caused by wind, thorns or pruning. Furthermore, infection through wounds requires only 1/100 to 1/1,000 of the inoculum dose required for infection through stomata openings (Bergamin-Filho and Hughes 2002). As a consequence, in the state of São Paulo (Brazil), where the leafminer was first reported in 1996, the number of disease foci increased from 25 in 1995 to 4,180 in 1999 (Bergamin-Filho et al. 2002), and the dispersal function of the disease changed (Bergamin-Filho et al. 2000).

Chemical control of the CLM usually lasts only a short period of time. Furthermore, studies have shown that *P. citrella* can develop a high degree of resistance to a broad range of insecticides (Villanueva-Jiménez and Hoy 1998). Therefore, it is essential to develop alternative management strategies for leafminers. Preventive control strategies that provide environmentally sound and sustainable solutions for pest control while keeping production and income levels as high as possible form the basis of integrated pest management (IPM) programmes. As such, resistant varieties remain the most economical means of pest control. Their use reduces the costs of chemicals, machine, fuel and labour associated with pesticide spray while also decreasing possible exposure to hazardous chemicals and pesticide contamination of soil and ground water, thereby alleviating pressure on the environment.

Breeding for resistance against pests would be a new tool in integrated citrus production. To date, very little is known about plant resistance to CLM. Differential susceptibility among varieties has been found in India (Batra and Sandhu 1983), Australia (Wilson 1991), Spain (Jacas et al. 1997) and elsewhere (Heppner 1993). In some of these studies, the existence of antibiotic mechanisms of resistance has been indicated (Batra and Sandhu 1983; Batra et al. 1984; Jacas et al. 1997), while in others, a plant phenology leading to *P. citrella* avoidance has been described (Singh et al. 1988; Padmanaban 1994; Jacas et al. 1997). Although complete resistance against *P. citrella* has been recorded in only two citrus-related species (Fletcher 1920; Jacas et al. 1997), laboratory experiments with different citrus hybrids have revealed the existence of significant differences in oviposition behaviour and larval viability among them (Jacas et al. 1997). Insect resistance in these hybrids is inherited as a quantitative trait, but evaluation of resistance to insects in citrus is labour-intensive and time-consuming. Therefore, conventional citrus breeding strategies for pest resistance are not affordable.

Molecular markers have been used to study the quantitative inheritance of resistance to insects in several crops species, including maize, tomato, potato, rice, barley and soybean (Rector et al. 1999; Yencho et al. 2000) but not in citrus. Quantitative trait loci (QTLs) analysis can also be used to study the inheritance of other quantifiable traits that might be related to the biochemical and/or physical mechanisms of insect resistance, thereby increasing understanding of the genetic and physiochemical mechanisms of plant defence. The objective of the study reported here was to genetically analyse the resistance to CLM and two other traits that might be related—the deciduous behaviour and the leaf area of the tree—by using the linkage maps of each parent (Asins et al. 2004), both of which include several resistance gene analogues (RGA) in a progeny derived from the cross between two species with different susceptibility to CLM, *C. aurantium* L. and *P. trifoliata* (L.) Raf.

Materials and methods

Stock colonies: plant and insect production

Rearing techniques are described by Urbaneja et al. (1999). Colonies of *Phyllocnistis citrella* were initiated with insects collected from citrus plants grown in commercial orchards around the city of Valencia (eastern Spain). Field-collected insects were regularly added to the colonies. Voucher specimens were deposited at the Insect Collection of the Instituto Valenciano de Investigaciones Agrarias (IVIA). Insect colonies and plants used in this study were maintained in a glasshouse at 25 ± 5°C and 60 ± 10% relative humidity. No artificial light was supplied.

Citrus trees were pruned to obtain homogeneous young leaf flushes. The optimal state for introducing these plants into the *P. citrella* rearing units was attained when the oldest leaves of the new flushes measured about 4 mm in length. Plants used for the routine rearing of the leafminer were 2-year-old potted sour orange trees.

Adult moths for the infestation of new plants were obtained from a continuous culture. Groups of four plants were introduced weekly into a screened cage (80×110×120 cm) where rearing took place. The plants were left undisturbed for 3 weeks. This long period allowed adults to emerge within the cage so that artificial re-infestation was not necessary. Adult moths were fed a mixture of honey and water (1:3) that was sprayed directly onto the plants. Adults were collected as needed and used in the assays.

Experimental plants and their infestation

Part of a segregating population consisting of 42 randomly chosen hybrids was used for infestation experiments with *P. citrella*. These hybrids were 3 years old and belong to a family of 104 hybrids derived from the

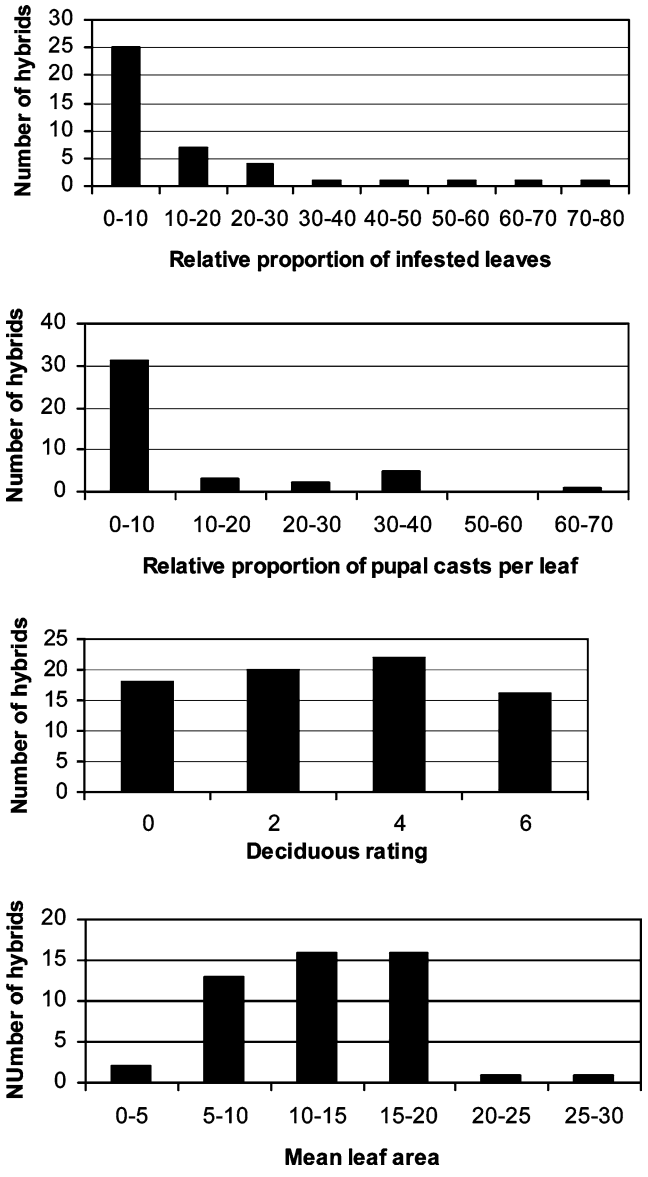

Fig. 1 Distribution of trait values

cross between sour orange (*Citrus aurantium* L.) and trifoliate orange (*Poncirus trifoliata*) that was used to construct the linkage map reported by Asins et al. (2004).

Experimental plants were exposed to approximately 250 moths in cages (same dimensions as above). Three cages were set up simultaneously and hybrids were randomly assigned to any of them. Two control plants were included in every cage: Afin Verna sour orange, the female parent of the progeny, as the susceptible control, and *P. trifoliata* cv. Flying Dragon, the male parent, as the tolerant control. One day later, plants were removed from those cages and kept undisturbed for about 21 days. By then, most *P. citrella* had completed their development and abandoned the mines. At this time, all of the leaves were collected and classified as infested or uninfested, and the number of pupal casts on infested leaves was counted. These results were later expressed as a percentage of those registered on their respective susceptible control tree.

QTL analysis

We used the linkage maps for *P. trifoliata* based on 63 markers and for *C. aurantium* based on 157 markers, as reported by Asins et al. (2004), with the only exception being linkage group (LG)A (7+3+4) (LOD 6.0) that was considered here to be three separate groups, identified at LOD of 8.0, 10.0 and 10.0, respectively. Most of the molecular markers were microsatellites and inter-retrotransposon amplified polymorphisms (IRAP). Some RGAs and expressed sequences were also included for candidate gene analysis. Polymorphic loci were distributed on 11 and 9 linkage groups in the *C. aurantium* and *P. trifoliata* maps, respectively. The total lengths of these maps determined using JOINMAP V. 2.0 (Stam and van Ooijen (1996) are 495.028 and 348.527 cM, respectively. The nomenclature used for linkage groups was described as described by Ruiz and Asins (2003), in which the first letter indicates which parent the linkage group belongs to: A (*C. aurantium*) and Pa (*P. trifoliata* in the progeny derived from *C. aurantium* as female parent). All groups also have a designated number: a Roman number indicates that no homology was found with respect to the linkage groups of other maps, while an Arabic number indicates that this group presents two or more markers that are common to another linkage group of another linkage groups. The inclusion of new markers (not used by Ruiz and Asins 2003) has made it possible, in some cases, to merge two linkage groups; this is indicated by linkage groups containing a "+" sign.

To distinguish true *P. citrella* resistance QTLs from those related to leaf size or the flushing pattern of the tree, we also carried out QTL analysis of these traits. The flushing pattern was evaluated as the deciduous degree of each hybrid in winter, in the field, on a scale from 0 (evergreen plants as sour orange) to 6 (completely deciduous as trifoliate orange). We so evaluated 43 of the 104 original hybrids of the mapping population. Eighty hybrids of the same population but grafted on Carrizo rootstock and located in the same field plot were also evaluated for this trait. Since differences regarding the QTLs involved might arise from the effect of the rootstock, both types of trees were analysed separately.

Three to five new, completely expanded leaves from 49 hybrids of the same mapping population growing on their own roots were used to measure leaf area (in square centimeters) with a Li-Cor Model 3100 area meter (Li-Cor, Lincoln, Neb.). Therefore, the evaluated trait here is mean area of a single leaf, not the total leaf area of the whole plant.

Putative QTLs were identified using several statistical methodologies. Since CLM resistance traits heavily

Table 1 Linkage groups and markers most significantly associated with the traits investigated

Linkage group	Marker[a]	K[b]	P value	aa[c]	ab[c]	Trait	LOD (IM)[d]	centiMorgans (IM)[d]
Pa7	**CR7**	13.02	0.0005	4.09	23.53	Infested leaves	2.1	5
Unmapped (P)	TAA52	5.55	0.05	7.35	25.13	Infested leaves	–	–
Pa7	**CR7**	14.4	0.0005	0.86	14.87	Pupal casts/leaf	2.84	0
Pa4	C1_300	6.96	0.01	3.79	1.9	Deciduous degree	2	0
Pa9	**5R-4R_1110**	7.03	0.01	2.42	4	Deciduous degree[e]	1.34	0
Pa9	**5R-4R_1110**	6.02	0.05	11.9	18.9	Leaf area	0.44	0
Pa1	6F-6R_850	7.56	0.01	15.25	11.05	Leaf area	1.54	0
A7	**3F-4R_650**	12.615	0.0005	6.68667	30.5683	Infested leaves	2.34	0
A7	**CR12_700**	11.288	0.001	31.4023	9.15808	Infested leaves	2.13	0
A3	CL1.40_1650	12.533	0.0005	24.614	6.44125	Infested leaves	0.85	0
AII	CL2.26	5.424	0.05	8.35316	18.8381	Infested leaves	0.87	0
Unmapped (A)	6F-6R_220	10.006	0.005	10.4912	29.8373	Infested leaves	–	–
A7	**3F-4R_650**	5.905	0.05	3.85125	17.8967	Pupal casts/leaf	1.53	0
A3	5R-4R_520	6.357	0.05	13.5409	3.15385	Pupal casts/leaf	0.54	0
A3	CL1.40_1650	6.201	0.05	13.31	3.3775	Pupal casts/leaf	0.67	0
A(10 + 5b)	S2-AS4_800	6.893	0.01	16.2089	4.37524	Pupal casts/leaf	1.89	0
A7	**3F-4R_650**	9.319	0.005	2.26316	3.8125	Deciduous degree[e]	2.2	5
A7	**CR12_700**	10.162	0.005	3.93548	2.30769	Deciduous degree[e]	2.41	4.5
A7	CL1.40_280	7.05	0.01	2.41	3.75	Deciduous degree[e]	1.34	12.6
A14	CR22_180	6.656	0.01	2.33333	3.82609	Deciduous degree[e]	1.26	41.6
A(10 + 5b)	CAC23	5.131	0.05	3.48571	2.21429	Deciduous degree[e]	1.04	1.8
A7	**CR12_700**	5.188	0.05	4	2.2963	Deciduous degree	1.15	9.2
A(10 + 5b)	CAC23	4.331	0.05	3.6	2.10526	Deciduous degree	0.89	9.1
A7	**CR12_700**	10.14	0.01	16.39	11.63	Leaf area	1.8	3.7
A(8 + 6)	3F-3R_650	5.84	0.05	14.72	11.83	Leaf area	1.5	0

[a] K is Kruskal-Wallis (KW) statistic
[b] Bold markers correspond to the location of most relevant QTL found
[c] Mean of citradias carrying a or b allele
[d] Main interval mapping results. LOD score and genetic distance in centiMorgans (cM) from the KW most significantly associated marker
[e] Citradias grafted on Carrizo rootstock

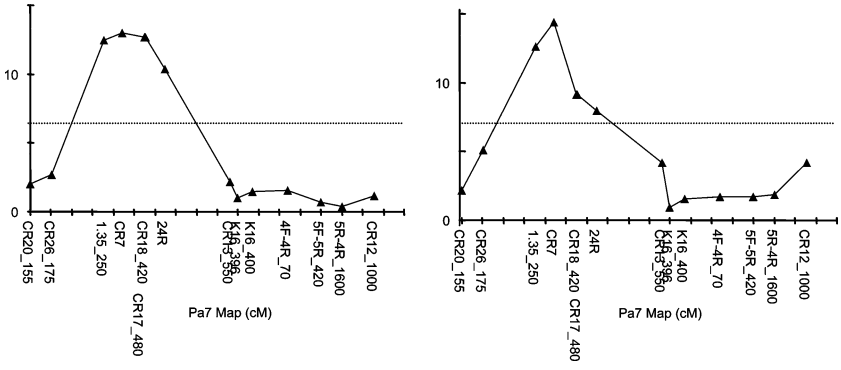

Fig. 2 The strongest effect-QTL for percentage of infested leaves (18.8%, *left*) and pupal casts/leaf (26.5%, *right*) at LG Pa7, suggesting the presence of an important antibiosis QTL in *Phyllocnistis trifoliata*. The curve shows the K statistic values. A *horizontal dashed line* has been placed at the $P < 0.01$ significance level

depart from normality (Fig. 1), a non-parametric genomic scan based on the Kruskal-Wallis (KW) methodology was considered in addition to the standard interval mapping for both *C. aurantium* and *P. trifoliata* linkage maps, separately, using the MAPQTL 3.0 computer programme (Van Ooijen and Maliepaard 1996). For markers segregating as a backcross [as in the pseudo-test-cross design described by Grattapaglia et al. (1996): $M_iM_j \times M_iM_i$ for the *C. aurantium* genetic map, or $M_iM_i \times M_iM_j$ for the *P. trifoliata* one], trait values were used for QTL analysis by both methodologies for comparison purposes and to have an estimate of the percentage of phenotypic variance explained by the QTL.

Results

Some variation was observed among cages for the genotype used as the susceptible control and for the genotype used as the tolerant control. To minimize random differences among cages, we transformed every value into a proportion relative to the value of the susceptible control within the lot. The distributions of the CLM resistance traits are far from normality (Fig. 1). The transformation suggested by Leonards-Schippers et al. (1994) to approach normality did not further improve the distributions of CLM resistance traits.

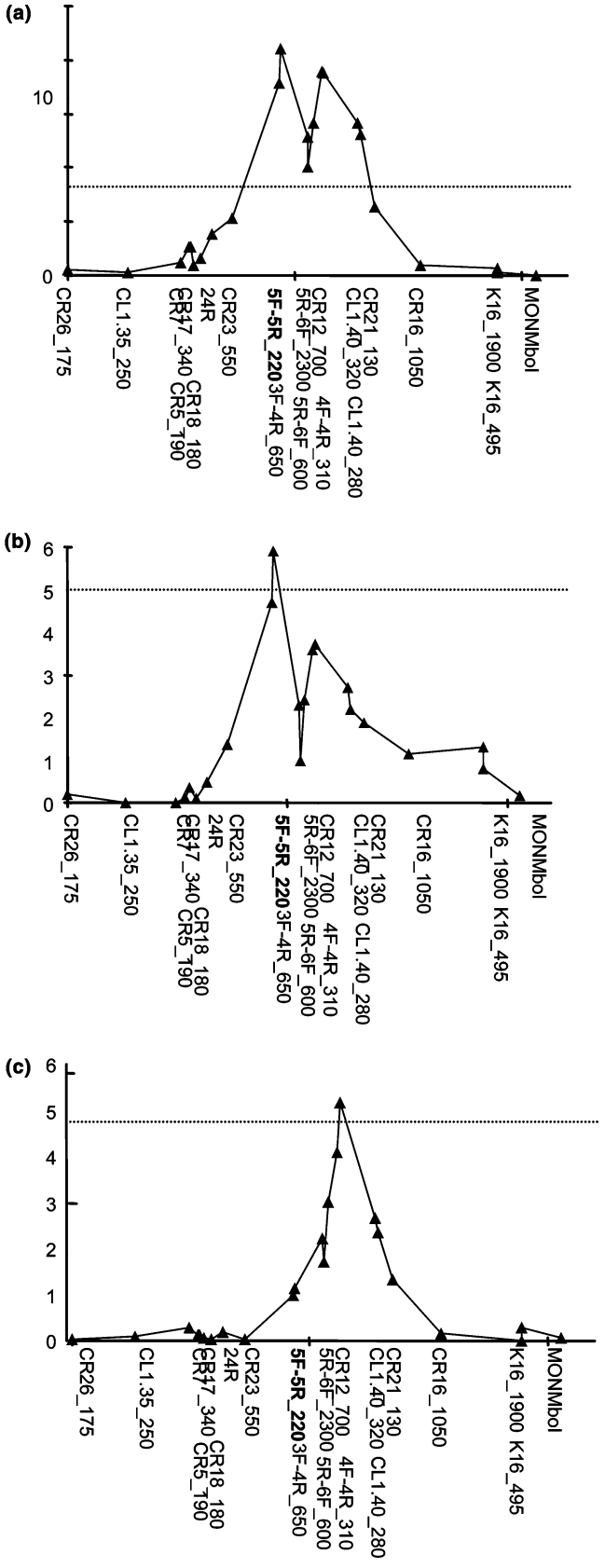

Fig. 3 Sour orange QTLs located at LG A7 for percentage of infested leaves (**a**), pupal casts/leaf (**b**) and deciduous degree (**c**). A *horizontal dashed line* has been placed at the $P < 0.05$ significance level

Fig. 4 Infested and non-infested leaves of sensitive and tolerant citradias, respectively

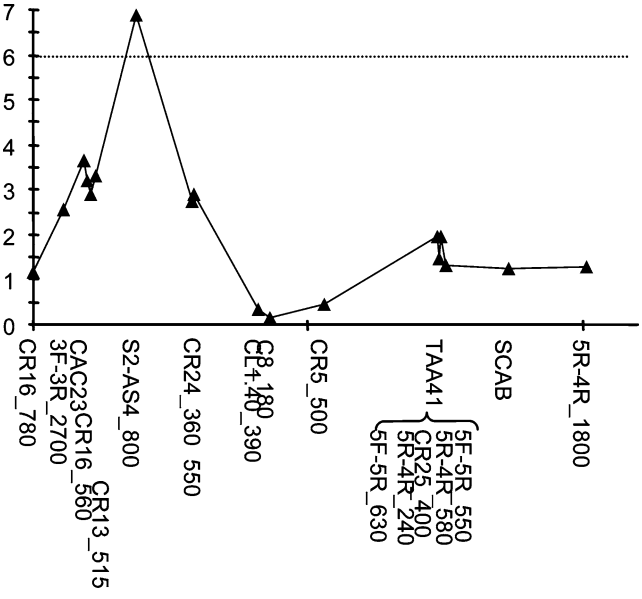

Fig. 5 The strongest effect-QTL for pupal casts/leaf at sour orange LG A(10 + 5b). Maximal value of the *K* statistic is located at S2AS4_800, a resistant gene analogue. A *horizontal dashed line* has been placed at the $P < 0.05$ significance level

Therefore, original variables were considered for the present study.

The markers within each linkage group showing the highest significant association with CLM susceptibility (percentage of infested leaves and number pupal casts per leaf), evergreen/deciduous degree and leaf area are presented in Table 1. This table also includes results from interval methodology given that when both

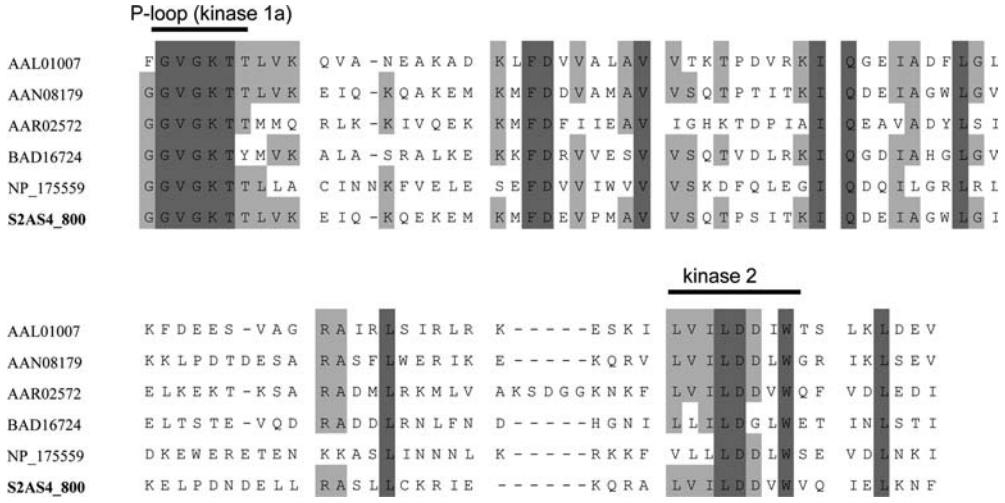

Fig. 6 Alignment of translated sequences that are most similar to the corresponding sequence of S2AS4_800, a RGA isolated from sour orange that is candidate of an antibiosis QTL. P-loop and kinase 2 are two conserved motifs within the NB-ARC domain. *AAN08179* Putative citrus disease resistance protein Pt19 (*C. grandis* × *P. trifoliata*): 167 amino acids (aa); 6e-23. *AAL01007* NBS/LRR resistance protein-like protein (*Theobroma cacao*): 170 aa; 3e-09. *BAD16724* Rapid transcriptional activation of a potato CC-NB-LRR class gene by inoculation with an incompatible race of *Phytophthora infestans* (*Solanum tuberosum*): 1,036 aa; 6e-04. *NP_175559* Putative disease resistance protein (CC-NBS-LRR class) (*Arabidopsis thaliana*): 941 aa; 9e-05. *AAR02572* Resistance protein candidate RGC2 (*Lactuca sativa*): 1,923 aa; 2e-04

approaches agree, the QTL detected becomes reinforced. The strongest effects of CLM susceptibility were found to locate at LGs 7 of both sour and trifoliate orange (Figs. 2, 3) and LG 3 of sour orange. Nevertheless, CLM-susceptibility QTLs at LGs 7 do not locate at the same markers. In the case of Pa7, the strongest significance is associated with microsatellite CR7 (Fig. 2), while in the case of A7, two QTLs appear to be located around 3F-4R_650 and CR12_700. The latter is also associated with the evergreen/deciduous degree and leaf area (Fig. 3).

Only two relevant ($P < 0.02$) CLM susceptibility QTLs (6F-6R_220 and S2-AS4_800) associate with just one of the CLM traits (either percentage of infested leaves and number pupal casts per leaf). Other markers (CR7, 3F-4R_650, CR12_700 and CL1.40_1650) with high K values (13.02, 12.61, 11.29 and 12.53, respectively) associate with QTLs affecting both CLM traits simultaneously, although generally not at the same significance level. More QTLs were detected for percentage of infested leaves than for number of pupal casts per leaves. When coincident, the value of the *K* statistic was larger for percentage of infested leaves, with the only exception being CR7.

Two QTLs detected for the deciduous degree of hybrids grafted on Carrizo were also detected when they are on their own roots. The position of the nearest marker from one of them (CR12_700) is coincident with that of a QTL affecting the number of infested leaves and with another involved in leaf area (Fig. 3). Since the QTL allele associated with fewer number of infested leaves is also associated with smaller leaf area (and not larger leaf area), this CLM susceptibility QTL might be considered to be an antixenosis QTL (Fig. 4)—i.e., moths show preference for larger leaves.

The most significant QTL affecting number of pupal casts per leaf in the sour orange linkage map presents its maximum value for the *K* statistic at marker S2-AS4_800 (Fig. 5). This marker was one out of the five markers obtained using the degenerate primers described by Mago et al. (1999) that were designed on the basis of nucleotide-binding site (NBS) motifs conserved among resistance genes. Sequence analysis of this fragment revealed that its in silico translation product shows regions with high similarities to putative disease resistance NBS/LRR (leucine-rich repeat) proteins (Fig. 6) of several species, including *Citrus* (AAN8179, 6e-23), Cacao (AAL01007, 3e-09), potato (BAD16724, 6e-04), *Arabidopsis* (NP_175559, 9e-05) and *lactuca* (AAR02572, 2e-04).

Discussion

Antibiosis, antixenosis and tolerance are the three principal modes of plant resistance to insects (Painter 1951; Kogan and Ortman 1978; Wieseman 1999). Antibiosis describes a type of resistance in which the insect's normal relationship with a host plant causes physiological or developmental detriment to the insect, whereas antixenosis, or nonpreference, describes resistance in which the insect is either repelled from or not attracted to its normal host plant. In this context, a genotype might appear resistant because the insect prefers larger sized or tenderer leaves. Finally, tolerance characterizes plants with normal yields in the presence of a damage that would affect the yield of non-tolerant plants. The parents of the progeny analysed here are different with respect to CLM susceptibility but also for leaf area and deciduous behaviour. Sour orange presents large unifoliate leaves during the whole year,

whereas trifoliate orange is the only deciduous species within the orange subfamily and its trifoliate leaves are characteristic of the genus *Poncirus* and its hybrids. Thus, one allele of CR12_700, at LG A7, is associated with lower percentage of infested leaves, smaller leaves of the largely deciduous hybrids. This putative antixenosis QTL might be related to a leaf type, similar to that of *P. trifoliata* var. *Flying Dragon*. Five other antixenosis QTLs have also been detected—four in the sour orange genome and one associated with an unlinked marker of trifoliate orange (TAA52). From those of sour orange, two sour orange QTLs, on LGs A7 and A3, are also associated with a lower percentage of pupal casts per leaf that might be explained by their large effect on the number of infested leaves. To complete the picture, two antibiosis QTLs have been detected. The most important of these is located at marker CR7 from the *Poncirus* LG Pa7 and is independent from any segregating QTL involved in its deciduous behaviour. It explains 26.3% of trait variance (estimated from IM), and its antibiotic effect might be so large that it would explain its additional effect on the number of infested leaves. Conversely, the effect of the other antibiosis QTL located at the sour orange LG A(10 + 5b) seems to be smaller since the corresponding marker (S2-AS4-800) does not appear to be significantly associated with a lower percentage of infested leaves. The sequence of this marker is highly similar to several NBS-LRR-type resistance genes, including one of citrus. The activation of the defence response mediated by this gene might involve antinutritive elements or pathogenesis-related (PR) proteins exhibiting chitinase or B-1,3-glucanase activities (Legrand et al. 1987; Kauffmann et al. 1987). Another explanation is that the RGAs might be part of a cluster of resistance genes that confer resistance to distinct pathogens, as the case reported by Van der Vossen et al. (2000). Future experiments that focus on the possible expression of this gene following CLM and *Phytophthora* attack are necessary.

Despite the small number of hybrids (42) that were evaluated for insect resistance in the present study, several putative antibiosis and antixenoxis QTLs were detected, probably due to their relative large contribution to the total variation for CLM susceptibility present in the progeny of the cross *C. aurantium* × *P. trifoliata*. Fortunately, these QTLs with a large contribution are the most important ones for implementation of marker-assisted selection schemes in breeding programmes (Asins 2002). Additionally, once a major gene or QTL has been identified and mapped, map-based gene cloning experiments can be initiated. In Table 1, the detection of the QTLs is presented for several levels of significance; given that no protection for multiple comparisons is directly used, the *P* values of 0.05 are merely suggestive. Nevertheless, these values were not ignored because a low-power test was used and the sample size was small.

As in many other QTL analyses, we have shown the presence of valuable alleles in the "bad" parent—in our case *C. aurantium*—which is important in the context of germplasm characterization and efficient utilization of citrus genetic resources towards the obtaining of tolerant cultivars that will allow an IPM of the culture. Our results have shown that the percentage of infested leaves in the tolerant control (1.59 ± 0.96; mean ± standard error) represents a 92.1% decrease in comparison to the susceptible control. This decrease could be larger since resistant cultivars should be used in combination with other control components of IPM (Wiseman 1999). Therefore, because of the enormous effect of CLM on citrus canker epidemics (Bergamin-Filho and Hughes 2002; Bergamin-Filho et al. 2002; Gottwald et al. 2002), even small differences in susceptibility against the CLM could have a dramatic impact on the spreading of citrus canker infections in the field.

How the QTL information here reported could be used as a source of resistance for commercial citrus varieties needs further investigation because it is difficult to transfer those QTLs into productive cultivars by conventional citrus breeding strategies. Marker-assisted selection using DNA markers tightly linked to QTLs conditioning resistance to insects would greatly enhance the efficiency of citrus breeders to obtain resistant cultivars from a segregant progeny. Nevertheless, we do not think this is feasible using a progeny derived from a cross involving *P. trifoliata* because it is a distantly related wild species with long juvenility and very unpalatable fruits. Fortunately, there are new ways to obtain segregant progenies where each plant contains just a small part of the genome of the donor parent, such as in asymmetric-protoplast fusion experiments (Wang et al. 2003).

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