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C. Caranta · A. Palloix · V. Lefebvre
A. M. Daubèze

QTLs for a component of partial resistance to cucumber mosaic virus in pepper: restriction of virus installation in host-cells

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Abstract Ninety four doubled-haploid (DH) lines obtained from the F_1 between Perennial, a cucumber mosaic virus (CMV)-partially resistant *Capsicum annuum* line, and Yolo Wonder, a CMV-susceptible *C. annuum* line, were analysed with 138 markers including mostly RFLPs and RAPDs. Clustering of RAPD markers was observed on five linkage groups of the intraspecific linkage map. These clusters could correspond to the centromeric regions of pepper chromosomes. The same progenies were evaluated for restriction of CMV installation in pepper cells in order to map quantitative trait loci (QTLs) controlling CMV resistance. This component of partial resistance to CMV was quantitatively assessed using a CMV strain that induced necrotic local lesions on the inoculated leaves. The number of local lesions gave an estimation of the density of the virus-infection sites. Genotypic variance among the DH lines was highly significant for the number of local lesions, and heritability was estimated to be 0.94. Using both analysis of variance and non-parametric tests, three genomic regions significantly affecting CMV resistance were detected on chromosomes Noir, Pourpre and linkage group 3, together explaining 57% of the phenotypic variation. A digenic epistasis between one locus that controlled significant trait variation and a second locus that by itself had no demonstrable effect on the trait was found to have an effect on CMV resistance. For each QTL, the allele from Perennial was associated with an increased resistance. Implications of QTL mapping in marker-based breeding for CMV resistance are discussed.

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C. Caranta (✉) · A. Palloix · V. Lefebvre · A. M. Daubèze
Institut National de la Recherche Agronomique (INRA),
Station d'Amélioration des Plantes Maraîchères, BP 94,
84143 Montfavet Cedex, France

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Introduction

Partial resistance to a virus, which decreases or prevents the normal development of the cycle of virus infection, is of obvious value in the control of economically important plant viruses such as cucumber mosaic virus (CMV) in pepper (*Capsicum annuum* L.). Todate, no complete resistance to any strain of CMV has been discovered; only components of partial resistance that interact with different steps of the cycle of virus infection have been demonstrated (Pochard 1977). The three main components are: (1) restriction of virus installation in the host-cells (Lecoq et al. 1982) (2) restriction of virus multiplication (Nono-Womdim et al. 1993a) and (3) inhibition of systemic virus movement (Dufour et al. 1989; Nono-Womdim et al. 1993b). In open fields or under artificial conditions (mechanically inoculated plants) partial resistance was expressed by an ability to escape the primary virus infection, to restrict the virus multiplication in the inoculated organ, or to prevent the long distance movement of the virus in the whole plant, especially when inoculum pressure is low. In order to assess these components of partial resistance to CMV in pepper, to study their inheritance, and to use them for CMV resistance breeding, distinct methods of resistance evaluation have been defined (Pochard 1977; Pochard and Daubèze 1989; Nono-Womdim et al. 1993a; Dogimont et al. 1994).

Restriction of virus installation, i.e. entry of the virus into the host-cells, could be directly and quantitatively assessed on the inoculated leaves with the Fulton CMV/N strain (Troutman and Fulton 1958) that induces necrotic local lesions on a large range of hosts including pepper. It has been shown that the number of

local lesions induced on the inoculated leaves gave an estimate of the density of the primary infection sites (Troutman and Fulton 1958) and was highly correlated with the number of plants that escaped or tolerated the infection (Pochard 1977; Lecoq et al. 1982). Other examples of partial resistance to virus installation have been demonstrated (Gibb et al. 1989; Wilson and Jones 1992), suggesting that this is a common defense mechanism against viruses in plants. However, the inheritance of this trait has not yet been analysed. A restriction of the number of local lesions induced by the CMV/N strain has been revealed in Perennial, a pungent small-fruit Indian *C. annuum* line. After inoculation with the CMV/N strain, Perennial produced ten times fewer local lesions than Yolo Wonder, a CMV-susceptible *C. annuum* line (Lecoq et al. 1982). The restriction was highly correlated with tolerance (mild or no detectable disease symptoms) to a systemic CMV (TL) strain.

Interestingly, a restriction of CMV multiplication in the inoculated leaves was also identified in this same pepper line (Nono-Wondim et al. 1993 a). Moreover, Perennial accumulates resistance factors against other important pathogens of pepper including several potyviruses (Caranta and Palloix 1996; Caranta et al. 1996) and the soilborne fungus *Phytophthora capsici* (Lefebvre and Palloix 1996). From the F_1 obtained from the cross between Perennial and Yolo Wonder, a doubled-haploid (DH) progeny was developed. This kind of population is ideal not only for studying the genetics of multiple disease resistance (several phenotypic evaluations with several pathogens can be performed) but also for constructing a molecular linkage map with the continuous addition of new markers (Lefebvre et al. 1995). This map would allow us to locate major genes and QTLs involved in each resistance and to study the genomic organization of the resistance factors from a multi-resistant pepper line.

We report here the use of molecular markers and DH progeny for identifying genomic regions involved in the restriction of CMV installation in pepper cells and for evaluating the quantitative effects and the mode of gene action of these QTLs. The potentialities of RAPD markers for pepper mapping and the implications of QTL mapping in marker-based breeding for CMV resistance are also discussed.

Material and methods

Plant material

The segregating DH progeny includes 94 lines obtained by *in vitro* androgenesis (Dumas de Vaulx 1981) from the F_1 hybrid of a cross between Perennial (supplied by J. Singh, Punjab University, Ludhiana, India) and Yolo Wonder, two *C. annuum* lines. Pepper plants were grown in a sterilized peat/soil mixture in the greenhouse using standard horticultural practices with the help of the experimental team of INRA-Montfavet.

RFLP and RAPD analysis

Plant genomic DNA isolation as well as RFLP and RAPD assays were carried out as described by Lefebvre et al. (1995). The TG, CD and CT probes used in this study were provided by S. D. Tanksley (Cornell University, New York, USA) and the PG probes by M. M. Kyle (Cornell University, New York, USA). The previous linkage map from the same cross (Lefebvre et al. 1995) was mainly composed of RFLP markers. In the present study, we chose to add RAPDs in order to assess the potentialities of these markers for pepper mapping. Two hundred and eight primers (Operon Technologies, Alameda, Calif.) were tested for polymorphism between Perennial and Yolo Wonder. The Mapmaker software (version 3.0b) (Lander et al. 1987), with Kosambi mapping function (Kosambi 1944), was used to add new markers to the existing linkage map. A framework was constructed using a minimum LOD score of 3.0 and a maximum recombination fraction of 0.3. New markers were then added with the 'try' command. To check the order suggested by the 'try' command, we used the 'ripple' and the 'compare' commands.

Resistance to CMV installation assay

The CMV/N strain (Troutman and Fulton 1958) was maintained at $+4\,^{\circ}C$ according to the Bos (1969) procedure and multiplied on *Vinca rosea*. Virus inoculum was prepared as described previously (Dogimont et al. 1994). In all experiments, plants were inoculated at the 5–6-leaf stage by manually rubbing the third leaf with the sap extract. The seedlings were rinsed 5 min after inoculation to remove excess inoculum. After inoculation, the plants were grown in a growth chamber (22°C, 12 h light per day). Resistance was assessed 4 days after inoculation by scoring the parental lines, the F_1, and the DH lines for the number of local lesions induced by the CMV/N strain on the inoculated leaves. The number of local lesions varied from 0 to more than 100 depending on the plant and the genotype. To increase the reproducibility, and particularly to simplify the checking procedure, a disease score ranging from 1 to 4 was defined depending on the number of local lesions: '1' = 0 to 5 lesions, '2' = 6 to 20 lesions, '3' = 21 to 50 lesions and '4' = more than 50 lesions. Beyond 50 lesions it appeared difficult to count because the lesions become coalescent.

The DH progeny were assessed for resistance during two independent tests of four and five plants per DH line, respectively. Variance components were estimated by using analysis of variance (ANOVA, GLM procedure, SAS Institute Inc 1989) and the heritability value was calculated using the following formula: $h_n^2 = \sigma_G^2/(\sigma_G^2 + \sigma_E^2/n)$, where σ_G^2 is the genetic variance, σ_E^2 is the environmental variance and n is the number of independent repeats.

Mapping quantitative resistance loci

The association of each marker genotype with the resistance value was assessed by comparing the phenotypic means of the two marker classes using a single-factor analysis of variance (ANOVA, GLM procedure, SAS Institute Inc 1989). A significant association between a DNA marker and CMV resistance was declared if the probability was equal to or less than 0.005 in order to minimize the detection of false positives. In order to detect QTLs with minor additive effects, a two-factor ANOVA was performed with the first component corresponding to the marker linked to a QTL with the most important effect (detected with the single-factor ANOVA). The same significance level as for single-factor ANOVA was used. Finally, two-factor ANOVAs with an interaction component between pairs of markers were used to detect digenic epistasis with a significance level of $P < 0.00005$. QTL effects were estimated as the percentage of phenotypic variation explained by the QTL (R^2, ratio of marker to the total sum of squares).

Residuals from the ANOVA were not normally distributed even after resistance data transformations. Consequently, association of each marker genotype with the resistance value was performed using the non-parametric Wilcoxon rank sum test. Digenic epistasis was tested using the non-parametric Kruskal-Wallis test (NPAR1WAY procedure, SAS Institute Inc 1989). The same significance levels as for the analysis of variance were chosen ($P < 0.005$ and $P < 0.00005$ respectively).

Results

Phenotypic evaluation of parents, the F_1 and DH lines

Since the two independent tests were not significantly different at the 5% level ($P = 0.0158$), the mean values of the nine plants per DH line were used in further analysis. After inoculation with the CMV/N strain, Perennial always developed less than five local lesions (average disease score = 1) on the inoculated leaves, whereas Yolo Wonder always developed more than 35 lesions (average disease score = 3.4). The behaviour of F_1 individuals from the cross between Perennial and Yolo Wonder was intermediate to that of the parental lines (average disease score = 2.3). The observed distribution of the DH lines in the disease-score classes (Fig. 1) suggested that several genetic factors were involved in this partial resistance. The DH progeny response was distinctly skewed toward susceptibility; about 70% of the DH lines were positioned in the right part of the distribution (disease score > 2.5). The genetic variance of DH lines was highly significant ($P < 0.0001$) and the heritability of the genotypic mean values for CMV resistance was estimated to be 0.94. This high value indicated that most of the variation observed between the DH lines was due to genetic factors.

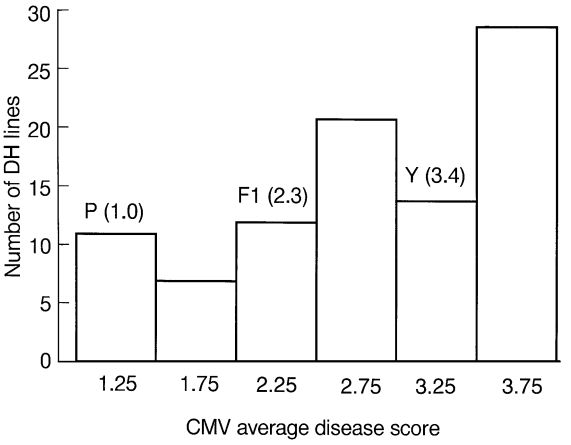


Fig. 1 Distribution of the DH lines for partial resistance to CMV in pepper evaluated by the number of local lesions induced by the CMV-N strain on inoculated leaves. (*P* perennial, *Y* Yolo Wonder, and F_1 hybrid between Perennial and Yolo Wonder)

Current status of the pepper intraspecific linkage map

Previous pepper linkage maps (Tanksley 1984; Tanksley et al. 1988; Prince et al. 1993; Lefebvre et al. 1995) were mostly composed of isozyme and RFLP markers. In order to investigate the relative potential of RAPD markers for pepper mapping, 208 oligonucleotide primers were tested as possible RAPDs. Among them, 60 produced polymorphic bands suitable for segregation analysis and allowed us to add 84 RAPD markers to the existing intraspecific pepper linkage map. One hundred and thirty eight markers (84 RAPDs, 51 RFLPs and 3 phenotypic markers) were assigned to 14 linkage groups that span a map distance of 1013.3 cM with an average interval length between markers of 8.8 ± 10.7 cM (Fig. 2). Seven markers remain unlinked. Some of the linkage groups were assigned to chromosomes (designated by French color names) thanks to the location of markers for which the assignment to a trisomic has been described (Pochard 1970; Lefebvre et al. 1995). This map was estimated to cover 80% of the pepper genome according to the Chakravarti et al. (1991) formula (Caranta 1995). An obvious clustering of markers can be observed on the chromosomes Brun, Pourpre, LG3, LG4 and LG10 (Fig. 2). Only one cluster per linkage group was observed and they were mostly composed of RAPD markers. The ratio of RAPDs (42/84) involved in clusters was significantly different ($P = 0.0012$) from the ratio of clustered RFLPs (8/51).

Association of markers with partial resistance to CMV

A significance level of $P < 0.005$ was chosen for detecting QTLs with additive effect and $P < 0.00005$ for digenic epistasis. One hundred and forty five ANOVA tests were performed for detecting additive QTLs and 10 440 tests for detecting epistatic QTLs, so 0.5 QTLs would be detected by chance alone (false positives). Moreover, this false-positive number is over-estimated since the markers are not independent and several RAPD markers were shown to be very tightly linked. Three distinct genomic regions involved in partial resistance to CMV were identified using the different ANOVA models (Fig. 2). The location and effect of QTLs affecting the number of local lesions induced by the CMV/N strain are summarized in Table 1.

The single-factor ANOVA allowed the detection of six markers that fulfilled the significance level chosen ($P < 0.005$). The markers P11_0.8, P15_1.3, PG140 and I07_1.4 were mapped on linkage group 3 and were linked within 23.9 cM, whereas AG03_2.1 and TG124 (linked within 13.8 cM) were located on the chromosome Noir. Since ANOVA did not permit the precise

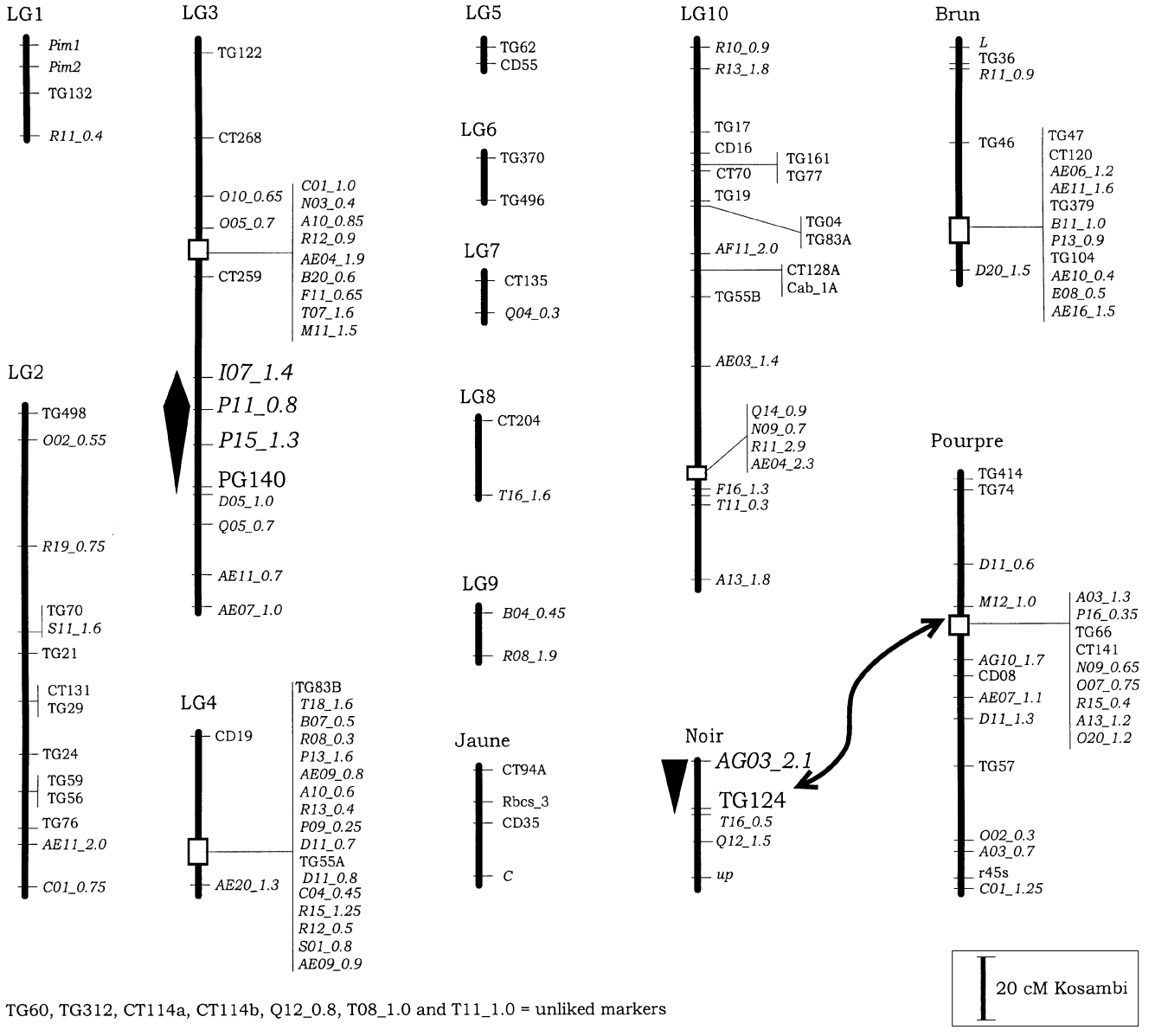


Fig. 2 Intraspecific linkage map of pepper showing markers linked to QTLs involved in the restriction of CMV installation in pepper cells and the clustering of markers. *Vertical bars* represent linkage groups (*LG*). Some of them were assigned to chromosomes designated by French color names (Pochard 1970; Pochard and Dumas de Vaulx 1982). *Designations* on the right of linkage groups represent marker names. RFLP markers are prefixed with '*TG*' for loci detected by random genomic clones of tomato, by '*CT*' or '*CD*' for loci detected by random tomato cDNA clones, and by '*PG*' for the locus detected by random genomic clones of pepper. Clones corresponding to known genes are designated by their specific names. Nomenclature for RAPD loci (*in italics*) indicates the Operon primer kit designation and the relative molecular weight, in kb, of the visualized RAPD band. *White boxes* indicate clusters of molecular markers. The *black triangle and rhomboid* represent genomic regions with additive effects (detected by single-factor ANOVA and the Wilcoxon rank sum test) associated with partial resistance. The broad part indicates the marker that displayed the highest R^2 value. *Arrows* indicate epistatic effects between loci (detected by two-factor ANOVA with an interaction component between pairs of markers and by the Kruskal-Wallis test)

location of the QTL, nor the separation from the presence of one or several QTLs in a genomic region, we considered that a single QTL was identified when several linked markers were significantly associated with the trait and that the QTL was localized near the marker that displayed the highest R^2 value. Nineteen per cent of the total variation in the disease response was explained by the QTL on LG3, and 24 per cent was explained by the QTL on the chromosome Noir. The comparison of means of CMV disease score observed between the two genotypic classes for markers P11_0.8 (LG3) and AG03_2.1 (chromosome Noir) are presented in Fig. 3A.

The two-factor ANOVA with AG03_2.1 or P11_0.8 as the first component did not permit the detection of new markers involved in partial resistance to CMV. Finally, one digenic epistasis was found to have

Table 1 Biometrical parameters of markers significantly affecting partial CMV resistance from the phenotypic means of 94 DH lines of the F_1 of the cross between Perennial and Yolo Wonder obtained using analysis of variance

Marker	Localization	Probability	R^{2a}	Favourable genotype[b]
QTLs with additive effect – single-factor ANOVA – $P<0.005$				
P11_0.8	LG3	0.0000535	0.19	P
P15_1.3	LG3	0.0001992	0.16	P
PG140	LG3	0.0030072	0.11	P
I07_1.4	LG3	0.0026858	0.11	P
AG03_2.1	Noir	0.0000045	0.24	P
TG124	Noir	0.0019122	0.12	P
QTLs with epistatic effect – Two-factors ANOVA with an interaction – $P<0.00005$				
TG124-TG66[c]	Noir-Pourpre	0.00001315	0.33	P-P

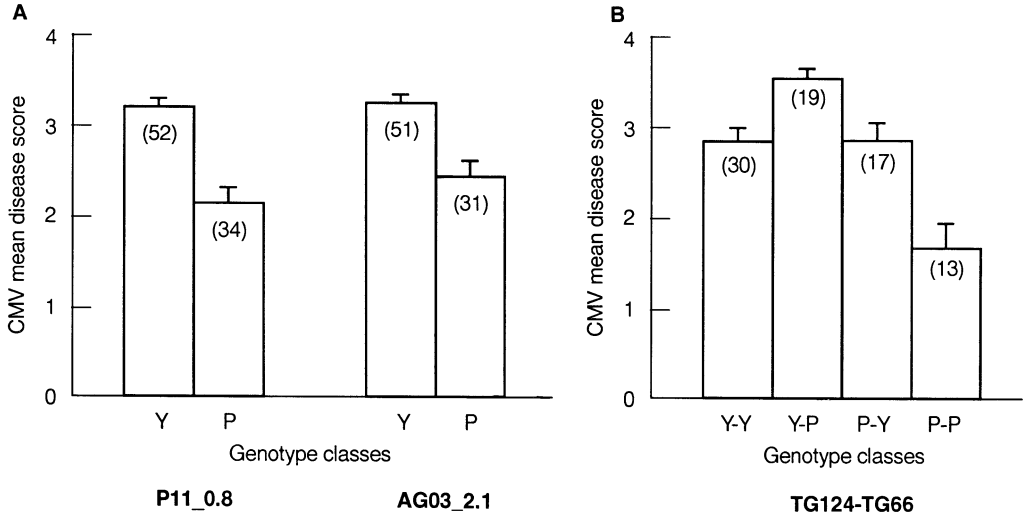
[a] Coefficient of determination
[b] Favourable genotype: P indicates that the Perennial allele decreases the disease index
[c] Closely linked markers were also significantly associated with the trait

a significant effect on CMV resistance ($P < 0.00005$). It occurred between TG124 (chromosome Noir) and TG66 mapped on the chromosome Pourpre. This interaction accounted for 33% of the observed variation and involved one allele that alone controlled significant trait variation (TG124) and a second one that by itself had no demonstrable effect on the trait (TG66). The comparisons of the mean of CMV disease score for the four genotypic classes at the significant pair of markers are presented in Fig. 3B.

With a global ANOVA model (that allows both for the markers with main effects, i.e. P11_0.8 and AG03_2.1, and the interacting markers, i.e. TG66 with TG124), 57% of the observed phenotypic variation was explained. For each QTL, the Perennial allele increased the level of resistance (Fig. 3).

Since ANOVA assumes a normal distribution of the residuals, and this assumption was violated in the present study (skewness: 0.04; kurtosis: 0.90), the ANOVA results were verified by performing non-parametric tests (the Wilcoxon rank sum test to detect association between each marker and the quantitative trait and the Kruskal-Wallis test to detect digenic epistasis). The markers P11_0.8, P15_1.3 and AG03_2.1 were significantly associated with CMV resistance (with $P = 0.0003$, 0.0012 and 0.0004 respectively). Interaction between TG124 (chromosome Noir) and TG66 (chromosome Pourpre) was also significant ($P = 0.000039$). These results confirmed that genomic regions on LG3, chromosome Noir and chromosome Pourpre were involved in CMV partial resistance. However, the probabilities (type-I error) observed for the non-parametric test were always higher than the probabilities obtained by ANOVA. Consequently, PG140, I07_1.4 and TG124 which appeared significant with ANOVA, were not significant with the non-parametric test ($P = 0.0137$, 0.0087 and 0.0104 respectively).

Fig. 3A, B Comparison of CMV disease score for different genotypic classes at the significant markers. Mean disease score and standard error for two (**A**) or four (**B**) genotypic classes are shown (*Y* Yolo Wonder allele at the marker, *P* perennial allele at the marker. The *numbers* in brackets represent the number of DH lines in each genotypic class)



Discussion

Clustering of the marker loci

We have detected non-random distribution of genetic markers on the pepper intraspecific linkage map and it appeared that RAPDs contributed more to this

clustering than did RFLPs. Clustering of markers has been reported in several linkage maps independently of the method used to assay polymorphism and several explanations have been proposed to account for this (for examples see Adam-Blondon et al. 1994; Grattapaglia and Sederoff et al. 1994; Kesseli et al. 1994). In the present study, two main reasons can be postulated for the clustered location of markers:

(1) Clustering could result from a reduction or suppression of genetic recombination in genomic regions flanking the centromeres (Mather 1938; Roberts 1965) and/or in regions flanking the telomeres (Tanksley et al. 1992). Two of the pepper map clusters seemed to correspond with the supposed centromeric regions of tomato chromosomes where clustering of markers was also observed. Indeed, the marker CT259, that flanked the cluster on linkage group 3 of the pepper map, was localized in the putative centromeric region of tomato chromosome 5 (Tanksley et al. 1992). The cluster of chromosome Pourpre includes two RFLP markers (TG66 and CT141) that flanked the putative tomato chromosome-3 centromere. These two regions could also correspond to pepper centromeres. For the other clusters, comparison with tomato was not possible because of the lack of common RFLP markers.

(2) Since clusters were mostly composed of RAPDs, they could result from the ability of these markers to detect polymorphisms in high-copy number DNA regions (Plomion et al. 1995). Interestingly, in tomato, RAPD markers as well as GATA microsatellites preferentially mark centromeres (Grandillo and Tanksley 1996). For a more conclusive statement about the origin of clusters, mapping more RFLP markers that are known to flank the tomato centromeres and an identification of the kind of sequences revealed by the RAPDs would be necessary. Thus, although RAPD markers did not provide the full genome coverage necessary for QTL mapping and marker-based breeding in pepper, they could be used to localize centromeres.

QTL mapping

Although there are numerous reports of the characterization of the mechanism(s) involved in partial resistance to viruses, little data are available on the inheritance of these partial resistances (Shahjahan et al. 1990; Barker et al. 1994). To our knowledge, our study is the first published report of QTL mapping for a component of partial resistance that interacts with a step of the cycle of virus infection. Overall, three genomic regions involved in partial restriction of CMV installation in pepper cells were located using molecular markers. All the resistance alleles originated from the resistant parent, Perennial.

Several authors (Kreike et al. 1993; Young et al. 1993; Maliepaard et al. 1995) suggested the use of a combination of both parametric (ANOVA and/or interval mapping, Lander and Botstein 1989 for example) and non-parametric methods when the trait under study did not fit a normal distribution. The results we obtained by ANOVA were confirmed by non-parametric tests but we noted that the probabilities observed for the non-parametric test were always higher than those obtained by ANOVA. In the present situation, the convergent results obtained with the different methods of QTL detection strongly indicate that the genomic regions detected were involved in partial resistance to CMV. Fifty seven per cent of the measured variation could be explained by the detected QTLs. This may result from the moderate size of our sample (94 lines), which did not allow low-effect QTLs to be detected, or to the incomplete coverage of the pepper linkage map. This may also result from an overestimation of the heritability of the trait due to the definition of a disease score.

In addition to QTLs with additive effect, digenic epistasis seemed to play an important role in the control of partial resistance to CMV. In this case, trait variation at one locus (TG66 on chromosome pourpre) seems to be conditioned by the presence of a specific allele with an additive effect at another locus (TG124 on chromosome Noir). Since the interaction between AG03_2.1 (linked to TG124 and detected with both single-factor ANOVA and the non-parametric test) and TG66 was not significant ($P = 0.0015$), we may hypothesise that this genomic region contains two resistance factors. Whatever the case, interaction between TG124 and TG66 needs to be more precisely evaluated on a larger population because the four genotypic classes used in the tests contained few lines (Fig. 3). Concerning QTLs involved in disease resistance, epistasis was more often reported between loci both of which showed an individual effect (Nodari et al. 1993; Schon et al. 1993; Wang et al. 1994; Webb et al. 1995) probably because these authors tested interactions only between QTLs with additive effect. The importance of epistasis between loci without an additive effect has been previously emphasized by Lefebvre and Palloix (1996) and here we provided a rationale for looking for interaction effects between all the markers.

Because tomato probes were used to map the pepper genome, it is possible to examine synteny for genomic regions involved in disease resistance between these related species and to test the hypothesis that these loci have evolved from a common ancestor. Interestingly, the epistatic QTL on chromosome Pourpre (marker TG66) for CMV resistance was located in the same genomic region as a minor QTL involved in Tomato Yellow Leaf Curl virus resistance (Zamir et al. 1994). This observation constitutes a new example of the fact that resistance factors (major genes but also QTLs) to different pathogens have been located in the same

genomic region – hot spots for disease resistance factors – in different solanaceous crops (i.e. tomato and pepper or potato) (Kreike et al. 1993; Lefebvre and Chevre 1995). This may indicate that a common mode of action is shared by these genes; in the present situation, this common mode of action could be a common mechanism of resistance to virus installation in the host cells.

Besides the restriction of virus installation, other partial resistance components have been described in other pepper lines: namely, reduction of virus multiplication and inhibition of systemic virus movement. For each component, the different resistance sources were recombined in our laboratory and separately introgressed into populations with improved agronomic characteristics in three distinct recurrent systems. Recombination between the components of partial resistance is underway, in order to increase the level of resistance and consequently to limit the spread of CMV. When partial resistance to installation is combined with partial resistance to systemic movement, more than 50% of the plants remained symptomless 35 days after inoculation at the plantlet stage with a moderately aggressive CMV strain, whereas 100% of the parental plants showed mosaic symptoms under the same conditions (Pochard and Daubèze 1989). These observations suggested that distinct loci were involved in these components and that both these components are efficient against naturally occurring CMV strains. But they did not exclude the occurrence of common loci involved in several components. For instance, loci controlling partial restriction of CMV multiplication in Perennial were also mapped in the DH progeny (Caranta 1995). Only three genomic regions with minor effect appeared to be significantly involved in the restriction of virus multiplication. Among them, the region flanking the marker TG124, already involved in restriction to virus installation, was detected. Involvement of this region with two resistance components may result from linked QTLs, but also from an interaction between the two components, both of them resulting in weak virus detection by serological tests. From a breeding point of view, the construction of genotypes with high resistance levels to CMV will be promoted by the identification of molecular markers linked to components of partial resistance. Indeed, QTL mapping now provides the most efficient way to distinguish between genomic regions involved in a single component and genomic regions common to several components of partial resistance, to choose the best combination of genes controlling transgressions for resistance and finally to avoid the difficulties of phenotypic screening.

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