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QTLs involved in the restriction of cucumber mosaic virus (CMV) long-distance movement in pepper

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Abstract Partial restriction of cucumber mosaic virus (CMV) long-distance movement originating from the *Capsicum annuum* inbred line 'Vania' was assessed in a doubled-haploid progeny using two screening methods: the first allowed one to assess the resistance of adult plants decapitated above the fourth leaf and inoculated on the third leaf using a common CMV strain, and the second allowed one to assess CMV resistance to long-distance movement on seedlings inoculated using an atypical CMV strain. For both resistance tests, the behavior of the F_1 hybrid between 'Vania' and the susceptible line 'H3' indicated that partial resistance is inherited as a dominant trait. Phenotypic data from the two screening methods were correlated but the one performed on seedlings was much more severe. A subset of 184 molecular markers well-distributed over the pepper genome was selected for QTL mapping using the composite interval mapping (CIM) method. A total of seven genomic regions, including one major effect and several minor effect QTLs, were shown to be associated with partial restriction of CMV long-distance movement. These results are compared with those already obtained in pepper and also in other solanaceous crops, potato and tomato.

Keywords *Capsicum annuum* · *Cucumber mosaic virus* (CMV) · Partial resistance · QTL mapping · Solanaceae

Introduction

Cucumber mosaic virus (CMV) causes important damage worldwide in many crop species of different families including pepper (*Capsicum* L.) and tomato (*Lycopersicon* Mill.) from the family Solanaceae (Watterson 1993). Unfortunately, no complete resistance capable of a restriction of CMV replication in inoculated cells has been found in both pepper and tomato. Only partial resistance controlled by several genetic factors was identified in *Capsicum baccatum*, *Capsicum annuum* and *Capsicum frutescens* (Dufour et al. 1989; Nono-Wondim et al. 1993; Caranta et al. 1997). In order to combine and to use these resistance factors for CMV resistance breeding in pepper, distinct methods of resistance evaluation were defined, each of them enabling the assessment of one component of partial resistance (Pochard 1977; Pochard and Daubèze 1989). Three main components, corresponding to three steps of disruption of the virus cycle in the plants, where shown to confer partial resistance to CMV in distinct *Capsicum* accessions: restriction of virus installation in the host-cells (Lecoq et al. 1982; Caranta et al. 1997), restriction of virus multiplication in the whole plant (Nono-Wondim et al. 1993) and restriction of long-distance movement (Dufour et al. 1989). This last mechanism of resistance, although qualified as partial resistance, was shown to be very efficient in field conditions (Nono-Wondim et al. 1993) and led to the first resistant large-fruited cultivars.

The limited systemic spread of CMV in *C. baccatum* 'Pen 3–4', and related *C. annuum* inbred lines, was shown to be a phloem-associated resistance (Dufour et al. 1989; Nono-Wondim et al. 1991). When inoculated to a leaf, the virus was detected, 10 to 15 days later, in internal and external phloem of the petiole and in the stem of susceptible lines; in partially resistant plants, the virus remained restricted to the inoculated leaf and its petiole. Depending on the resistance level and environment, it may spread to the axillary shoot associated with the inoculated leaf, but never spread to the other parts of the plant. This resistance results from an inhibition of virus translocation into or via the vascular system, preventing or delaying the virus transport over long distances and its spread in tissues of secondarily infected organs. This is in agreement with data about the directional flow of pho-

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toassimilates and viruses in *Capsicum* species, i.e. movement down the stem occurs via external phloem while movement up the stem occurs via internal phloem (Guerini and Murphy 1999).

Breeding for restriction of CMV long-distance movement in pepper and analysis of the genetic determinism of this resistance require screening methods applicable to a large number of plants. Two methods are available: the first allows one to assess the resistance of pepper plants decapitated above the fourth leaf and inoculated on the third leaf (Pochard 1977). Later, Dogimont et al. (1994) developed a method to discriminate resistance to CMV migration at an early stage of plant development using the CMV-N strain (Troutman and Fulton 1958). This CMV strain becomes systemic and induces the collapse of very young seedlings when inoculated on cotyledons of susceptible genotypes, whereas it remains restricted to the inoculated cotyledons and expressed as local lesions in the resistant genotypes.

The objective of this paper was to characterize the genetic determinism of resistance to long-distance movement of CMV from the *C. annuum* line 'Vania', evaluated using two distinct procedures, and to localize the CMV resistance factors on the pepper genome.

Materials and methods

Plant material

'Vania', the CMV-resistant genotype, is a bell-pepper inbred line from INRA, obtained from recurrent selection using *C. baccatum* 'Pen 3–4' as a primary genitor and *C. annuum* 'Antibois' and 'Bastidon' as secondary genitors for resistance to CMV (Pochard and Daubèze 1989). Resistance factors from these different genotypes were cumulated in 'Vania' during the selection process. The *C. annuum* cultivar 'H3' from Ethiopia was used as the CMV-susceptible parent. Anthers from the F_1 hybrid (H3×Vania) were cultivated in vitro using the method described by Dumas de Vaulx (1990) to produce 101 doubled-haploid (DH) lines. The 101 DH lines were analyzed for both CMV resistance and molecular markers.

Partial resistance to CMV long-distance movement assay

Partial resistance to CMV long-distance movement was assessed using two distinct procedures and CMV strains. The CMV-MES (isolated from *C. annuum*) and the CMV-N (Troutman and Fulton 1958) strains were maintained at +4°C according to the Bos (1969) procedure and multiplied on *Cucumis sativus* and *Vinca rosea*, respectively. Virus inoculum was prepared as described by Dogimont et al. (1994).

The CMV-MES strain was inoculated according to the method described by Pochard and Daubèze (1989): pepper plants (grown in a sterilized peat soil mixture in 8 cm-diameter pots under a temperature-controlled greenhouse) at the 6 leaf-stage were cut below the 5th developed leaf. Four-days later, plants were inoculated by manually rubbing the upper surface of the third leaf. Resistance was assessed 16, 21, 25 and 30 days after inoculation according to the following disease score: 1=no symptom on the third and the fourth axillary shoots, 2=systemic necrosis on the shoot and/or mosaic on the leaves of the third axillary shoot (corresponding to the inoculated leaf), 3=systemic necrosis on the shoots and/or mosaic on the leaves of both axillary shoots. The sum of disease score obtained for eight plants per DH line was used in further analysis. The sum for each DH line were not significantly different between 25 and 30 days after inoculation (data not shown), indicating a stabilization of the disease.

The CMV-N strain was inoculated by manually rubbing the two cotyledons of 12-day old seedlings. Immediately after inoculation, a 48-h dark-treatment was applied. Resistance to CMV long-distance movement was assessed 6, 8, 15, 20 and 30 days after inoculation by scoring the percentage of plants with apical necrosis for each DH line. Percentages were transformed into degrees using the formula Arcsin $\sqrt{(\times/100)}$ in order to stabilize the variance. Three independent tests on 20 plants per DH line were performed. The mean values of the three independent tests was used in further analysis. Mean values for each DH line were not significantly different between 20 and 30 days after inoculation (data not shown) indicating a stabilization of the disease.

In order to take the disease progression into account, the Area Under the Symptom Progress Curve (AUSPC) was determined for the parental lines, the F_1 hybrid and the DH lines, and for both methods of resistance assessment, using the following formula: $AUSPC=\Sigma_i\ [(x_i+x_{i+1})/2].t_i$ with x_i=mean value of disease score at date i, and t_i=time (in days) between scoring date i and scoring date i+1.

Molecular marker analysis, linkage map and QTL detection

Genomic DNA isolation, Southern blots, probe preparation, and RFLP and RAPD assays were carried out as described by Lefebvre et al. (1995). Genomic DNA and cDNA probes from tomato (named TG for tomato genomic DNA, CD and CT for tomato cDNA) and pepper (named PG for pepper genomic DNA) were acquired from S.D. Tanksley and M.K. Jahn (Cornell University, Ithaca, N.Y.). Decamer oligonucleotide primers for the RAPD procedure were purchased from Operon Technologies (Alameda, Calif.). AFLP markers were generated by Keygene n.v. (Wageningen, The Netherlands) using the procedure of Vos et al. (1995). Two phenotypic markers were mapped in the DH progeny: the *L* gene for resistance to *Tobacco mosaic virus* (TMV) and the *pvr2* gene for resistance to *Potato virus Y* pathotype 0.

The MAPMAKER/EXP v3.0b program (Lander et al. 1987) was used for map construction with a maximum recombination fraction of 0.3 and a minimum LOD score of 5. Distances were computed using the Haldane mapping function.

QTL detection was performed using linear regression (LR), interval mapping (IM) and composite interval mapping (CIM) with the QTL Cartographer software (Basten et al. 1997). Five markers with the highest *P*-values (selected using a forward-backward stepwise regression) were added as cofactors in the CIM procedure (model 6 with a window size of 10 cM). Significance thresholds were evaluated for IM and CIM by 1,000 permutations and conducted to the definition of a LOD-threshold of 2.57 and 2.68 for IM and CIM, respectively (for a type-I-error α=0.1). When two QTLs were detected by CIM within less than 20 cM, only the most significant was retained. Epistatic interactions between pairs of markers of the framework map were tested using a two-factor analysis of variance (ANOVA) with a fixed interaction model [GLM procedure of Statistical Analysis System (SAS), 1989, SAS/STAT, User guide version 6.11]. A significant interaction was detected when $P<5.10^{-5}$. The percentage of phenotypic variation explained by the markers linked to the QTL (R^2) was estimated in the different models.

Results

Response of parental lines and the F_1 hybrid

The parental line 'Vania' never presented typical CMV symptoms on both axillary shoots 30 days post-inoculation (p.i.) using the CMV-MES strain, indicating that the

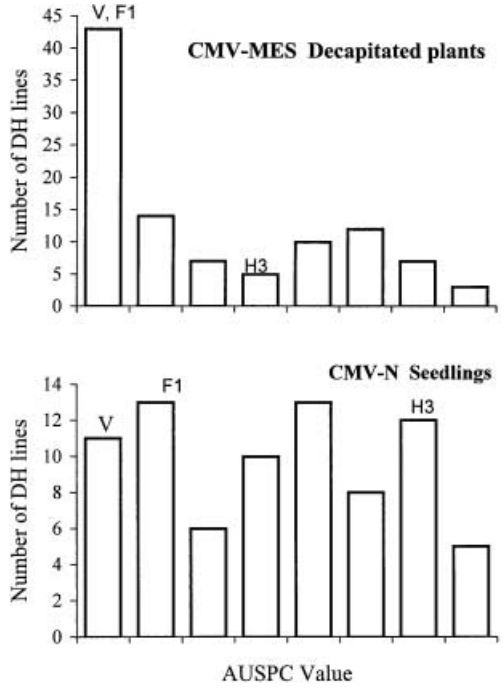

Fig. 1 Distribution of the doubled-haploid (DH) lines assessed by the CMV/MES and the CMV/N strains for partial resistance to CMV long-distance movement. (*V* 'Vania', *H3* 'H3', and F_1 hybrid between 'Vania' and 'H3'; because of missing data, only 78 DH lines were assessed using the CMV/N strain)

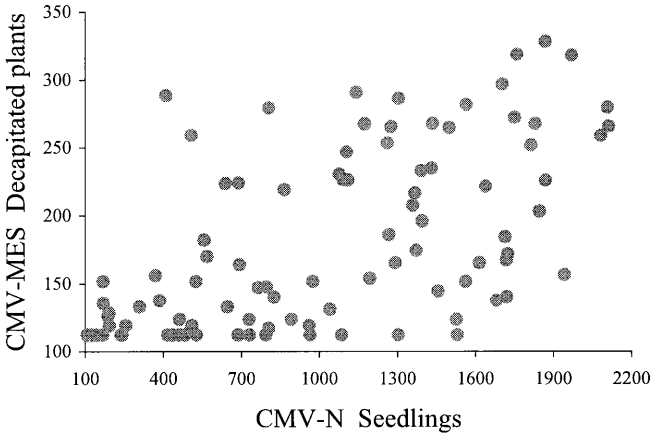

Fig. 2 Relationships between the two methods of resistance evaluation. Partial resistance was evaluated using the AUSPC criterion for both methods. The AUSPC value for each DH line varied from 100 (the most resistant) to 2,000 (the most susceptible) using the CMV-N strain on seedlings, and from 100 to 350 using the CMV-MES strain on decapitated plants

virus did not move to long-distance. Mosaic symptoms on leaves of the third axillary shoot became visible 16 days p.i. on half of the 'H3' plants. Thirty days p.i., all 'H3' plants presented mosaic symptoms on the third axillary shoot (corresponding to the inoculated leaf), 25% showed symptoms on both the 3rd and 4th shoots. The CMV-N strain inoculated on young 'Vania' seedlings became systemic on 18% of the plants 30 days p.i., whereas this percentage reached 84% when this strain was inoculated on the line 'H3'. For both resistance tests, the F_1 hybrid between 'Vania' and 'H3' showed a level of resistance similar to 'Vania', indicating that partial resistance is inherited as a dominant trait (Fig. 1).

Progeny distribution and relationship between the two resistance evaluation methods

In the progeny screening, only the DH lines inoculated with the CMV-MES strain and without symptoms on both axillary shoots 30 days p.i. could be considered as resistant. With this as a criterion, the DH progeny segregated 22 'Resistant': 79 'Susceptible'. This ratio did not fit with simple genetic models and validated the use of the Area Under the Symptom Progress Curve (AUSPC) criterion. For both resistance evaluation procedures, and according to the AUSPC criterion, the distribution of the DH lines was continuous (Fig. 1). The distribution of the DH lines assessed using the CMV-MES strain on decapitated plants suggested the intervention of a major genetic factor in resistance to CMV long-distance movement. However, the 'H3' parent presented an intermediate level of susceptibility and the occurrence of numerous transgressive DH lines indicated the segregation of secondary genetic factors. The distribution of the DH lines assessed using the CMV-N strain on seedlings highlighted a more complex genetic basis.

The relationship between the two resistance evaluation methods on the DH progeny is presented in Fig. 2. Data are correlated (correlation coefficient=0.61) but several DH lines classified as 'Resistant' using the CMV-MES strain on decapitated plants moved to susceptibility when inoculated with the CMV-N strain at an early plant stage, suggesting that this last method is much more severe.

Status of the intraspecific (H3×Vania) map and QTL mapping

Among the 543 loci mapped on the (H3×Vania) progeny, a subset of 184 (93 AFLP, 51 RFLP, 38 RAPD and two phenotypic markers) well distributed over the genome and used to anchor three pepper genetic linkage maps was selected for QTL mapping. The 184 markers were mapped onto 20 linkage groups (LGs), spanning a Haldane map distance of 1,600 cM. Alignment with other intraspecific linkage maps allows one to group the 20 LGs into 14 large and four small linkage groups.

The QTL results did not differ significantly using the three methods, LR, IM and CIM. All the QTLs detected by LR and IM were significant with CIM ; two additional QTLs were detected only by CIM but, in these cases, the LR and IM LOD-values were close to the significance threshold (data not shown). Since CIM increases the precision of the QTL location and the R^2 evaluation,

Table 1 QTLs detected for partial restriction of CMV long-distance movement in pepper assessed using two distinct procedures

Markers/QTLs	Location[a]	Trait	LOD score (CIM) Proba (epistasis)	R^2 (%)[b]	Allele[c]
QTLs with additive effect[d]					
E33/M48–132 *cmv 12.1*	P12 (Noir)	CMV-MES	13.43	45.0	V
		CMV-N	25.0	63.6	V
P14/M59–137 *cmv 11.2*	P11 (Brun)	CMV-N	4.62	7.8	V
L cmv 11.1	P11 (Brun)	CMV-N	2.68	4.0	V
E35/M48–475 *cmv 5.1*	P5	CMV-MES	4.11	13.1	H
QTLs with epistatic effect[e]					
NBS-GC148*P13/M47–361	P12-P1a	CMV-N	$0.24.10^{-5}$	28.8	H-H
P18/M47–285[f]*E32/M49–203	P5-P1b	CMV-MES	$4.9.10^{-5}$	25.4	H-H

[a] P numbers indicate the linkage groups in agreement with the nomenclature described in Livingstone et al. (1999) and color names refer to linkage groups assigned to chromosomes according to the pepper trisomic nomenclature
[b] Percentage of phenotypic variation explained
[c] V and H indicate that the Vania or the H3 allele increases the resistance
[d] Detected by composite interval mapping
[e] Detected by two-factor ANOVA with an interaction factor
[f] P18/M47–285 is linked to E35/M48–475

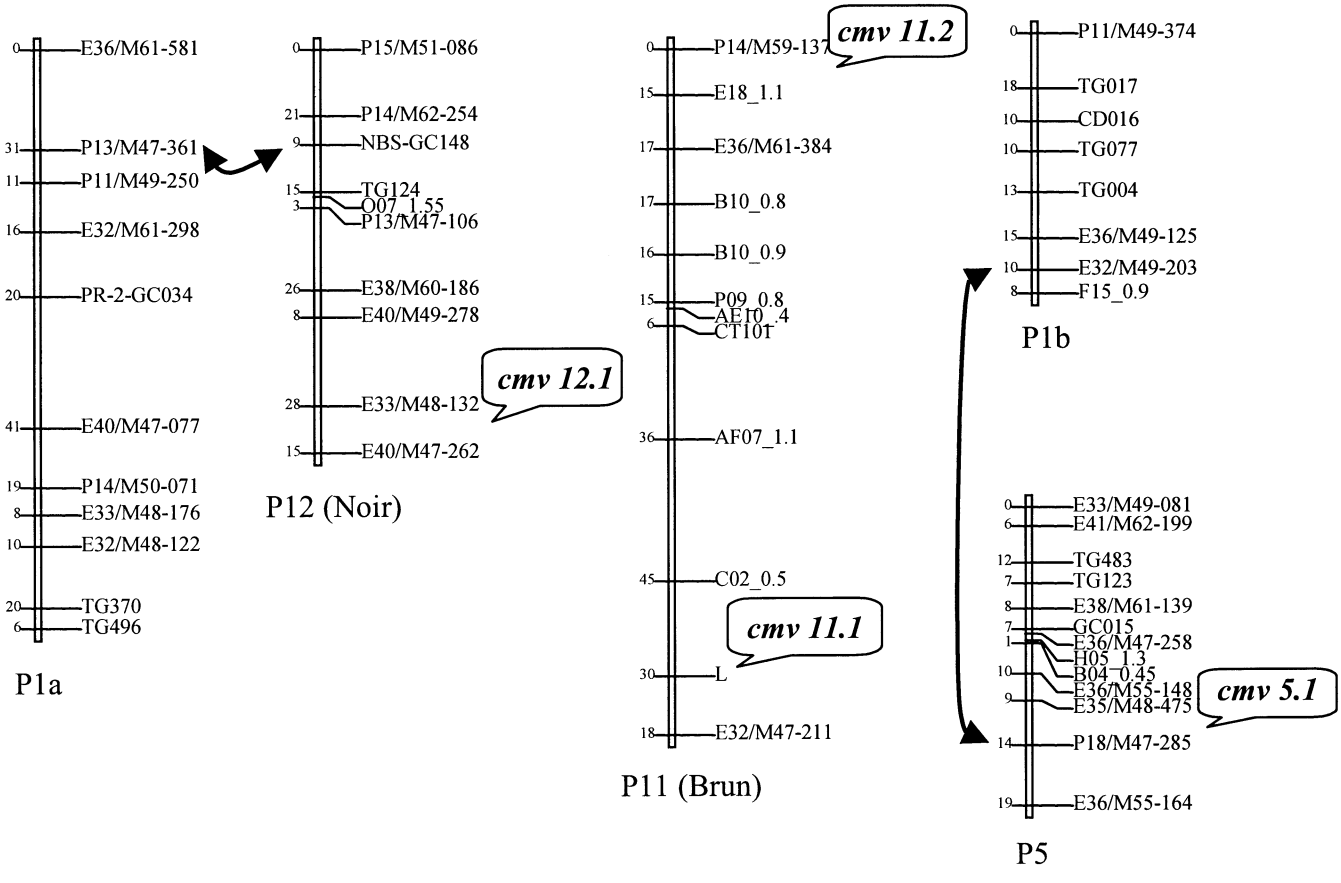

Fig. 3 Map location of quantitative trait loci involved in partial restriction of CMV long-distance movement on the (H3×Vania) intraspecific map of pepper. *Vertical bars* represent linkage groups (LGs). *P* (for pepper) *numbers* under the vertical bars indicate the chromosome number in agreement with the nomenclature described in Linvingstone et al. (1999). *Color names* are given to LGs assigned to chromosomes according to the pepper trisomic nomenclature. Only LGs holding QTLs associated with CMV resistance are shown. *Marker names* (RFLP, AFLP, RAPD and morphological markers) are indicated on the right. AFLP markers were generated with the primer combinations *Eco*RI-*Mse*I (E/M) or *Pst*I-*Mse*I (P/M); the molecular weight of the markers is indicated in base pairs at the end of the name. Two known genes were mapped on P1a and P12, respectively: PR-2-GC034 encodes for β-1,3 glucanase (Pflieger et al. 2001) and NBS-GC148 is a nucleotide binding site (NBS)-containing sequence (Pflieger et al. 1999). *Boxes on the right* indicate the position of additive effect QTLs involved in CMV resistance (maximum LOD score position according to CIM) and the *curved arrows* indicate digenic interactions between loci

and allowed more than one QTL to be mapped on the same chromosome (Zeng 1994), we focussed only on results obtained with this method.

A total of seven pepper genomic regions were identified as involved in partial restriction of CMV long-distance movement (Table 1, Fig. 3). Using both CMV-MES and CMV-N strains, a major-effect QTL named *cmv 12.1* and localized near the AFLP markers E33/M48–132 and E40/M47–262 was detected. It explained between 45 and 63.6% of the phenotypic variation depending on the evaluation procedure. The resistant parent allele contributed to the resistance. The other QTLs were specific from the method of resistance evaluation and presented a lower effect on the phenotype. Two genomic regions were associated with CMV resistance assessed using the CMV-MES strain: a minor-effect QTL named *cmv 5.1* on pepper LG P5 and a digenic interaction between this QTL and another one located near the AFLP marker E32/M49–203 on LG P1b. For both QTLs, the allele from the susceptible parent 'H3' increased the resistance. Four additional genomic regions were associated with CMV resistance, assessed using the CMV-N strain: two minor-effect QTLs located on pepper LG P11 (*cmv 11.1* and *cmv 11.2*) with the 'Vania' allele increasing the resistance, and a digenic interaction between the AFLP marker P13/M47–361 on LG P1a and the marker NBS-GC148 [Nucleotide Binding Site (NBS)-containing sequences] on the LG P12. In this latter case, the 'H3' allele for both loci increased the resistance.

Discussion

Restriction of CMV long-distance movement was inherited as a dominant trait and seven pepper genomic regions (with additive and/or epistatic effect) were associated with the resistance assessed using two distinct evaluation methods. Overall, partial restriction of CMV long-distance movement resulted from the association of one major-effect plus several minor-effect QTLs. The major-effect QTL *cmv 12.1* linked to the AFLP marker E33/M48–132 was detected using both evaluation methods, while the detection of the others (minor additive effect QTLs and digenic interactions) depends on the evaluation method. Moreover, alleles from both parents contributed to the resistance. This observation may explain *a posteriori* why the level of resistance to long-distance movement was continuously increased during the recurrent breeding process leading to the line 'Vania' (Pochard and Daubèze 1989): unknown QTLs from lines considered as 'Susceptible' were retained thanks to the phenotypical selection, promoting recombinations between the main resistance genitor *C. baccatum*, that brought the major QTL *cmv 12.1*, and the secondary QTLs from large-fruited *C. annuum* parents. Large transgressions for resistance were also obtained from the association of the different partial resistance mechanisms against CMV, and markers will help in a more-exhaustive exploitation of this large quantitative variability.

One QTL for the restriction of CMV installation in host-cells (Caranta et al. 1997) was mapped in the same genomic region as the major-effect QTL *cmv 12.1* identified in this study. Because of the distinct mapping population used for studying the restriction of CMV installation in host-cells and the restriction of long-distance movement, and the confidence interval around the position of the QTLs, we cannot assume if they are distinct, linked, or the same. However, two results argue for the linkage hypothesis: the *C. annuum* line 'Vania' is highly susceptible to CMV installation into the inoculated leaves, and 'Perennial' is highly susceptible to the long-distance movement of CMV (unpublished data); breeding data showed that large transgressions for resistance were obtained from the association of the two resistance components (Palloix et al.1997).

Another study identified four major-effect QTLs and three genomic regions involved in digenic interactions associated with tolerance to CMV in the *C. annuum* line 'Perennial' (Ben Chaim et al. 2001). In this case, partial resistance was assessed by visually scoring the intensity of the symptoms induced by the virus, 3 weeks after inoculation. Due to the lack of shared markers between the mapping populations and the difficulties to align common AFLP markers, only two QTLs among the four detected by Ben Chaim et al. (2001) can be reported on the intraspecific maps. The minor-effect QTL *cmv 11.1* linked to the *L* gene for resistance to *Tobacco mosaic virus* identified in this study was also detected by Ben Chaim et al. (2001). However, this QTL explained a larger part of tolerance to CMV than the restriction of long-distance movement, and the CMV resistance QTL from 'Perennial' was shown to be in repulsion phase with the *L* gene for TMV resistance, whereas, in this study, the CMV resistance QTL *cmv 11.1* from 'Vania' is transmitted together with the *L* resistance allele. The *C. annuum* line 'Perennial' was not included in the breeding program leading to the 'Vania' large-fruited variety, and the QTL *cmv 11.1* was inherited from *C. baccatum* or *C. annuum* resistance genitors also possessing the *L* allele. These data suggested that the CMV resistance factors are distinct but linked to the *L* locus for resistance to TMV and, consequently, they can be recombined in the same genotype. The second QTL detected in two different studies is *cmv 13.1* identified by Ben Chaim et al. (2001). This QTL seems to correspond to the QTL participating in the digenic interaction with marker TG66 and involved in the restriction of CMV installation in host cells (Caranta et al. 1997).

Overall, at least three pepper genomic regions were identified from distinct studies (using distinct resistance sources and/or distinct resistance evaluation methods) to have an effect on partial resistance to CMV: *cmv 12.1* and *cmv 11.1* from 'Vania' and 'Perennial', and *cmv 13.1* from 'Perennial'.

Resistance to the long-distance movement of CMV is a common resistance mechanism in Solanaceous crops, and hypersensitive or extreme resistance was never observed against this virus. As in pepper, a locus with a

major effect was identified to be involved in resistance to CMV long-distance movement in potato (Valkonen and Watanabe 1999). In tomato, partial resistance to CMV was also identified in wild species and one dominant factor (*Cmr*) was mapped (Stamova and Chetelat 2000). Comparative mapping analysis show that none of the QTLs identified in this study are orthologous to the *Cmr* locus. Co-localization with resistance gene analogs (RGA) was also surveyed in those two species. In our study, only the minor QTL on LG P12 involved in a digenic interaction with the AFLP marker P13/M47–361 was shown to co-segregate with a cluster of nucleotide binding site – leucine rich repeat (NBS-LRR) sequences. None of the major QTLs of pepper or tomato displayed such co-segregation with RGA (Pfieger et al. 1999; Pan et al. 2000). These data, together with the recent cloning of the *RTM1* and *RTM2* locus from *Arabidopsis* involved in restriction of the long-distance movement of the tobacco etch potyvirus (Chisholm et al. 2000; Whitham et al. 2000), suggest that genes controlling restriction of CMV long-distance movement include distinct classes of resistance genes.

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