Theor Appl Genet (2001) 102:1213–1220

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Identification of quantitative trait loci associated with resistance to cucumber mosaic virus in *Capsicum annuum*

Received: 17 July 2000 / Accepted: 16 October 2000

Abstract QTL analysis for resistance to cucumber mosaic virus (CMV) was performed in an intraspecific *Capsicum annuum* population. A total of 180 F3 families were derived from a cross between the susceptible bell-type cultivar Maor and the resistant small-fruited Indian line Perennial and inoculated with CMV in three experiments carried out in the USA and Israel using two virus isolates. Mostly RFLP and AFLP markers were used to construct the genetic map, and interval analysis was used for QTL detection. Four QTL were significantly associated with resistance to CMV. Two digenic interactions involving markers with and without an individual effect on CMV resistance were also detected. The QTL controlling the largest percentage (16–33%) of the observed phenotypic variation (*cmv11.1*) was detected in all three experiments and was also involved in one of the digenic interactions. This QTL is linked to the *L* locus that confers resistance to tobacco mosaic virus (TMV), confirming earlier anecdotal observations of an association between resistance to CMV and susceptibility to TMV in Perennial. An advanced backcross breeding line from an unrelated population, 3990, selected for resistance to CMV was analyzed for markers covering the genome, allowing the identification of genomic regions introgressed from Perennial. Four of these introgressions included regions associated with QTL for CMV resistance. Markers in two genomic regions that were identified as linked to QTL for CMV resistance were also linked to QTL for fruit weight, confirming additional breeding observations of an association between resistance to CMV originating from Perennial and small fruit weight.

Keywords *Capsicum* · Disease resistance · CMV · Molecular markers

Communicated by J. Dvorak

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Introduction

Cucumber mosaic virus (CMV) has been described as one of the five most important viruses infecting vegetable species worldwide. In pepper (*Capsicum* spp.), CMV can cause severe systemic mosaic symptoms, leaf distortion and fruit lesions, thereby drastically reducing marketable yield (Palukaitis et al. 1992). A broad host range that includes many weed species and a large number of insect vectors make cultural control of CMV difficult. Genetic resistance in the host plant is an ideal primary line of defense against CMV, since it requires neither exogenous chemical inputs nor transgenic manipulation and may be stable and long-lasting. Although resistance to CMV has been reported in several pepper accessions (Dogimont et al. 1994), the genetic control of resistance is complex, and commercial varieties with an adequate level of CMV resistance are not yet available (Green and Kim 1991; Monma and Sakata 1997).

Capsicum annuum var. Perennial has been described as resistant to CMV by numerous researchers (Rusko and Csillery 1980; Pochard and Daubeze 1989; Nono-Womdim et al. 1993a, b; Lapidot et al. 1997). Several resistance mechanisms have been proposed for Perennial: these include a partial resistance to initial viral infection as evidenced by reduced development of local necrotic lesions (Caranta et al. 1997a), a reduction in viral replication (Nono-Womdim et al. 1993a) and a restriction of long-distance movement of the virus (Nono-Womdim et

al. 1993b). Resistance in Perennial has been reported to be controlled by one to several genes that show recessive or partially dominant inheritance (Lapidot et al. 1997; Rusko and Csillery 1980; Singh and Thakur 1977). The wide range of resistance mechanisms and the reported differences in inheritance may be a consequence of the effects of environmental conditions, CMV isolates or genetic backgrounds.

Many agronomically important traits in crop plants exhibit a continuum of phenotypic variation in a segregating population, suggesting that they are under the control of several genes, each of which may account for only a small portion of the existing phenotypic variation. The identification of regions of the genome containing quantitative trait loci (QTL) and subsequent analysis to reveal which QTL makes the largest contribution across multiple environments are likely to facilitate combination of the most useful QTL in advanced genotypes. Tightly linked markers are required for effective marker-assisted selection and are also the starting point for map-based cloning of alleles at QTL of interest. In pepper, the QTL identified and mapped thus far include those involved in resistance to *Phytophthora capsici* (Lefevbre and Palloix 1996), potato virus Y (Caranta et al. 1997b) and CMV (Caranta et al. 1997a). More recently, QTL controlling several fruit and horticultural characteristics such as fruit shape, color, yield and soluble solids content have been mapped in the same population described in this paper (Ben Chaim et al. 2001).

The primary objective of the study reported here was to identify QTL associated with resistance to CMV derived from the variety Perennial in two environments. We report on the identification of a major QTL for resistance to CMV linked to the *L* gene that confers resistance to tobacco mosaic virus (TMV). This QTL and the *L* gene occur in a cluster of resistance genes located on chromosome 11 in a position that apparently overlaps with resistance gene clusters in tomato and pepper (Grube et al. 2000). Further, whole-genome fingerprinting was used to determine whether genomic regions containing the identified QTL had been introgressed from var. Perennial into the CMV-resistant breeding line 3990 that has been described by Lapidot et al. (1997).

Materials and methods

Plant material

Capsicum annnum Maor, an inbred sweet bell pepper variety, was obtained from Dr. C. Shifriss (The Volcani Institute, Israel) and used as the CMV-susceptible, TMV-resistant (L^1/L^1) genotype. The CMV-resistant, TMV-susceptible (*l*/*l*) genotype, *C. annuum* var. Perennial, is an inbred pungent small-fruited Indian variety and was provided by Dr. A. Palloix (INRA, France). Individual plants from the intraspecific (Maor×Perennial) F2 population resulted from a single F1 plant; these were used for molecular genotyping and were self-pollinated in a closed greenhouse to yield 220 F3 families from which 180 families were chosen at random for CMV inoculation and mapping. The CMV-resistant BC5F6 line 3990 was developed using Perennial as the donor of CMV resistance crossed with Maor and additional bell-type lines as the recurrent parents, as described by Lapidot et al. (1997).

Evaluation of resistance to CMV

Three inoculation experiments were performed to evaluate CMV resistance. Two experiments were performed in 1997 and 1998 at the Volcani Institute (Israel) using a subgroup-I isolate of CMV described by Lapidot et al. (1997). A third experiment was performed at Cornell University (Ithaca, N.Y., USA) in 1997 using the subgroup-I isolate CMV-V27 (provided by John Hubbard, Seminis Vegetable Seeds). For each experiment, at least 20 plants from each of the 180 (Maor×Perennial) F3 families as well as both parents and their F1 were inoculated. At the Volcani Institute, the first true leaf of plants seeded in Speedling trays (Speedling, Sun City, Fla.) were inoculated as described by Lapidot et al. (1997). Briefly, 1 g of inoculum obtained from CMV-infected cucumber plants in a 20 m*M* potassium phosphate buffer, pH 7.0 was applied by rubbing carborundum-dusted leaves. At Cornell University, plants were seeded in covered seeding trays and transplanted into 6×12 Speedling trays. The first and second true leaves were rub-inoculated with inoculum consisting of 1 g of systemically infected squash leaf tissue ground in 10 ml of 50 m*M* potassium phosphate buffer, pH 7.0. Plants were rinsed with water within 30 min after inoculation.

At the Volcani Institute, resistance to CMV was evaluated 3 weeks after inoculation, at the six to eight true leaf stage. Plants were examined visually and rated according to the severity of the mosaic and leaf distortion (0–no symptoms, 1–mild mosaic, no leaf distortion; 2–strong mosaic, mild leaf distortion; 3–severe mosaic and distortion). Individual plant ratings were used to calculate F3 family means. At Cornell University, plants were monitored daily for symptom development, and data were first collected 3 weeks post-inoculation. Each plant was rated for mosaic symptoms (0 – no mosaic, 5 – severe systemic mosaic) and leaf distortion (0 – no leaf distortion, 5 – severe systemic leaf distortion). The mosaic and distortion ratings were summed for each plant and these values were averaged over all plants in each F3 family to give an overall rating of symptom severity for the progenitor F2 plant.

TMV inoculation

Both parents and 20 seeds of each 180 F3 families were sown in a Speedling tray at the Volcani Institute in Spring 2000. Four-week-old seedlings were mechanically inoculated with TMV strain P0 maintained in tomato by rubbing the third and fourth true leaves with a diluted sap from infected tissue after dusting the plants with carborundum powder. Plants were scored for the presence of local lesions observed on resistant plants and for a mosaic response that appeared on susceptible plants within 3–6 days or 2–3 weeks after inoculation, respectively.

Marker and statistical analyses

Map construction and QTL analyses for this population have been described in detail by Ben Chaim et al. (2001). To summarize briefly, tomato and pepper restriction fragment length polymorphism (RFLP), codominantly scored amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers were used for genotypic analysis, and the genetic map was constructed using MAPMAKER/EXP v. 2.0 (Lander et al. 1987). The *L* gene that confers resistance to TMV was added to the map by scoring the population as described previously. QTL mapping was performed using interval analysis, with LOD 3.6 as a threshold for QTL detection. This threshold corresponded to an experimental-wise significance of 0.05 as determined by permutation analysis of 1000 datasets performed by QGENE software (Nelson 1997). Estimation of gene action and the magnitude of QTL effects were performed using QGENE. Interactions between all mapped codominant markers were detected by two-way ANOVA tests using SAS/STAT v. 6 (SAS Institute 1989) and JMP v. 3.0 Macintosh. Each significant interaction was partitioned into four components (additive×additive, additive×dominance, domi-

Table 1 Means, standard errors (SE) and heritabilities of resistance to CMV in the parental, F_1 and F_3 generations

Cmv screen	Mean[a]	SE	Mean	SE	Mean	SE	Mean	SE	Heritability
	Maor		Perennial		F_1		F_3		
Volcani 97	2.8	0.03	0.17	0.04	1.63	0.1	2.0	0.04	0.42
Volcani 98	3.0	–	0	–	–	–	1.4	0.03	0.38
Cornell 97	7.1	0.24	0.3	0.14	3.5	0.38	3.3	0.09	0.44

[a] The scale of resistance in the Volcani 97 and Volcani 98 screens was from 0 to 3; the scale of resistance in the Cornell 97 screen was from 0 to 10

Table 2 List of QTL for resistance to CMV detected in the F_3 progeny of Maor×Perennial

CMV screen	QTL	Marker	LG	Direction[a]	*F*	Variation explained		*P* value	Effect[b]		D/A
						Locus	Trait		Additive	Dominance	
Volcani 97	cmv 4.1	E48/M49–270	4	Perennial	8.7	0.10		0.0003	0.21	–0.18	–0.9
	cmv 6.1	$OPA12_{1700}$	6	Perennial	14.8	0.08		0.0002	0.24	0	0.0
	cmv 11.1	E35/M48–101	11	Perennial	24.8	0.22	0.34	0	0.33	–0.14	–0.4
Volcani 98	cmv 11.1	*L*	11	Perennial	17.1	0.16		0	0.21	–0.12	–0.6
	cmv 13.1	$OPP11_{500}$	UL[c]	Perennial	12.7	0.07	0.25	0.0005	0.19	0	0.0
Cornell 97	cmv 11.1	E35/M48–101	11	Perennial	42.7	0.33		0	0.82	–0.26	–0.3

[a] Indicates the parent which contributes to an increased level of resistance

[b] A positive sign of the additive effect indicates that the allele originating from Perennial increases the level of resistance

[c] UL, Unlinked

nance×additive and dominance×dominance). These components were estimated using an orthogonal test performed using JMP v. 3.0 as described by Yu et al. (1997). The genotype of the advanced BC5F6 CMV-resistant line 3990 (Lapidot et al. 1997) was determined for all AFLP markers used to construct the Maor×Perennial genetic map by Keygene (Wageningen, The Netherlands). DNA samples from 3990, Maor and Perennial were also screened with 180 RAPD primers (kits A, B, C, E, F, G, H, J and P, Operon Technology) as described by Paran et al. (1998) to identify those markers that were present in both Perennial and 3990 and absent in Maor. Correlations among the results obtained for the three experiments and narrow-sense heritabilities obtained by determining the components of variance in the F3 generation were estimated as described by Ben Chaim and Paran (2000).

Results

Maor and Perennial exhibited large differences in their response to inoculation with CMV in all experiments (Table 1). Most Maor plants had severe mosaic and leaf distortion, while most Perennial plants were free of symptoms. A few Perennial plants developed mild mosaic symptoms but no leaf distortion, which is consistent with previous reports that the CMV resistance in Perennial is incompletely penetrant (Lapidot et al. 1997; Grube 1999). The F1 of Maor×Perennial had an intermediate phenotype, indicating incomplete dominance of the resistance response.

Responses to inoculation with CMV were only moderately correlated for the three experiments; furthermore, the two Volcani experiments showed only a slightly stronger association with each other (r=0.56) than either did with the Cornell 97 experiment (r=0.52 and 0.44 with Volcani 97 and Volcani 98, respectively). Similarly, narrow-sense heritability values estimated for each experiment were also only moderate (Table 1).

A total of four CMV resistance QTL were identified (Table 2, Fig. 1). For all QTL, the alleles originating from Perennial contributed to an increased level of resistance. A single QTL found on linkage group (LG) 11, *cmv 11.1*, was identified in all three experiments. In the Volcani 97 experiment, *cmv 11.1* explained 22% of the phenotypic variation for the trait, and two additional QTL were detected on LG4 and LG6. Multiple regression on all three QTL gave a R^2 value of 0.34. In the Volcani 98 experiment, *cmv 11.1* explained 16% of the observed phenotypic variation. An additional QTL, associated with the unlinked RAPD marker $OPP11_{500}$, was also detected. The two QTL together accounted for 25% of the phenotypic variation observed. *cmv11.1* was the only QTL detected for the Cornell 97 experiment, and it explained 33% of the variation for this inoculation.

TG36, linked to *cmv11.1* in the present study, was previously identified as being linked to the *L* gene that confers resistance to TMV (Lefebvre et al. 1995). In order to determine the linkage relationships between the resistance genes for CMV and TMV, we scored the F3 population for reaction to TMV. *L* and the AFLP marker E35/M48–101 were found to be less than 2 cM apart (Fig. 1). While E35/M48–101 had the highest *F* value at *cmv11.1* in the Volcani 97 and Cornell 97 experiments, *L* had the highest *F* value at this QTL in the Volcani 98 experiment (Table 2).

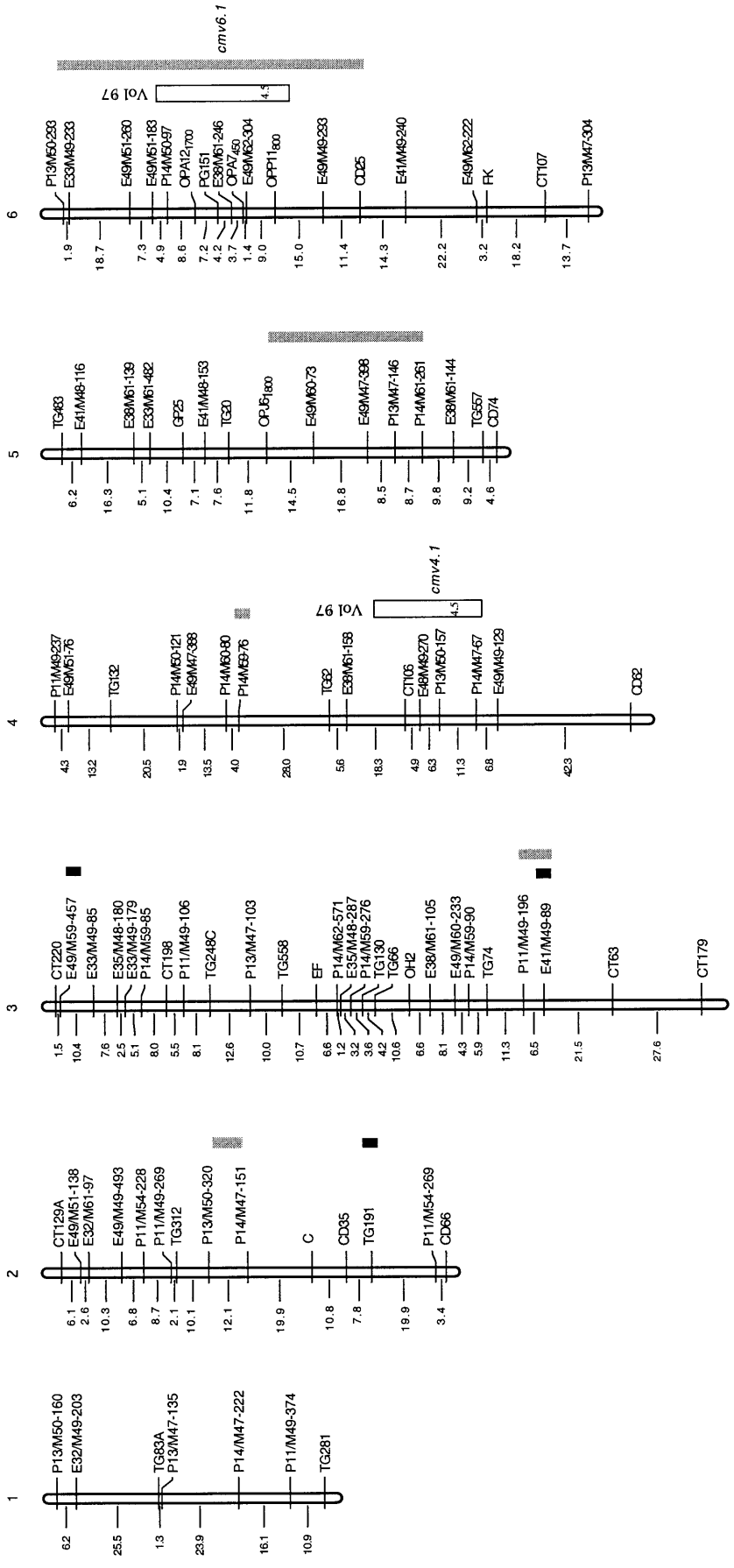

Fig. 1 QTL mapping for CMV resistance in *C. annuum*. Marker types are as follows: *TG*, *CT*, *CD* (*G*) tomato RFLP clones, *PG* pepper RFLP clones, *GP* potato RFLP clones. AFLP markers are presented as the primer combination followed by the size of the mapped fragment. RAPD markers are presented as *OP* followed by the primer name and band size. Morphological markers were *C* (pungency) and *up* (erect fruit type). *EF* and *B2* are pepper RFLPs obtained from Dr. U. Bonas (Martin Luther University, Germany). *pN18C* is a tobacco gene provided by B. Baker (USDA, Albany, Calif.). *OH2* is a tomato beta-carotene hydroxylase cDNA obtained from Dr. J. Hirschberg (The Hebrew University of Jerusalem). *FK* is potato fructokinase cDNA obtained from Dr. D. Granot (The Volcani Institute, Israel). Distances in centiMorgans are to the *left* of each linkage group. QTL detected for CMV resistance in each experiment are indicated as follows. Main-effect QTL are presented as *white bars* to the *right* of the linkage group; the *number* in each bar is the LOD peak value; the *length* of the bar represents a 1-LOD support interval from the peak LOD of the QTL. *Black bars* represent QTL with no main effect that were detected in digenic interactions. *Shaded bars* represent introgressions from cv. Perennial present in the advanced backcross CMV-resistant line 3990. Trait abbreviations are: Vol 97 Volcani 97, Vol 98 Volcani 98; Cor 97 Cornell 97

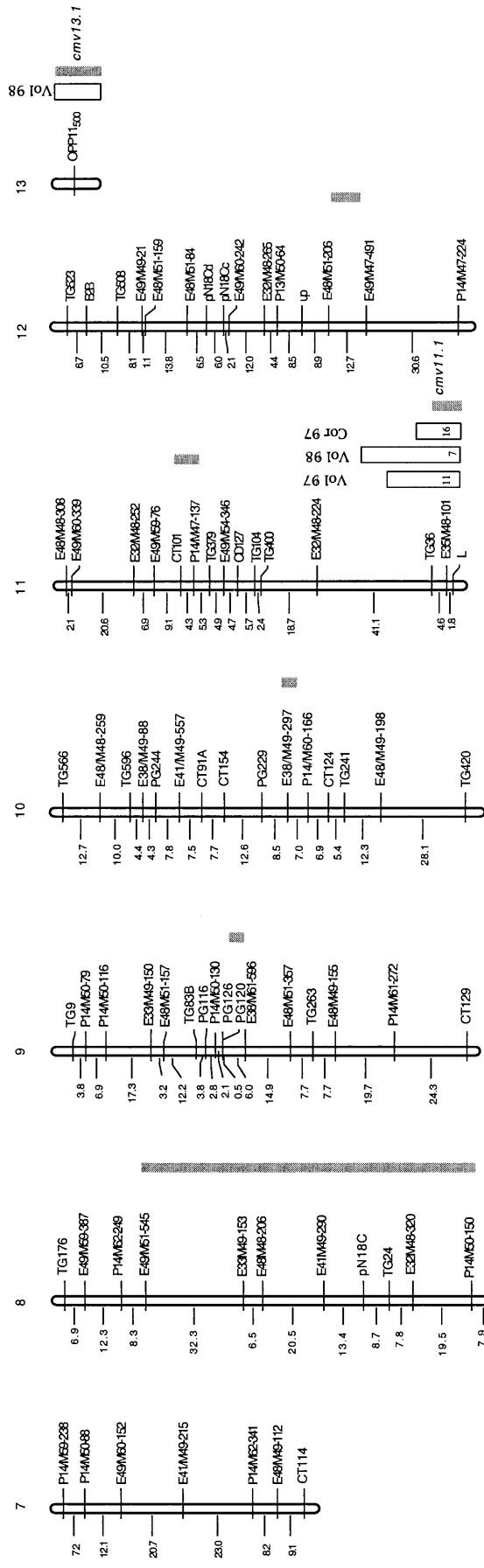

Fig. 1 (continued)

Table 3 Two-locus interactions for markers associated with CMV resistance (*LG* linkage group)

CMV screen	Marker 1	LG	Marker 2	LG	Type [a]	*P* [b]	R^2 [c]
Volcani 97	TG191	2	E35/M48–101 [d]	11	AD	0.0001	0.35
Volcani 98	TG191		E35/M48–101		AD	0.002	0.25
Cornell 97	TG191		E35/M48–101		AD	0.004	0.46
Volcani 97	E41/M49–89	3	E49/M59–457	3	AD	0.0009	0.13
Cornell 97	E41/M49–89		E49/M59–457		AD	0.0001	0.17

[a] Interaction type: AD, additive×dominance
[b] Probability for the interaction type derived from parameter estimates in the Fit Model function of JMP
[c] Variance explained by the interaction
[d] Marker had a main QTL effect

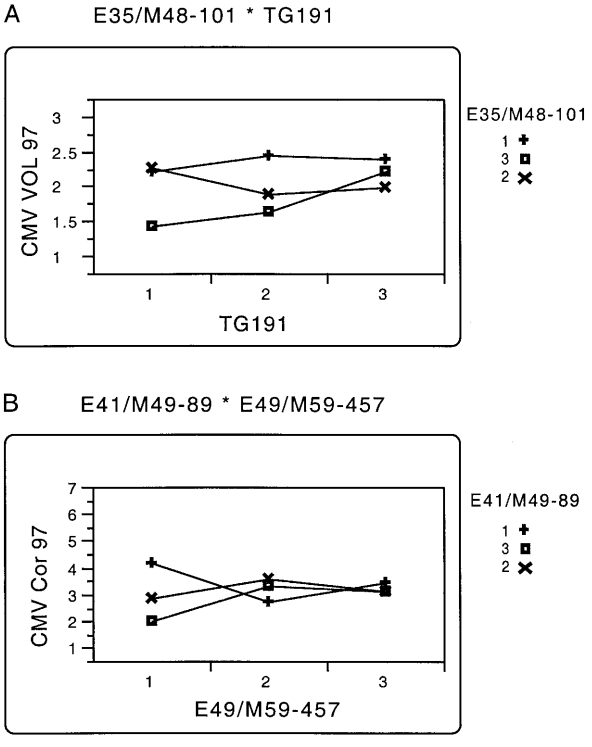

Fig. 2A, B Plots of phenotypic means for each of the marker classes in the digenic interactions. **A** Interaction between E35/M48–101 and TG191. **B** Interaction between E41/M49–89 and E49/M59–457. The numbers *1*, *2*, and *3* represent the three genotypic classes: *1* homozygote cv. Maor allele, *2* heterozygote, *3* homozygote var. Perennial allele

Two-way ANOVA for all mapped codominant markers allowed the detection of epistatic effects ($P<0.001$) on CMV resistance (Table 3, Fig. 2). Two digenic interactions were detected in more than one experiment. One interaction involved a marker (E35/M48–101) linked to QTL with main effect, *cmv11.1*, and the RFLP marker, TG191, located on LG2. This interaction was detected in all three experiments. The total amount of phenotypic variability explained by this interaction and the main effect of *cmv11.1* was approximately 10% higher than that attributed to *cmv11.1* alone. The highest level of resistance was observed when the Maor allele at TG191 was present with the Perennial allele at E35/M48–101. The second interaction was detected for two markers on LG3, neither of which were associated with CMV resistance. As in the first interaction, the combination of a Maor allele at one locus (E49/M59–457) with a Perennial allele at the second locus (E41/M49–89) was associated with a higher level of resistance (Fig. 2).

The advanced pepper breeding line 3990 has a CMV resistance response similar to Perennial, and was therefore analyzed with RAPD and AFLP markers throughout the genome to determine which genomic regions had been introgressed through the backcross breeding program from Perennial. Out of 180 RAPD primers that were screened, four (OPA7, OPA12, OPP11 and OPJ6) amplified fragments were present in both Perennial and 3990 and absent in Maor. $OPA7_{450}$, $OPA12_{1700}$ and $OPP1_{1800}$ were linked to each other and located in the region of LG6 containing the *cmv6.1* QTL (Fig. 1). A second locus ($OPP11_{500}$) amplified by OPP11 was identified as the QTL *cmv13.1*. $OPJ6_{1800}$ mapped to LG5 and was not significantly associated with CMV resistance in any of the experiments conducted.

AFLP fingerprinting of 3990 detected introgressions from Perennial in 10 of the 12 linkage groups (Fig. 1). Except for three introgressions in LG5, 6 and 8 that included large portions of the chromosomes, all other introgressions were small and did not exceed 15 cM in length. Four of the introgressions, located on LG3, LG6, LG11 and the unlinked marker $OPP11_{500}$ contained markers linked to QTL for resistance to CMV, including the epistatic marker E41/M49–89 in LG3.

Discussion

This study has identified QTL associated with resistance to CMV in the *C. annuum* line Perennial. Previous QTL analysis in a doubled haploid population derived from a cross between a susceptible *C. annuum* cultivar Yolo Wonder and Perennial identified three genomic regions associated with resistance to the Fulton CMV strain that induces necrotic local lesions upon infection (Caranta et al. 1997a). None of these QTL can be definitively assigned to positions on the interspecific comparative map of pepper generated in our lab (Livingstone et al. 1999) because of the minimal number of shared markers used in the two mapping populations. However, one QTL identified by Caranta et al. (1997a) likely corre-

sponds to *cmv6.1* identified in the present study, because both QTL are linked to the same RAPD marker amplified by primer OPP11. A second QTL identified by Caranta et al. (1997a) was linked to the *up* locus causing an erect fruit type. In the current study, the *up* locus mapped to LG12 and was not significantly associated with CMV resistance. However, when we used a larger data set of 220 F3 families from our same mapping population, *up* was significantly associated with CMV resistance (P=0.001 and 0.005 in one-way ANOVA for Volcani 97 and Cornell 97, respectively). Furthermore, line 3990 contained a small introgression from Perennial linked to *up*. The third QTL identified by Caranta et al. (1997a) was linked to TG66. This marker is located in LG3 in the present map but did not have an effect on the resistance to CMV.

The incomplete correspondence between the QTL identified in the present study and those identified by Caranta et al. (1997a) may be the result of using different CMV strains and resistance evaluation methods. While the QTL study conducted by Caranta et al. (1997a) was focused on a specific resistance mechanism, i.e. restriction of virus entry into the host cell, the present study did not focus on a specific resistance mechanism but rather on an overall resistance response as indicated by diminished disease symptoms presumably resulting from a combined effect of several resistance components.

The major QTL identified in the present study was *cmv11.1*, linked to the AFLP marker E35/M48–101 and to *L*. This region was detected in all three experiments and was also involved in a digenic interaction with the marker TG191, explaining a total of 25–46% of the total observed phenotypic variation for the resistance. A linkage between resistance to CMV and susceptibility to TMV in Perennial was reported by Pochard et al. (1983), who noted that, out of 78 doubled haploid lines subsequently used in the QTL study by Caranta et al. (1997a), all of the CMV-tolerant lines were susceptible to TMV. Our QTL data indicating linkage in *cis* between the CMV resistance allele at *cmv11.1* and the susceptibility allele at *L* confirm this earlier observation.

Further support for our QTL mapping results came from fingerprinting the CMV-resistant breeding line 3990. Perennial alleles at marker loci linked to three of the four QTL with major effects on resistance in the mapping population (*cmv6.1*, *cmv11.1* and *cmv13.1*) were present in 3990. Furthermore, the introgressions from Perennial resembled the pattern of the digenic interactions identified in the QTL analysis, i.e. the presence of Maor and Perennial alleles at TG191 and E49/M59–457 and *cmv11.1* and E41/M49–89, respectively.

Genetic linkage between loci affecting fruit size and CMV resistance has been proposed as a cause of the relatively slow progress in developing CMV-tolerant large-fruited pepper genotypes by conventional breeding methods (Lapidot et al. 1997; C. Shifriss, The Volcani Institute, Israel, personal communication). Support for this hypothesis was obtained by performing QTL analysis for resistance to CMV and for horticultural traits including fruit weight in this same mapping population (Ben Chaim et al. 2001). Two QTL for fruit weight, *fw3.2* and *fw4.1* linked to E41/M49–89 and P14/M47–67, respectively, were found in the same regions as QTL conferring resistance to CMV (Ben Chaim et al. 2001). In both cases, Perennial alleles were associated with reduced fruit weight and increased CMV resistance. Analysis of the resistant line 3990 revealed the presence of Perennial alleles in the regions containing *fw3.2* and *fw8.1* linked to E33/M49–153, both associated with decreased fruit weight. Line 3990 had intermediate-sized fruits (Lapidot et al. 1997) despite intense selection, probably as a result of the unfavorable linkages described. The availability of linked molecular markers to these regions will facilitate the identification of recombinant genotypes that would otherwise be difficult to identify phenotypically.

The genomic region containing *cmv11.1* and *L* in pepper also contains a QTL for resistance to *Phytophthora capsici* (Lefebvre and Palloix 1996). In addition, the corresponding region in tomato and potato contains resistance genes to different pathogens (Grube et al. 2000). In tomato, the gene *I2* conferring resistance to *Fusarium oxysporum* is tightly linked to TG105, which in turn is linked to TG36 (Sarfatti et al. 1989). The *Ty-2* gene conferring resistance to tomato yellow leaf curl virus was also identified as being linked to both TG105 and TG36 (Hanson et al. 2000). In potato, genes conferring resistance to *Phytophthora infestans* (R3, R6 and R7) were mapped to markers linked to TG105 (El-Kharbotly et al. 1996). Fine comparative mapping and sequence comparison of BAC clones containing the resistance genes in the three Solanaceae species will allow definite determination of the evolutionary relationships among these different resistance genes.

The availability of molecular markers linked to genes controlling resistance to CMV in pepper could facilitate marker-assisted selection in breeding programs aiming to transfer the resistance from Perennial to elite breeding lines. The possibility of obtaining plants resistant to both TMV and CMV remains to be determined. Our F_3 progeny screening identified one recombinant TMV-resistant (L^1/L^1) F_2 plant that was heterozygous at E35/M48–101 and had good level of resistance to CMV. If recombination has occurred between *L* and *cmv11.1*, it will be possible to fix plants that are homozygous Maor and homozygous Perennial at *L* and *cmv11.1*, respectively. Knowledge of the locations of QTL controlling both CMV resistance and important horticultural traits in crosses with Perennial will allow the design and implementation of more efficient selection schemes to develop CMV-resistant pepper genotypes.

Acknowledgments We thank V. Lackney, J. Jantz and G. Moriarty for technical assistance and L. Landry for critical review of the manuscript. This research was supported by research grant no. IS-2389–94 from BARD, The United States-Israel Binational Agricultural Research and Development Fund and USDA NRI Award no. 94–37300–0333 and the California Pepper improvement Foundation/California Pepper commission. RCG was supported by a DOE/NSF/USDA interagency Research Training Group grant.

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