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Inheritance and genetic mapping of cucumber mosaic virus resistance introgressed from *Lycopersicon chilense* into tomato

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Abstract Cucumber mosaic virus (CMV) infects a wide variety of crop plants and in tomato (*Lycopersicon esculentum* Mill.) causes significant economic losses in many growing regions, particularly the Mediterranean. The objective of the present study was to identify the number and map locations of genes controlling resistance to CMV in breeding lines (BC_1–inbreds) derived from the related wild species *L. chilense*. These lines also carried the gene *Tm-2*^{a} for resistance to ToMV, which facilitated the interpretation of disease symptoms. The segregation for CMV resistance in the BC_2F_1 and BC_2F_2 generations, following mechanical inoculation with subgroup-I isolates, was consistent with expectations for a single dominant gene, for which the symbol *Cmr* (cucumber mosaic resistance) was given. Resistant and susceptible BC_1-inbreds were analyzed with RFLP and isozyme markers to identify genomic regions introgressed from *L. chilense*. The only *L. chilense*-specific markers found were on chromosome 12; some resistant lines contained a single introgression comprising the entire short arm and part of the long arm of this chromosome, while others contained a recombinant derivative of this introgression. The chromosome 12 markers were significantly associated with CMV resistance in both qualitative and quantitative models of inheritance. The qualitative analysis, however, demonstrated that CMV resistance was not expressed as a reliable monogenic character, suggesting a lack of penetrance, significant environmental effects, or the existence of additional (undetected) resistance factors. In the quantitative analysis, the marker interval TG68 – CT79 showed the most significant association with CMV resistance. No association between CMV resistance and the *Tm-2*^{a} gene was observed. These breeding lines are potentially useful sources of CMV resistance for tomato improvement, in which context knowledge of the map location of *Cmr* should accelerate introgression by marker-assisted selection.

Key words CMV · *Lycopersicon esculentum* · Disease resistance · Breeding · *Cmr*

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Introduction

Cucumber mosaic virus (CMV) is an important disease on tomatoes in temperate regions and is the most destructive virus in some areas. CMV has been identified as the causal agent of several disease epidemics (Palukaitis et al. 1992), including severe outbreaks in eastern France (Gebre et al. 1990), in southern Italy (Crescenzi et al. 1993), and in Bulgaria (Stamova et al. 1990). CMV and potato virus Y (PVY) are considered to be the two most important virus diseases in the Mediterranean countries (Parella et al. 1997). In the U.S., outbreaks of CMV on tomatoes have been reported in Alabama (Sikora et al. 1998) and the San Joaquin valley of California (R. Gilbertson, personal communication), a state that produces approximately 50% of the world's supply of processing tomatoes.

CMV has an exceptionally wide host range, infecting more than 800 plant species (Palukaitis et al. 1992). It is aphid-transmitted and consists of many strains that differ in the disease symptoms they elicit and/or in their host range. The manifestations of CMV infection on tomato are quite diverse, ranging from no symptoms to severe stunting, fern leaf, 'shoestring' (filiform) leaves, and necrosis. Symptoms vary depending on the virus strain, presence of satellite RNAs, and host genotype.

Control of CMV is complicated by (1) its broad host range, which allows many crops and also non-crops (e.g., weeds and ornamentals) to serve as virus reservoirs, (2) its broad aphid transmissibility, and (3) a lack of natural genetic resistance in many crops and/or a failure to incorporate resistance into commercial varieties. In contrast to host plant sources, pathogen-derived resistance has been successfully used to develop CMV-resistant varieties. For example, transgenic tomato plants

expressing the coat protein gene of CMV were highly resistant to infection (Xue et al. 1994). CMV satellite RNA and replicase genes also confer resistance or immunity in transgenic tomatoes (Gal-On et al. 1998; McGarvey et al. 1994).

The availability of commercial varieties possessing disease resistances is a major factor underlying the success of tomato cultivation under diverse growing conditions, providing excellent control for many diseases and reducing the need for pesticides. Because of limited genetic diversity within the cultivated tomato, related wild species have been valuable sources of many economic traits, particularly disease resistances, virtually all of which have been derived from exotics (Rick and Chetelat 1995). Most of these resistances are controlled by a single gene, usually dominant or incompletely dominant.

Environmental conditions and stage of plant development often influence the effectiveness of resistance genes. For example, CMV resistance in *Lycopersicon hirsutum* is influenced by temperature and plant age (Parella et al. 1997). Durability of resistance also varies. For instance, the resistance to leaf mold (*Cladosporium fulvum*) encoded by *Cf* genes in tomato has been broken relatively quickly by the appearance of new races of the pathogen. On the other hand, resistance genes such as *Tm-2*^{a} and *Mi*, conferring resistance to tomato mosaic virus (ToMV) and root knot nematodes, respectively, have been in use for decades and remain highly effective.

Several wild tomato species are resistant or tolerant to CMV, including *L. pimpinellifolium, L. peruvianum, L. hirsutum, L. cheesmanii* var. *minor*, *L. chilense* and *Solanum lycopersicoides* (Gebre et al. 1990; Nitzany 1972; Parella et al. 1997; Phills et al. 1977; Stamova 1993; Stoimenova et al. 1992). Despite the availability of these genetic resources, no resistant or tolerant cultivars derived therefrom are presently available. The genetic basis of CMV resistance in these species is largely unknown. However, in the related nightshade *Capsicum annuum*, CMV tolerance is a quantitative character, with incomplete dominance, for which several quantitative trait loci (QTLs) have been mapped (Caranta et al. 1997; Lapidot et al. 1997).

In the study reported here, we examined the genetic basis of CMV resistance in breeding lines derived, by backcrossing and selection, from *L. chilense*. The objective was to determine the number and map locations of resistance genes and thereby to develop molecular markers to facilitate the eventual incorporation of this resistance into improved varieties.

Materials and methods

Plant material

These investigations made use of lines derived from a cross between the cultivated tomato (*Lycopersicon esculentum*, putatively cv. Ace) and its wild relative *L. chilense* (accession LA0458), which were previously reported to be resistant to Bulgarian and Hungarian isolates of CMV (Stamova et al. 1990). LA0458 was collected by C.M. Rick near Tacna, Dept. Tacna, Peru, and was obtained from the Tomato Genetics Resource Center at UC-Davis. *L. chilense* has the same chromosome number as tomato (2n = 2x = 24) and is a self-incompatible outcrossing species possessing significant diversity within and between populations (Rick 1988). The original interspecific F_1 was made at the Maritza Vegetable Crops Research Institute in Plovdiv, Bulgaria, by crossing *L. esculentum* as female parent to *L. chilense* (Manuelyan et al. 1975). A single backcross to *L. esculentum* was made, followed by at least 14 generations of self-pollination and selection. During inbreeding, selection was applied for horticultural traits such as fruit traits (shape, size, color, and setting ability) and resistance to several diseases, including ToMV, powdery mildew (*Leveillula taurica*), as well as CMV. Resistance to CMV was found in a subset of the *L. taurica*-resistant lines (L. Stamova, personal communication).

For the present experiments, the most advanced backcross-inbred generation (approx. BC_1F_{15}, hereinafter referred to as BC_1-inbred), was crossed to CMV-susceptible *L. esculentum* cv. VF36, resulting in a BC_2F_1. Two resistant BC_2F_1 plants were selfed to create segregating BC_2F_2 populations. The BC_1-inbreds were also surveyed with molecular markers to identify *L. chilense* introgressions (see below). The BC_2F_1 and BC_2F_2 were used to study the inheritance and dominance of CMV resistance and to determine the map location of the underlying gene(s). The *L. esculentum* varieties used in this study were VF36, Ace, Earlipak and Vendor-Tm2^a, all of which are susceptible to CMV. The first three cultivars are also susceptible to ToMV, which is prevalent in our greenhouses and sometimes produces symptoms that mimic those of CMV infection, whereas cv. Vendor-Tm2^a carries the *Tm-2*^{a} gene for resistance to ToMV.

Inoculations

Three isolates from California, CMV-113A (subgroup I, from Huron), CMV-KCII9 (subgroup I, from Kettleman City), and CMV-S (subgroup II) were provided by R. Gilbertson (UC-Davis). These isolates were maintained by rub-inoculation of small sugar pumpkin seedlings at the cotyledon to second true leaf stage. Leaves were first dusted with abrasive powder (Celite®, J.T. Baker), then rubbed between fingers dipped in inoculum, which consisted of a 1:10 (w/v) ratio of young symptomatic leaves in freshly prepared ice-cold grinding buffer (1:1 0.03 *M* potassium phosphate buffer, pH 7.0, and 0.1% sodium sulfite). The same inoculation procedure was used for determining the resistance or susceptibility of tomato genotypes, except that seedlings were inoculated at the first to second true leaf stage, and a second inoculation was performed a week later in order to reduce the chance of escapes. Disease symptoms were scored visually 2–3 weeks after the last inoculation.

CMV resistance tests

A five-point disease severity index (DSI) scale was used to represent the degree of disease symptom development, where 0 = no symptoms, 1 = mild leaf mottling, 2 = mottling, rugose surface and some filiform or fern leaves, 3 = fern or some shoestring leaves, and 4 = severe shoestring symptoms. Plants with scores of 0–1 were considered resistant; those with scores of 2–4 were considered susceptible (see example in Fig. 1). This classification was used because plants with a score of 1 showed only mild symptoms, whereas plants with scores greater than 1 showed more pronounced symptoms that clearly indicated a systemic infection.

Seeds from eight resistant BC_1-inbred lines, ten BC_2F_1, two BC_2F_2 families, and susceptible controls (cvs. VF36, Vendor-Tm2^a, Earlipak and Ace) were treated with half-strength commercial bleach for 15 – 20 min for surface sterilization and to improve germination. Seeds were incubated on germination paper in plastic boxes in an incubator at 25°C. Seedlings were transplanted at the cotyledon stage into an artificial soil mix (approx. 1 part peat

moss: 1 part redwood bark: 1 part sand) in wooden flats (60 × 40 × 10 cm, 40–50 seedlings per flat). Seedlings were grown in the greenhouse at 65°–70°C day/55°–60°C night.

Susceptible *L. esculentum* controls were included in each experiment and were inoculated with CMV (5–24 plants each) or buffer only (5–10 plants each). Both ToMV-resistant and -susceptible varieties were included in each test in order to control for ToMV contamination. This is done because ToMV infection can cause symptoms similar to CMV, potentially resulting in a misclassification of CMV response.

Those BC_1-inbred lines (13-A-1, 27-A-3, 17-A-1, and 30-A-2) showing the highest level of CMV resistance were selected for molecular work. The BC_2F_1 that we continued working with was 27-A-3 × VF36. This BC_2F_1 was selected because it had the highest percentage (100%) of resistant plants among the BC_2F_1 populations tested; also, the parental line 27-A-3 was one of the most resistant BC_1-inbreds.

Molecular marker analysis

The BC_1-inbreds were genotyped with restriction fragment length polymorphism (RFLP) and isozyme markers to detect *L. chilense* introgressions. DNA isolations were performed as previously described (Chetelat and DeVerna 1991). Survey blots were used to accelerate the search for *L. chilense*-specific markers and contained the following bulk DNA samples: four CMV resistant BC_1-inbred bulks representing lines 13-A-1, 27-A-3, 17-A-1, and 30-A-2 (10–17 plants per bulk), *L. chilense* LA0458 (a bulk of 6 plants), VF36, and Vendor-Tm2[a]. BC_1-inbred bulks consisted only of symptomless plants. For *L. chilense*, random uninoculated plants were chosen for the bulk in order to obtain a representative sample of its allelic diversity.

Each DNA sample (20 µg) was digested separately with the restriction enzymes *Eco*RI, *Eco*RV, *Hind*III, and *Xba*I according to the manufacturer's instructions for 6–18 hours at 37°C. Restriction fragments were size-fractionated on 0.9% agarose gels in 1 × NEB buffer (Neutral Electrophoresis Buffer – 0.1 *M* TRIS, 0.001 *M* EDTA, 0.012 *M* Na-acetate, pH 8.0) for approximately 20 hours at 30 V (75—100 mA). Ethidium bromide-stained gels were photographed with Polaroid B&W film on a UV-light box. DNA was transferred to nylon filters (Hybond-N^+, Amersham) according to the manufacturer's instructions for capillary transfer. Filters were pre-hybridized for 4 h at 65°C in 7% SDS, 0.5 *M* sodium phosphate, pH 7.2, and 1% BSA. DNA probes consisted of previously mapped tomato genomic (TG) and cDNA clones (CT and CD) chosen to provide maximum genome coverage according to the map by Tanksley et al. (1992). Probes were polymerase chain reaction (PCR)-amplified then labeled by the random hexamer primer method as previously described (Chetelat and DeVerna 1991). The labeled probes were purified through Bio-Gel P-30 spin columns (Bio-Rad Laboratories). Hybridizations were performed for 12–18 h at 65°C. Blots were washed three times at 2 ×, 1 × and a final stringency of 0.5 × SSC in 0.1% SDS at 65°C. Blots were exposed to BioMax MS-1 Autoradiography Film (Kodak) at –80°C for several hours to 2 days.

Isozyme analysis was performed by starch gel electrophoresis as previously described (Chetelat and DeVerna 1991). The enzymes aconitase (ACO), 6-phosphogluconate dehydrogenase (6PGDH), Malate dehydrogeanse (MDH), esterase (EST), glutamate oxaloacetate transaminase (GOT), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), peroxidase (PRX), alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), malic enzyme (MAE), and acid phosphatase (APS) were tested for polymorphism between *L. esculentum* and *L. chilense* and/or between *L. esculentum* and the BC_1-inbreds.

Statistical analysis

Conformity of segregation ratios with expected values was tested with the LINKAGE-1 program (Suiter et al. 1983). Single-locus goodness-of-fit tests were performed as well as contingency X^2 analysis to test for linkage between segregating locus pairs. Recombination fractions were estimated using the maximum likelihood method. MAPMAKER 2.0 (Lander et al. 1987) was employed to create a partial map of chromosome 12. The "LOD-table" and "ripple" commands were used to find the most likely marker order. The "map" command was used to create partial chromosome 12 map. The Kosambi mapping function was used to calculate centiMorgans. The QGENE program (Nelson 1997) was employed for interval mapping of resistance QTLs.

ELISA tests

Enzyme-linked immunosorbent assay (ELISA) kits from Agdia® (Elkhar, Ind.) were used to detect CMV *in planta* according to the manufacturer's instructions. The Agdia kit is a modified Double Antibody Sandwich ELISA with a polyclonal antibody used for capture and a mixture of monoclonal antibodies for detection. The wells of the microtiter plates were pre-coated with capture antibodies by the manufacturer. A 100-µl aliquot of sap from young leaves of plants to be tested was added to each well and incubated for 2 h at RT. Plates were washed with PBST, then incubated for 2 h with a detection antibody conjugated with alkaline phosphatase. Plates were again washed with PBST, and the enzyme substrate was then added. In the presence of virus, the enzyme-substrate reaction results in a yellow-colored product. The intensity of the color on the ELISA plate was determined colorimetrically with an ELISA Plate Reader at a wavelength of 405 nm. ELISA readings at 405 nm were corrected for the absorbance of the negative plant control as follows: corrected ELISA reading = initial A_{405} – 3 x A_{405} of negative plant control. Corrected values above zero were considered positive for the presence of CMV; values below zero were considered negative. ELISAs were performed primarily on plants without symptoms. Plants showing symptoms were assumed to carry the virus, as confirmed by the few ELISAs performed on this group.

Virus recovery tests

Recovery tests on small sugar pumpkins were carried out in order to confirm ELISA results. Eight tomato plants from BC_1-inbred line 27-A-3 that had been inoculated with CMV-KCII9 were tested. Plant tissue from CMV-inoculated tomato seedlings was used for rub-inoculation of three to five small sugar pumpkins at the cotyledon to first true leaf stage. The negative small sugar pumpkin controls were inoculated with buffer only or with leaves from uninoculated tomato seedlings.

Results

Hybridizations

Fertility problems were encountered when trying to backcross CMV-resistant lines as female parents to *L. esculentum.* Fruit set from these crosses was very poor, with only around 10% of flowers setting fruit. Few hybrid seeds per fruit were obtained, and many fruits were parthenocarpic. The average number of seeds per fruit following selfing was 26 for the BC_1-inbred lines and 44 for the BC_2F_1 27-A-3 × VF36; in contrast, the *L. esculentum* cultivars averaged 132 seeds per fruit. Following pollinations with VF36, the BC_1-inbreds produced an average of 17.8 seeds per fruit, while the BC_2F_1 yielded 16.6.

Using the aniline blue method for pollen-tube staining (Martin 1959), we observed normal germination of *L. es-*

Table 1 Reaction of BC_1-inbred derivatives of *L. esculentum* × *L. chilense*, BC_2F_1 hybrids, and susceptible controls to subgroup I and II isolates of CMV. Classification of plants was based on a disease severity index (DSI) score, wherein a score of 0–1 was treated as resistant, and 2–4 as susceptible

Genotype	Isolate	CMV Sub-group	*n*	Percentage resistant plants
BC_1-inbreds				
27-A-3	113 A	I	34	100.0
27-A-3	KCII9	I	8	100.0
27-A-3	S	II	27	77.8
30-A-2	113 A	I	22	100.0
13-A-1	113 A	I	32	96.9
27-A-2	113 A	I	18	94.4
17-A-1	113 A	I	22	90.9
32–1	113 A	I	22	90.9
30-A-1	113 A	I	21	90.5
31-A-2	113 A	I	25	76.0
BC_2F_1s				
17-A-1 × Vendor-Tm2[a]	113 A	I	10	100.0
27-A-3 × VF36	113 A	I	20	100.0
27-A-3 × Vendor-Tm2[a]	113 A	I	12	83.3
13-A-1 × Vendor-Tm2[a]	113 A	I	24	95.8
31-A-2 × Vendor-Tm2[a]	113 A	I	8	62.5
31-A-2 × VF36	113 A	I	24	58.3
30-A-1 × VF36	113 A	I	24	58.3
27-A-2 × VF36	113 A	I	24	54.2
32—1 × VF36	113 A	I	24	50.0
30-A-2 × Vendor-Tm2[a]	113 A	I	11	45.5
Susceptible controls				
Ace	113 A	I	16	12.5
Vendor-Tm2[a]	113 A	I	50	10.0
VF36	113 A	I	40	7.5
Vendor-Tm2[a]	KCII9	I	23	8.7
VF36	KCII9	I	8	0.0
Earlipak	KCII9	I	8	0.0
Vendor-Tm2[a]	S	II	25	28

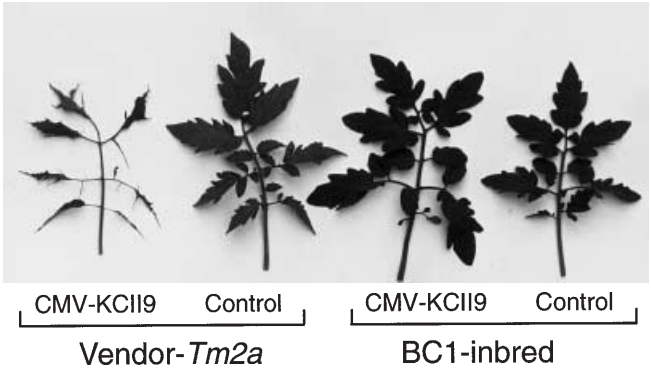


Fig. 1 Leaves of susceptible variety Vendor-Tm2[a] and resistant BC_1-inbred line 27-A-3 following inoculation with CMV isolate KCII9, compared to uninoculated controls

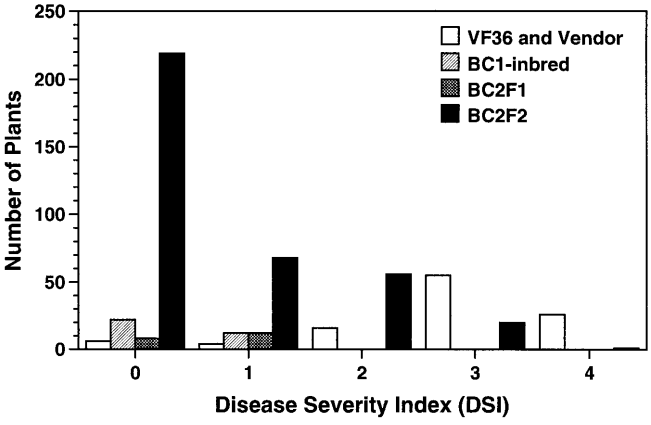


Fig. 2 Histograms of disease severity scores following inoculation with CMV isolate 113 A, for BC_1-inbred 27-A-3, BC_2F_1 (made to VF36), BC_2F_2, and susceptible parents (VF36 and Vendor-Tm2[a])

culentum pollen grains on stigmas of BC_1-inbreds. Microscopic examination revealed that the pollen tubes grew through the stigma and style but that very few had penetrated the ovules. This observation suggests some kind of incompatibility that impedes fertilization of the BC_1-inbred lines by *L. esculentum* pollen. Fruit set from the reciprocal cross was also very poor, indicating low pollen viability and/or production in the BC_1-inbreds.

Inheritance of CMV resistance

The responses of susceptible controls, BC_1-inbred lines and BC_2F_1 hybrids to three isolates of CMV were evaluated (Table 1). Overall, 94% of the *L. esculentum*-susceptible controls inoculated with the subgroup I isolates CMV-113A and CMV-KCII9, including cv. Vendor-Tm2[a], VF36, Ace, and Earlipak, developed disease symptoms ranging from mottling to severe filiform leaves (Figs. 1, 2; Table 1). The approximate 6% of the *L. esculentum* control genotypes that did not develop symptoms were assumed to represent escapes (i.e., unsuccessful inoculation), but they may have developed symptoms later on. In contrast, an average of 93% of the BC_1-inbred plants inoculated with CMV subgroup I isolates were scored as resistant (Table 1). For some of the BC_1-inbred lines, no susceptibles were detected, whereas others had a few plants that developed symptoms (Table 1, Fig. 2).

Slower growth rates were observed for all plants inoculated with subgroup II isolate CMV-S, including the BC_1-inbred line 27-A-3 and Vendor-Tm2[a] (Table 1). 27-A-3 plants showed mild symptoms, with reduced leaf area and growth rate compared to uninoculated controls, but no leaf margin distortion. In contrast, the symptoms on Vendor-Tm2[a] plants were severe, consisting of leaf margin distortion (filiform to severe shoestring) and pronounced growth retardation. The average DSI was 0.6 for 27-A-3 and 2.8 for Vendor-Tm2[a]. Up to 78% of the 27-A-3 plants were resistant to isolate CMV-S, while 28% of Vendor-Tm2[a] plants did not develop symptoms (Table 1). However, the DSI scores reflect primarily leaf coloration (mottling) and shape (distorted margins), rather than leaf area or growth rate, hence do not convey the full range of responses to this isolate.

The situation with the BC_2F_1s, all inoculated with subgroup I isolates, was similar to that of the BC_1-inbreds. Some families were scored as 100% resistant, whereas others had only a few plants that developed symptoms (Table 1). Some BC_2F_1 families appeared to segregate for resistance in a 1R: 1S ratio ($P < 0.05$), and the group as a whole averaged 71% resistant plants. The results indicate resistance is dominant or partially dominant in these crosses.

Detection of CMV *in planta*

Compound direct ELISA was performed primarily on selected plants from the BC_1-inbred, BC_2F_1, and BC_2F_2 populations that did not show CMV symptoms (data not shown). Resistance was not directly correlated with the presence or absence of virus. Some plants that were scored as resistant had detectable virus, whereas in others no virus was detected. However, most plants with symptoms tested positive for CMV by ELISA.

CMV recovery tests performed on small sugar pumpkins found virus in all 8 symptomless 27-A-3 plants tested, as well as from the control susceptible plants Vendor-Tm-2[a] and VF36, which showed severe CMV symptoms (data not shown). Virus was not recovered from the negative controls (i.e., tomato plants inoculated with buffer only). The virus recovery test results therefore confirmed the ELISA data indicating the presence of virus in symptomless (i.e., nominally resistant) BC_1-inbred plants.

Segregation for CMV response in BC_2F_2

Two independent BC_2F_2 families (98L8099 and 98L8100) representing the same parents (27-A-3 ×VF36) were screened for response to CMV, and individual plants were classified as resistant or susceptible according to DSI values. In both families, the segregations were consistent with the 3:1 (resistant : susceptible) ratio expected for a monogenic dominant trait. A homogeneity test ($X^2 = 1.19$) indicated that the data from these two groups could be pooled. The combined BC_2F_2 population consisted of 287 plants scored as resistant and 77 scored as susceptible, also consistent with a 3:1 ratio ($X^2 = 2.67$). The results therefore suggest that CMV resistance is governed by a single dominant gene, for which the symbol *Cmr* (cucumber mosaic resistance) was proposed (Stamova et al. 1998).

Identification of *L. chilense* introgressions in CMV resistant lines

A group of the most CMV-resistant lines were analyzed with molecular markers in order to find *L. chilense* introgressions. A total of 138 RFLP and 20 isozyme markers were used to cover the tomato genome. The high-density RFLP map of tomato, which is based on an F_2 *L. esculentum* × *L. pennellii* population (Tanksley et al. 1992; Fulton et al. 1997) was used for choosing the tomato genomic and cDNA probes for the RFLP analyses. Probes were chosen to provide maximum genome coverage, and the average distance between markers was 9.8 cM.

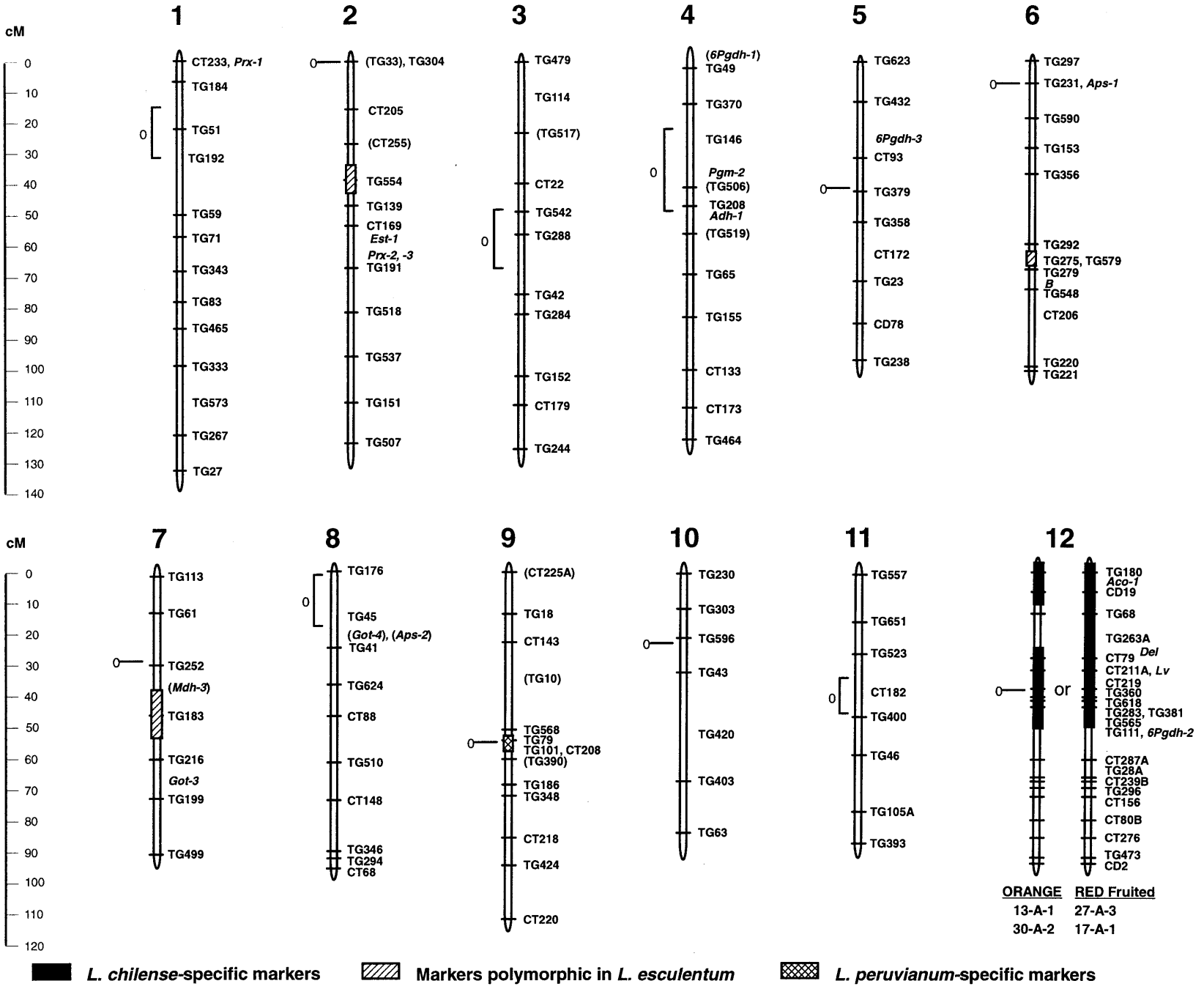
130 of the RFLPs and 5 of the isozyme markers were informative in that at least one restriction enzyme (out of 4 tested) revealed a polymorphism either between *L. esculentum* and *L. chilense* and/or between *L. esculentum* and the BC_1-inbred lines (Fig. 3). There were 8 RFLPs (TG33, CT255, TG517, TG506, TG519, TG10, CT225 A and TG390) and 15 isozyme markers (*6Pgdh-1*; *Mdh-1, –3*; *Got-3, –4*; *Pgi-1*; *Pgm-2*; *Prx-1, –2, –3*; *Aps-1, –2*; *Adh-1*; *Fdh-1*; *Mae-1*) for which the *L. chilense* controls were unreadable. Although the BC_1-inbred lines were homozygous for the *L. esculentum* alleles at all these loci, they were considered potentially uninformative (Fig. 3). The overall rate of polymorphism revealed between *L. esculentum* and *L. chilense* was approximately 79%. Of the restriction enzymes used, *Eco*RV revealed the greatest number of polymorphisms (83%), and *Hind*III revealed the least (74%).

Chromosome 12

The only *L. chilense*-specific markers detected in the BC_1-inbreds were on the short arm and a small part of the long arm of chromosome 12 (Fig. 3). Twelve informative *L. chilense* RFLPs (TG180, CD19, TG68, TG263A, CT79, CT211A, CT219, TG360, TG618, TG283, TG381, and TG565) and 1 isozyme marker (*Aco-1*) were identified on this chromosome. The CMV-resistant lines 27-A-3 and 17-A-1 were homozygous for the *L. chilense* alleles of each of these markers. In the CMV-resistant lines 13-A-1 and 30-A-2, 2 of these markers (TG68 and TG263 A) were homozygous for the *L. esculentum* alleles (Fig. 3). The maps for chromosome 12 indicate one to two introgressed segments, depending on the BC_1-inbred line. These introgressions included marker TG565, which on the Tanksley map is situated distal to TG111, a marker for which all the lines were homozygous *L. esculentum*; since this configuration would require two additional cross-over events (unlikely), and considering TG565 is a low LOD marker on the Tanksley map, we infer its true location is proximal to TG111 as shown (Fig. 3). This interpretation is supported by recombination within the *L. chilense* introgression that showed tight linkage between TG381 and TG565 (see below). The chromosome 12 segments were the only *L. chilense* introgressions detected in the BC_1-inbreds and represent 2.9% of the genome in 13-A-1 and 30-A-2 and 4% in 27-A-3 and 17-A-1.

Chromosome 9

Three markers (TG79, TG101 and CT208) not specific to *L. chilense*, nor to the putative recurrent parent (Ace), were found on chromosome 9 (Fig. 3). The same TG79,


cM
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CT233, Prx-1
TG184
TG51
TG192
TG59
TG71
TG343
TG83
TG465
TG333
TG573
TG267
TG27
2
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CT205
(CT255)
TG554
TG139
CT169
Est-1
Prx-2, -3
TG191
TG518
TG537
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TG507
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TG114
(TG517)
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TG542
TG288
TG42
TG284
TG152
CT179
TG244
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(6Pgdh-1)
TG49
TG370
TG146
Pgm-2
(TG506)
TG208
Adh-1
(TG519)
TG65
TG155
CT133
CT173
TG464
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TG623
TG432
6Pgdh-3
CT93
TG379
TG358
CT172
TG23
CD78
TG238
6
TG297
TG231, Aps-1
TG590
TG153
TG356
TG292
TG275, TG579
TG279
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TG548
CT206
TG220
TG221
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TG61
TG252
(Mdh-3)
TG183
TG216
Got-3
TG199
TG499
8
TG176
TG45
(Got-4), (Aps-2)
TG41
TG624
CT88
TG510
CT148
TG346
TG294
CT68
9
(CT225A)
TG18
CT143
(TG10)
TG568
TG79
TG101, CT208
(TG390)
TG186
TG348
CT218
TG424
CT220
10
TG230
TG303
TG596
TG43
TG420
TG403
TG63
11
TG557
TG651
TG523
CT182
TG400
TG46
TG105A
TG393
12
or
TG180
Aco-1
CD19
TG68
TG263A
CT79 Del
CT211A, Lv
CT219
TG360
TG618
TG283, TG381
TG565
TG111, 6Pgdh-2
CT287A
TG28A
CT239B
TG296
CT156
CT80B
CT276
TG473
CD2
ORANGE
13-A-1
30-A-2
RED Fruited
27-A-3
17-A-1
L. chilense-specific markers
Markers polymorphic in L. esculentum
L. peruvianum-specific markers

TG101, and CT208 polymorphisms were also seen in Vendor-Tm2^a, which carries the *Tm-2^a* gene that is also on chromosome 9 (closely linked to TG101) (Young et al. 1988), suggesting the markers were introgressed along with *Tm-2^a*, originally from *L. peruvianum* (Alexander and Hoover 1955). This hypothesis is supported by the fact that the lines were selected for ToMV resistance (L. Stamova, personal communication) as well as from the lack of symptoms of ToMV infection on the lines in the greenhouse over several years. The origin of the *Tm-2^a* gene in the BC_1-inbred lines is unknown but presumably traces to the *L. esculentum* parents used in the original breeding program, which suggests Ace (which lacks *Tm-2^a*) was not the only cultivar used. The existence of *Tm-2^a* in the BC_1-inbreds was nonetheless useful, as it eliminated the possibility of accidental coinfection with ToMV, which would have confused the expression of CMV symptoms.

Chromosomes 2 and 7

L. esculentum-specific polymorphisms for markers TG554 on chromosome 2 and TG183 on chromosome 7 were also seen in the BC_1-inbreds (Fig. 3). The *L. esculentum* cultivars Vendor-Tm2^a and VF36 carried alleles that were different from those of Ace. The CMV-resistant lines carried the same allele as Vendor-Tm2^a and VF36 for both markers. This is further evidence that Ace was not the only *L. esculentum* cultivar used in the original breeding program.

Fruit color variation

Fruit color and softness characteristics varied among the CMV-resistant lines. The color of the BC_1-inbred lines 27-A-3 and 17-A-1 were red, whereas lines 13-A-1 and 30-A-2 were bright orange and softened rapidly. The BC_2F_1s derived from red-fruited BC_1-inbred lines gave red fruits, whereas those derived from orange-fruited BC_1-inbred lines gave intermediate-colored fruits (reddish orange). Because the BC_2F_1s derived from orange-fruited BC_1-inbred lines were intermediate for fruit color, the trait must be incompletely dominant. However, we were not able to test the inheritance or linkage relations of this character because the BC_2F_2 populations grown were from a red-fruited line. It is interesting to note that the orange-fruited lines contained a different pattern of *L. chilense* markers on chromosome 12 than did the red-fruited lines (Fig. 3).

The genetic basis of orange fruit in these lines is unclear. One possibility is that the orange-fruited BC_1-inbred lines carried the *B* gene (for high beta-carotene, low lycopene content in ripe fruit), which is located on the long arm of chromosome 6. Alleles of *B* have been found in several of the wild *Lycopersicon* spp. (Mackinney et al. 1954; Tomes 1963). For the chromosome 6 marker TG579, the red-fruited lines had the Vendor allele, whereas the orange-fruited lines carried the Ace allele. However, none of the lines carried the *L. chilense* alleles for TG579 or any of the other chromosome 6 RFLP markers (Fig. 3).

Alternatively, the orange fruit trait may be due to an allele of the *Del* gene on the short arm of chromosome 12 (Vulkova et al. 1992). Mature fruit of *Del* appear orange due to a reduction in lycopene and increase in delta-carotene concentrations. Surprisingly, the orange-fruited lines displayed only the *L. esculentum* alleles for markers TG68 and TG263A, whereas the red-fruited lines carried the *L. chilense* alleles (Fig. 3), just the reverse of what would be expected if fruit color were controlled by an allele of *Del* from *L. chilense*.

Association of CMV resistance with chromosome 12 markers in BC_2F_2

A total of 364 BC_2F_2 plants were scored for 7 of the *L. chilense*-specific RFLP markers (TG180, CD19, TG68, CT79, CT219, TG381, and TG565) on chromosome 12. The marker TG101 (closely linked to the *Tm-2^a* gene on chromosome 9) was also scored to evaluate the possible co-segregation of CMV and ToMV resistance as well as to predict plants that would be ToMV resistant (in homozygous or heterozygous condition) or susceptible. Single-locus goodness-of-fit tests were performed for each marker to test for conformity to the expected 1:2:1 ratio. All markers segregated in a manner consistent with a 1:2:1 ratio at the 95% confidence level (data not shown). CMV resistance was analyzed both as a monogenic trait using LINKAGE-1, and as a quantitative trait using QGENE.

For the qualitative analysis, association between CMV resistance (i.e., inferred *Cmr* genotype) and RFLP marker genotypes was tested by contingency chi-square analysis using LINAKGE-1. The association between marker TG101 (chromosome 9, near *Tm-2^a*) and *Cmr* was not significant ($X^2 = 3.01$, $P < 0.1$), consistent with the hypothesis that CMV resistance is not a pleiotropic effect of the *Tm-2^a* gene for ToMV resistance. In contrast, all 7 *L. chilense*-specific markers on chromosome 12 showed significant association with CMV resistance, with maximum values recorded for CT79 ($X^2 = 18.0$, $P < 0.001$) and TG565 ($X^2 = 17.8$, $P < 0.001$). However, estimates of the recombination frequencies between the chromosome 12 markers and *Cmr* were not additive,

◀ **Fig. 3** Genetic map showing RFLP and isozyme markers used in this study and the locations of *L. chilense* introgressions and other polymorphic regions detected in the CMV-resistant BC_1-inbred lines. The order and position of markers and centromeres are from published maps (Fulton et al. 1997; Tanksley et al. 1992). Markers in *parenthesis* were uninformative in the BC_1-inbreds. The genes *Lv* for resistance to *L. taurica*, *B* for increased fruit β-carotene, and *Del* for increased δ-carotene concentrations are also indicated. *Solid bars* indicate *L. chilense* introgressions, *hatched bars* regions with putative *L. peruvianum*- or *L. esculentum*-specific polymorphisms. All other markers were monomorphic for the *L. esculentum* alleles in the lines

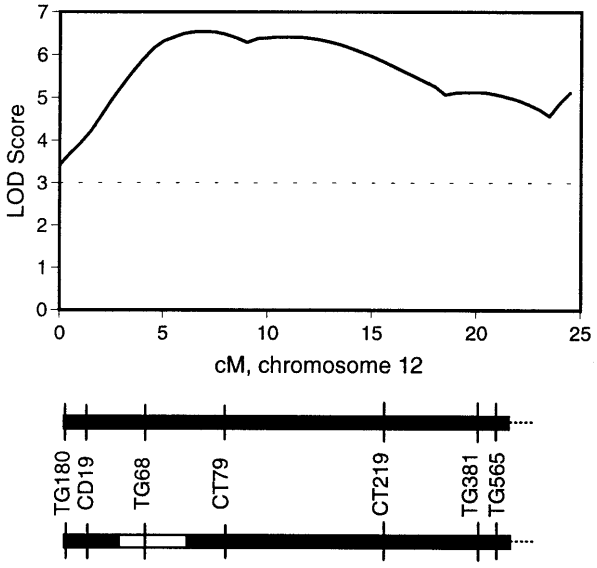

Fig. 4 Plot of LOD values indicating the association of chromosome 12 markers with CMV resistance, as determined by QTL analysis in the BC_2F_2 population. The solid line represents the threshold for significance (LOD = 3.0). The two different genotypes of the BC_1-inbred lines are presented below the chart with the *black bars* representing *L. chilense* DNA, *white bars L. esculentum* DNA. Map distances (centiMorgans, cM) are based on recombination within the introgressed region as observed in this study

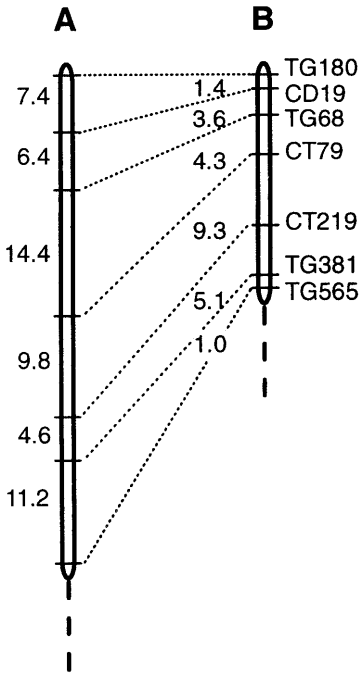

Fig. 5A, B Comparison of partial chromosome 12 maps from F_2 *L. esculentum* × *L. pennellii* (Tanksley et al. 1992) and BC_2F_2 *L. esculentum* × *L. chilense* (**B**) from the present study. Map distances are in centiMorgans

Table 2 Single-point analysis of BC_2F_2 population for association of marker genotype with CMV resistance based on DSI values (*A* additivity, *D* dominance)

Marker	Chromosome	R^2	*P*	A	D
TG180	12	0.0451	0.0007	0.28	0.03
CD19	12	0.0507	0.0001	0.30	0.01
TG68	12	0.0763	0.0000	0.36	–0.04
CT79	12	0.0771	0.0000	0.36	–0.07
CT219	12	0.0650	0.0000	0.34	0.00
TG381	12	0.0575	0.0000	0.31	0.05
TG565	12	0.0644	0.0000	0.34	0.02
TG101	9	0.0044	0.4638	–0.06	–0.08

hence the resistance gene could not be unambiguously assigned to a particular marker interval. This result suggests some plants were misclassified for response to CMV, either due to unsuccessful inoculation (i.e., susceptible genotypes scored as resistant) or incomplete penetrance (i.e., resistant genotypes scored as susceptible). These observations indicate that CMV resistance in this study did not behave like a reliable monogenic character.

Another possible explanation for the difficulties in precisely mapping *Cmr* could be segregation for the *Tm-2*^{a} gene, combined with accidental inoculation of some susceptible plants with ToMV. For this reason, a subset of ToMV-resistant plants (*Tm-2*^{a}/- = 3/4 of the BC_2F_2 population) was used to calculate recombination estimates between *Cmr* and the chromosome 12 markers. The results did not differ significantly from those for the whole population, suggesting that the ToMV susceptibility of some BC_2F_2 plants did not affect the scoring of the CMV phenotype. Furthermore, none of the *L. esculentum* controls inoculated with buffer showed only ToMV symptoms, indicating little if any contamination with this virus.

Another possibility is that CMV resistance is governed by more than one gene, possibly on *L. chilense* introgressions not detected in the present study. Therefore, resistance was also treated as a quantitative trait, and the location of QTLs was analyzed with QGENE. Single-point analysis was utilized for determining the significance of associations between markers and CMV response. TG101 (chromosome 9) was not associated with resistance (Table 2). However, all 7 *L. chilense*-specific markers on chromosome 12 markers were associated with resistance ($P \leq 0.0007$, Table 2). The correlation coefficient (R^2) was greatest for marker CT79, consistent with the qualitative analysis; however this marker accounted for only about 7.7% of the total phenotypic variation (DSI scores) in the experiment. Interval analysis revealed significant LOD scores (range: 3.69–6.56) for all 7 chromosome 12 markers, with a peak between TG68 and CT79 (Fig. 4).

Recombination within the introgressed segment

From the BC_2F_2 segregation of 364 plants, a partial chromosome 12 linkage map was constructed, based on

recombination within the introgressed region, and compared with the *L. esculentum* × *L. pennellii* map (Tanksley et al. 1992) (Fig. 5). The order of markers was consistent with the Tanksley map, except for TG565, which was placed as a low LOD marker between TG111 and CT287A; in our BC_2F_2 population, TG565 mapped very close to TG381. Recombination was suppressed approximately 58% over the introgressed region compared to the Tanksley map (Fig. 5).

Discussion

L. chilense has been a rich source of disease resistance genes for tomato improvement. In particular, *L. chilense* expresses high levels of resistances to several important viral diseases, including tomato spotted wilt virus (Stevens et al. 1994), Taiwan tomato leafcurl virus (Green et al. 1994), tomato yellow leaf curl virus (TYLCV) (Zamir et al. 1994), tomato mottle virus (Scott and Schuster 1991), curly top virus (Martin 1970), as well as CMV (Stamova et al. 1990). Resistance to TYLCV is controlled by a single gene with partial dominance (*Ty-1*), which was mapped to chromosome 6 (Zamir et al. 1994). The genetic basis for the other viral resistances is largely unknown.

The present study revealed potentially useful resistance to CMV in backcross-inbred derivatives of *L. chilense*. The effectiveness of this resistance to at least two subgroup I isolates was demonstrated. Resistance to the single subgroup II strain tested was somewhat less effective. Additional testing is required to obtain a more complete picture of the range of CMV strains this resistance may protect against. Furthermore, since all tests relied on mechanical inoculations in the greenhouse, we cannot draw conclusions as to the performance of these lines under field conditions where repeated insect-vectored inoculation at different developmental stages may occur.

Single gene hypothesis

The results of our inheritance studies suggest that CMV resistance is determined by a single dominant gene, given the symbol *Cmr*. The resistance expressed by the BC_2F_1 populations indicated a dominant mode of action of the resistance gene. The phenotypic segregation in BC_2F_2 fit the 3R:1S ratio at the 95% confidence level, consistent with the hypothesis of a single dominant gene. However, the *Cmr* gene could not be scored reliably as a qualitative character since (1) not all plants fell into discrete classes, and (2) inoculations were less than 100% successful in some cases, as demonstrated by occasional symptomless plants among the susceptible controls. Therefore, the existence of additional genes cannot be ruled out.

Also, occasional susceptible plants were found in the homozygous BC_1-inbred lines as well as in the BC_2F_1 hybrids. A possible explanation of this observation could be that there is incomplete penetrance of the resistance gene or an environmental influence on its expression. Further evidence of the importance of environmental conditions was the lack of uniformity we observed in the response to CMV of clones of an F_1 *L. esculentum* × *S. lycopersicoides* hybrid, synthesized by Chetelat et al. (1997). It was previously reported that *S. lycopersicoides* possessed some level of CMV resistance or tolerance (Phills et al. 1977). We observed an apparent segregation of 6 resistant and 6 susceptible plants following inoculation of the F_1 with isolate CMV-113A. Genotypic segregation can be ruled out since this intergeneric hybrid was vegetatively propagated, and all 12 inoculated plants had exactly the same genotype. Environmental and physiological factors influencing the expression of CMV resistance were reported by Parella et al. (1997) who found resistance in *L. hirsutum* (PI 247087) to be highly influenced by growth temperature and plant age at time of inoculation.

Another possible explanation for the above-mentioned observations is that the CMV resistance is a complex trait governed by more than one gene. This by itself still does not explain the different resistance phenotypes of plants of the same genotype (e.g., the F_1 *L. esculentum* × *S. lycopersicoides*). Furthermore, the absence of *L. chilense* markers on chromosomes other than number 12 in the BC_1-inbreds does not support the multigenic hypothesis.

Mapping the resistance gene(s)

The results of the RFLP analysis of the CMV-resistant BC_1-inbred lines revealed the presence of *L. chilense*-specific markers only on the short arm and part of the long arm of chromosome 12, presumably spanning the centromere. All 7 chromosome 12 *L. chilense* markers tested in the BC_2F_2 were significantly associated with CMV resistance by qualitative and quantitative models. Interval analysis indicated a LOD peak between markers TG68 and CT79.

Two genotypes of CMV-resistant BC_1-inbred lines were observed. One was homozygous for a single, large introgressed *L. chilense* segment (the BC_2F_2 population was derived from such a line). The other type contained a recombinant, apparently derivative introgression with two homozygous *L. chilense* segments flanking a region of *L. esculentum* DNA. Since these lines were also resistant, we infer that the resistance gene(s) are situated in one or both of the *L. chilense* segments, but not in between. An additional consideration is that the recombinant introgression presumably resulted from a double cross-over, a rare event and unlikely to be homozygous without selection. Therefore, the marker data suggest the possibility of two resistance genes, one on each segment. This would help explain the lack of a clear association of resistance with any one chromosome 12 marker. However, QTL analysis of the large introgression did not reveal two LOD peaks, as would be expected in the digenic

model. This might be partially explained by the reduced level of recombination observed within the introgressed region, which would have reduced the ability to detect two linked QTLs.

CMV resistance mapped to the same general region of the genome (short arm of chromosome 12) in the present study as the gene *Lv*, controlling resistance to *L. taurica*, which was previously mapped by Chunwongse et al. (1994). In fact, the chromosomal segment introgressed from *L. chilense* was the same in both studies, not surprising considering that the CMV resistance reported herein was first discovered in *L. taurica* resistant lines (L. Stamova, personal communication). Whereas the *Lv* gene mapped to the proximal interval between CT211A and CT219 (Chunwongse et al. 1994), CMV resistance in the present study was most strongly associated with the more distal markers TG68 and CT79. This is consistent with observations that not all *Lv*-containing lines were resistant to CMV (L. Stamova, personal communication), suggesting that linkage between these resistance genes may not be very tight. These results nonetheless suggest the presence of a resistance gene cluster on chromosome 12, comprising at least *Lv* and *Cmr*. Such clusters are well-known in tomato (Lindhout 1995) and other plants and are thought to arise by a process of gene duplication, followed by the evolution of new specificities (Meyer et al. 1998).

Nature of the resistance

Some symptomless plants tested positive for CMV by ELISA, a result confirmed by recovery tests on indicator plants, demonstrating they harbored the virus. Furthermore, no correlation between the level of phenotypic resistance and ELISA virus titer could be found in the present study. This may reflect natural fluctuations of virus levels *in planta* during development of a systemic infection. Therefore, resistance in this germplasm is a form of tolerance rather than immunity, in that at least some resistant plants harbor the virus, yet show relatively mild symptoms relative to susceptible genotypes. Similar results were reported for CMV resistance in *S. lycopersicoides* (Phills et al. 1977), as well as in tolerant breeding lines of pepper (Lapidot et al. 1997).

Genetic basis of orange fruit trait

Because some BC_1-inbred lines were orange-fruited, additional markers were scored in the vicinity of the *B* gene for high beta-carotene and low lycopene content in ripe fruit, which is located on the long arm of chromosome 6. The absence of *L. chilense* alleles at flanking marker loci did not support the hypothesis that the orange-fruited BC_1-inbred lines carry an allele of *B*. Furthermore, the intermediate color of heterozygotes in the BC_2F_1 is inconsistent with the dominant gene action of *B* (Mackinney et al. 1954). On the other hand, the *Del* gene on chromosome 12 exhibits incomplete dominance (Tomes 1963), and alleles have been detected in the genomes of several green-fruited *Lycopersicon* species (Chalukova and Stoeva 1988), although not expressed therein due to the absence of carotenoids in ripe fruit. This finding is also consistent with the location of *Del* on chromosome 12 between markers TG263A and CT79 (Ronen et al. 1999).

However, the fact that our orange-fruited lines had an interstitial segment from *L. esculentum* (between markers CD19 and CT79), whereas the red-fruited lines contained the large *L. chilense* segment, suggests the possibility of a two-gene system: *Del*, located between TG263 A and CT79, and a suppressor gene (suppressor of *Del*), also from *L. chilense* and located between the markers CD19 and CT79.

In conclusion, we have mapped a putatively monogenic CMV resistance trait (*Cmr*) to chromosome 12 of tomato. Knowledge of the map location of this gene should facilitate marker-assisted selection for CMV resistance in breeding programs. Also, the size of the *L. chilense* introgression containing *Cmr* can now be systematically reduced, possibly eliminating deleterious traits such as orange fruit and reduced fecundity exhibited by these lines. This may be the first reported mapping of a naturally occurring CMV resistance gene in tomato.

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