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High-Resolution Mapping of the Physical Location of the Tomato *Cf-2* Gene

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To isolate the tomato *Cf-2* resistance gene by map-based cloning, plants recombinant for RFLP markers close to *Cf-2* were selected by exploiting the flanking morphological markers *yv* (yellow virescent) and *tl* (thiaminless). Using these recombinants, a high-resolution linkage map of the region encompassing the *Cf-2* gene has been generated containing several new RFLP markers. Mapping of two YAC clones carrying *Lycopersicon esculentum* and *L. peruvianum* DNA, indicates that in both genotypes the physical distance between the two closest flanking markers is less than 40 kb. This study also positions *Cf-2* relative to the *Mi* gene, which confers resistance to root-knot nematodes.

Additional keywords: Cladosporium fulvum, tomato leaf mold.

The genetics of the interaction between dominant resistance (R) genes in plants and dominant avirulence (Avr) genes in pathogenic organisms are relatively well understood (Flor 1946). Generally, they imply that resistance to pathogens is a consequence of a specific recognition event, which induces a number of plant defense mechanisms, resulting in the reduction of growth and spread of the pathogen (Lamb et al. 1989). The biochemistry of R gene function remains elusive. To date, the sequences of five disease resistance genes, *HM1*, *Pto, RPS2, N,* and *Cf-9* have been published (Johal and Briggs 1992; Martin et al. 1993; Mindrinos et al. 1994; Bent et al. 1994; Whitham et al. 1994; Jones et al. 1994). The *HM1* gene confers resistance in maize to the fungal pathogen *Cochliobolus carbonum* Nelson race 1 and encodes an enzyme which inactivates the HC toxin from *C. carbonum* (Johal and Briggs 1992). This interaction does not conform to Flor's gene-for-gene hypothesis, and is therefore distinct from interactions that involve R genes that may couple recognition of specific pathogen races to defense-response reactions. The *Pto* gene which confers resistance in tomato to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* is part of a small linked gene family and encodes a cytoplasmically located, but potentially membrane associated, protein kinase (Martin et al. 1993). It is not clear how this kinase might accomplish race-specific recognition, although genetic analysis suggests the involvement of a second gene, *Prf* (Salmeron et al. 1994). The three other R genes, *RPS2, N,* and *Cf-9*, all contain leucine-rich repeat (LRR) motifs coupled to features indicative of a role in signaling. The *RPS2* gene from *Arabidopsis thaliana* confers resistance to *Pseudomonas syringae* strains carrying *avrRpt2*, the tobacco *N* gene confers resistance to tobacco mosaic virus and the tomato *Cf-9* confers resistance to *Cladosporium fulvum*. The isolation of *Pto, RPS2, N,* and *Cf-9* provides a key step towards the identification of host signal transduction systems involved in the activation of plant defense mechanisms.

The interaction of tomato (*Lycopersicon esculentum* Miller) and the causal agent of leaf mold *C. fulvum* Cooke provides a good system for the study and isolation of additional R genes. Several different R genes which confer resistance to different races of *C. fulvum* have been reported (Stevens and Rick 1988), of which, *Cf-2, Cf-3, Cf-4, Cf-5,* and *Cf-9* have received particular attention (Hammond-Kosack and Jones 1994). The majority of these have been identified in wild species and bred into cultivated tomato (*L. esculentum*). A comprehensive integrated classical marker and restriction fragment length polymorphism (RFLP) map exists for tomato (Tanksley et al. 1992), which, together with the relatively small genome size, makes this system amenable to the isolation of R and other genes. *Cf-4* and *Cf-9* are tightly linked or allelic and map to the short arm of chromosome 1 between the molecular markers TG301 and TG236 (Jones et al. 1993; Balint-Kurti et al. 1994). The *Cf-2* gene, which has been bred into *L. esculentum* from *L. pimpinellifolium*, maps to a position approximately 2 centimorgans (cM) from *yv* and less than 1 cM from *tl* (Jones et al. 1993). Both of these morphological markers have been mapped cytologically (Khush and Rick 1968). Available evidence indicates that *Cf-2* maps between these two visible markers (Jones et al. 1993), therefore allowing simple selection for informative recombinational events either side of the resistance gene. On the molecular map, *Cf-2* maps between *Aps-1* (a cloned isozyme marker encoding acid phosphatase-1 that cosegregates with the classical marker *yv*) and GP79 (Dickinson et al. 1993). The *Cf-5* gene, derived from *L. esculentum* var. *cerasiforme*, has been assigned to the same map location and is either allelic or very closely linked to *Cf-2* (Jones et al. 1993; Dickinson et al. 1993). A third R gene, *Mi*, which confers resistance to three species of root-knot nematode, has been placed in a similar map location but is known not to be allelic to *Cf-2* (Mess-

The work contributed by M.S.D. and D.A.J. should be considered equal.

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eguer et al. 1991; Ho et al. 1992; Dickinson et al. 1993). Additionally, *Ty-1* which provides tolerance to tomato yellow leaf curl virus maps approximately to the same region of chromosome 6 (Zamir et al. 1994).

In this paper, we localize *Cf-2* between two closely linked RFLP markers by analysis of selected recombinant individuals. A cosmid contig which spans the region containing these flanking RFLP markers was assembled from a yeast artificial chromosome (YAC) clone derived from a susceptible variety of tomato. This not only defines the physical distance but also provides DNA probes which should enable the *Cf-2* gene to be directly isolated.

RESULTS

Selection for recombination near *Cf-2*.

Recombination events near *Cf-2* were selected from several F_2 populations generated from parents with *Cf-2* in coupling phase with either of the adjacent morphological marker genes *tl* or *yv* (Table 1). All plants were scored for resistance to *C. fulvum* race 5 by inoculation with fungal spore suspensions (see Materials and Methods). For a cross between the parents GCR472 (*tl*) and Cf2 (a near-isogenic line [NIL] of Moneymaker carrying the *Cf-2* gene), the recombinant classes in the F_2 population are phenotypically resistant to *C. fulvum* and thiaminless (RT) or susceptible and thiamine wild-type (STWT) (indicating the ability to produce thiamine endogenously). Likewise, for crosses involving *yv* and Cf2, the F_2 recombinant classes are resistant and yellow virescent (RY) or susceptible and green (SG).

RFLP mapping.

Most of the recombinant plants listed in Table 1 were screened with the RFLP markers listed in Table 2 and the results are summarized in Figure 1. All the recombination breakpoints were assigned to an approximate map location. No plants were identified that had undergone more than a single recombination event within this region. Breakpoints in recombinant plants involving *yv* always mapped on the opposite side of *Cf-2* to the breakpoints in *tl* recombinant plants, confirming the location of *Cf-2* between these two morphological markers.

The RFLP screening of plants recombinant between *tl* and *Cf-2* was complicated by the fact that the original thiaminless mutant, which gave rise to the line GCR472, arose in a stock which was selected for *Mi* (Langridge and Brock 1961; Giles and Hutton 1957) and therefore likely to carry *L. peruvianum* DNA in the region examined in this study. This was confirmed by a comparison of GCR472 with Cf0 (a Moneymaker line lacking any detectable resistance genes for *C. fulvum*) and VFNT-cherry, a line carrying *Mi* on a large introgressed segment from *L. peruvianum* known to include GP79 and *Aps*-1 (Ho et al. 1992). RFLP analysis clearly shows GCR472 to be *L. peruvianum*-like at both the CT119 (Fig 2A) and GP79 loci (data not shown).

Conversion of well-characterized cloned RFLP markers to cleaved amplified polymorphic (CAPs) markers provides a more efficient procedure for the scoring of genomic DNA at the corresponding map locations (Konieczny and Ausubel 1993). By sequencing the ends of a cloned RFLP probe, PCR primers can be synthesized which specifically amplify the corresponding genomic DNA. Such reactions frequently generate products which are of the same size for related plant species as judged by conventional agarose gel electrophoresis. However, differences between these products may be revealed by subsequent restriction enzyme digestion. This was carried out for two of the most closely linked RFLP markers, CT119 and GP79, and was employed for screening some of the recombinant plant DNA samples (see Materials and Methods). Restriction enzymes which reveal polymorphisms between the amplification products for each marker were determined (Table 2).

The RFLP marker MG112, which maps between GP79 and CT119, is a 3.1-kb clone derived from screening a root cDNA library with radioactive probes derived from YACs that map to the *Mi* region. The library was made from the cultivar Mogeor, which has the same size introgressed *L. peruvianum* segement as Motelle. MG112 maps outside of the *Mi* region (Ganal et al., in preparation). This marker hybridizes to several bands on a genomic Southern blot of DNA digested with any of seven restriction enzymes tested (*Ase*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Ssp*I, and *Xba*I), suggesting that it detects a small multigene family (Fig. 2B). The most intensely hybridizing bands always cosegregated when tested on the *Cf-2*/*tl* and *Cf-2*/*yv* recombinant plants, indicating that these cluster at single complex locus. All seven restriction enzymes reveal

Table 1. Segregating populations used for mapping *Cf-2*[a]

Tester	Marker	Phenotype +R	+S	mR	mS	Recombination (%)[b]
GCR472[c]	*tl*	1,479	8	3	484	0.6 ± 0.2
GCR472[d]	*tl*	604	4	5	185	1.2 ± 0.4
GCR472[e]	*tl*	913	3	3	326	0.5 ± 0.2
LA1190[c,f]	*yv*	1,370	28	21	381	2.9 ± 0.4
LA1178[c]	*yv*	1,692	36	29	523	2.9 ± 0.4

[a] All data refer to segregating F_2 populations except as indicated. + = Wild type for the visible marker; m = mutant for the visible marker; R = resistant; S = susceptible.
[b] Calculated by the maximum likelihood method.
[c] Continuation of cross detailed in Jones et al. 1993.
[d] F_1 produced by crossing a *yv Cf-2* recombinant (RY200) to GCR472.
[e] F_3 population.
[f] Includes F_2 data detailed in Dickinson et al. 1993.

Table 2. RFLP probes and CAPs markers used for the mapping of *Cf-2*[a]

RFLP	Source	Polymorphism Cf0/Cf2	Cf2/GCR472	Cf0/VFNT-cherry
GP79	CG	*Eco*RV	*Eco*RV	*Eco*RV
MG112	MG	*Eco*RV	All	*Eco*RV
CT119	MG	*Eco*RV	*Xba*I	*Xba*I
RAPD2	MG	*Eco*RV	*Eco*RI	*Eco*RV
CAPs marker				
GP79		ND	*Rsa*I/*Hin*fI	ND
CT119		*Rsa*I	*Rsa*I	*Rsa*I

[a] RFLP probes were tested on DNA digested with the restriction enzymes *Dra*I, *Eco*RI, *Eco*RV, *Hae*III or *Xba*I. Many restriction enzymes which have four base pair recognition sequences were tested for the ability to reveal polymorphisms for CAPs. In each case only those enzymes which gave the clearest polymorphisms are listed. All indicates that for this marker all enzymes tested gave clear polymorphisms. The sources of the probes were Christina Gebhardt (CG) (Gebhardt et al. 1991), Martin Ganal (MG). ND = not determined.

polymorphisms between DNA of Cf0, Cf2, Cf5, and plants carrying *L. peruvianum* DNA at this locus (Fig. 2B). This is the only RFLP marker to reveal any polymorphisms between Cf0 and Cf5. The *Cf-5* gene was introgressed from a closely related subspecies, *L. escultum* var. *cerasiforme*, resulting in a general lack of polymorphic DNA.

The data summarized in Figure 1 clearly place *Cf-2* between the two RFLP markers MG112 and CT119 which are tightly linked genetically. A single recombinant (STWT201) separates MG112 from *Cf-2* and only two recombinants (RY204 and RY244) separate CT119 from *Cf-2*.

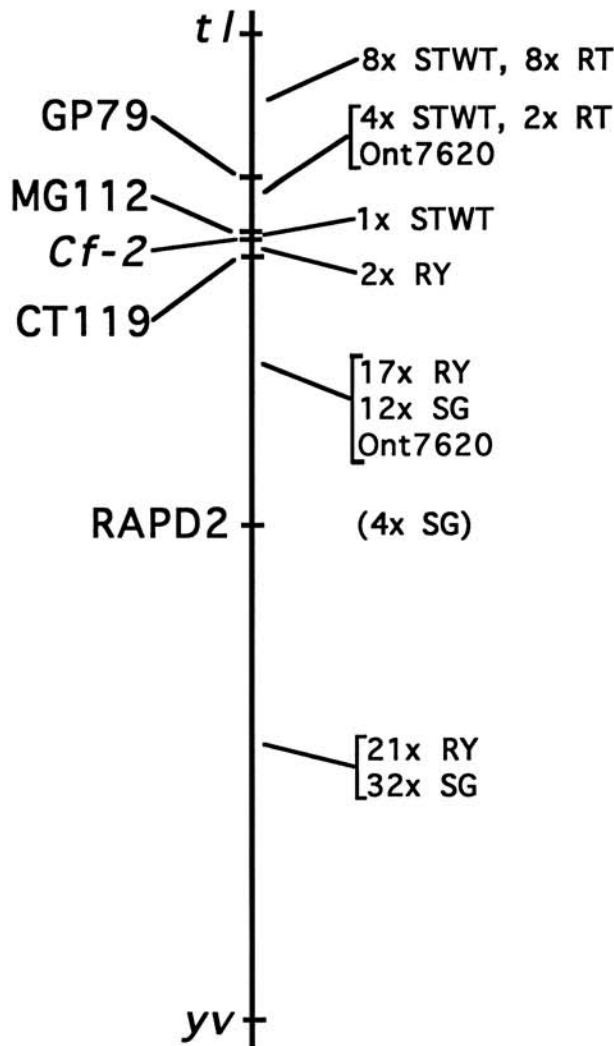

Fig. 1. Integrated linkage map of the region encompassing *Cf-2*. The map depicts the order of restriction fragment length polymorphism (RFLP) probes together with the two morphological markers *tl* and *yv*. The total numbers of recombinant plants with breakpoints between the various markers are grouped by phenotype and listed accordingly. The distances between each marker are a reflection of the number of recombinants placed in that interval. Breakpoints for the four plants listed in parentheses have been placed below CT119, but have not been placed with respect to RAPD2. Susceptible thiamine wild type (STWT); resistant thiaminless (RT); susceptible green (SG) and resistant yellow virescent (RY). Ont7620 = the line Ontario 7620 which has an introgressed segment of *Lycopersicon pimpinellifolium* carrying the *Cf-2* gene, the limits of which have been mapped between the RFLP markers indicated (see text).

Cf-2 maps between *Mi* and *tl*.

One recombinant line, Ontario 7620 (Kerr et al. 1980), carries *Cf-2* and a second gene *Mi*, which confers resistance to three species of root-knot nematode. The parental *Mi* line for Ontario 7620 was Anahu, which carries a large segment of *L. peruvianum* DNA that includes GP79 but does not extend as far as the *Aps-1* locus (Messeguer et al. 1991). The same study showed that a second line, Motelle, carrying a very small introgressed segment of *L. peruvianum* DNA, places *Mi* between GP79 and *Aps-1*, a similar location to that of *Cf-2* (Dickinson et al. 1993). Screening of DNA from different isogenic lines carrying *Mi*, with RAPDs (randomly amplified polymorphic DNA) resulted in a single copy marker (RAPD2) specific for the introgessed segment of Motelle. This marker maps approximately 200–250 kb below CT119 (Ganal et al., in preparation). Here, RFLP analysis of Ontario 7620 shows that this line carries the *L. esculentum* form of the GP79 locus, the *L. pimpinellifolium* form of the CT119 and MG112 loci (Fig. 2B) and the *L. peruvianum* form of RAPD2. Excluding the unlikely possibility of multiple recombination events in the generation of the original Ontario 7620 line, these latest data strongly suggest that the gene order is *tl–Cf-2–Mi–yv*.

Location of RFLP markers on YAC 302.H4.

The ordering of RFLP markers using a set of selected recombinant plants has positioned *Cf-2* to between two closely linked markers, CT119 and MG112. To define the physical distance between these markers, two YACs from a library derived from the tomato cultivars VFNT-cherry and Rio

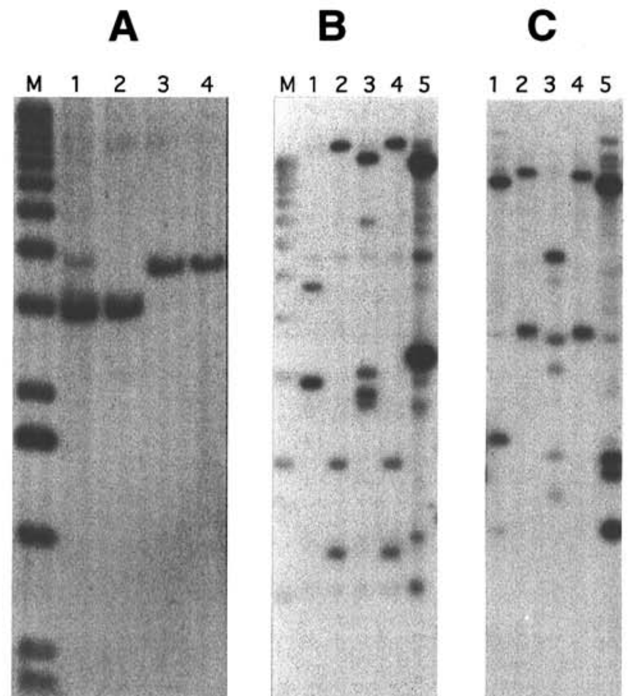

Fig. 2. Southern blots revealing restriction fragment length polymorphisms for probes CT119 and MG112. **A,** DNA digested with *Xba*I and probed with CT119. Lane 1, Cf0; lane 2, Cf2; lane 3, VFNT-cherry; lane 4, GCR472. **B and C,** DNA digested with *Xba*I or *Eco*RV, respectively, and probed with MG112. Lane 1, Cf0; lane 2, Cf2; lane 3, Ontario 7620; lane 4, VFNT-cherry; lane 5, Cf5..

Grande-PtoR were exploited (Martin et al. 1992). One YAC (YAC302.H4) that carries 340 kb of *L. esculentum* DNA from Rio Grande-PtoR was identified by hybridization to CT119, MG112, and GP79. The other YAC which includes RAPD2, CT119, and MG112 carries 290 kb of *L. peruvianum* DNA derived from VFNT-cherry.

Several of the RFLP markers were placed on a restriction map of YAC302.H4, and the results are shown in Figure 3. Both the markers MG112 and CT119 hybridize to a 60-kb *Bss*HII fragment localized at one end of this YAC. This indicates, at least in *L. esculentum* lacking the *Cf-2* gene, that the physical distance in this region is sufficiently small to allow detailed characterization using standard molecular techniques. Similar mapping of the overlapping YAC derived from *L. peruvianum* DNA substantiates this result, suggesting no major rearrangements or deletions of YAC302.H4.

Two other probes, SC29 and SC3-8, derived from Cf2 leaf RNA were placed on the YAC302.H4 restriction map (Fig. 3). Both probes are partial cDNA clones directly selected with YAC302.H4 using a biotin streptavidin capture system (Morgan et al. 1992). SC3-8 and SC29 are both present as single copy loci on YAC302.H4. However, within the tomato genome they are present as low (fewer than 10 copies) and highly repetitive sequences, respectively. The repetitive nature of these probes makes them unsuitable for use as RFLP markers, though both are valuable as additional markers for the characterization of YAC302.H4.

Construction of a *L. esculentum* cosmid contig.

Placement of the RFLP markers MG112 and CT119 on the restriction map of YAC302.H4 indicates that in *L. esculentum* the maximum distance between these probes is 60 kb. Subcloning of this intervening region into cosmids provides a more accurate estimate of this distance and also generates DNA probes suitable for the isolation of the corresponding region of *L. pimpinellifolium* that carries the *Cf-2* gene. Total DNA prepared from the yeast strain carrying YAC302.H4 was partially digested with *Mbo*I and size fractionated on a sucrose gradient. DNA of approximately 20 to 25 kb was further purified and ligated into the cosmid vector pLAFR4. As the library includes the entire *Saccharomyces cerevisiae* genome as well as YAC302.H4, approximately 600 colonies should represent one haploid genome equivalent. Several genome equivalents were screened with the probes MG112 and SC3-8 and 14 independent nonchimeric cosmids were purified. These were mapped and assembled into a single contig which included the marker CT119 (Fig. 4).

Hybridization with the marker MG112 revealed the presence of two loci, one of which hybridizes strongly, the other at a much reduced level. These loci are referred to as MG112a and MG112b, respectively, and the hybridizing regions are separated by only 3 kb of intervening sequence. The cosmid contig confirms the close location of MG112 and CT119, localizing them to a region of slightly over 30 kb.

DISCUSSION

RFLP screening of selected recombinants from segregating F_2 populations has provided detailed information for the ordering of several genes and new RFLP markers. The order *tl*–GP79–MG112–*Cf-2*–CT119–RAPD2- *yv* is consistent with previously published data (Messeguer et al. 1991; Ho et al. 1992; Dickinson et al. 1993; Jones et al. 1993). In conjunction with the analysis of the line Ontario 7620, *Mi* can be placed between CT119 and *yv* but not with respect to RAPD2.

Six different populations were used to generate recombinants around *Cf-2* which prevents the use of these data for the calculation of accurate recombination distances. Previous studies using similar stocks place *Cf-2* approximately 2 cM from *yv* and less than 1 cM from *tl* (Jones et al. 1993). Therefore, it is clear from the large number of recombinants examined in this study that the genetic distance between MG112 and CT119 must be relatively small.

The presence of *L. peruvianum* DNA in crosses involving the marker gene *tl* may have resulted in a suppression of recombination within this region as suggested by Ho et al. (1992). This issue was not addressed here but may have resulted in the selection of a reduced number of recombinants on the *tl* side of *Cf-2*. Generation of a new stock carrying *tl* where most of the DNA between *tl* and the markers GP79 has been replaced with *L. esculentum* DNA may overcome this limitation.

The RFLP marker MG112 reveals at low-stringency several bands on genomic Southern blots indicative of a small gene family. Identification of a second related locus (MG112b) in the *L. esculentum* cosmid contig confirms this. In the analysis of genomic DNA from recombinant plants, only the MG112a locus, which provides the most intense RFLPs, was examined. Consequently, the localization of *Cf-2* between the markers MG112a and CT119 leaves the markers MG112b and SC3-8 as candidate *Cf-2* homologs. Additionally, MG112 is the first and only RFLP probe to reveal polymorphisms between the NILs Cf0 and Cf5. In allelism tests, no susceptible plants were recovered among 484 progeny from a Cf2/5 test cross indicating allelism or very close linkage (Jones et al. 1993). Parallels can be drawn with other resistance genes like the *Mla* locus of barley and the *Rp1* locus of maize where alleles exist as sets of closely linked and functionally related genes (Jorgensen 1992; Hulbert and Bennetzen 1991).

Several R genes hybridize to small gene families whose members in several cases have been shown to be clustered. For example, the *Pto* gene hybridizes to a small gene family containing at least six different members, all but one of which map to the same 400-kb YAC (Martin et al. 1993). Similar data exist for both *N* and *Cf-9* (Whitham et al. 1994; Jones et

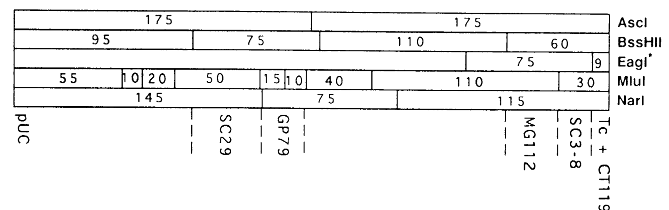

Fig. 3. Restriction enzyme map of the *Lycopersicon esculentum* YAC302.H4. The map was compiled by hybridization of all of the probes to Southern blots of total DNA from the yeast strain carrying YAC302.H4 digested to completion (all enzymes) or partially digested (*Bss*HII, *Mlu*I). * = Incomplete data. Tc is a 1,050-bp *Bam*HI to *Ava*I fragment purified from pBR322 which hybridizes to one end of the YAC vector pYAC4. pUC118 was used as a probe to the other end of the vector pYAC4.

al. 1994). The gene probes for *Pto*, *N*, and *Cf-9* hybridize to sequences in the respective disease-sensitive plants (Martin et al. 1993; Whitham et al. 1994; Jones et al. 1994). Therefore, probes derived from susceptible plants could, in theory, be used to isolate R genes from their resistant counterparts. Only slightly more than 30 kb of DNA separates MG112a and CT119 in *L. esculentum*, so few other sequences remain as candidate *Cf-2* and *Cf-5* homologs. Duplications and rearrangements could have expanded these regions in *L. pimpinellifolium* and *L. escultenum* var. *cerasiforme*. The systematic characterization of the corresponding regions should allow the direct cloning of both the *Cf-2* and *Cf-5* genes.

MATERIALS AND METHODS

Plant materials and scoring for disease resistance

The NILs of the *L. esculentum* cultivar Moneymaker carrying either *Cf-2* (designated Cf2) or *Cf-5* (designated Cf5) and the original Moneymaker line carry no detectable resistance genes for *C. fulvum* (designated Cf0) were obtained from R. Oliver (University of East Anglia, Norwich, UK). The Cf2 and Cf5 NILs were developed at the Centre of Genetic Resources (CPRO, Netherlands) by backcrossing the donor parent five times to Moneymaker, the recurrent parent, and selfing for a further five generations (Tigchelaar 1984). LA1190 (*yv*), LA1178(*yv,coa,c*), *L. pimpinellifolium* LA100 and VFNT-cherry were provided by C. Rick (Tomato Genetic Resource Center, Davis, California). GCR472(*tl*) was obtained from J. Maxon-Smith (HRI, Littlehampton, UK). Ontario 7620 was provided by H. Laterrot (INRA, Montfavet, France).

Assays for disease resistance were performed exactly as described in Dickinson et al. 1993.

DNA preparation and RFLP analysis.

DNA extractions were performed as described by Carroll et al. 1994. Genomic DNA (5 μg) was digested with the appropriate restriction enzyme using manufacturer's buffers in 200 μl at 37°C overnight. Samples were concentrated by ethanol precipitation, separated on 0.8% agarose gels and Southern blotted onto Hybond N membranes (Amersham). DNA was fixed to the membranes by exposure to 120 mJ of ultraviolet irradiation using a Stratalinker 2400 (Stratagene, La Jolla, California). Filters were probed with DNA labeled with ^{32}P-dCTP by the random hexamer method (Sambrook et al. 1989) using an Oligolabeling Kit (Pharmacia). Hybridizations were carried out according to Church and Gilbert (1984), final washes were at 65°C in 40 mM sodium phosphate, pH 7.2, 0.1% sodium dodecyl sulfate.

Conversion of RFLP markers to CAPs.

CT119 is a 600-bp tomato cDNA clone and only required limited sequencing for conversion to a CAPs marker. However, GP79 is a potato-derived genomic DNA clone (Gebhardt et al. 1991) and the synthesis of corresponding oligonucleotides generated primers which failed to amplify the correct specific product in tomato. To overcome this problem, the potato probe was used to isolate a cosmid clone from the *L. esculentum* derived YAC302.H4. Restriction enzyme analysis was performed on this clone, and a 500-bp *Hin*dIII fragment which hybridized strongly to GP79 was identified and cloned into pUC119. This clone was sequenced and CAPs primers corresponding to the *L. esculentum* form of GP79 were synthesized. The synthetic oligonucleotides used as CAPs primers were; CT119F, 5′-TCA GGT ATC GAA CCA AAA CC-3′; CT119R, 5′-TAA AAG GTT CAT CCT AAT AC-3′; GP79F, 5′-TGT TCT CTA GTA TCT CAT CC-3′; GP79R, 5′-GGA TTG TGA TGT CGA GTT GC-3′.

PCR and CAPs analysis.

PCR was performed on extracted DNA in a volume of 20 μl in the presence of 0.25 μM of each of the primers, 250 mM dNTPs (Pharmacia) in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet P-40 and 1.0 to 2.5 units of "AmpliTaq" thermostable DNA polymerase (Perkin Elmer Cetus). Cycling conditions were 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min for 35 cycles, followed by a 10-min extension at 72°C. Restriction polymorphisms in the reaction products were revealed by digesting 10-μl aliquots with an equal volume of 1× restriction buffer (supplied by enzyme manufacturers) and an excess of the appropriate restriction enzyme. After incubation at 37°C digestion products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Pulsed-field gel electrophoresis and YAC mapping.

Yeast chromosomes were prepared from yeast cells embedded in blocks of 1% InCert agarose (FMC) made up in 1 M

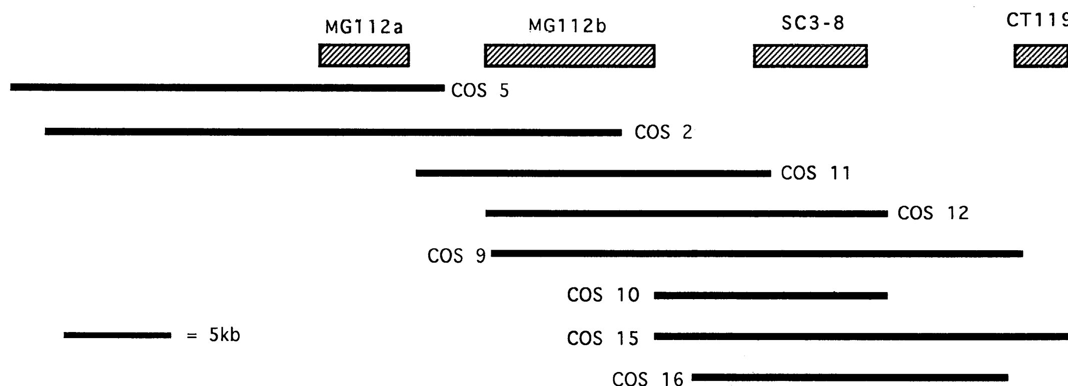

Fig. 4. Cosmid contig covering the MG112/CT119 region of YAC302.H4. Informative cosmids are represented as solid bars and the regions of hybridization to the various probes are indicated by hatched boxes. The scale bar represents 5 kb.

sorbitol. These were incubated at 50°C in at least 10 volumes of a solution of 0.5 mg/ml proteinase K (Sigma) in 100 mM EDTA, pH 8.0, 1% sarkosyl for at least 16 h. Blocks were finally treated for 2 h with at least 10 volumes of TE containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then several changes of TE before storage at +4°C.

Pulsed-field gel electrophoresis was performed using a Bio-Rad CHEF-DRII electrophoresis system. Longer than standard gels were run on this apparatus by casting gels onto a glass plate. To prevent gels moving in the circulating buffer, Velcro tape was attached to the glass plate prior to casting of the gel. All gels were run under standard conditions of 0.8% agarose with 0.5× TBE electrophoresis buffer at 12°C, the voltage was 200V, pulse times were from 2 to 20 seconds and the run time was 20 h. Gels were Southern blotted using standard procedures except that the DNA was nicked by exposure to 400 mJ of ultraviolet light in a StrataLinker 2400 (Stratagene).

Construction of cosmid library.

High molecular weight DNA from the yeast culture carrying the YAC302.H4 was prepared according to the method of Cryer et al. (1975) except that zymolase was replaced with Novozym 234 (Novo Biolabs, Bagvaerd, Denmark). Further purification in a caesium chloride/ethidium bromide gradient, partial digestion with *Mbo*I, size fractionation, and subsequent cloning were performed using standard procedures (Sambrook et al. 1989). The cosmid cloning vector pLAFR3 was provided by F. Ausubel (Harvard, Cambridge, Massachusetts). Ligated DNA was packaged according to manufacturers instructions using Gigapack XL and plated on the *E. coli* strain SURE Tet^{S} (Stratagene).

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