Construction of a 1.2-Mb contig including the citrus tristeza virus resistance gene locus using a bacterial artificial chromosome library of *Poncirus trifoliata* (L.) Raf.

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Abstract: The citrus tristeza virus resistance gene (*Ctv*) is a single dominant gene in *Poncirus trifoliata*, a sexually compatible relative of citrus. To clone this gene, a bacterial artificial chromosome (BAC) library has been constructed from an individual plant that was homozygous for *Ctv*. This library contains 45 696 clones with an average insert size of 80 kb, corresponding to 9.6 genome equivalents. Screening of the BAC library with five chloroplast DNA probes indicated that 0.58% of the BAC clones contained chloroplast-derived inserts. The chromosome walk across the *Ctv* locus was initiated using three closely linked genetic markers: C19, AD8, and Z16. The walk has been completed and a contig of ca. 1.2 Mb was constructed. Based on new data, the genetic map in the *Ctv* region was revised, with *Ctv* being located between AD8–Z16 and C19 at distances of 1.2 and 0.6 cM, respectively. Utilizing DNA fragments isolated from the contig as RFLP markers, the *Ctv* locus was further mapped to a region of ca. 300 kb. This contig contains several putative disease-resistance genes similar to the rice *Xa21* gene, the tomato *Cf-2* gene, and the *Arabidopsis thaliana RPS2* gene. This library will therefore allow cloning of *Ctv* and other putative disease-resistance genes.

Key words: *Poncirus*, citrus tristeza virus, chromosome walk, resistance gene.

Résumé : Le gène *Ctv* conférant la résistance au tristeza est un gène unique et dominant chez le *Poncirus trifoliata*, une espèce apparentée et sexuellement compatible avec les agrumes. Afin de cloner ce gène, une banque de clones BAC (chromosomes bactériens artificiels) a été produite à partir d'une plante homozygote pour *Ctv*. Cette banque comprend 45 696 clones dont la taille moyenne est de 80 kb ce qui confère une redondance de 9,6 équivalents génomiques. Le criblage de la banque avec cinq sondes d'ADN chloroplastique a indiqué que 0,58 % des clones contenaient des inserts d'origine chloroplastique. Une marche chromosomique jusqu'au locus *Ctv* a été initiée à partir de trois marqueurs génétiques fortement liés : C19, AD8 et Z16. La marche a été complétée et a produit un contig d'environ 1,2 Mb. En fonction de données nouvelles, la carte génétique de la région où se trouve *Ctv* a été révisée, ce locus étant maintenant situé entre AD8–Z16 et C19, à des distances de 1,2 et 0,6 cM respectivement. En utilisant des fragments d'ADN comme sondes RFLP, le locus *Ctv* a pu être assigné à une région faisant environ 300 kb. Ce contig contient plusieurs gènes de résistance potentiels présentant de l'homologie aux gènes *Xa21* du riz, *Cf-2* de la tomate et *RPS2* d'*Arabidopsis thaliana*. Cette banque permettra ainsi de cloner *Ctv* de même que d'autres gènes de résistance putatifs.

Mots clés : *Poncirus*, tristeza, marche chromosomique, gène de résistance.

[Traduit par la Rédaction]

Introduction

Citrus tristeza virus (CTV) is one of the most important citrus diseases worldwide (Bar-Joseph et al. 1989) and causes economic losses by killing trees or reducing fruit size. CTV is transmitted by several species of aphids, with *Toxoptera citricida* Kirk (the brown citrus aphid) being the most efficient CTV vector (Rocha-Pena et al. 1995). *Toxoptera citricida* has already reached Florida and Mexico and threatens California, Texas, and Arizona, the major cit-

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rus growing states in the United States. Most citrus species are susceptible to CTV. However, *Poncirus trifoliata* (L.) Raf. (the trifoliate orange), a close relative of citrus, can provide an effective resistance to a broad range of CTV strains (Yoshida 1985; Garnsey et al. 1987; Gmitter et al. 1996; Mestre et al. 1997). This resistance has been characterized and is controlled by a single dominant gene called *Ctv* (Gmitter et al. 1996).

Molecular markers linked with the *Ctv* gene have been developed in several laboratories (Gmitter et al. 1996; Deng et al. 1997; Mestre et al. 1997; Fang et al. 1998). A total of eight random amplified polymorphic DNA (RAPD) markers linked to *Ctv* were initially identified by Gmitter et al. (1996). The closest markers flanked *Ctv* at distances of 0.4 and 1.8 centi-Morgans (cM) in a consensus map of the *Ctv* region based on four populations of 138 progeny. In 1997, Deng et al. incorporated 12 new RAPD markers into this linkage map. Sequence characterized amplified region (SCAR) markers were developed from seven RAPD markers that were closely linked with *Ctv*, to increase the precision of the genetic-distance estimates and to develop markers suitable for map-based cloning of the *Ctv* gene. The two closest SCAR markers on either side were SCAD08 (AD8) and SCO07, which flank *Ctv* at distances of 0.5 and 2.4 cM, respectively. Mestere et al. (1997) also identified seven RAPD markers linked to *Ctv* in two populations with a total of 90 plants, but these markers were at least 4 cM away from *Ctv*. Fang et al. (1998) developed a high-resolution map around *Ctv* based on 554 progeny of 10 populations derived from crosses between *P. trifoliata* and *Citrus* spp. In the consensus map, marker Z16 cosegregated with *Ctv*, while markers C19 and AD8 (Deng et al. 1997) flanked *Ctv* at distances of 0.5 and 0.8 cM, respectively. As each of these three markers was present in the *P. trifoliata* genome as a single copy (Fang et al. 1998), and considering the small genome size of citrus, this high-resolution genetic map was suitable as a starting point for map-based cloning of the *Ctv* gene.

Map-based cloning has revolutionized the molecular analysis of disease-resistance genes (Hammond-Kosack and Jones 1997). Disease-resistance genes can now be isolated with no knowledge of the biological function of the molecule involved, relying only on genetic inheritance or chromosomal perturbation as keys to isolating the gene. This process is usually divided into three steps: (1) development of high resolution genetic maps, (2) physical map construction by chromosome walking with closely linked markers, and (3) determination of the target gene through genetic complementation of a susceptible variety via genetic transformation. Physical map construction requires markers tightly linked to the gene and large insert genomic DNA libraries using vectors such as cosmids, yeast artificial chromosomes, bacterial artificial chromosomes (BACs), or P1-derived artificial chromosomes. Because of ease of manipulation, stability in host cells, and low levels of chimerism (Zhang and Wing 1997), BAC vectors have become the vectors of choice for generating large insert libraries.

The objectives of this study were to initiate the map-based cloning of the *Ctv* gene. In this paper, we describe: (*i*) the construction of a BAC library of the *P. trifoliata* genome; (*ii*) construction of a physical map encompassing the markers C19, Z16, AD8, and the *Ctv* gene, utilizing a chromosome walk and contig assembly process with clones from the BAC library; (*iii*) fine mapping of the region that must contain *Ctv* in the contig, utilizing progeny plants with recombination events between C19 and AD8 relative to markers derived from BAC clones between C19 and AD8; and (*iv*) the partial characterization of disease resistance gene analogs in the C19–*Ctv*–Z16–AD8 region of the *P. trifoliata* genome.

Materials and methods

BAC library construction and characterization

An individual plant of *P. trifoliata* cv. Pomeroy that was homozygous for *Ctv* was used (Fang et al. 1998). Leaves from this tree were collected for megabase-size DNA preparation (Zhang et al. 1995), and the BAC library was constructed essentially as previously described (Choi and Wing 2000). The nuclei were embedded in agarose microbeads. Size selection of *Hin*dIII partially digested megabase-size DNA was carried out in a 1% low melting point agarose gel at 4.0 V/cm, with a 5-s pulse, for 10 h at 11°C. Size-selected *Hin*dIII-digested DNA was ligated to dephosphorylated pBeloBAC11 (Shizuya et al. 1992) and used to transform DH10B (GIBCO BRL, Rockville, Md.), using a BRL Cell-Porator system. The transformed cells were resuspended in SOC medium (Sambrook et al. 1989), incubated at 37°C for 1 h, and plated onto LB agar plates containing 12.5 mg/L chloramphenicol, 0.5 mM isopropyl β-D-thiogalactoside, and 40 mg/L 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. White colonies were picked and transferred into 384-well microtiter plates containing LB freezing medium (Woo et al. 1994) using a Genetix Q-Bot (New Milton, Hampshire, U.K.) located at the Clemson Genome Center in Clemson University. Plates were incubated at 37°C overnight and then stored at –80°C. High-density hybridization filters were spotted with colonies of the BAC library, prepared utilizing the same instrument, as described by the Q-Bot manufacturer.

The BAC DNA was isolated from 5-mL overnight cultures (LB plus 12.5 mg/L chloramphenicol), using standard alkaline lysis procedures (Sambrook et al. 1989), and resuspended in 33 µL TE (10 mM Tris-HCl plus 1 mM EDTA, pH 8.0). To determine the average insert size of the BAC library, BAC DNA was digested with *Not*I. The digested DNA was separated by electrophoresis in a 1% agarose gel in 0.5× TBE (1× TBE: 90 mM Tris-borate plus 2 mM EDTA, pH 8.0) at 11°C, using a Bio-Rad (Hercules, Calif.) CHEF Mapper set at 6 V/cm, with a linear pulse time ramping from 5 to 15 s, for 16 h. Alternatively, electrophoresis was performed in 1% agarose gels in 1× TAE (40 mM Tris-acetate plus 1 mM EDTA), using a programmable power inverter at 6 V/cm, with forward time 3–5 s and reverse time 1–3 s, for 16 h.

Chloroplast DNA probes for BAC library screening were amplified with primer pairs *trn*H [tRNA-His (GUG)] – *trn*K [tRNA-Lys (UUU) exon 1], cp1; *trn*K [tRNA-Lys (UUU) exon 1] – *trn*K [tRNA-Lys (UUU) exon 2], cp2; *psb*C [psII 44 kd protein] – *trn*S (tRNA-Ser (UGA)], cp5; and *trn*S [tRNA-Ser (GGA)] – *trn*T [tRNA-Thr (UGU)], cp8, using total DNA from *P. trifoliata* cv. Pomeroy as a template, as previously described (Demesure et al. 1995). Primer pair cpF (5′-GTCAGGAGTCCATTGATG-3′) – cpR (5′-ATGATCTGGCATGTACAG-3′) was designed on the basis of the sequence alignment of three chloroplast genomes: tobacco (NC_001879), rice (NC_001320), and *Spinacia oleracea* (NC_002202). A total of five chloroplast DNA fragments were amplified and used for BAC library screening.

High-density colony filters for BAC library screening were prepared as previously described (Tomkins et al. 1999). For each filter, 18 432 unique clones from 48 plates were spotted in duplicate. One set of two filters representing 36 864 unique clones from

Table 1. Probes used for the chromosome walk and BAC clones that hybridized with the corresponding probes.

Probe	Size (kb)	Source	Isolation method[a]	BAC clone (size in kb)
Z16	0.65	Fang et al. 1998	RAPD-PCR	31L21 (30), 42N17 (210), 51N13 (55), 57P08 (90), 60P09 (50), 74C23 (130), 89K03 (145)
AD8	0.8	Fang et al. 1998	RAPD-PCR	02G12 (30), 10M17 (50), 42N17 (210), 51N13 (55), 57P08 (90), 73I04 (160), 74C23 (130), 89K03 (145), 94L10 (200)
C19	0.95	Fang et al. 1998	RAPD-PCR	13M06 (70), 14N13 (90), 45P23 (100), 74F14 (150), 78J03 (30), 93C03 (130)
74B	5	74F14	Subclone (*Bgl*II)	74F14 (150), 85K20 (30)
42A	1.5	42N17	Subclone (*Bgl*II)	02N02 (55), 42N17 (210), 75J18 (90), 86N13 (60)
42B	0.5	42N17	Subclone (*Eco*RV)	42N17 (210), 73I04 (160), 79A06 (100), 94L10 (200)
75B	4	75J18	PCR (*Xmn*I)	24E04 (35), 28N09 (220), 31H19 (45), 39D01 (45), 44B18 (40), 54J14 (150), 75J18 (90), 93I04 (55), 94C08 (50)
28B	0.3	28N09	PCR (*Eco*RV)	01O09 (40), 28N09 (220), 31G10 (35), 42P03 (45), 68N12 (40), 84F05 (120)
84B	2.2	84F05	PCR (*Hpa*I)	83D17 (60), 84F05 (120)
84F	8.0	84F05	Fragment	83D17 (60), 84F05 (120)
83F	2.2, 2.5, 4.0	83D17	Fragment	20J24 (150), 74O14 (30), 83D17 (60), 92F12 (75), 95L19 (70)
20A	2	20J24	PCR (*Hpa*I)	12J24 (200), 20J24 (150), 54I17 (95), 55B01 (30), 67D19 (65), 96C04 (45)
12B	0.7	12J24	PCR (*Xmn*I)	12J24 (200), 27A14 (160), 65A14 (150)
65B	2.6	65A14	PCR (*Hpa*I)	14N13 (90), 24E07 (40), 38P01 (90), 47B12 (45), 81P19 (40), 94H09 (30)
14F	4.7, 5.1	14N13	Fragment	14N13 (90), 24E07 (40), 36A15 (30), 38P01 (90), 47B12 (45), 65A14 (150), 81P19 (40), 94H09 (30)

[a]RAPD-PCR RFLP markers were developed from RAPD-PCR products as described by Fang et al. (1998); subclone probe DNA was subcloned as described in Materials and methods; PCR probe DNA was isolated with the PCR method described by Yang and Mirkov (2000) and the second restriction enzyme used is indicated in parentheses; fragment probe DNA was isolated from a *Hin*dIII-fingerprinting gel as described in Materials and methods.

plates 1–96 was used for library screening. Occasionally the third filter representing BAC clones from plates 97–119 was also used for library screening. Standard techniques (Sambrook et al. 1989) were used for filter processing, BAC library screening, and Southern hybridization, except that Church's buffer (Church and Gilbert 1984) was used.

Fingerprinting of BAC clones

Markers C19, Z16, and AD8 were hybridized to sets of high-density colony filters to initiate a chromosome walk. After hybridization, BAC clones were picked for fingerprinting, to confirm sequence overlap and identify clones that represented the end of the existing contig to be used for the next walk step. Fingerprinting of BAC clones was performed as previously described, with some modifications (Marra et al. 1997). For each BAC DNA sample, individual restriction digests consisted of 75 μL of H_20, 10 μL of 10× buffer II (New England Biolabs, Beverly, Mass.), 5 μL of *Hin*dIII (20 U/μL), and 10 μL of miniprep BAC DNA. Digestion was achieved by incubation at 37°C for 1 h. After digestion, DNA was precipitated and resuspended in 10 μL of water and separated in a 1% agarose gel. The comb formed 20 wells, with each well 6 mm wide by 0.5 mm thick. Samples were electrophoresed at 50 V for 14–15 h at room temperature with circulated 1× TAE buffer. After electrophoresis, gels were stained with ethidium bromide and photographed. DNA in the gel was Southern-transferred to membranes (Hybond N+; Amersham, Piscataway, N.J.) by downward alkaline blotting (Koetsier et al. 1993). The membrane of the *Hin*dIII-fingerprinting gel was either stored or used directly for Southern hybridization.

Table 2. Probes used for the fine mapping and their BAC source.

Probe	Size (kb)	Source	Enzyme[a]
20A	2	20J24	*Hpa*I
31A	0.8	31G10	*Stu*I
39B	2.1	39D1	*Hpa*I
54A	2.7	54J14	*Hpa*I
55B[b]	0.9	55B1	*Hin*dIII
81A	0.8	81P19	*Hpa*I
83A[c]	0.25	83D17	*Hae*III
94A	1.7	94C8	*Stu*I
107B	2.3	107I19	*Stu*I
116B	2.8	116N19	*Stu*I

Note: Probe DNA was isolated from BAC clones using the PCR method described by Yang and Mirkov (2000) unless otherwise noted.

[a]The restriction enzyme used to digest the BAC DNA.

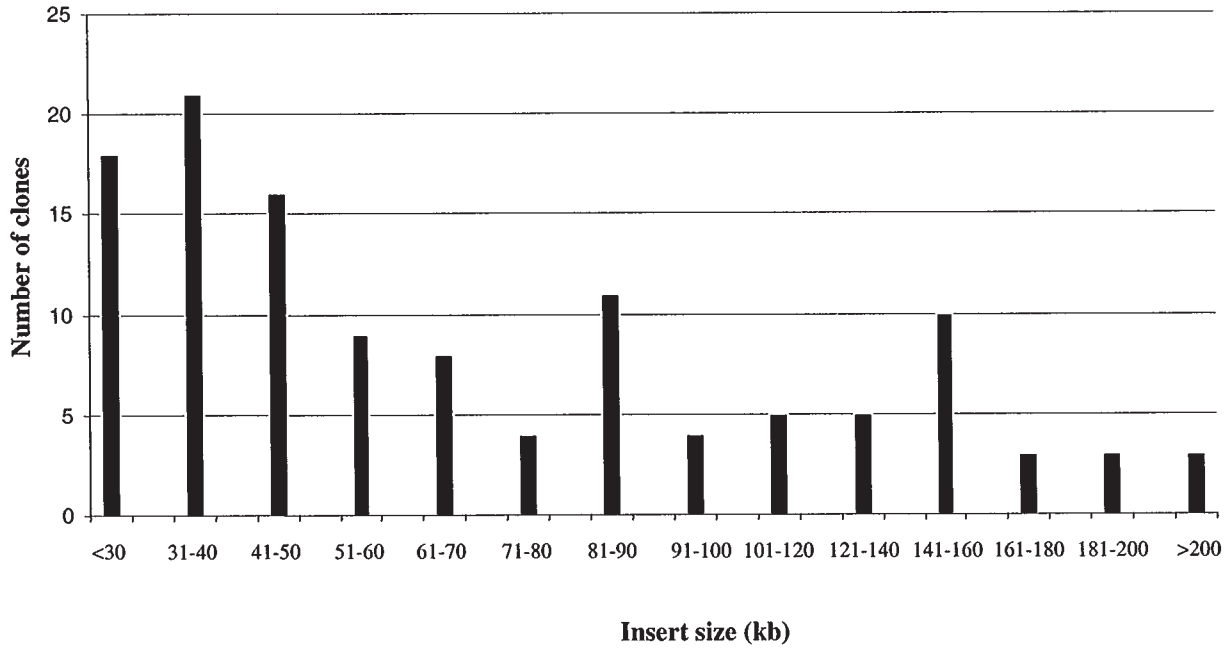
[b]The DNA fragment was isolated from the *Hin*dIII-fingerprinting gel of BAC clone 55B1.

[c]The DNA fragment was isolated using inverse PCR as described in Meyers et al. (1998).

Probe DNA isolation for chromosome walking

After the BAC clone representing the end of the contig was determined from the *Hin*dIII-fingerprinting results, BAC ends were isolated from the extreme BAC clone as a probe for the next step in the chromosome walk. At the beginning of this work, BAC ends were subcloned from BAC clones 42N17 and 74F14. After the

Fig. 1. Insert-size distribution of BAC clones of the *Poncirus trifoliata* BAC library. A total of 124 BAC clones were randomly selected and digested with *Not*I. The insert sizes were determined by CHEF (contour-clamped homogeneous electric field) electrophoresis and plotted against frequency of occurrence.



BAC insert was released by *Not*I digestion and gel-purified, it was digested with either *Eco*RV, *Bgl*II, or *Nsi*I, as these restriction enzymes do not cut in the region between the *Not*I sites and the *Hin*dIII insertion site but do cut the vector outside the *Not*I sites. The digested DNA was ligated to the *Eco*RV–*Not*I or *Bam*HI–*Not*I sites of pBluescript II (SK+) or the *Nsi*I–*Not*I sites of pGEM 5Z (+), correspondingly. The left arm of the vector has multiple cloning sites and the right arm of the vector has a *Pvu*II site. This allows discrimination between the left and the right ends. The left end of a BAC clone was designated "A" and the right end "B." The complete clone designation also included the plate number where a BAC clone was located in the library preceding these symbols. For example, the left and right ends of BAC 42N17 were 42A and 42B.

As the subcloning method is time consuming and labor intensive, most BAC ends were isolated using the polymerase chain reaction (PCR) method as described in Yang and Mirkov (2000). After gel-purification, the PCR product was used to hybridize with the Southern blot of the *Hin*dIII-fingerprinting gel, to determine which BAC end would be used for the next step in the chromosome walk. In those few cases where no PCR product representing the extreme BAC ends could be obtained, DNA fragments were isolated from *Hin*dIII-fingerprinting gels directly and used as probe DNA (Table 1). For BAC clones 42N17, 74F14, and 65A14, the entire insert released by a *Not*I digestion was used as a DNA probe to screen the BAC library. With these particular BAC inserts, hybridization with repetitive DNA has not been encountered.

Chromosome walk and contig assembly

After *Hin*dIII-fingerprinting of the BAC clones, overlapping BAC clones, including the distal BAC clones, were identified and their ends isolated for use in the next walk step. However, it is difficult to identify overlapping clones if the probe DNA contains a repetitive sequence, as it hybridizes with many BAC clones. In this case, BAC clones with a strong hybridization signal were picked and *Hin*dIII-fingerprinted. The probe used to screen the BAC library was hybridized to the *Hin*dIII-fingerprinting gel again. Overlapping clones must have at least one band identical to a band of the original BAC clone. When it was difficult to select overlapping BAC clones because a probe contained repetitive sequence that hybridized with a large number of BAC clones, a "two-step walking method" was employed (Nakamura et al. 1997). DNA fragments were isolated from the original distal BAC clone of the *Hin*dIII-fingerprinting gel and used as a probe to screen the BAC library again.

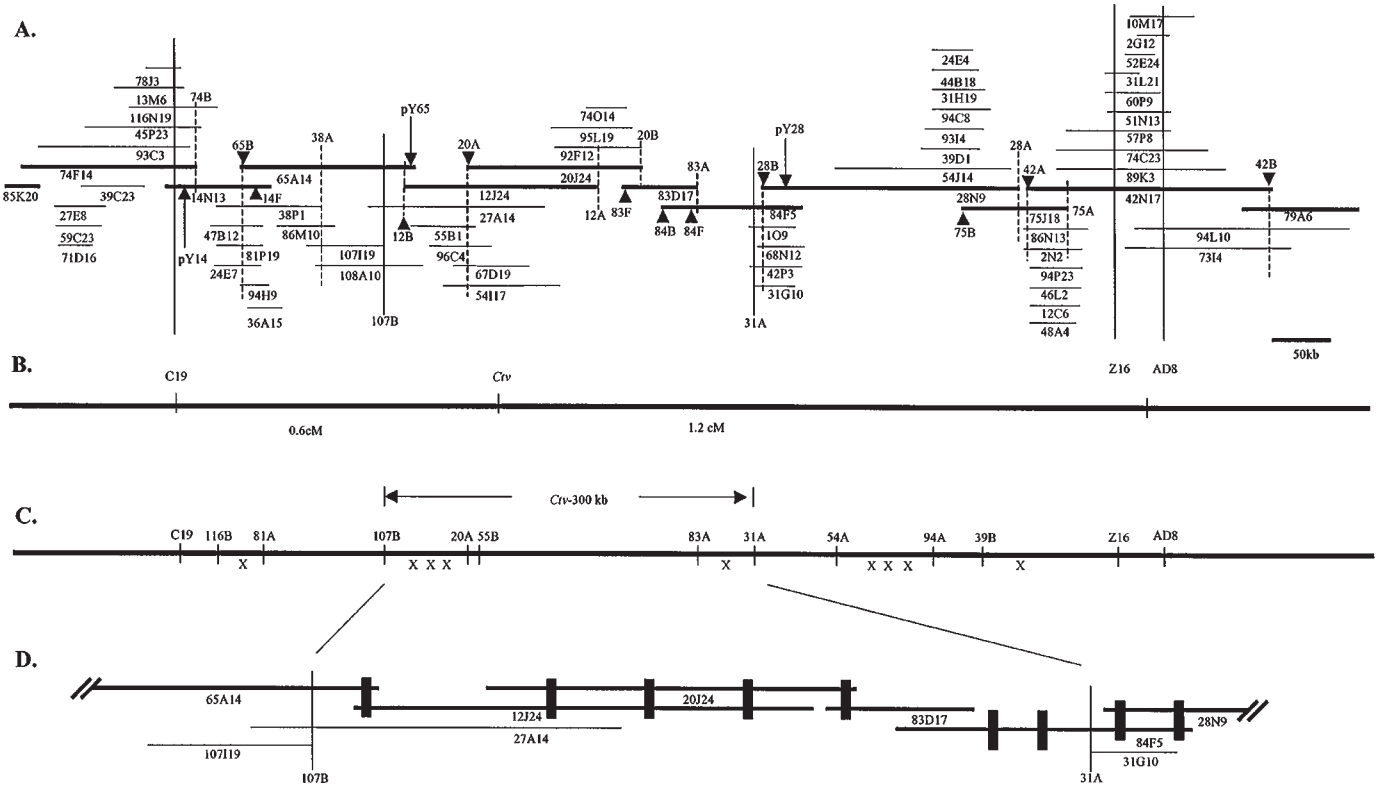
For simple digestion patterns, contigs were assembled manually. For more complex patterns with more than five clones, contigs were assembled using the computer programs Image and FingerPrint Contig (FPC) from The Sanger Center.

Map revision and fine mapping

As the chromosome walk was in progress, the genetic map constructed by Fang et al. (1998) was revised. The populations segregating for CTV resistance and fine mapping were previously described (Fang et al. 1998). The scoring of markers AD8, C19, Z16, and T9 was checked again. Some scoring errors and progeny with ambiguous scores were found. These four markers were then rescored for all progeny. All progeny scored as having recombination events between AD8 and C19 were also retested for the CTV-resistance phenotype, as previously described (Fang et al. 1998).

Meanwhile, BAC ends isolated from the BAC clones were mapped, to confirm that the walk had not jumped to other regions of the genome and to further narrow the region containing the *Ctv* gene. A total of 15 BAC ends were isolated using the PCR method of Yang and Mirkov (2000). After gel-purification, DNA fragments were hybridized with the *Hin*dIII-digested genomic DNA of sweet orange and *P. trifoliata*. A total of 10 DNA fragments that hybridized with a single strong band (Table 2) were used for RFLP mapping, as previously described (Fang et al. 1998). There were nine progeny with recombination events between markers C19 and Z16 in our populations. These new markers were genotyped on the nine recombinant progeny.

Fig. 2. Construction of a 1.2-Mb BAC contig with 61 BAC clones around the *Ctv* locus encompassing markers C19, Z16, and AD8. (A) Overlapping BAC clones forming the contig. The chromosome walk was initiated from markers Z16 and AD8 toward C19. BAC ends or DNA fragments used for the chromosome walk are indicated with small arrows. The major BAC clones are presented in bold-faced type. The reconfirmation of overlapping regions among major BAC clones is shown with broken vertical lines. The long vertical arrows represent the three resistance gene analogs (pY14, pY65, and pY28). The solid vertical lines represent the three markers (C19, Z16, and AD8) used for initiating the chromosome walk and the markers 107B and 31A that define the 300-kb contig that must contain *Ctv*. (B) Revised genetic map of the *Ctv* locus and the closely linked markers C19, Z16, and AD8. (C) Physical map of the contig. DNA fragments isolated from the contig for fine mapping are indicated. The recombination break points are shown below the solid line (X) and the region that must contain *Ctv* is shown above the solid line. (D) Fine-mapped region of the *Ctv* locus showing putative resistance genes that hybridized with pY65 and pY28 (indicated by ▮).



Characterization of putative disease resistance gene analogs

A pair of degenerate oligonucleotide primers, NBSF (5′-GGIGGGIGTIGGIAARACIAC-3′) and NBSR (5′-YCTAGTTGT-RAYDATDAYYYTR-3′), were designed on the basis of the primers s1 and s2 (Leister et al. 1996) and primer NBS-R1 (Yu et al. 1996). Each 50-μL PCR reaction comprised 1 μL of a 1:100 dilution of a BAC DNA miniprep from one of the 12 clones in the contig; 0.2 μg of each primer; 5 μL of 10× PCR reaction buffer containing 15 mM $MgCl_2$ (QIAGEN, Valencia, Calif.); 5 μL of 2 mM dNTPs; and 2.5 U of Taq polymerase (QIAGEN). Thirty-five cycles consisting of denaturation at 95°C for 1 min, reannealing at 35°C for 1 min, and extension at 72°C for 1 min were performed in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.). PCR products were cloned using a TOPO TA cloning kit from Invitrogen (Carlsbad, Calif.). Cloned DNA fragments were hybridized with the *Hin*dIII-fingerprinting gel of 12 overlapping clones that formed the 1.2-Mb contig as described above, except that the washing temperature was 50°C. Cloned DNA fragments were sequenced with T7 and T3 primers that were located on either side of the cloning site. Sequences were assembled using the "GCG" program "GelAssemble" (Devereux et al. 1984). BLAST searches (Altschul et al. 1997) were used for sequence-similarity comparison in the GenBank database.

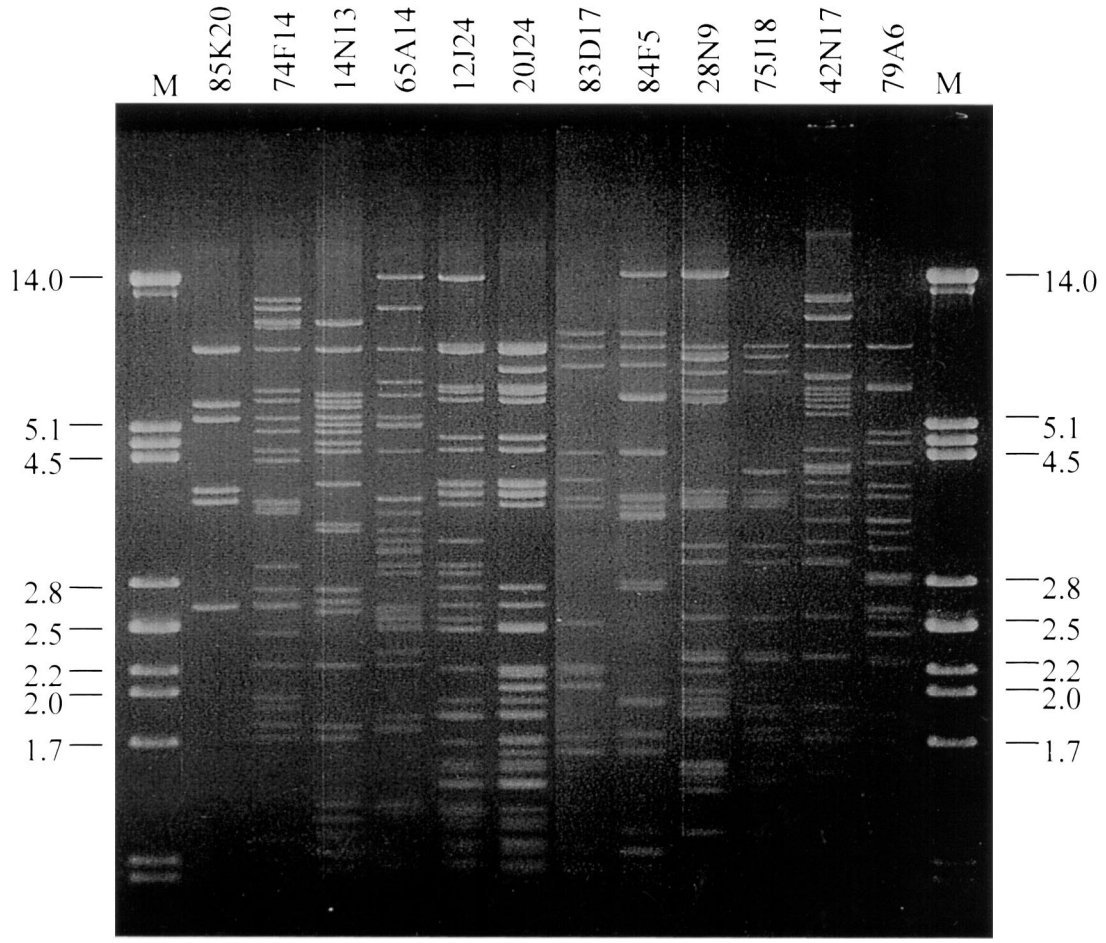
Results

BAC library construction and characterization

To facilitate the map-based cloning of the *Ctv* gene, a BAC library was constructed of *P. trifoliata* cv. Pomeroy. The source of high molecular weight DNA was leaf nuclei from an individual plant that was homozygous for codominant markers flanking *Ctv* (Fang et al. 1998) and therefore predicted to be homozygous for C*tv*. The BAC library contains 45 696 clones arrayed in 119 plates with 384 clones for each plate. To determine the average insert size and insert-size distribution of the library, BAC DNA was isolated from 124 random BAC clones. One clone had no insert, while the rest contained insert DNA ranging in size from 30 to 220 kb, as shown in Fig. 1. The average insert size was approximately 80 kb. As the haploid-DNA content was estimated to be 380 Mb (Arumuganathan and Earle 1991), the genome coverage of this BAC library was estimated to be approximately 9.6 genome equivalents.

To determine the proportion of chloroplast-DNA clones in the library, five chloroplast-DNA probes were isolated using PCR. The PCR products for cp1, cp2, cp5, cp8, and cp10

Fig. 3. *Hin*dIII-fingerprinting gel of the major BAC clones in the 1.2-Mb contig that covers the markers C19, Z16, and AD8 and the *Ctv* locus (M, DNA size marker λ-*Pst*I (kb)). The BAC clone is indicated at the top of each lane.



were 1.8, 2.6, 1.6, 1.4, and 0.8 kb, respectively. Based on the tobacco-chloroplast sequence, the cp1, cp2, cp5, and cp8 probes correspond to nucleotide positions 14 to1825, 1810 to 4372, 35551 to 37142, and 48180 to 48546, respectively. The PCR product of cp10 corresponds to nucleotide positions 98497 to 99244. These probes hybridized with 215 BAC clones. As one set of BAC filters represents 36 864 unique clones, this indicated that 0.58% of the BAC clones in this library were from chloroplast DNA.

Chromosome walk and contig assembly

Based on the linkage map of Fang et al. (1998), Z16 cosegregated with *Ctv*, and C19 and AD8 flank *Ctv* at distances of 0.5 and 0.8 cM, respectively. As these markers are present as single copies in the *P. trifoliata* genome, they were used to initiate a chromosome walk. AD8 and Z16 hybridized with nine and seven BAC clones, respectively. Five of them were common to the two probes (Table 1). The *Hin*dIII-fingerprinting of clones that hybridized with Z16 showed that 42N17 was the largest BAC clone and that all other clones were contained within it (Fig. 2A). BAC ends were subcloned from 42N17. BAC ends 42A (1.5 kb) and 42B (0.5 kb) were derived from *Bgl*II and *Eco*RV digestions, respectively, of the insert of BAC DNA released by *Not*I digestion. When the right end (42B) was used to screen the BAC library, it hybridized with BAC clones 42N17, 73I04, 75J18, and 94L10. As 73I4 and 94L10 only hybridized with AD8 and could not hybridize with Z16, it can be concluded that 42B was the AD8 side of Z16. The left end of 42N17 (42A) was therefore chosen as the probe to initiate the walk toward C19.

Four BAC clones hybridized with 42A when it was used to screen the BAC library. *Hin*dIII-fingerprinting of these four BAC clones indicated that 75J18 was positioned at the extreme end of the contig of the four BAC clones, on the C19 side of Z16. BAC end 75B was obtained using PCR and determined to be the extreme end located on the C19 side of 75J18. The chromosome walk from 75J18 to 28N9 and then to 84F5 progressed in a fashion similar to that from 42N17 to 75J18. BAC end 84B was isolated and determined to be the extreme end located on the C19 side of 84F5. This probe hybridized with more than 1000 BAC clones, suggesting that it contained sequence repeats present throughout the *P. trifoliata* genome. A total of 15 BAC clones that hybridized with 84B were picked and *Hin*dIII-fingerprinted. None of these clones had a *Hin*dIII fragment the same size as the 84F5 *Hin*dIII fragment when probe 84B was used to hybridize with the *Hin*dIII-fingerprinting Southern blot again. This substantiated the evidence that none of these clones overlapped with 84F5 and that probe 84B contained repetitive

Table 3. Fine maps of nine recombinant chromosomes (Chr) for the *Ctv* region, showing the genotype of each chromosome for 14 marker loci (ordered from the physical map) and the phenotype of the individual plant for CTV (s, susceptible; r, resistant).

	Marker locus															
Chr	E20	C19	116B	81A	107B	20A	55B	83A	31A	54A	94A	39B	Z16	AD8	T9	CTV
R2	a	a	a	a	a	a	a	a	a	a	r	r	r	r	r	s
R4	a	a	a		a	r	r						r	r	r	r
R5	a	a	a		a	r	a	a	r	r			r	r	r	s
R6	r	r	r		a		a	a	a				a	a	a	s?
R8	a	a	a	a	a	a	a	a	a	a	r	r	r	r	r	s
R9	r	r	r	r	r	r	r	r	r	r	a	a	a	a	a	r
R10	r	r	r	r	r	a	a	a	a				a	a	a	s
R11	r	r	r	r	r	r	r	r	r	r	r	r	a	a	a	r?
R12	r	r	r		r		a?						a	a	a	s

Note: The second chromosome in each individual was nonrecombinant and did not carry a *Ctv* resistance allele; "r" and "a" indicate markers from chromosomes that originally carried the resistant and susceptible alleles of *Ctv*, respectively.

sequences. Thus, probe 84F (Fig. 2A) was isolated from a *Hin*dIII-fingerprinting gel of BAC clone 84F5 and was used to screen the BAC library again. Using this "two-step walking method," overlapping BAC clone 83D17 was obtained. Since no PCR product representing the left end of the contig was obtained from BAC clone 83D17, a *Hin*dIII DNA fragment, 83F, was isolated directly from BAC clone 83D17. This probe also hybridized with more than 1000 BAC clones. However, some BAC clones showed very strong hybridization signals. A total of 12 BAC clones, including 83D17, were picked and *Hin*dIII-fingerprinted. Fingerprint analysis confirmed that only four of these clones were overlapping with 83D17, and that 20J24 extended to the most C19 proximal side of the contig. The C19 proximal end of 20J24 (20A) was isolated and used to screen the BAC library. The walk from 20J24 to 65A14 was similar to the walk from 42N17 to 75J18. As 27A14 was not identified in the initial screening, 12J24 was determined to represent the C19 proximal end of the contig formed by clones 12J24, 55B1, 96C4, 67D17, and 54I17. As probe 65B contained repetitive sequences, a methodology similar to that used with the 83F probe was used with BAC clones identified by 65B hybridization. BAC clones with very strong hybridization signals were picked and *Hin*dIII-fingerprinted, and overlapping clones 14N13, 38P1, 47B12, 81P19, 24E7, and 94H9 were identified.

The final steps of the chromosome walk were completed by initiating a hybridization step using the marker C19. C19 hybridized with seven BAC clones (Table 1). The *Hin*dIII-fingerprinting of these clones showed that 74F14 and 14N13 extended to opposite ends of the contigs that spanned C19. Probes from these two clones were chosen to walk in both directions, since we initially could not determine a single orientation in which to proceed. The BAC ends of 74F14 were subcloned. Of the three digestions (*Bgl*II, *Eco*RV, and *Nsi*I) of the insert of BAC clone 74F14 released by *Not*I digestion, BAC end 74A was derived from the *Bgl*II digestion. Probe 74A hybridized only with 85K20 and itself when it was used to screen the BAC library. Two *Hin*dIII fragments were isolated from 14N13 to be used as probes, and were hybridized with the same seven BAC clones that hybridized with probe 65B, confirming that the walk from AD8 to C19 had been completed.

The assembled contig of 61 BAC clones was approximately 1.2 Mb and is shown in Fig. 2A; the major BAC clones used for the chromosome walk are presented in boldfaced type. The contig covers markers C19, Z16, and AD8 and the predicted location of the *Ctv* locus. The overlapping regions between major BAC clones in the contig were confirmed by hybridization of the *Hin*dIII-fingerprinting gel of BAC clones located in the overlapping regions with related probes. The *Hin*dIII-fingerprinting gel of the12 major clones is shown in Fig. 3. Thirteen probes, including those used for the chromosome walk, were used, as indicated with broken vertical lines in Fig. 2A. Of these, 84B, derived from BAC 84F5, appeared to include regions of sequence repeated throughout the *P. trifoliata* genome.

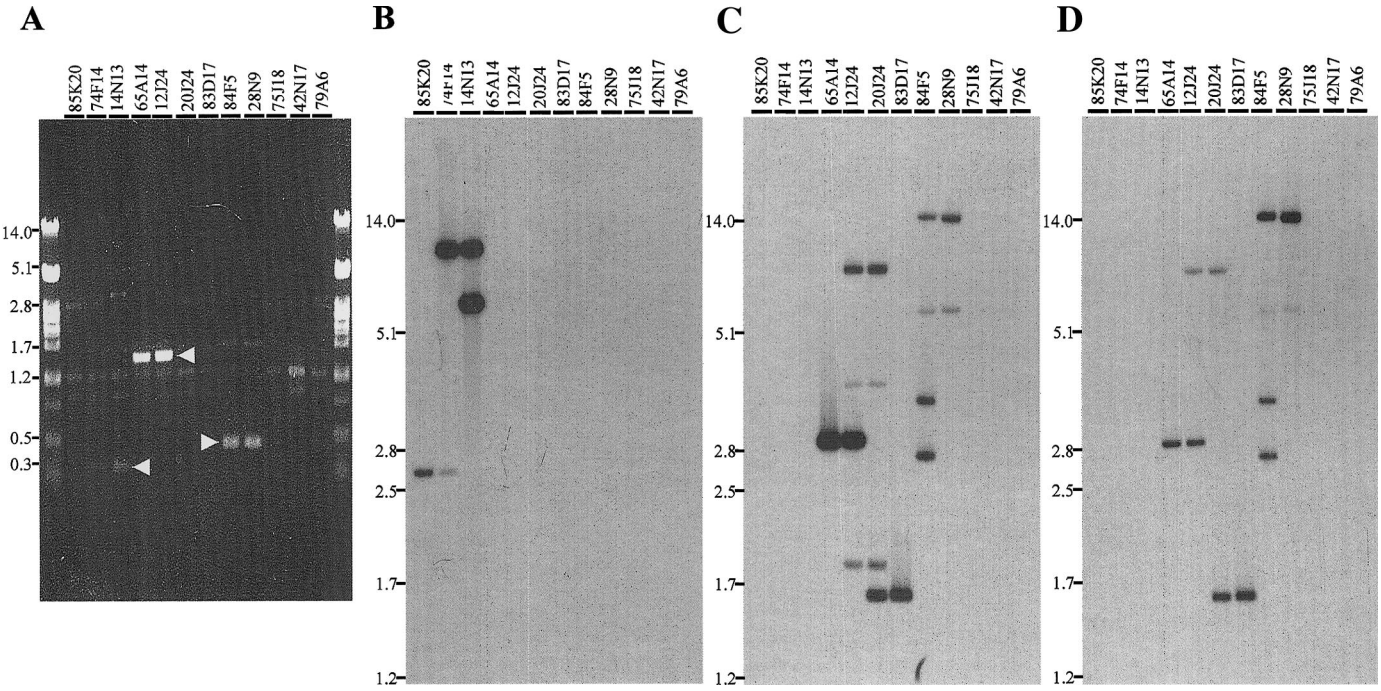
Linkage-map revision

Of the five clones that hybridized with both AD8 and Z16, 51N13 was the smallest at 50 kb. This indicated that the physical distance between Z16 and AD8 could not be more than 50 kb. Further digestion of this clone with different restriction enzymes and hybridization with probes Z16 and AD8 showed that the physical distance between Z16 and AD8 was about 30 kb. This was a surprising result, as the genetic distance between these two markers was 0.8 cM. To determine whether we had located a hot spot of recombination or that errors in phenotype scoring had been made, we rechecked the scoring of phenotypic and genotypic data. Six errors in scores for marker Z16 were found. The three markers (C19, Z16, and AD8) flanking *Ctv* were then rescored for all progeny. Two additional errors were found. Some progeny with ambiguous scores were reanalyzed to confirm their marker genotypes. A revised map after correcting for all erroneous data and missing values for ambiguous data is shown in Fig. 2B. This map does not change the marker order from that published previously (Fang et al. 1998), however, markers AD8 and Z16 now map to the same location, with *Ctv* located between AD8–Z16 and C19 at distances of 1.2 and 0.6 cM, respectively (Fig. 2B).

Fine mapping

The entire contig established is about 1.2 Mb, with the *Ctv* gene located between markers C19 and Z16, which are approximately 850 kb apart. To further refine the location of

Fig. 4. Disease resistance gene candidates in the *Ctv* region. PCR amplification products obtained from 12 major BAC clones using degenerate primer pair NBSF and NBSR (A). The PCR products pY14, pY65, and pY28, indicated with arrows, were used to hybridize with the *Hin*dIII-fingerprinting Southern blot of the 12 major BAC clones (B, pY14; C, pY65; D, pY28). Sizes are indicated in kilobases.



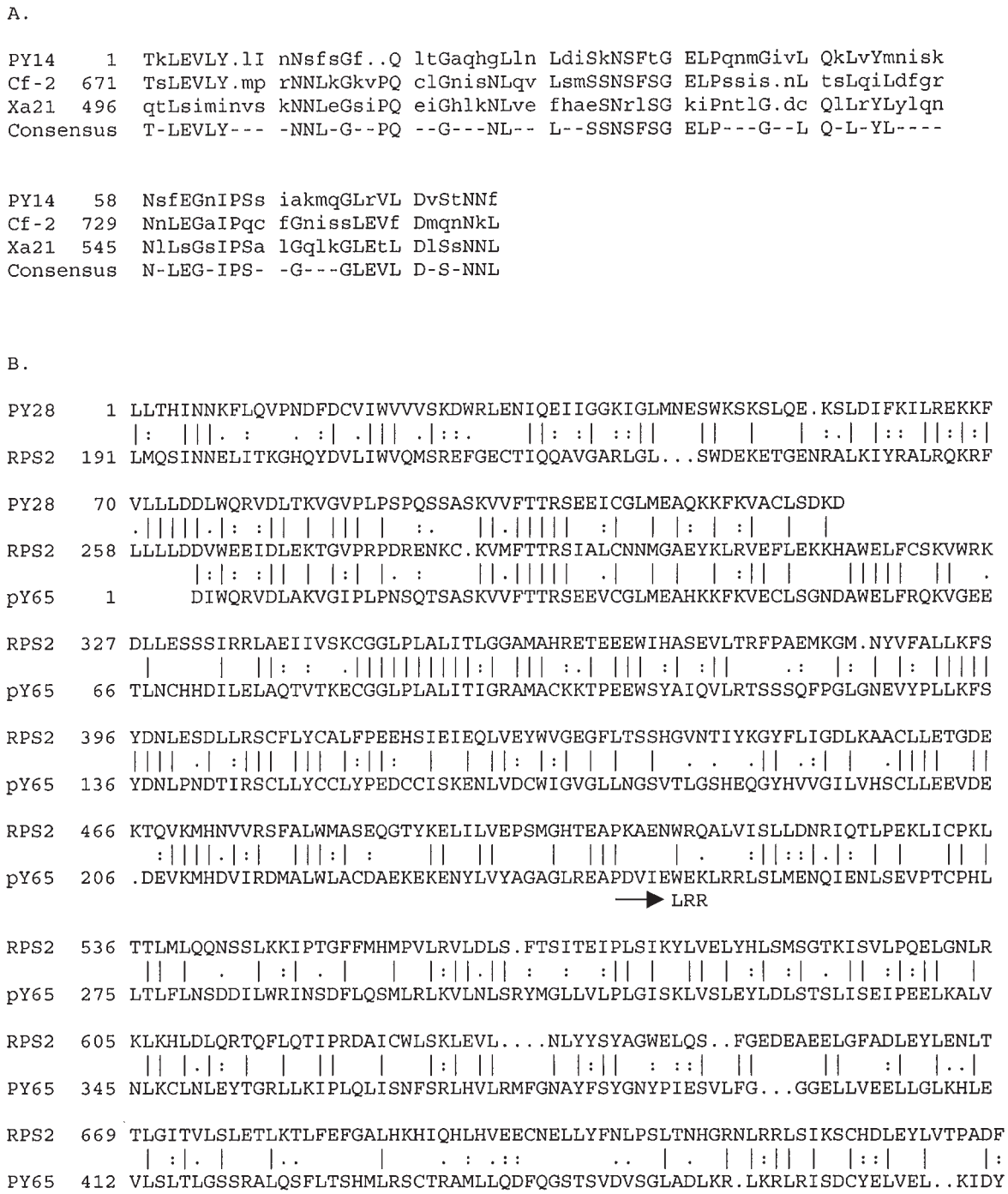
Ctv within this 850-kb region, isolated DNA fragments obtained from BAC clones using the PCR method of Yang and Mirkov (2000) were used as markers. Ten DNA fragments (Table 2) that were present as single copies in the *P. trifoliata* genome as determined by Southern hybridization were selected for RFLP analysis. There are nine plants with recombination events between markers C19 and Z16 in our populations, and these new markers were genotyped on the nine recombinant progeny, as shown in Table 3. Three progeny (R4, R10, and R12) had recombination events between markers 107B and 20A and one progeny (R5) had a recombination event between markers 83A and 31A (Table 3; Fig. 2C). There was no recombination event between markers 20A, 55B, 83A, and the *Ctv* locus (Fig. 2C), indicating that markers 20A, 55B, and 83A cosegregated with the *Ctv* gene. Therefore, the *Ctv* gene must be located between markers 107B and 31A. The physical distance between markers 107B and 31A is about 300 kb. This region is spanned by BAC clones 27A14, 20J24, 83D17, and 84F5 (Fig. 2D). This analysis also showed that the order of markers on the linkage map is consistent with the physical map in this region.

Analysis of putative resistance genes in the contig

Using the degenerate primers NBSF and NBSR and 12 major BAC clones in the contig as templates, three DNA fragments (pY14, pY65, and pY28) were cloned (Fig. 4A). The 0.3 kb DNA clone pY14 was from an amplification product of BAC clones 74F14 and 14N13; the sequence was deposited in GenBank under the accession number AF278856. A BLAST search showed that this sequence was very similar to the tomato disease resistance gene *Cf-2* (accession number U42444) and the rice disease resistance gene *Xa21* (accession number U37133). Clones pY14 and *Cf-2* had 38.7% similarity and 35.5% identity at the amino acid level, while pY14 and *Xa21* had 41.9% similarity and 32.3% identity. The amino acid sequence alignments of pY14, *Cf-2*, and *Xa21* showed that pY14 contained four leucine-rich repeats (LRRs; Fig. 5A). When this DNA fragment was hybridized with the *Hin*dIII-fingerprinting Southern blot of the 12 major clones in the contig, it hybridized with three *Hin*dIII fragments in BAC clone 74F14. Two of these three fragments were located in the overlapping regions of 85K20 and 74F14, and 74F14 and 14N13 (Fig. 4B).

The 1.4 kb DNA clone pY65 was from an amplification product of BAC clones 12J24 and 65A14, and the 0.4 kb DNA clone pY28 was from an amplification product of BAC clones 84F5 and 28N9 (Fig. 4A); these sequences were deposited in GenBank under accession numbers AF278857 and AF278858, respectively. BLAST search results indicated that both of these clones were very similar to the *Arabidopsis thaliana* disease resistance gene *RPS2*. The amino acid sequence comparison showed that pY65 and *RPS2* were 51.5% similar and 40.0% identical, while pY28 and *RPS2* were 57.7% similar and 41.5% identical. The amino acid sequence of pY28 contained part of a nucleotide binding site (NBS), and the amino acid sequence of pY65 contained part of a NBS and nine LRRs (Fig. 5B). Clone pY65 hybridized with nine *Hin*dIII fragments from BAC clones 65A14, 12J24, 20J24, 83D17, 84F5, and 28N9 (Fig. 4C), and pY28 hybridized with seven DNA fragments (Fig. 4D). All the DNA fragments that hybridized with pY28 were identical to those that hybridized with pY65. Seven of these DNA fragments were distributed between markers

Fig. 5. Amino acid sequence comparisons between PCR products (pY14, pY65, and pY28) and known disease-resistance genes (*Cf-2*, *Xa21*, and *RPS2*). (A) Amino acid sequence alignment among pY14, *Cf-2*, and *Xa21*. The alignment was generated using the GCG PileUp and Pretty programs (Devereux et al. 1984). (B) Amino acid sequence comparisons between pY28 and *RPS2* and between pY65 and *RPS2* using the GCG GAP program. The start of the leucine rich repeat region in pY65 is indicated with an arrow. Numbers at the left indicate amino acid positions.



107B and 31A, which is the region in which the *Ctv* gene is located (Fig. 2D).

Discussion

We constructed a BAC library from a seedling of *P. trifoliata* cv. Pomeroy, a relative of citrus. The average insert size of approximately 80 kb is relatively small compared with some BAC libraries, such as 100 kb for tomato (Folkertsma et al. 1999), 130 kb for sugarcane (Tomkins et al. 1999), and 155 kb for rice (Nakamura et al. 1997). However, the 9.6× genome coverage, low chloroplast DNA content, and the successful completion of a 1.2 Mb walk indicate that this library is suitable for map-based cloning of the *Ctv* gene and other disease-resistance genes. In the 1.2-Mb contig, the physical distance of 850 kb between C19 and

Z16 corresponds to the genetic distance of 1.8 cM, and it may be estimated that, in this region, 1 cM is equivalent to about 470 kb, which is higher than the 185 kb/cM estimated for *Arabidopsis* (Schmidt et al. 1995) or the 250–300 kb/cM estimated for rice (Nakamura et al. 1997).

The primer pair NBSF and NBSR were designed on the basis of the NBS sequence of resistance genes *RPS2* and *N* (Mindrinos et al. 1994; Whitham et al. 1994). The amino acid sequences of NBSF and NBSR are GGVGKT and CKVMFTTR, and the expected PCR products correspond to amino acid positions 184–291 of the *RPS2* gene. Amino acid sequence comparisons show that clones pY28 and pY65 correspond to amino acid positions 184–314 and 262–770 of the *RPS2* gene, respectively. Clone pY14, however, contains only the sequence corresponding to the LRRs of the *Xa21* (amino acid positions 496–571) and *Cf2* (671–744) genes that do not contain NBSs. The low annealing temperature (35°C) used for PCR in this work could have led to less-specific annealing of the primers to the templates. Thus, the primer sequences were not included in the sequences deposited in GenBank or in the sequence comparisons in Fig. 5.

Clone pY28 only contains a NBS sequence and hybridized with seven DNA fragments from 12J24, 20J24, 83D17, and 84F5. This suggests that this region might contain clustered disease resistance gene analogs or pseudogenes, whose members are similar to the *Arabidopsis* disease resistance gene *RPS2*. Based on the hybridization of pY14 with the *Hin*dIII-fingerprinting Southern blots of the 12 major BAC clones, at least two homologous sequences of pY14 must be present. Since clones 85K20 and 14N13 are approximately 100 kb apart, and disease-resistance genes are not usually larger than 10 kb, it seems unlikely that these homologous sequences and pY14 belong to a single gene with a similar sequence repeat, although the probe pY14 might not detect some homologous sequences, owing to the stringency of the hybridization. Given this preliminary data, we predict that there is another disease resistance gene cluster around marker C19, which is likely similar to the rice disease resistance gene *Xa21* and the tomato disease resistance gene *Cf-2*. *Xa21* and *Cf-2* belong to a disease resistance gene class that encodes proteins with LRRs, and *RPS2* belongs to another disease resistance gene class that encodes proteins with a leucine zipper (LZ) motif, NBS, and LRR (Hammond-Kosack and Jones 1997). Based on the sequences of two markers, Fang et al. (1998) speculated that there might be two or more classes of resistance genes in this region, and our preliminary data support this contention. The two classes of our putative disease resistance gene analogs are distributed between BACs 85K20 and 84F5, which span a physical distance of about 700 kb (Fig. 2C). Resistance-gene clusters have been postulated to have arisen from recombination–duplication and rearrangement events, to produce novel specificity for different pathogens (Hammond-Kosack and Jones 1997). If further genomic-sequence and gene-expression studies confirm the existence of these disease resistance gene classes in this 700-kb region, its structure might provide further insight into disease resistance gene evolution.

To date, several virus resistance genes have been cloned and characterized. The *N* gene isolated from tobacco and the *HRT* and *RPP* genes isolated from *Arabidopsis* control the hypersensitive response (HR) of resistances to tobacco mosaic virus and turnip crinkle virus, respectively (Whitham et al. 1994; Cooley et al. 2000; Kachroo et al. 2000). Both the resistance to potato virus X (PVX) in potato, controlled by the *Rx* gene, and the resistance to tobacco etch virus in *Arabidopsis*, controlled by *RTM1* and *RTM2*, are not associated with the HR (Bendahamane et al. 1999; Chisholm et al. 2000; Whitham et al. 2000). *Rx*-mediated resistance is the rapid arrest of PVX accumulation in the initially infected cell (Bendahamane et al. 1999), and the primary structure of the *Rx* gene is similar to that of *RPS2*. *RTM1* and *RTM2* are necessary for restriction of the long-distance movement of tobacco etch virus in *Arabidopsis* (Chisholm et al. 2000; Whitham et al. 2000). The *RTM1* gene encodes a protein similar to the lectin jacalin and to a large family of related proteins containing one or more repeats of a jacalin-like domain (Chisholm et al. 2000). The *RTM2* gene product is a multidomain protein containing an N-terminal region with high similarity to plant small heat shock proteins (Whitham et al. 2000). No HR has been observed in the resistance of the *Ctv* gene to CTV infection. In this work, the *Ctv* gene has been finely mapped to a region of 300 kb, where several disease resistance gene candidates similar to the *Arabidopsis RPS2* gene were located. Is the *Ctv* gene one of the clustered resistance gene candidates similar to *RPS2* and *Rx* or some other genes in the region without the NBS and LRR domains like *RTM1* and *RTM2*? The cloning of this gene will elucidate the structure and may give some indication of the function of this gene.

Four BAC clones (27A14, 20J24, 83D17, and 84F5) span the 300-kb region where the *Ctv* gene is located. Based on the published sequences of *Arabidopsis* (Kotani et al. 1998) and rice (Tarchini et al. 2000), the average density of genes in the genome is one every 4–10 kb. The genome size of *P. trifoliata* is larger than that of *Arabidopsis* and smaller than that of rice (Arumuganathan and Earle 1991), therefore, the 300-kb region may contain 30–75 genes. Sequence comparisons with known disease-resistance genes may further reduce the number of candidate genes to be confirmed through genetic transformation. However, it can be expected that there may be as many as seven disease resistance gene candidates, based on the hybridization of pY65 and pY28 with BAC clones in the region. Genetic transformation of all candidate genes to CTV-susceptible varieties is time consuming and labor intensive. Once transgenic shoots have been obtained, it takes another 3–12 months to test for CTV resistance (Fang et al. 1998). Further fine mapping to delimit the region containing *Ctv* is, therefore, the more rapid alternative. Since there are no recombination events between markers 20A and 83A, it is difficult to shorten the contig with the populations used for the *Ctv* mapping (Fang et al. 1998). Additional populations (totaling more than 240 progeny) are now available for fine mapping and the sequencing of the four BAC clones is in progress.

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