High-Resolution Linkage Analysis and Physical Characterization of the *Pto* Bacterial Resistance Locus in Tomato

Gregory B. Martin, M. Carmen de Vicente, and Steven D. Tanksley

Department of Plant Breeding and Biometry, 252 Emerson Hall, Cornell University, Ithaca, NY 14853-1902, U.S.A.
Received 15 July 1992. Accepted 2 October 1992.

Resistance to *Pseudomonas syringae* pv. *tomato* in tomato is conferred by a single dominant locus, *Pto*. We are attempting to isolate the *Pto* locus using a multistep positional cloning strategy. Towards this goal, a high-resolution linkage map containing 18 molecular markers and spanning 20 centiMorgans of tomato chromosome 5 was developed for the region containing *Pto*. Genome-wide mapping and two methods of targeting markers to specific regions were used to identify a total of 28 markers located in the *Pto* region. A subset of these markers was analyzed on an F_2 population derived from a cross between near-isogenic lines that were susceptible or resistant to *P. s.* pv. *tomato*. A total of 1,200 F_2 plants were prescreened using an organophosphate insecticide, fenthion, known to cause necrotic lesions on plants containing the dominant *Pto* allele. Only those plants showing insensitivity to the insecticide (251 total) were analyzed further. Individual plants carrying crossover events in the *Pto* region were identified using flanking restriction fragment length polymorphism markers, and a high-resolution map was constructed. One marker identified, TG538, cosegregated with *Pto* and therefore provides a starting point for chromosome walking. An estimate of physical distance between TG538 and another marker 0.4 cM away revealed that they lie no further than 435 kb apart. The existence of a relatively small physical distance in this region, the identification of a marker that cosegregates with *Pto*, and the availability of localized crossovers provide the foundation for the positional cloning of this locus.

Additional keywords: *Lycopersicon esculentum*, physical mapping, random amplified polymorphic DNA (RAPD).

Natural variation for resistance to plant pathogens has been identified by plant breeders and pathologists in most crop species and is of great agricultural importance. Despite this commercial significance, little is known about the molecular basis of disease resistance (DR) gene function. Based on genetic evidence, Flor postulated that disease resistance may be induced by the interaction of single genes in both the pathogen and the host (Flor 1947, 1971). Since this initial hypothesis, many plant DR genes have been mapped to single loci, and individual avirulence genes have been isolated from bacterial and fungal pathogens (Ellingboe 1976; Staskawicz *et al.* 1984; Gabriel *et al.* 1986; Kobayashi *et al.* 1989; Debener *et al.* 1991; Hulbert and Bennetzen 1991; Keen and Buzzell 1991; Messeguer *et al.* 1991; van Kan *et al.* 1991). Despite this progress, the molecular isolation of plant DR genes has been hindered by the fact that little is known of the gene products encoded at these loci. Recently, precise knowledge of the location of a gene on a high-resolution genetic linkage map has permitted the "positional cloning" of several human disease genes, and it is likely that this approach will prove useful in the isolation of plant DR genes (Rommens *et al.* 1989; Wallace *et al.* 1990).

The interaction of tomato with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* provides a particularly attractive experimental system for attempting to positionally clone a DR gene. First, resistance to *P. s.* pv. *tomato* in tomato is encoded by a single locus (*Pto*) that displays dominant gene action (Pitblado and MacNeill 1983). As with many commercially important traits in cultivated tomato (*Lycopersicon esculentum*), the resistance was identified in a wild tomato species (*L. pimpinellifolium*; Pitblado and MacNeill 1983). Since the *Pto* gene was introduced into tomato by introgression from a wild species, the region around the locus is polymorphic with respect to *L. esculentum* DNA. This polymorphism has been exploited by using a strategy relying on near-isogenic lines to identify molecular markers closely linked to *Pto* (Martin *et al.* 1991). A second advantage of the tomato–*P. s.* pv. *tomato* system is that the pathogen is amenable to molecular analysis and that a gene inducing resistance in tomato (*avrPto*) was recently isolated (Ronald *et al.* 1992). The availability of the cloned *avrPto* gene will facilitate experiments designed to assess the role of *Pto* in conferring resistance to tomato. Thirdly, the unusual observation that an organophosphate insecticide, fenthion, elicits necrotic lesions similar to a hypersensitive response only on tomato lines carrying the *Pto* gene offers an easy screening method for the gene and eventually may provide insight into its function (Laterrot 1985; Laterrot and Moretti 1989). Aside from these favorable attributes for experimentation, the disease caused by *P. s.* pv. *tomato* (bacterial speck) is responsible for serious agricultural losses and is becoming an increasing problem in tomato-growing regions world-

Direct correspondence to G. Martin. Current address: Department of Agronomy, Lilly Hall of Life Sciences, Purdue University, W. Lafayette, IN 47907-1150.

MPMI Vol. 6, No. 1, 1993, pp. 26-34
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wide (Anonymous 1990).

Positional cloning of a gene proceeds in several steps. First, molecular markers are identified that are tightly linked to the target gene, and these markers are then ordered on a high-resolution linkage map. Secondly, the physical distance is determined between the closest markers and the target gene using pulsed-field gel electrophoresis techniques (Ganal and Tanksley 1989). This step reveals whether the markers are physically close enough to the gene to make positional cloning feasible. Thirdly, a chromosome walk is undertaken by using various genomic libraries constructed in lambda or yeast artificial chromosome (YAC) libraries (Burke *et al.* 1987; Martin *et al.* 1992). The final step requires the comparison of the isolated gene with a variant allele or, in plants, complementation of the recessive phenotype by transformation (McCormick *et al.* 1986).

In this paper we describe the genetic and physical characterization of a 20-centiMorgan (cM) region of tomato chromosome 5 that contains the *Pto* resistance locus. Our data indicate that positional cloning of this DR locus appears feasible.

RESULTS

Identification of markers in the *Pto* region.

Three approaches were used to identify a total of 28 markers linked to the *Pto* gene (Table 1). First, a genome-wide mapping effort in this laboratory to place 1,000 RFLP markers on the tomato map identified 19 markers in the general *Pto* region that were then surveyed on the *Pto* near-isogenic lines (NILs) to identify informative clones (Fig. 1; Tanksley *et al.* 1992). Secondly, surveys of the resistant and susceptible NILs were probed with pools of five random clones (600 total clones) to identify polymorphic probes ("multiprobing"; Young *et al.* 1988). Finally, random amplified polymorphic DNA (RAPD) analysis with 150 primers of arbitrary sequence (each amplifying about four products) was used to identify additional linked markers. The markers identified by the latter two approaches were initially placed on the whole genome map (population 86T64; Tanksley *et al.* 1992) to confirm their placement to the *Pto* region. Each marker was then hybridized to survey filters of NILs DNA digested with six restriction enzymes to detect the general level of polymorphism exhibited by the clone and to identify the most easily scored RFLP for mapping purposes (Fig. 1, Table 1). From the 28 markers placed in the *Pto* region, 18 were found to detect an RFLP between the *Pto* NILs with at least one enzyme (64%, Table 1). Most informative markers detected RFLPs with one to three enzymes on these surveys, although two markers—TG538 and R53—detected polymorphisms with all enzymes tested. Subsequently, TG538 was found to detect RFLPs with an additional seven restriction enzymes (data not shown). Overall, marker representation was three cDNAs, four

Table 1. Molecular markers located in the *Pto* region

Marker	Type[a]	Enzyme(s) showing RFLP between near-isogenic lines[b]	Reference
CD31A	cDNA	EV, X	Bernatzky and Tanksley 1986
CT63A	cDNA	none	Tanksley *et al.* 1992
CT104A	cDNA	none	This study
CT155	cDNA	H	Tanksley *et al.* 1992
CT201A	cDNA	none	Tanksley *et al.* 1992
CT202	cDNA	none	Tanksley *et al.* 1992
CT260A	cDNA	B, X	Tanksley *et al.* 1992
R11	RAPD	E, EV	This study
R53	RAPD	B, D, E, EV, H, X	This study
R110	RAPD	B, E	Martin *et al.* 1991
RS120	RAPD	E, H	Martin *et al.* 1991
TG96	Sheared genomic	E, EV, X	Tanksley *et al.* 1992
TG100	Sheared genomic	B, H	Tanksley *et al.* 1992
TG318	*Pst*I genomic	none	Tanksley *et al.* 1992
TG344B	*Pst*I genomic	H	This study
TG358	*Pst*I genomic	none	Tanksley *et al.* 1992
TG363	*Pst*I genomic	none	Tanksley *et al.* 1992
TG379	*Pst*I genomic	none	Tanksley *et al.* 1992
TG448	*Pst*I genomic	E	This study
TG475	*Pst*I genomic	D, H	This study
TG478B	*Pst*I genomic	none	Tanksley *et al.* 1992
TG503	*Pst*I genomic	none	Tanksley *et al.* 1992
TG504	*Pst*I genomic	B	This study
TG538	*Pst*I genomic	B, D, E, EV, H, X	This study
TG606	*Pst*I genomic	B, D, E, RV	This study
TG619	*Pst*I genomic	E, X	Tanksley *et al.* 1992
TG638	*Pst*I genomic	EV	This study
TM5	MADS box gene	B, D, E, H, X	Pnueli *et al.* 1991

[a] RAPD, random amplified polymorphic DNA; *Pst*I, genomic fragments from *Pst*I digest.
[b] RFLP, restriction fragment length polymorphism. Restriction enzymes: B, *Bst*NI; D, *Dra*I; E, *Eco*RI; EV, *Eco*RV; H, *Hae*III; X, *Xba*.

RAPDs, 10 RFLP markers, and one known gene (TM5; Pnueli *et al.* 1991). The number of informative markers identified from each approach outlined above was genome-wide mapping (9 markers), multiprobing (4), and RAPD analysis (4).

Development and screening of populations segregating for *Pto*.

The majority of the identified markers cosegregated when placed on population 86T64, presumably because of a combination of small population size, lower recombination in this wide cross, and close proximity of the markers (Fig. 2A). To determine the order of the markers and to estimate linkage distances between them and the *Pto* gene, we developed two populations that were segregating for *P. s.* pv. *tomato* resistance conferred by *Pto*. Earlier work had placed *Pto* between two morphological markers *af* and *tf*, and we initially sought to use these mutations as flanking markers to identify recombinants in the *Pto* region (Pitblado *et al.* 1984). Thus an F_2 population of 36 plants was analyzed from a cross between LA1444, an *af-tf* marker stock, and Rio Grande-PtoR (Table 2). However, linkage analysis in this population using *af, tf, Pto*, and various RFLP markers showed that *Pto* placed outside of the *af-tf* interval, and this population was not used further (Fig. 2A; Tanksley *et al.* 1992).

A second F_2 population of approximately 1,200 plants was developed from a cross between NILs (Table 2). Since *Pto* displays dominant gene action, it is necessary to test progeny of any plants resistant to *P. s.* pv. *tomato* with potential recombination events in the *Pto* region to determine the allelic state at the *Pto* locus. In order to avoid progeny testing a large number of plants, we chose to identify and analyze only those plants that were homozygous recessive (*pto/pto*). To accomplish this, we relied on the unusual observation made by French plant breeders that an organophosphate insecticide, fenthion, elicits small necrotic lesions on tomato plants carrying the dominant *Pto* locus (Fig. 3; Laterrot 1985; Laterrot and Moretti 1989). It is unknown whether this reaction is a pleiotropic effect of the *Pto* gene or the result of a tightly linked gene, termed *Fen* (Fig. 3). Whatever the case may be, no plant showing recombination between insensitivity to fenthion and susceptibility to *P. s.* pv. *tomato* has been identified in populations of over 650 plants, making this an ideal screen for identifying homozygous susceptible plants (G. Martin, unpublished data).

Approximately 1,200 F_2 plants were treated with fenthion, and only those healthy plants (251 total) showing insensitivity (no necrotic lesions; Fig. 3) were selected for follow-up. Subsequent work showed that 82% of the plants initially scored as insensitive to fenthion were susceptible to *P. s.* pv. *tomato* (*pto/pto*). Another 16% were heterozygous at *Pto*, and 2% were homozygous resistant (*Pto/Pto*). A second screen of the 18% misscored plants found that they were in fact sensitive to fenthion. Thus, prescreening with the insecticide was not absolutely predictive of the *Pto* allelic state but did greatly reduce the amount of progeny testing required. A subsequent screening of a segregating population of 419 plants with fenthion, where the treatment was modified by dipping the plants in a solution of fenthion instead of spraying them, resulted in a 97% accurate prediction of *pto/pto* plants (14 misscored plants; G. Martin, unpublished data). Of the 14 misscored plants that were resistant to *P. s.* pv. *tomato*, all exhibited fenthion sensitivity when rescreened.

High-resolution linkage analysis.

The 251 selected plants were transplanted in the field and analyzed with flanking markers CD31 and TG619 to detect recombinants in the *Pto* region (Fig. 2B). A total of 85 such plants were identified and these were then

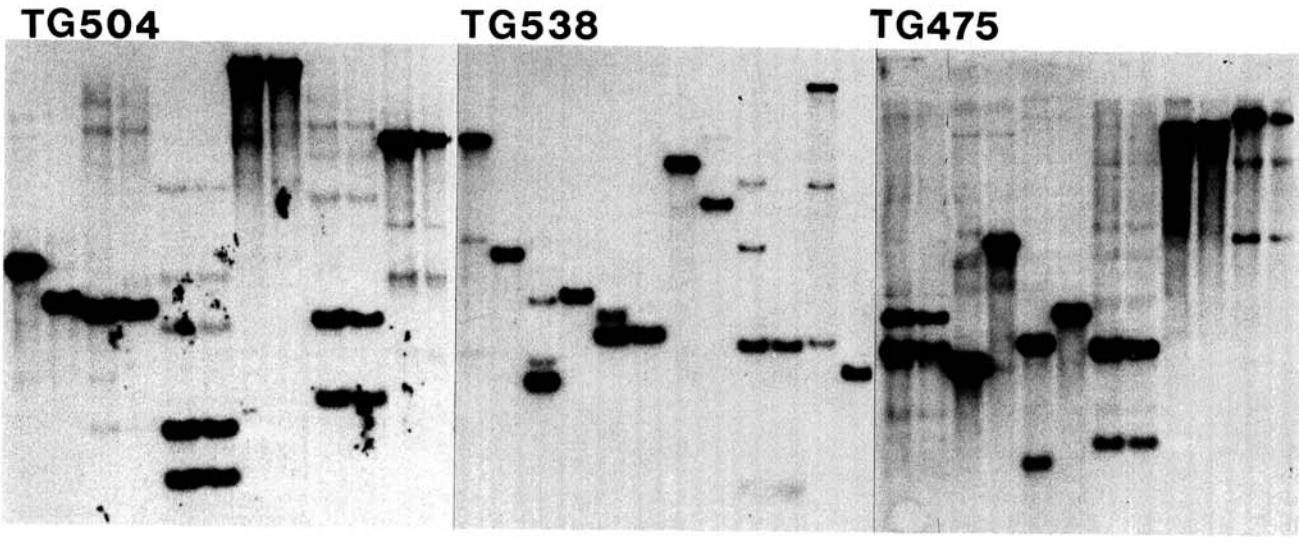

Fig. 1. Hybridization of radiolabeled markers TG504, TG538, and TG475 to DNA from the near-isogenic lines Rio Grande-PtoR and Rio Grande. The surveys consist of pairs of DNA samples of Rio Grande-PtoR (left lane of each pair) and Rio Grande (right lane). Three micrograms of DNA was digested with one of six restriction enzymes, separated on a 1.0% agarose gel, and blotted onto Hybond N+ membrane. The paired digests, in order from left to right, are *Bst*NI, *Dra*I, *Hae*III, *Eco*RI, *Eco*RV, and *Xba*I.

analyzed with the remaining 16 informative markers described above. The 18 markers mapped to nine loci and span a region of almost 20 cM (Fig. 2B). Notably, crossover events were identified between many markers that cosegregated in the 86T64 population, and the NILs map (population 90GM251) displays almost 10-fold expansion in the *Pto* region (Fig. 2). The cause of this map expansion is unknown but may be the result of increased homology between the *L. esculentum* and *L. pimpinellifolium* DNA as compared with the *L. esculentum* and *L. pennellii* parents in population 86T64. Both morphological data and RFLP analyses show *L. esculentum* and *L. pimpinellifolium* to be closely related phylogenetically (Miller and Tanksley 1990). Over one-half of the map expansion can be accounted for by the distance between TG504 and TG538 (12 cM; Fig. 2B). The question of whether this large genetic distance translates into a large physical distance awaits further study. It is also possible that this region exhibits exceptionally high recombination. In contrast to the TG504–TG538 interval, 13 of the markers were found to cluster in a nearby 0.6-cM region. Again, it is not yet known whether this clustering is due to poor recombination or tight physical linkage of these markers on the chromosome. Interestingly, the *Pto* region appears to be close to a region of high marker density on chromosome 5. A comparison with the pachytene chromosome karyotype suggests this region may correspond to the centromere (Tanksley *et al.* 1992).

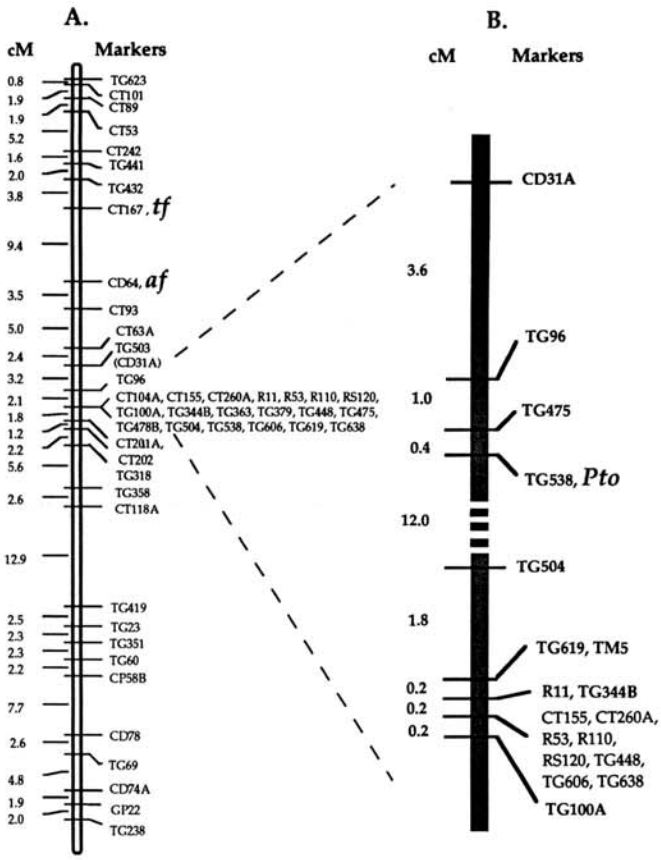

Fig. 2. Linkage maps of tomato chromosome 5 showing location of *Pto*, *af*, *tf*, and molecular markers. **A,** Linkage map developed from F_2 population 86T64 derived from a cross between *Lycopersicon esculentum* and *L. pennellii* (Tanksley *et al.* 1992). Loci by ticks are ordered with a LOD score greater than 3. Loci following commas cosegregate and loci in parentheses have been located to corresponding intervals with LOD scores of less than 3. **B,** Linkage map of 20-cM region of chromosome 5 developed from F_2 population 90GM251 derived from a cross between near-isogenic lines Rio Grande-PtoR and Rio Grande. CT and CD = cDNA clones; TG = tomato genomic clones; R and RS = RAPD markers. (See Material and Methods for details).

The linkage analysis also revealed that one marker, TG538, cosegregated with the *Pto* locus. Considering the size of the population and the corresponding standard error, TG538 lies less than 0.6 cM (95% confidence interval) from *Pto*. This discovery of tight linkage to *Pto* is especially interesting in light of the fact that TG538 is derived from a region highly divergent between *L. esculentum* and *L. pimpinellifolium*, as evidenced by the high level of polymorphism detected by this marker (Fig. 1). Since no crossover event was detected between TG538 and *Pto*, it is unknown whether the locus is contained in the TG475–TG538 interval or in the TG538–TG504 interval (Fig. 2B).

Determination of physical distance in the *Pto* region.

Because our goal was to use the linkage map to isolate the *Pto* gene, we used pulsed-field gel electrophoresis to estimate the maximum physical distance encompassed by the intervals on each side of TG538. A total of eight rare-cutting restriction enzymes were surveyed (*Bss*HI, *Nar*I, *Nru*I, *Mlu*I, *Sac*II, *Sal*I, *Sfi*I, and *Sma*I). The five enzymes that gave fragments between 100 and 900 kb when probed with TG538 were further investigated by probing with TG475 and TG504 (Fig. 4, Table 3).

The experiments revealed that TG538 and TG475 detected nine identical restriction fragments ranging in size from 340 kb to more than 800 kb (Fig. 4). Unique fragments were identified in only two enzyme digests, *Mlu*I and *Sfi*I. In all cases, the two NILs were distinguished by RFLPs

Table 2. Populations used in the construction of the high-resolution map around the *Pto* locus

Population	Parent 1 Species	Parent 2 Species	Generation	No. of progeny	Segregating for *Pto*
86T64	VF36-Tm2a *Lycopersicon esculentum*	LA716 *L. pennellii*	F_2	67	no
89GM36	Rio Grande-PtoR *L. esculentum* / *L. pimpinellifolium*	LA1444 *L. esculentum*	F_2	36	yes
90GM251	Rio Grande-PtoR *L. esculentum* / *L. pimpinellifolium*	Rio Grande *L. esculentum*	F_2	1,200 (251 selected)	yes

using pulsed-field gel electrophoresis. For TG475, the degree of polymorphism was even higher than when 6-bp recognition enzymes were used (Tables 1 and 3). Although the smallest fragment hybridizing to both TG538 and TG475 was 340 kb (with *Nru*I), this fragment only occurred in the *P. s.* pv. *tomato*–susceptible line, Rio Grande. Since different insertions or deletions could exist between the resistant and susceptible lines in this region, we were primarily interested in the smallest common fragment that existed in Rio Grande-PtoR. The analysis showed that two fragments of 435 and 450 kb were in common between TG475 and TG538 (*Sal*I and *Sfi*I digests) in Rio Grande-PtoR. Thus, these two markers are located no further apart than this distance on the chromosome. The minimum distance between them cannot be determined from these data.

If the large genetic distance between TG538 and TG504 is even moderately reflected in the physical distance, we thought it would be unlikely to detect common restriction fragments between the two markers. In fact, the survey with TG504 of eight rare-cutting enzymes found no fragments in common with TG538 and, in addition, uncovered very few fragments smaller than 900 kb (Fig. 4, Table 3). Possible explanations for the latter observation are that the sites are highly methylated around TG504 (the enzymes used are methylation-sensitive) or that there are no recognition sites close to this marker. Since the same filter was probed with all three markers, we can discount the possibility that incomplete digests are responsible for this result.

DISCUSSION

Although DR loci have been identified in most crop species, there have been few successes in cloning these important genes (Johal and Briggs 1991). We have chosen

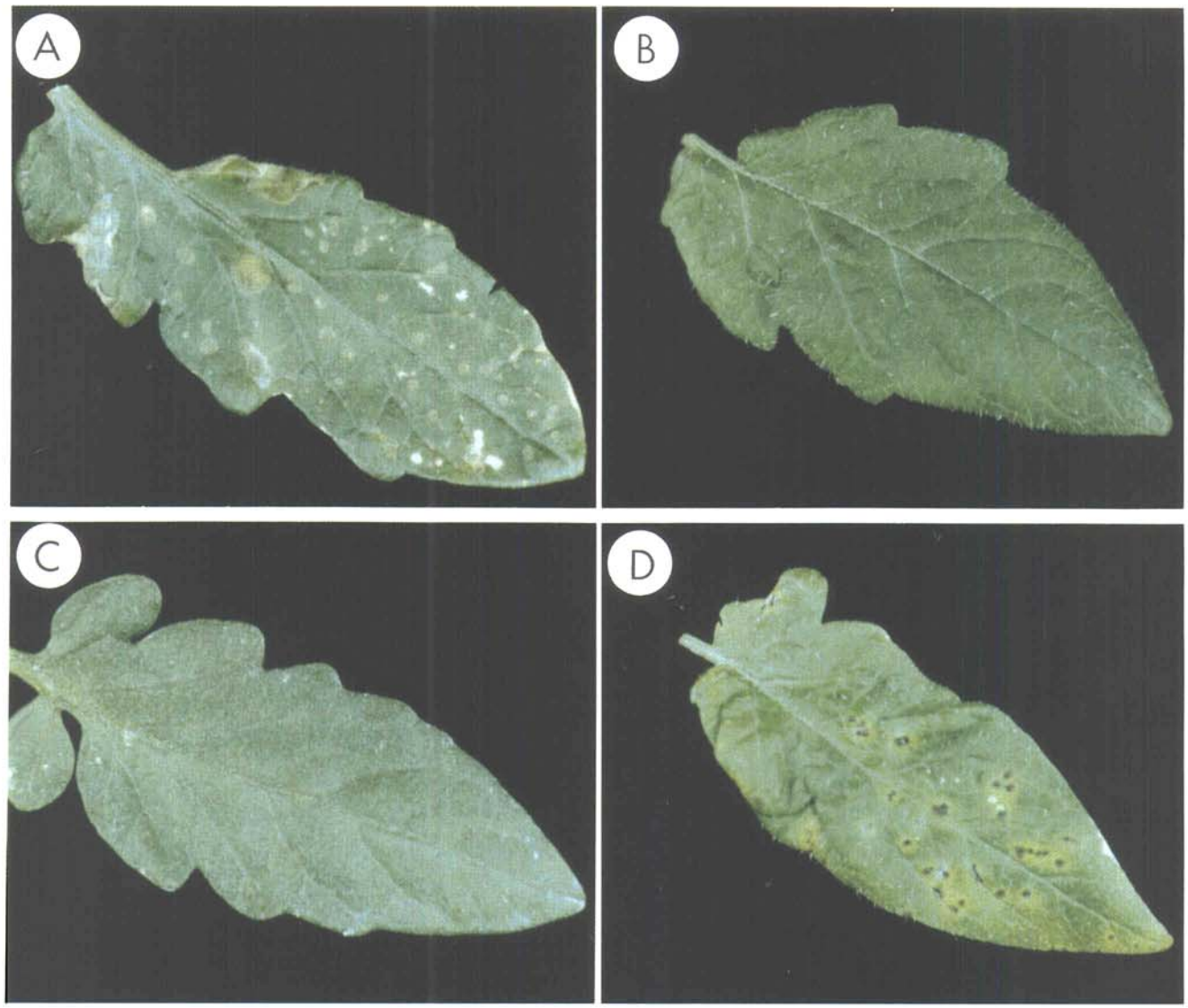

Fig. 3. Phenotypes of the near-isogenic lines Rio Grande-PtoR (**A and C**) and Rio Grande (**B and D**) treated with fenthion and strain PT11 of *Pseudomonas syringae* pv. *tomato*. **A and B,** Leaves were sprayed with 0.15% fenthion/0.05% Silwet L-77 and photographed 3 days after treatment. **C and D,** Leaves were inoculated with *P. s.* pv. *tomato* strain PT11 using 10^6 colony-forming units per milliliter in 0.05% Silwet L-77, 10 mM $MgCl_2$. Photographs of *P. s.* pv. *tomato* symptoms were taken 7 days after inoculation. (See Materials and Methods for details).

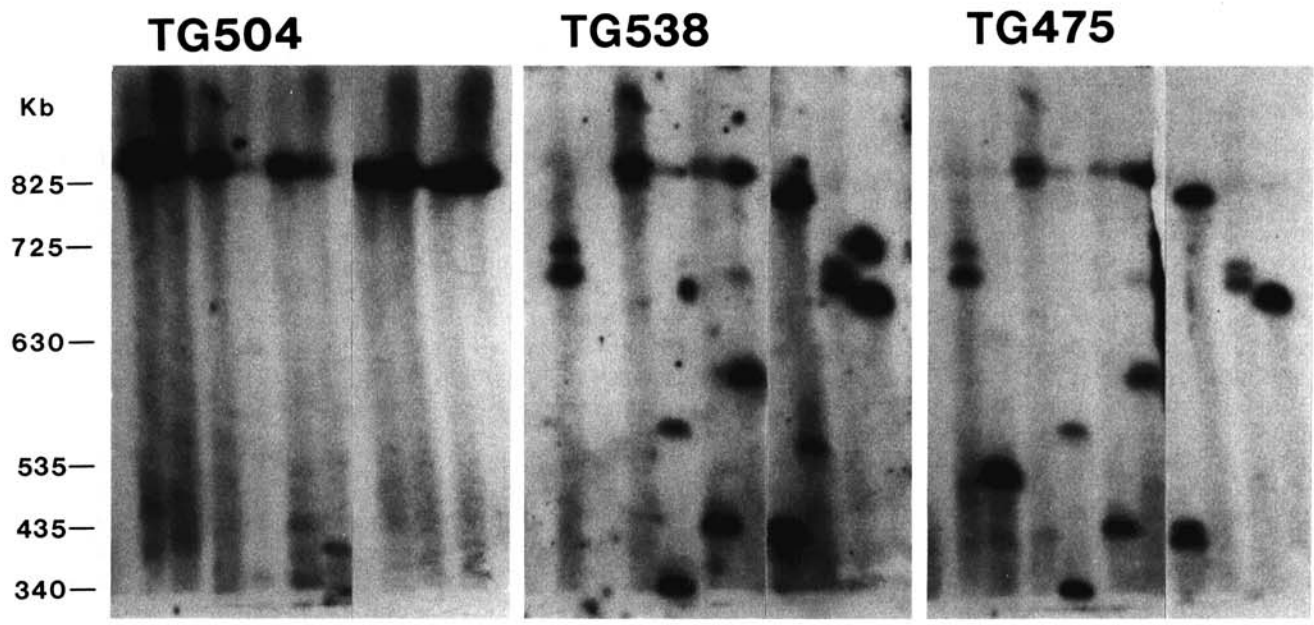

Fig. 4. Hybridization of radiolabeled markers TG504, TG538, and TG475 to pulsed-field gel electrophoresis blots of DNA from the near-isogenic lines Rio Grande-PtoR and Rio Grande. High molecular weight protoplast DNA from Rio Grande-PtoR (left lane of each pair) and Rio Grande (right lane) were separated on a 1.0% contour-clamped homogeneous electric field gel, blotted to Hybond N+, and probed with the indicated markers. The same filter was used in each experiment after stripping and reexposing to film to ensure that all previous signal was removed. The paired digests, in order from left to right, are *Mlu*I, *Nru*I, *Sal*I, *Sfi*I, and *Sma*I. The molecular weight size standards indicated in kilobases (kb) were determined by comparison with lambda concatemers (48.5-kb ladder) and yeast chromosomes from strain AB1380 run on the gel.

to develop tomato as a model system for positional cloning of DR genes because of its many favorable attributes for this approach. Tomato has a relatively small genome (900 Mb) and a small percentage of repetitive DNA (Zamir and Tanksley 1988; Arumuganathan and Earle 1991). Recently, more than 1,000 RFLP markers were placed on the tomato map, and a YAC library consisting of 22,000 clones is now available to expedite chromosome walking (Martin *et al.* 1992; Tanksley *et al.* 1992). Most importantly, because tomato has been the subject of more than 50 years of plant breeding, over 27 DR loci have been identified that confer resistance to many agriculturally important fungi, nematodes, viruses, and bacteria (Watterson 1986; Jones *et al.* 1991). We targeted the *Pto* gene for positional cloning because of its potential for elucidating the gene-for-gene hypothesis and its economic importance.

The *Pto* region on chromosome 5 was saturated with markers using three different strategies: genome-wide mapping, multiprobing, and RAPD analysis. Although genome-wide mapping resulted in the most markers and the closest marker (TG538), this was largely a reflection of the great effort expended on this approach (more than 1,400 markers mapped). Multiprobing of 600 RFLP markers yielded four markers with an investment of about 1 person-year. In contrast, RAPD analysis yielded four markers with a time investment of 1 mo by one person. Most marker identification in this project was accomplished before RAPD markers became available and, since preliminary mapping showed very close linkage with TG475 and TG538, only 150 RAPD primers were screened on the *Pto* NILs. Nevertheless, it is clear that the ability to quickly assay over 4,000 loci using 800–1,000 RAPD primers will greatly speed the future discovery of markers linked to any gene scorable in a segregating population (Giovannoni *et al.* 1991; Michelmore *et al.* 1991). It is unclear why the four RAPD markers identified clustered together in a 0.6-cM region. One possible explanation for this is that there could be a sequence preference for RAPD primer annealing in certain regions of the genome (the RAPD primers used were 50–60% guanine plus cytosine). However, this region also contained the preponderance of other markers, and such clustering could be the result of recombination suppression in a region of large physical size—possibly near the centromere (Tanksley *et al.* 1992).

The serendipitous observation by French researchers that fenthion elicits necrotic lesions in *Pto*-containing to-

Table 3. Size determination of fragments hybridizing to markers in the *Pto* region (all sizes in kilobases)

Marker	Tomato line[a]	Restriction enzyme				
		*Mlu*I	*Nru*I	*Sal*I	*Sfi*I	*Sma*I
TG475						
	R	670	>900	450	435	660
	S	510	560, 340	600	800	650
TG538						
	R	670	900	450	435	660
	S	ND[b]	560, 340	600	800, 530	650
TG504						
	R	>900	>900	>900	>900	>900
	S	>900	>900	>900, 390	>900	>900

[a] Tomato lines: R, Rio Grande-PtoR; S, Rio Grande.
[b] No fragment was detected greater than 100 kb.

mato plants both raises interesting questions of the underlying mechanism of this reaction and also provides an effective tool to screen segregating populations (Laterrot and Moretti 1989). Two genetic explanations are possible for the corresponding sensitivity to fenthion and resistance to *P. s.* pv. *tomato*. First, the two responses may be caused by separate genes that are very tightly linked. Although this remains a formal possibility, no recombinants have been observed between the necrotic reaction to fenthion and resistance to *P. s.* pv. *tomato* in large F_2 populations (over 650 plants, G. Martin, unpublished data). Based on this observation, if *Fen* and *Pto* are different genes, they are separated by less than 0.2 cM (95% confidence interval). Given the similarity of the fenthion reaction to a hypersensitive resistance reaction elicited by high titers of *P. s.* pv. *tomato*, a more likely explanation is that the two reactions are a pleiotropic response conferred by *Pto*. Ultimately, the isolation of the *Pto* (and/or the *Fen*) locus will permit the discrimination between these two possibilities. Regardless of the underlying mechanism of reaction to fenthion, our work demonstrates its utility in easily identifying *P. s.* pv. *tomato*–susceptible (*pto/pto*) plants for use in high-resolution mapping.

It is notable that the markers are not evenly distributed throughout the mapped region. The possibility that this clustering reflects changes in physical distance and recombination hot or cold spots has already been mentioned. Two other explanations for the paucity of markers in certain regions (e.g. TG504–TG538) are possible. First, these regions may be less polymorphic between the two NILs, and thus RFLPs were not identified with the six survey enzymes used. Since only low-copy RFLP markers were selected for mapping, a second possibility is that the barren regions contain high percentages of repeated sequences. If this is the case, it might be expected that further RAPD analysis will identify markers in the TG504–TG538 interval, since it is thought that such markers exhibit less bias against repeated sequences (Williams *et al.* 1990).

For purposes of positional cloning, the most valuable resources identified in this study are the markers most closely linked to *Pto*, TG475 and TG538, and the recombinant plants. The markers can now be used as probes for the identification of YAC clones derived from this region. In addition, the identification of plants that contain crossover events around the *Pto* locus will allow the accurate placement of new closely linked markers, including end probes isolated from the YAC clones. Ultimately, the pinpointing of *Pto* to a small physical interval should be possible using the crossovers as delimiting points on the chromosome.

The study of physical distance between TG475 and TG538 showed that the maximum distance between these markers is 435 kb. Based on the estimate of total genetic distance of tomato of 1,276 cM, and the size of the tomato genome (900 Mb), the average physical distance per centiMorgan in tomato is calculated to be 750 kb (Tanksley *et al.* 1992). Although this estimate was derived from an *L. esculentum* × *L. pennellii* cross, similar estimates arise from the analysis of other crosses that show localized map expansion in some regions compared with this cross (e.g. *L. esculentum* × *L. cheesmanii*; Tanksley, unpublished data). Recognizing that this is only an average, the genetic distance (0.4 cM) between TG538 and TG475 is nevertheless predicted to encompass approximately 300 kb—close to our estimate for this region. We cannot assume that the kilobase-centiMorgan relationship between TG475 and TG538 can be extrapolated to the interval between TG504 and TG538, and thus the physical distance between these markers is not known. Despite this uncertainty, the key factor for positional cloning will be the proximity to TG538 of the nearest crossover event in this interval. These distances will be uncovered as we begin to map YAC end clones derived from this region. Our estimate of 435-kb maximum for the TG538–TG475 interval means that if the two crossovers between these markers are spaced randomly, they will divide this region into three segments of no more than 145 kb (435 kb/3), and the closest crossover should occur near this distance from TG538.

The work reported here forms the foundation for the positional cloning of the *Pto* gene using the closely linked markers TG475 and TG538. In order to use these markers in isolating large segments of the chromosome derived from this region, we developed a YAC library of greater than 22,000 clones (Martin *et al.* 1992). The average insert size of the clones is approximately 140 kb, and the library thus represents a threefold coverage of the genome. We have already identified YACs hybridizing to TG475 (Martin *et al.* 1992), but the current linkage analysis revealed that TG475 is not the closest marker to *Pto*. Recently, we have identified YAC clones from this library that hybridize to TG538. We are currently isolating the ends of these clones and will place them on the linkage map described here. The identification of recombinant plants and the existence of a small physical distance between our markers gives us confidence that the positional cloning of *Pto* is feasible.

MATERIALS AND METHODS

Plant material and segregating populations.

An F_2 population (86T64) derived from an interspecific cross of *L. esculentum* 'VF36-Tm2a' × *L. pennellii* 'LA716' was used initially to assign markers to the *Pto* region (Table 2; Tanksley *et al.* 1992). To order the markers with respect to *Pto*, an F_2 population (90GM251) derived from a cross between two NILs differing for *P. s.* pv. *tomato* susceptibility was used (Table 2). Rio Grande-PtoR derives its resistance from *L. pimpinellifolium* and has undergone six backcrosses to Rio Grande and a final selfing generation (Martin *et al.* 1991). An F_2 population derived from a cross between Rio Grande-PtoR and a tomato marker stock, LA1444, served as the mapping population to place *Pto* with respect to *af* and *tf* (Tomato Genetics Cooperative, University of California, Davis, CA).

Scoring plant reactions to fenthion and to *P. s.* pv. *tomato*.

Approximately 100 seeds were sown per flat in the greenhouse (20–25° C) in a 1:2:1 (v/v) mixture of peat, loam, and perlite. Six weeks after germination the plants were sprayed with a solution of 0.15% fenthion (Mobay Corp., Kansas City, MO) and 0.05% Silwet L-77 (Union Carbide, Southbury, CT, [Whalen *et al.* 1991]) dispersed in sterile

distilled water. After 3–4 days, small necrotic lesions (1–2 mm) were visible on controls known to be either homozygous or heterozygous at the *Pto* locus. A razor blade was used to cull all F_2 seedlings showing the necrotic lesions, and 1 wk later the fenthion treatment and culling were repeated. Those seedlings that remained symptom-free after two fenthion treatments were placed in the field and scored with flanking RFLP markers to identify plants having crossover events in the *Pto* region.

F_3 progeny from F_2 plants having crossovers near the *Pto* locus were screened for their reaction to *P. s.* pv. *tomato* (strain PT11, provided by L. Walling) in the greenhouse as described by Martin *et al.* (1991), except that instead of using cotton swabs the plants were dipped in a solution of 10^6 colony-forming units per milliliter of *P. s.* pv. *tomato* strain PT11/0.05% Silwet L-77/10 mM $MgCl_2$ dispersed in sterile distilled water. Between 20 and 30 F_3 plants were sown as described above and treated at the three- to four-leaf stage. Reaction to the pathogen was scored after 7 days as either susceptible (indicated by numerous necrotic specks surrounded by chlorotic halos) or as resistant (indicated by the absence of necrotic specks on the inoculated leaves).

Molecular analysis.

DNA was prepared from fresh leaves as described by Bernatzky and Tanksley (1986). Methods used for probe preparation, Southern hybridization, and autoradiography were the same as previously published, with the exception that the polymerase chain reaction was used to amplify clone inserts before radiolabeling by the random hexamer method (Feinberg and Vogelstein 1983; Bernatzky and Tanksley 1986). RAPD analysis was as described previously (Williams *et al.* 1990; Martin *et al.* 1991). The sequences for the primers used to amplify the four RAPD markers are as follows (5′–3′): R11, GGAAGTAGTG; R53, GTACGTTCTG; R110, CAACCACGA; RS120, ATCCGCGTG.

Pulsed-field gel electrophoresis.

Preparation of tomato protoplasts (cvs. Rio Grande-PtoR and Rio Grande), isolation of high molecular weight DNA, and digestion in agarose blocks were performed as described in Ganal and Tanksley (1989). Contour-clamped homogeneous electric field gels (Chu et al 1986) were used to separate the digested high molecular weight DNA. Gels were prepared in 0.5× TBE (1× TBE = 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) at an agarose concentration of 1%. For DNA blotting, the gels were treated with UV light (254 nm for 5 min, using a Fotodyne Transilluminator model 3-4400) and then blotted onto Hybond N+ (Amersham Co.) using the recommended alkaline (0.4 N NaOH) blotting procedure. The molecular weight size standards included lambda concatemers (48.5-kb ladder, FMC Bioproducts, Rockland, ME) and yeast chromosomes from strain AB1380 (Burke *et al.* 1987).

Linkage analysis.

The linkage map for the cross between *L. esculentum* and *L. pennellii* was constructed using Mapmaker software on a Sun II workstation as described previously (Lander *et al.* 1987; Tanksley *et al.* 1992). All markers shown placed with a LOD (log of the odds) score of greater than 3. The ordering of the markers for the other two populations was determined using Mapmaker, and the ripple command gave $\Delta LOD > 2.9$ for all alternative triple-point placements. Recombination frequencies between markers were calculated manually using the maximum likelihood estimators of Allard (1956).

ACKNOWLEDGMENTS

We thank Nick Van Eck, Brian Heist, Lynn Miller, Eliana Yglesias, and Gui Xia Yu for excellent technical assistance; Martin Ganal, Jim Giovannoni, and Louise O'Donoughue for comments on the manuscript; Jon Watterson for tomato seeds; Linda Walling and Eric Stockinger for *P. s.* pv. *tomato* strain PT11 and helpful advice; and Henri Laterrot, Pam Ronald, John Salmeron, and Brian Staskawicz for interesting discussions over the past three years. This work was supported, in part, by grants from the National Research Initiative Competitive Grants Program, USDA nos. 91-37300-6418 and 91-37300-6565, and by the Binational Agricultural Research and Development Fund (no. IS-1822-90C). GBM was supported by a 1989 NSF Postdoctoral Research Fellowship in Plant Biology.

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