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Genetic Mapping of *Ph-2*, a Single Locus Controlling Partial Resistance to *Phytophthora infestans* in Tomato

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Late blight caused by the fungal pathogen *Phytophthora infestans* is one of the most important diseases of potato (*Solanum tuberosum)* and tomato (*Lycopersicon esculentum*). Genetic analysis of the resistance to this pathogen was performed on an F_2 progeny of 322 plants derived from a cross between the tomato line *L. esculentum* var. Hawaii7996 susceptible to late blight and the resistant wild relative *L. pimpinellifolium* WVa700. Qualitative and quantitative analyses of the resistance with restriction fragment length polymorphism markers spanning the genome showed that this resistance was controlled by a single, incompletely dominant allele, *Ph-2,* present on the distal part of the long arm of chromosome 10 in an interval of 8.4 cM flanked by markers CP105 and TG233. Genetic analysis of F_2 progeny from a second cross between an *L. esculentum* introgression line IL10-3 carrying a homozygous *L. pennellii* segment spanning the distal part of the long arm of chromosome 10 and WVa700 confirmed the map location, but high suppression of recombination was observed in this cross in the introgressed fragment. A high-resolution genetic linkage map of the chromosomal region surrounding *Ph-2* was initiated to permit future map-based cloning of this gene. Amplified fragment length polymorphism markers closely linked to *Ph-2* were screened by bulked segregant analysis.

Additional keyword: tolerance.

Late blight of solanaceous plants, caused by the fungal pathogen *Phytophthora infestans* (Mont.) de Bary, is one of the most important diseases of the cultivated potato and tomato (Robertson 1991). Epidemics of late blight, favored by wet weather and cool temperatures, can be sudden and highly destructive in the field or in unheated glasshouses. Prevention of epidemics requires frequent applications of fungicides. This is expensive and undesirable for the environment, and leads to the emergence of resistant isolates with increased fitness (Ko 1994).

Breeding for resistance to *P. infestans* in tomato began 50 years ago (Richards and Barratt 1946), leading to the transfer, from the wild relative *L. pimpinellifolium,* of a dominant gene, *Ph-1,* conferring specific resistance to *P. infestans* race 0 (Bonde and Murphy 1952). *Ph-1* was rapidly overcome by a new race of *P. infestans,* referred to as tomato race 1 (Conover and Walter 1953). Breeding efforts have therefore been concentrated on a partial resistance found in the *L. pimpinellifolium* accession West Virginia 700 (Gallegly 1960). As with many partial resistances, expression of this character is dependent to some extent on the environmental conditions, the physiological stage of the plants (Turkensteen 1973), the aggressiveness of the *P. infestans* isolate (Laterrot 1975), and the plant organ (Turkensteen 1973). Thus, resistance to *P. infestans* in the line WVa700 is difficult to measure accurately, which explains previous, controversial results about the genetic nature of this partial resistance. Continuous variation of this character (Gallegly 1960) suggested that the resistance was quantitative, presumably polygenic in nature, as are many partial resistances (Nelson 1978). However, this resistance increased significantly after plants were 6 to 8 weeks old, giving rise to a more discrete variation in progenies (Turkensteen 1973). Consequently, partial resistance to *P. infestans* in the line WVa700 was redefined to be controlled by a single, incompletely dominant gene named *Ph-2* (Laterrot 1975; Turkensteen 1973).

An effective approach for studying complex disease resistances is provided by the use of molecular markers (reviewed by Young 1996). With the development of saturated linkage maps, all regions of the genome can be assayed for the presence of a locus underlying a particular trait (Tanksley 1993), and estimation of the phenotypic variation associated with this locus can be performed (Paterson et al. 1988). Frequently, quantitative resistance has been found to be governed by a few major resistance loci (reviewed by Young 1996) and even single loci were found to control, per se, quantitative resistance in some cases, such as to *Stemphylium* in tomato (Behare et al. 1991), to *Globodera pallida* in *Solanum spegazzinii* (Kreike et al. 1994), or to cyst nematode in soybean (Concibido et al. 1996). Absence of discrete segregation in these cases could be due to the presence of genes with minor effects that were be-

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low the threshold of significance for detection of linkage, or may indicate a large environmental influence on the expression of the resistance.

In tomato, the construction of a saturated restriction fragment length polymorphism (RFLP) linkage map (Tanksley et al. 1992) has permitted numerous disease resistance genes to be mapped (reviewed by Chagué et al. 1996). In addition to breeding applications, molecular markers tightly linked to a target gene can be used as starting points for physical mapping and subsequent cloning of the gene (Tanksley et al. 1995). Such a strategy has been successfully used in tomato for the isolation of *Pto* and *Prf*, two genes involved in tomato resistance to *Pseudomonas syringae* pv. *tomato* (Martin et al. 1993; Salmeron et al. 1996), *Cf-2,* a gene conferring resistance to *Cladosporium fulvum* expressing the *Avr2* gene (Dixon et al. 1996), and *I2C,* a gene conferring resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2 (Ori et al. 1997).

The recent cloning of several race-specific disease resistance genes has permitted new insights into the mechanism of resistance underlying host-pathogen "gene-for-gene" interactions (Baker et al. 1997). In these cases, host resistance, activated by a single gene, is expressed when the attacking pathogen expresses an appropriate avirulence gene (Flor 1971). Incompatible interactions, resulting in the absence of pathogen development, are often associated with a hypersensitive response, characterized by localized, rapid, host cell death at the infection site (Keen 1990). However, many plant-pathogen interactions do not fit Flor's model. Resistance has also been associated with a reduction in the time course of development of symptoms (partial resistance) or with the absence of symptoms while the development of the pathogen in the host may not be reduced (tolerance). Partial resistance is a desirable trait for plant breeders since it is often effective across a broad range of pathogen races or strains (Parlevliet 1979). The elucidation of the mechanisms of partial resistance at the molecular level requires, as for race-specific resistance, the cloning of the host genes implied in these interactions.

In the present study, we report on the genetic analysis of the partial resistance to *P. infestans* present in *L. pimpinellifolium* accession WVa700 and thereby place *Ph-2* on the tomato RFLP linkage map. High resolution mapping of the tomato chromosomal region containing *Ph-2,* a prerequisite for map-based cloning of this gene, was initiated with bulked segregant analysis (Giovannoni et al. 1991; Michelmore et al. 1991) and amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995).

RESULTS

Inheritance of resistance to *P. infestans*.

Initially, the inheritance of resistance to *P. infestans* was studied in a population of 200 F_2 plants derived from a cross between the susceptible cultivated tomato *L. esculentum* var. Hawaii7996 (H7996) and the resistant line *L. pimpinellifolium* WVa700 for which a genetic map was available (Thoquet et al. 1996). Evaluation of resistance to *P. infestans* was performed on individual rooted cuttings from 113 plants of this population (referred to as WH1), with a moderately aggressive isolate, originating from Almeria, Spain. F_1 hybrids showed a level of resistance intermediate between the resistant and the susceptible parents (Table 1), indicating that resistance is inherited as an incompletely dominant trait. However, one F_1 hybrid could not be unambiguously distinguished from the susceptible parent H7996; thus, in the progeny screening, only the plants from the phenotypic class 0 could be assigned as resistant. With this as a criterion, the F_2 plants segregated 30

Table 1. *Phytophthora infestans* resistance tests on parents, F_1 hybrids, and progenies from the crosses between *Lycopersicon esculentum* lines H7996 and IL10-3 (susceptible) and *L. pimpinellifolium* line WVa700 (resistant)

Cross	*P. infestans* isolates	Observed organ	Parents or generation	Disease index[a] 0	1	2	3	Total
				Plants per class (no.)				
H7996 × WVa700 (WH1)[b]	Almeria	Stem	WVa700	6				6
			H7996			5	1	6
			F1		11	1		12
			F2	30	38	34	11	113
H7996 × WVa700 (WH2)[c]	1306	Stem	WVa700	5				5
			H7996			2	3	5
			F2	24	24	21	19	88
		Petiole	WVa700	5				5
			H7996			1	4	5
			F2	16	26	15	31	88
		Leaf	WVa700	5				5
			H7996			1	4	5
			F2	18	28	18	24	88
IL10-3 × WVa700 (WIP)[c]	1306	Stem	WVa700	8				8
			IL10-3				8	8
			F2	23	14	21	18	76

[a] Symptoms were scored according to the following index: 0 = no symptoms or rare necrotic spots, 1 = small lesions, 2 = large expanding lesions, and 3 = more than 30% of the organ covered with lesions.
[b] Evaluation of resistance to *P. infestans* was performed on individual rooted cuttings from parents, F_1 hybrids, and F_2 plants derived from the cross between the *L. esculentum* line H7996 and *L. pimpinellifolium* line WVa700 (population WH1).
[c] Evaluation of resistance to *P. infestans* was performed on 5-week-old F_2 plants derived from the same cross (population WH2) and on F_2 plants derived from the cross between the *L. esculentum* introgression line IL10-3 and WVa700 (population WIP).

resistant and 83 susceptible, in accordance with a single gene inheritance (Table 2).

With a second population of 122 F_2 plants derived from the same cross (referred to as WH2), 88 plants were tested for resistance to *P. infestans* with the more aggressive fungal strain "1306." Tests were done on plants grown from seed in order to avoid physiological effects, due to the stress of propagation by cutting, that could interfere with expression of the resistance (Thoquet et al. 1996). Under these conditions, symptoms on the susceptible parent were more severe on stems, petioles, and flag leaves while the resistant WVa700 line did not develop symptoms (Table 1). Twenty-four out of the 88 F_2 progeny tested did not develop any symptoms on stems, supporting the hypothesis of single-gene inheritance (Table 2). Evaluation of resistance to *P. infestans* on petioles or leaves was later considered to be less reliable than on stems, since, in some experiments, symptoms were observed on petioles and leaves of the resistant parent WVa700 (data not shown). On the other hand, 19 F_2 plants showed severe symptoms on stems, consistent with the proportion of homozygous susceptible plants expected for the inheritance of an incompletely dominant gene. Nevertheless, the variations in symptoms observed in the parental susceptible plants indicated that misclassification of plants between heterozygous and homozygous susceptible plants was likely under the assumption of a single gene control.

With a third segregating progeny of 146 F_2 plants derived from the cross between *L. esculentum* introgression line IL10-3 (IL10-3) and WVa700 (referred to as population WIP), 76 plants were tested for resistance to *P. infestans*. IL10-3 was highly susceptible to *P. infestans* strain 1306 (Table 1). By comparison with parental lines, F_2 plants could be grouped into three distinct classes and the segregation ratio observed was in accordance with the hypothesis of a single, incompletely dominant resistance gene (Table 2).

Mapping of the resistance gene *Ph-2*.

Since genetic analysis of resistance to *P. infestans* in line WVa700 has been controversial in the literature and since a more complex mode of inheritance of the resistance could not be excluded from the genetic analysis presented above, we attempted to map the resistance character either as a qualitative trait, by attempting to regroup the plants into classes, or as a quantitative trait, with raw data obtained by scoring plants on a scale of symptom severity.

A genetic map of the tomato genome was previously constructed with the population WH1 (Thoquet et al. 1996). Out of 462 probes tested with 14 different restriction enzymes, only 6% of enzyme-probe combinations were found to be polymorphic in this cross. Seventy-five molecular markers, 62 RFLP and 13 random amplified polymorphic DNA (RAPD), were ordered on the 12 linkage groups, covering about 75% of the tomato genome (Thoquet et al. 1996). Since a preliminary analysis (see below) showed an association of the resistance character with markers on chromosome 10, all of the tomato and potato RFLP probes previously mapped on this chromosome (Gebhardt et al. 1991, 1994; Tanksley et al. 1992) were tested with 15 different restriction enzymes.

Thus, RFLP markers TG233, CT240, CT124, and CT126 were added to the previous chromosome 10 linkage group for the purpose of the present study (Fig. 1A). A partial genetic map, restricted to chromosome 10, was constructed with the population WH2, using RFLP markers CT203, CT124, TG229, GP87, CT240, CP105, and TG233. Linkage order and genetic distances between markers were in close accordance with results obtained with population WH1 and a common genetic map of the chromosome 10 was established (Fig. 1A). CT124 was found to cosegregate with TG229 in a total of 322 F_2 plants analyzed. Distortion of segregation was not observed for the 13 markers mapped on chromosome 10 (data not shown).

In a first attempt to map *Ph-2,* resistance to *P. infestans* was analyzed as a Mendelian character. In order to maximize linkage detection, plants from WH1 and WH2 (Table 1) were grouped into three genotypic classes despite the fact that erroneous assignment of the *Ph-2* genotype was likely for several plants with use of this classification (see above). Plants showing no symptoms (disease index 0) were considered to have the genotype *Ph-2/Ph-2* (homozygous for the WVa700 allele) whereas plants showing symptoms were assigned to the genotype *Ph-2/ Ph-2*$^+$ (disease index 1 or 2) and *Ph-2*$^+$*/Ph-2*$^+$ (disease index 3). Linkage analysis and mapping were performed with the software JoinMap (version 2.0) and the Kosambi mapping function. With this approach, the map position of *Ph-2* was determined in population WH1 to be in an interval of 8.4 cM bracketed by markers CP105 and TG233 on the distal part of the long arm of the chromosome 10. *Ph-2* was found to be significantly linked to both markers with a LOD score (log_{10} of the odds ratio) of 3.4 with TG233 and 2.9 with CP105. The genetic location of *Ph-2* was confirmed by the analysis of the population WH2. Whatever the organ considered for scoring the resistance, highly significant association was found between *Ph-2* and markers TG233 (LOD = 9.2 to 12.8), CP105 (LOD = 8.0 to 11.2), CT240 (LOD = 7.8 to

Table 2. Segregation ratios observed in F_2 progenies in response to *Phytophthora infestans* infection

Cross	*P. infestans* isolates	Observed organ	Phenotypic class[a]			Total	Expected ratio[b]	χ^2	*P*
			Resistant	Intermediate	Susceptible				
WH1	Almeria	Stem	30	83		113	1 : 3	0.14	0.70
WH2	1306	Stem	24	64		88	1 : 3	0.24	0.62
		Petiole	16	72		88	1 : 3	2.18	0.14
		Leaf	18	70		88	1 : 3	0.97	0.32
WIP	1306	Stem	23	35	18	76	1 : 2 : 1	1.13	0.57

[a] Plants evaluated for symptom severity on a scale of 0 to 3 (see Table 1) were grouped into two classes according to the absence (disease index 0) or presence (disease index 1, 2, or 3) of symptoms, or into three classes: resistant (disease index 0), intermediate (disease index 1 or 2), or susceptible (disease index 3).

[b] The χ^2 and the corresponding *P* value were calculated to test the probability that the data fit an expected ratio of 1:3 or 1:2:1 for segregation of a single locus conferring resistance to *P. infestans*.

10.1), GP87 (LOD = 5.3 to 7.0), and TG229/CT124 (LOD = 4.8 to 5.5). The most likely location of *Ph-2* was thus again between markers TG233 and CP105. Evaluation of the possible erroneous assignment of the *Ph-2* genotype was performed on this population with RFLP marker information. Out of the 88 plants surveyed, 73 were nonrecombinant between the two markers CP105 and TG233 flanking *Ph-2*. Among these 73 nonrecombinant plants, the resistance of two individuals was inconsistent with their flanking marker genotypes. These two plants were heterozygous for both marker loci, but one was evaluated to be $Ph\text{-}2^{+}/Ph\text{-}2^{+}$ (homozygous for the H7996 susceptible allele) and the other was *Ph-2/Ph-2* (homozygous for the WVa700 resistant allele) at the resistance locus. The probability of a double cross-over event in an interval of 8.4 cM was estimated to be 2.10^{-3} in an F_2 population. Thus, the two inconsistent plants were more probably misclassified regarding their phenotype and will be reevaluated by analyzing the segregation observed for their resistance character by analysis of their F_3 families.

As a second step toward mapping *Ph-2,* quantitative trait analysis of the resistance was done, using interval mapping (Lander and Botstein 1989) with the software MapQTL (Van Ooijen and Maliepaard 1996). In population WH1, where a complete genetic map was available, the possible presence of other loci contributing to the resistance could be assessed. With this approach, only the lower part of the chromosome 10 was found to be significantly associated with the resistance, using a threshold value of 1% (LOD = 2.9). To assess the genetic contribution of chromosome 10 segments to the resistance, interval mapping was performed on this chromosome, with either the WH1 or WH2 populations. In both populations, the most probable position of the resistance locus present on this chromosome was found to be located between the same markers CP105 and TG233 (Fig. 2), in accordance with qualitative analysis. In population WH1, markers CP105 and TG233 explained 17.2 and 17.8% of the total variance of the resistance character, respectively, and the putative contribution of the resistance locus located in this region was estimated to explain 20.4% of the total variation of the character. In population WH2, a much larger part of the phenotypic variance could be explained. CP105 was found to explain from 55.5 to 60.5% of the total variance, considering resistance

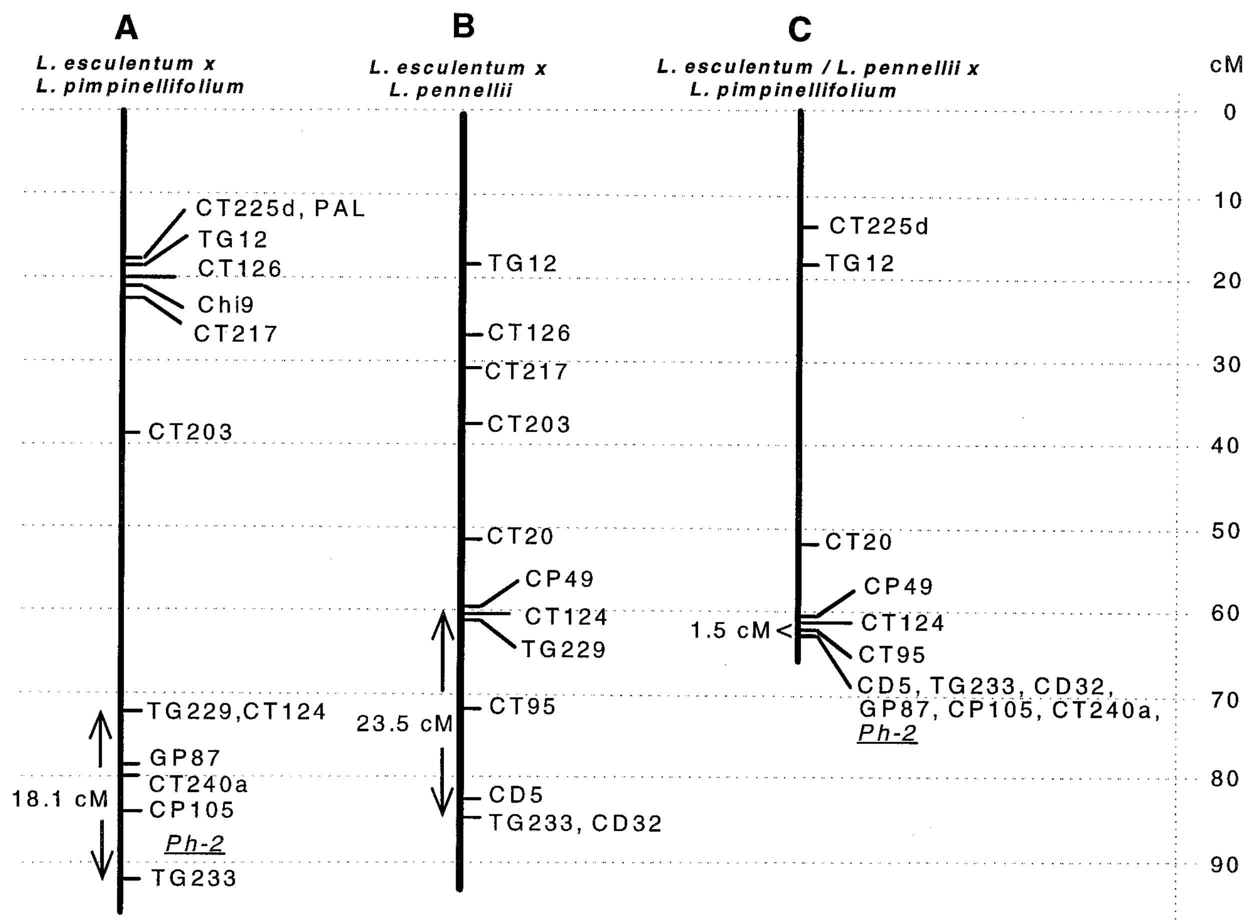

Fig. 1. A comparison of three restriction fragment length polymorphism (RFLP) linkage maps of tomato chromosome 10. **A,** Linkage map based on the cross *Lycopersicon esculentum* line Hawaii7996 × *L. pimpinellifolium* line WVa700. **B,** Linkage map based on the interspecific cross *L. esculentum* line VF36 × *L. pennellii* line LA716 (Tanksley et al. 1992). **C,** Linkage map based on the cross *L. esculentum* introgression *L. pennellii* line IL10-3 (Eshed and Zamir 1994) × *L. pimpinellifolium* line WVa700. Map distances depicted on the right of the maps are calculated in centiMorgans (Kosambi units) with the software JoinMap (Stam 1993). Genetic distances between markers CT124 and TG233, located on the ends of the *L. pennellii* introgressed fragment in line IL10-3, are indicated on the left of each map. Resistance locus *Ph-2* is underlined; its map location corresponds to the qualitative analysis of the resistance character in crosses **A** and **C**.

evaluation on stems, petioles, or leaves, while TG233 explained from 58.6 to 65.0% of the total variance. The putative resistance locus, located between these markers, was estimated to explain between 74.7 and 83.4% of the total variance of the resistance.

Fine mapping of *Ph*-2 with an introgression line.

In order to increase marker density in the vicinity of *Ph-2,* an F_2 population resulting from the cross between the *L. esculentum* introgression line IL10-3, carrying a chromosomal segment from *L. pennellii* spanning the distal portion of chromosome 10, and WVa700 (population WIP) was analyzed. Along the IL10-3 introgressed fragment, 68% of the enzyme/probe combinations tested were polymorphic with WVa700, compared with 6% that were found to be polymorphic between H7996 and WVa700 (data not shown). A partial genetic map was constructed with 12 probes and 148 F_2 plants (Fig. 1C). The genetic distances between TG12, CT20, and CP49, which map outside the introgressed fragment, agreed with previous work (Tanksley et al. 1992) and the present study (Fig. 1A). The remaining eight markers, which mapped inside the introgressed fragment, were closely linked in this cross, spanning 1.5 cM. None of them showed a distorted segregation (data not shown). The genetic distance between CT124 and TG233, markers that mapped on the ends of the introgressed fragment, was 18.1 cM in cross WH1/WH2 (*L. esculentum* × *L. pimpinellifolium*), 23.5 cM in cross *L. esculentum* × *L. pennellii* (Tanksley et al. 1992), and 1.5 cM in cross WIP (*L. esculentum* introgression *L. pennellii* × *L. pimpinellifolium*). The markers GP87, CT240, TG229, CP105, CD32, and TG233, spanning 12.5 cM in the distal part of the chromosome 10 in cross WH1/WH2, were found to cosegregate in WIP. The position of *Ph-2* was confirmed in this cross, since it cosegregated with these markers for 75 out of the 77 F_2 plants tested for *P. infestans* resistance (Tables 1 and 2). The two inconsistent plants were probably misclassified for the same reason as explained above for the WH2 population. The strong suppression of recombination along the introgressed fragment in WIP precludes use of this population for fine mapping of *Ph-2*.

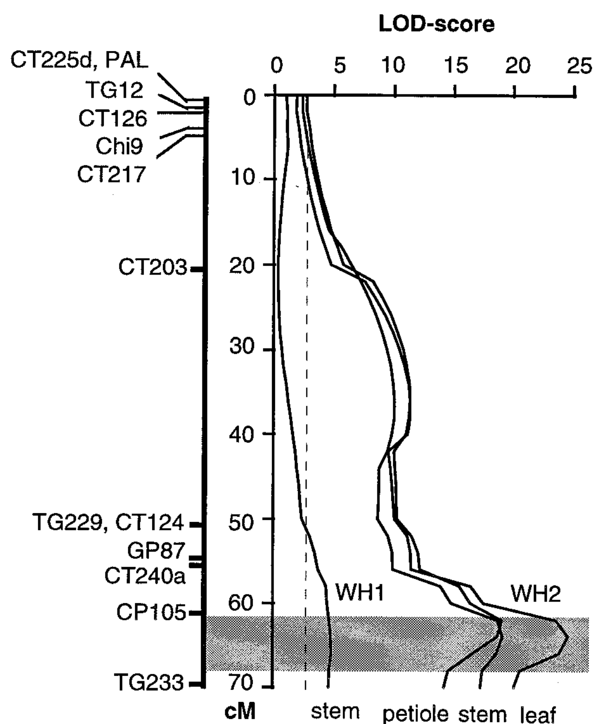

Fig. 2. MapQTL scans of chromosome 10 (vertical axis) showing location of the partial resistance locus *Ph-2* in quantitative analysis of the resistance to *Phytophthora infestans* in populations WH1 and WH2. The LOD score for the presence of a quantitative trait locus (QTL) at the corresponding position on the linkage group is shown on the horizontal axis. Dotted line at LOD = 2.9 indicates required significance level with a threshold value of 1% for declaring the presence of a QTL in population WH1. Shaded area indicates a 95% confidence interval in the map position of *Ph-2*, corresponding to a genomic segment where the LOD-score drops by 0.83 units from the peak (calculated according to Mangin et al. 1994) in quantitative analysis of symptoms scored on stem in population WH2.

Fine mapping of *Ph*-2 with AFLP markers and bulked segregant analysis.

A bulked segregant analysis was performed with the WH1/WH2 population and the AFLP technique to increase the number of markers in the vicinity of *Ph-2*. A DNA pooling strategy based on RFLP marker information available for the 322 segregating plants allowed us to control the genetic size of the targeted region. Four pools of 5 plants each were constructed, two pools homozygous for the H7996 allele at markers CP105 and TG233 (pools A1 and A2) and two pools homozygous for the WVa700 allele at the same markers (pools B1 and B2). Plants with recombination events just outside of the targeted chromosomal region were preferentially included in bulk to reduce the size of the genomic fragment accessible by bulked segregant analysis. Thus, two plants recombinant between markers CP105 and GP87, a marker located 4 cM away from CP105, were included in pools A1 and A2 and one plant recombinant between these markers was included in pool B1.

A total of 128 selective primer combinations (Table 3) were tested on the four pools and the two parents. From 11 to 98 fragments were reliably scored per primer combination, with an average of 38 fragments. Out of the 4,814 AFLP fragments surveyed, only 7.6% were polymorphic. Thus, considering each polymorphic AFLP fragment as a single genetic locus,

Table 3. Amplified fragment length polymorphism (AFLP) adapters and primers used to generate AFLP fingerprints

Designation	Nucleotide sequence
Adapters	
*Eco*RI	5′-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5′
*Mse*I	5′-GACGATGAGTCCTGAG TACTCAGGACTCAT-5′
Primers	
*Eco*RI+A	5′-AGACTGCGTACCAATTCA
*Mse*I+*C*	5′-GACGATGAGTCCTGAGTAAC
*Eco*RI+AXY[a]	5′-GACTGCGTACCAATTC+AXY
*Mse*I+CXY[b]	5′-GATGAGTCCTGAGTAA+CXY

[a] Eight different *Eco*RI+3 primers were used: *Eco*RI+AAC, *Eco*RI+AAG, *Eco*RI+ACA, *Eco*RI+ACC, *Eco*RI+ACG, *Eco*RI+ACT, *Eco*RI+AGC, and *Eco*RI+AGG.

[b] Sixteen different *Mse*I+3 primers were used: *Mse*I+CXY with X and Y = A, C, G, or T.

not taking into account possible allelism relationships, 367 loci were screened with 199 informative for the parent H7996 and 168 informative for the parent WVa700. No amplified fragments with clear polymorphism between the resistant and susceptible bulks were detected with the 128 primer combinations used. However, one fragment, amplified with the primer combination *Eco*RI-ACG/*Mse*I-CAC, is present in pools A1 and A2 and parent H7996, absent in pool B2 and parent WVa700, and present as a weak band in pool B1. Analysis of the individual plant DNA that was used to make the pools showed that out of the 10 plants present in pools B1 and B2 only one, B1.1, amplified this fragment (Fig. 3). This result agrees with RFLP data, since B1.1 was the only plant recombinant between CP105 and GP87 included in pools B1 or B2, indicating that AFLP1 should map in the vicinity of GP87. An attempt to score this AFLP marker as codominant, based on the different signal intensities observed on the autoradiographs, agreed with RFLP data for the 20 plants used in bulked analysis (data not shown). According to this notation, AFLP1 mapped between markers GP87 and TG229, approximately 1 cM away from GP87.

DISCUSSION

Compared with cultivated varieties of tomato, which are susceptible to *P. infestans,* the wild relative *L. pimpinellifolium* line WVa700 exhibits greatly reduced symptoms in controlled test conditions. At an early stage of the interaction, pale flecking symptoms were observable on WVa700 but these were not associated with necrosis and no typical hypersensitive reaction was observed (data not shown). Similar macroscopic phenotypes have been encountered in several plant-pathogen interactions, such as in wheat line carrying the resistance gene *Lr*34 following inoculation with the leaf rust fungus *Puccinia recondita* (Rubiales and Niks 1995).

Fungal sporulation occurs later during the infection process in line WVa700 than in susceptible tomato lines, indicating that this resistance is incomplete. In field experiments, delayed symptom development was consistently observed on line WVa700 (Laterrot 1975). These features are characteristic of partial resistance (Parlevliet 1979) defined as a resistance that retards epidemic development in the field, although plants show a compatible infection type. Further work needs to be done to characterize the tomato–*P. infestans* interaction in detail in order to investigate the mode of action of the resistance in line WVa700.

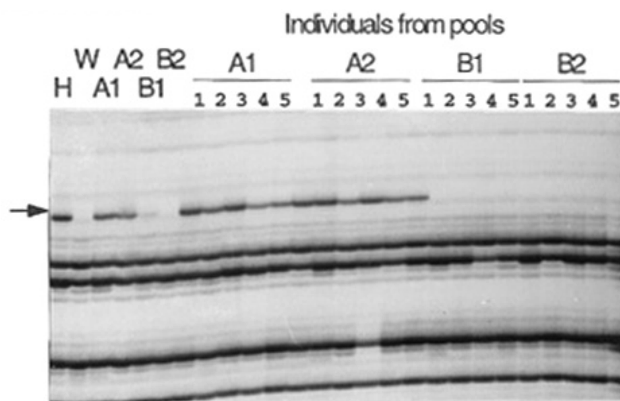

Fig. 3. Bulked segregant analysis of amplified fragment length polymorphism (AFLP) markers generated with the primer combination *Eco*RI-ACG / *Mse*I-CAC. Lanes H and W correspond to the susceptible (Hawaii7996) and the resistant (WVa700) parents, A1 to B2 correspond to DNA pools made of five individuals either homozygous for the H7996 allele at both markers TG233 and CP105 flanking the resistance gene *Ph-2* (pools A1 and A2), or homozygous for the WVa700 allele (B1 and B2), and individual analysis of the 20 F_2 plants used in pools. The AFLP1 marker is indicated by an arrow. B1.1 is the only plant from pools B1 or B2 where AFLP1 is present.

Previous studies of the genetic basis of this resistance gave controversial results concerning the number of genes involved in this trait. According to Gallegly (1960), the resistance was presumably polygenic in nature, whereas subsequently Turkensteen (1973) and Laterrot (1975) found that it was determined mainly by a single, incompletely dominant gene. In the present study, we investigated the resistance in different progenies, using 6-week-old plants. According to Turkensteen (1973) the resistance is not fully expressed at this stage, resulting in a continuously distributed trait in F_2 progeny and an intermediate level of resistance in F_1 hybrids. Our results are in general agreement with this observation (Table 1). Classical genetic analysis of such a trait relies heavily on the choice of the threshold for genotype classification and often results in ambiguous interpretation. However, segregation analysis of the resistance governed by WVa700 in two distinct crosses with two different *P. infestans* strains (Table 1) was in general accordance with the hypothesis of a single-gene mode of inheritance (Table 2).

Genetic analysis based on markers circumvents the classification of genotypes for the resistance trait. Thus, assuming the resistance to be a quantitative character and using RFLP markers spanning the genome, we investigated the presence of the quantitative trait locus (QTL) underlying the resistance trait without resorting to any a priori interpretation of the genetic basis of this character. Standard regression interval-mapping methodology was used to estimate the map location of QTLs and the magnitude of their effect on the resistance despite the non-normal distribution of this trait (Rebaï 1997). In population WH1, where a genetic map covering about 75% of the genome was available (Thoquet et al. 1996), only one region was found to be significantly associated with the resistance. However, this region explained no more than 20% of the variance of the resistance character. With the use of different test conditions and another *P. infestans* strain, the same genomic region in the same genetic background (population WH2) accounted for most of the genetic variation (75 to 85% of the variance). These observations are consistent with monogenic determination of the quantitatively inherited resistance to *P. infestans* in line WVa700. However, the magnitude of the phenotypic variance explained by this locus varied greatly from one population to the other, indicating a large influence of the test conditions and/or the aggressiveness of the fungal strain on the expression of the resistance. In other studies, analysis of several quantitatively inherited resistance traits with molecular markers has also revealed the unexpected presence of a single major locus (Behare et al. 1991; Concibido et al. 1996; Danesh et al. 1994; Kreike et al. 1994; Zamir et al. 1994).

The *Ph-2* locus was found to be located in an interval of 8.4 cM on the lower part of the long arm of chromosome 10 in qualitative (Fig. 1) or quantitative (Fig. 2) analyses. The same

genomic location was found for the resistance locus active against both the Almeria and the 1306 strains of *P. infestans.* In the present study, we cannot distinguish between the presence of a single resistance gene effective against both isolates or the clustering of two different genes at the *Ph-2* locus, each conferring resistance to a single isolate. Indeed, there is ample evidence in other pathosystems for the clustering of families of disease resistance genes (Richter et al. 1995; Staskawicz et al. 1995). To our knowledge, the specificity of the resistance in line WVa700 has not been extensively studied. However, WVa700 was found to be resistant to both T0 and T1 races of *P. infestans* (Gallegly 1960; Vartanian and Endo 1985), was effective to various extents to all isolates tested under controlled conditions (Laterrot 1975; Vartanian and Endo 1985; P. Moreau and N. Grimsley, *unpublished data*), and was effective in the field (Bagirova et al. 1996). The resistance of line WVa700 was introgressed into several tomato cultivars (Gallegly 1960; Laterrot 1975, 1994) and the derivative line WVa′63 was found to be one of the least susceptible tomato lines to the highly virulent *P. infestans* US7 clone (Fry et al. 1995).

The position of *Ph-2* on the long arm of chromosome 10 indicates that this gene is allelic neither with the previously mapped *P. infestans* resistance gene *Ph-1* on tomato chromosome 7 (Peirce 1971) nor with any of the mapped *P. infestans* resistance loci in potato, including R1 on chromosome 5 (Leonards-Schippers et al. 1992), and R3, R6, and R7 on chromosome 11 (El-Kharbotly et al. 1996), since the potato and tomato genomes are highly collinear, differing only by five chromosome arm inversions (Tanksley et al. 1992). Moreover, chromosome 10 did not carry any QTL associated with quantitative resistance to *P. infestans* in potato (Leonards-Schippers et al. 1994). These observations indicate that there are numerous genes in the two closely related species that confer either qualitative or quantitative resistance to *P. infestans*. In this respect, the *Ph-2* locus has interesting features, such as multi-isolate resistance. No definite relationship between the potato and tomato races of *P. infestans* has been found (Wilson and Gallegly 1955). Late blight of solanaceous plants therefore provides an interesting model for studying the relationship between quantitative and qualitative resistance, regarding their race specificities and their mechanisms of action. For instance, some QTLs detected in potato were found to be race specific (Leonards-Schippers et al. 1994). This is a characteristic typical of qualitative resistance. Qualitative and quantitative resistance may thus be related, the qualitative allele representing an extreme allelic form of a quantitative locus (Robertson 1985).

Recently, a resistance-gene-like sequence, St1.2.4, was mapped on chromosome 10 of potato (Leister et al. 1996). This clone was isolated by a polymerase chain reaction (PCR)–based approach and shares homology to the resistance genes RPS2, a gene conferring resistance to *P. syringae* in *Arabidopsis thaliana* (Bent et al. 1994; Mindrinos et al. 1994) and N, conferring resistance to tobacco mosaic virus in tobacco (Whitham et al. 1994). Although St1.2.4 maps close to the centromere in potato, an inversion in the comparative maps of the tomato and potato genomes in this region leaves open the possibility that it may be closely linked to *Ph-2*. Work is currently in progress to investigate genetic linkage between St1.2.4 and *Ph-2* on the tomato genome.

Positional cloning of a gene requires both the availability of closely linked DNA markers and a large segregating population to achieve fine mapping of these markers relative to the targeted gene. This objective is facilitated when the density of markers is sufficient to “land” on a single genomic clone (YAC or BAC) with markers tightly flanking the gene of interest (Tanksley et al. 1995). Out of the 322 F_2 plants derived from the cross H7996 × WVa700, 45 recombinants between the two markers CP105 and TG233 flanking *Ph-2* were observed. This represents, on average, one recombinant every 0.2 cM in the vicinity of *Ph-2*. Thus, assuming a 1 cM/750 kb ratio for the tomato genome (Tanksley et al. 1992), the size of this segregating population would be sufficient to map additional markers within approximately 200 kb of *Ph-2*. However, the low level of detectable polymorphism within this cross has hindered progress in finding more markers in this region. To circumvent this lack of sequence polymorphism between *L. esculentum* and *L. pimpinellifolium* lines, mapping experiments have been widely conducted on an interspecific cross of tomato with a wild relative *L. pennellii* line as a parent (Tanksley et al. 1992). However, the high level of phenotypic variation found in the progeny of a cross between WVa700 and *L. pennellii* line LA716 makes scoring of the *P. infestans* resistance phenotype much less reliable (P. Moreau, *unpublished data*). An alternative approach was assessed with an *L. esculentum* line containing a defined *L. pennellii* introgression (Eshed et al. 1992; Eshed and Zamir 1994). The high level of polymorphism in the chromosome 10 genomic segment introgressed from *L. pennellii* into the tomato line IL10-3 allowed us to map several RFLP markers in the vicinity of *Ph-2* with a progeny derived from the cross IL10-3 × WVa700. Scoring of late blight symptoms was reliable in this population. However, high suppression of recombination was observed in this cross along the introgressed fragment. Although some reduction of recombination frequencies was previously observed in this kind of material (Alpert et al. 1995), the extent of this in our material was unexpected and precluded its use for further studies.

Bulked segregant analysis with AFLP markers provides a powerful way for increasing marker density in a targeted region (Ballvora et al. 1995; Büschges et al. 1997; Cervera et al. 1996; Cnops et al. 1996; Meksem et al. 1995; Thomas et al. 1995). In order to maximize the detection of informative markers, pools of DNA were constructed according to RFLP marker information available in the vicinity of *Ph-2*. This strategy allowed us to (i) eliminate the possible inclusion of plants erroneously classified for their *Ph*-2 genotype, (ii) detect markers linked both in *cis* and *trans* to the target allele, since pool constitution based on the *Ph-2* genotype would have been restricted to disease-resistant plants due to the absence of a clear-cut distinction between intermediate and susceptible plants in progeny screening, (iii) narrow the interval around *Ph-2* within which linked markers could be identified by pooling plants that were recombinant just outside of the targeted chromosomal segment, and (iv) facilitate subsequent screening of YAC or BAC clone libraries with AFLP markers linked in coupling or repulsion to the resistant allele to be cloned (Vos et al. 1995). With the AFLP procedure, we detect only 7.4% polymorphism in this *L. esculentum* × *L. pimpinellifolium* cross, consistent with results obtained in a similar cross (Thomas et al. 1995), suggesting

these two species are closely related. However, with 128 primer combinations, 367 informative AFLP markers have been surveyed in bulked segregant analysis but none of them was found to map in the targeted chromosomal region. According to the linkage data, the genomic size of the region targeted by bulked segregant analysis was estimated to represent an interval of approximately 10 cM, corresponding to approximately 0.8% of the total tomato genome size of about 1,300 cM (Grandillo and Tanksley 1996; Tanksley et al. 1992). The absence of markers in the targeted fragment, where two or three were expected in theory, could be explained either by nonrandom distribution of AFLP markers on the genome, although this was not observed in potato (Van Eck et al. 1995) or in rice (Maheswaran et al. 1997), or from a lower ratio of the genetic/physical distances in the targeted fragment. The map position of *Ph-2* far away from the centromeric region, together with the observations of high variability in the genome-wide and local ratios of the genetic versus physical distance in tomato (Gorman et al. 1996), favors the latter hypothesis, and could explain the absence of markers in this region. However, increasing the number of primer combinations should allow us to map additional markers in the vicinity of *Ph-2*.

Over the last years, numerous resistance genes have been cloned from several species and most of them were found to encode proteins with striking structural similarities (reviewed in Baker et al. 1997). However, highly significant differences have been observed in the ability of two of them, *Cf-2* and *Cf-9,* to restrict *Cladosporium fulvum* growth in tomato (Hammond-Kosack and Jones 1994), indicating that resistance genes with a similar structure can confer different levels of resistance when challenged with the pathogen harboring the appropriate *Avr* gene. Furthermore, in a recent study, partial resistance to *F. oxysporum* f. sp. *lycopersici* race 2 was observed in tomato plants genetically transformed with the I2C-1 gene, a member of the multigene I2C family, cloned from the I-2 locus that normally confers complete resistance (Ori et al. 1997). The I2C-1 gene encodes a protein with a nucleotide binding site and a leucine-rich repeat (LRR), two conserved motifs also found in several resistance genes designated as the first R gene group (according to Baker et al. 1997). Thus, genes involved in partial resistance could be allelic to genes involved in complete resistance.

Ph-2 is another single locus conferring resistance to a large number of isolates. These loci could be composed of a cluster of tightly linked genes, each governing a unique isolate-specific resistance (Pryor and Ellis 1993) or, alternatively, could carry a single resistance gene conferring a broad spectrum resistance. In rice, the single *Xa*21 gene was found to confer resistance to 29 diverse isolates of *Xanthomonas oryzae* pv. *oryzae* and susceptibility to three (Wang et al. 1996). *Xa*21 is also a member of a multi-gene family and encodes a putative receptor kinase-like protein carrying an LRR motif in its presumed extracellular domain (Song et al. 1995). Although *Xa*21 conferred a complete resistance phenotype, transgenic plants expressing another family member encoding the extracellular LRR domain alone were found to confer incomplete resistance to the same spectrum as *Xa*21-containing plants, suggesting that the LRR domain is responsible for race specific recognition (G. L. Wang and P. C. Ronald, *in preparation*).

By studying the genetic and later the molecular basis of the partial resistance response to *P. infestans* in line WVa700, we hope to gain a better understanding of the mechanism underlying partial resistance to late blight in tomato. Whether or not *Ph-2* is similar to already characterized resistance genes remains to be elucidated.

MATERIALS AND METHODS

Plant material.

The susceptible *Lycopersicon esculentum* var. Hawaii7996 was crossed with the resistant accession *L. pimpinellifolium* WVa700. F_2 seeds were obtained from a single F_1 plant. Two hundred F_2 plants (population WH1) were maintained in a greenhouse and used for DNA extraction, F_3 progeny generation, and preparation of cuttings for resistance tests. Another set of 122 F_2 plants from the same cross (population WH2) were generated, from which 88 were tested for resistance to *P. infestans* at the 6-week-old stage, treated with mancozeb after scoring, and transferred to a greenhouse for DNA preparation and F_3 progeny generation.

The susceptible *L. esculentum* introgression line IL10-3 was also crossed with WVa700. IL10-3 was one of the 50 introgression lines generated as described by Eshed and Zamir (1994). IL10-3 carries a single chromosomal segment from the *L. pennellii* genome spanning about one third of the long arm of chromosome 10 in an otherwise *L. esculentum* var. M82 genome. One hundred forty-six F_2 plants (population WIP) were generated, from which 77 were evaluated for resistance to *P. infestans*, treated as above, and transferred to the greenhouse for DNA preparation and generation of F_3 progeny.

Resistance tests.

Population WH1 was evaluated for resistance to *P. infestans* as described by Laterrot (1975). Isolate Almeria, originating from Spain, was cultured on oats/chickpea medium (Laterrot 1975) for 2 weeks before sporangia were harvested and the concentration adjusted to 7.10^4 sporangia per ml. Inoculation was performed, by spraying to runoff on 6-week-old cuttings. Plants were transferred to a growth chamber at 18°C, 80 to 100% relative humidity, and illuminated for 10 h per day with cool fluorescent lights. The plants were covered with a transparent plastic sheet for the first 4 days after inoculation. Disease symptoms were scored on the plant stems 7 days after inoculation, according to the following index: 0 = no symptoms or rare necrotic spots, 1 = small lesions, 2 = large expanding lesions, and 3 = more than 30% of the stem covered with lesions.

F_2 plants from the populations WH2 and WIP were tested under different conditions. *P. infestans* isolate 1306, which is highly aggressive on tomato and also virulent on *Ph*-1 tomato variety New Yorker (H. S. Judelson, *personal communication*), was used. Resistance was evaluated on 6-week-old plants by spraying, on each plant, 4 ml of fresh sporangia suspension adjusted to 10^5 sporangia per ml. Plants were maintained in the same conditions as described above and severity of symptoms on stems, petioles, and flag leaves was evaluated 5 days after inoculation. The disease index used on stem or petiole was identical to that used for the resistance test on cuttings (see above). The two leaves closest to the apex were scored according the following index: 0 = no symptoms or

rare necrotic spots, 1 = a few expanding lesions, 2 = most of the leaves affected with expending lesions, 3 = more than 50% of the total leaf surface affected.

RFLP markers.

The RFLP procedure was described previously (Thoquet et al. 1996). Extensive screening for polymorphism between WVa700 and H7996 was performed with 15 restriction enzymes (*Eco*RI, *Eco*RV, *Bam*HI, *Asp*700, *Hin*dIII, *Bgl*II, *Sca*I, *Dra*I, *Rsa*I, *Alu*I, *Sau*3A, *Taq*I, *Hae*III, *Hin*fI, and *Bst*NI) with RFLP markers previously mapped on tomato (CT, CD, and TG clones; Tanksley et al. 1992) and potato (CP and GP clones; Gebhardt et al. 1991, 1994) to map additional markers on chromosome 10.

Genetic analyses.

RFLP maps were constructed with the software package JoinMap (Stam 1993) with the Kosambi mapping function. A consensus map of chromosome 10 was created by integrating the linkage maps from population WH1 (200 plants) and WH2 (122 plants). Qualitative analysis of resistance test data was done by grouping phenotypic classes and testing segregation ratios (1:2:1 or 1:3) by Chi-squared analyses. Quantitative analysis of the resistance was performed with the MapQTL software (Van Ooijen and Maliepaard 1996) version 3.1. Steps of 2 cM were used for interval mapping on chromosome 10 to find genomic regions associated with the resistance and to investigate the most probable position of the *Ph*-2 locus.

AFLP analysis.

AFLP markers were produced essentially as described by Vos et al (1995). DNA (0.25 µg) was digested with 2.5 U *Eco*RI and 2.5 U *Mse*I in 25 µl of 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM dithiothreitol, 0.005% bovine serum albumin (BSA), pH 7.5 for 2 h at 37°C. An equal volume containing 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter (Table 3), 0.005% BSA, 1 µl 10 mM ATP, and 1 U T4 DNA-ligase was added and incubation was continued for another 2 h.

The restriction-ligation mixture was diluted 10-fold with TE 10/0.1 (10 mM Tris-HCl, 0.1 mM EDTA) and aliquots were stored at –20°C. PCR amplification was done in a PHC-3 thermocycler (Techne, Cambridge, U.K.) with a 96-well microtiter plate. A preselective amplification was performed in 50 µl with 5 µl of template DNA diluted, 75 ng of each of the preselective primers (Table 3), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 U *Taq* DNA polymerase (GIBCO/BRL Life Technologies, Gaithersburg, MD) in PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl). The cycle profile was 30 s at 94°C, 30 s at 56°C, 60 s at 72°C, for 30 cycles. The preamplification mixture was diluted 50-fold in TE 10/0.1 and aliquots were stored at –20°C. The final amplification was performed in 20 µl with 5 µl of the diluted preamplified DNA, 5 ng of selective *Eco*RI+3 primer labeled by phosphorylating the 5′ end with [γ^{33}P]ATP and T4 polynucleotide kinase, 30 ng of selective *Mse*I+3 primer (Table 3), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 0.5 U *Taq* DNA polymerase in PCR buffer. The cycle profile was 13 cycles: 30 s at 94°C, 30 s at 65 to 57°C (lowered by 1°C every 2 cycles), and 60 s at 72°C; followed by 24 cycles: 30 s at 94°C, 30 s at 56°C. and 60 s at 72°C. The amplification products were mixed with 20 µl of loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and xylene cyanol), denatured for 3 min. at 90°C and quickly cooled on ice. Three microliters was loaded onto a denaturing 5% polyacrylamide gel (19:1 ratio acrylamide:bisacrylamide) and run for 1 h and 45 min at constant power (55 W). The gel was then fixed, rinsed, and dried on a glass plate prior to exposition on X-ray film for 12 to 48 h.

The AFLP System I kit (GIBCO/BRL Life Technologies) was also used to generate AFLP markers. No marked differences were observed in the results obtained.

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