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Molecular mapping of the *py-1* gene for resistance to corky root rot (*Pyrenochaeta lycopersici*) in tomato

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Abstract We report the molecular mapping of the *py-1* gene for resistance to corky root rot [*Pyrenochaeta lycopersici* (Schneider and Gerlach)] in tomato using RAPD and RFLP marker analysis. DNA from near-isogenic lines (NILs) of tomato differing in corky root rot resistance was screened with 575 random oligonucleotide primers to detect polymorphic DNAs linked to *py-1*. Three primers (OPW-04, OPC-02, OPG-19) revealed polymorphisms between the NILs. Twelve resistant and eight susceptible DNA pools derived from segregating F_3 families were used to confirm that the RAPD markers were linked to the *py-1* gene. Two of the linked amplified fragments, corresponding to OPW-04 and OPC-02, were subsequently cloned and mapped on the tomato molecular linkage map as RFLPs. These clones were located between TG40 and CT31 on the short arm of chromosome 3. Further analysis with selected RFLP markers showed that 7% (8.8 cM) of chromosome 3 of the resistant line 'Mobog-lan' was introgressed from the *L. peruvianum* donor parent. Three RFLP markers (TG40, TG324, and TG479) from the introgressed part of chromosome 3 were converted to cleaved amplified polymorphism (CAP) markers for use in a polymerase chain reaction (PCR) assay. These PCR markers will allow rapid large-scale screening of tomato populations for corky root rot resistance.

Key words CAP · *py-1* · RAPD · RFLP · Breeding

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Introduction

Pyrenochaeta lycopersici is the soil-borne fungus that causes corky root rot disease of tomato. After infection with the pathogen, tomato roots form lesions, become furrowed and eventually develop a corky texture (Jones et al. 1989). Therefore, corky root rot disease is characterized by the progressive deterioration of the root system of infected plants (Gerlach and Schneider 1964). Corky root rot can cause major crop losses in temperate areas; loss estimates as high as 70–75% have been reported (Hogenboom 1970; Campbell et al. 1982). Strategies to control the disease by soil fumigation with methyl bromide, chloropicrin or steam have been inadequate because such methods are usually expensive and often only practical for greenhouse culture of tomatoes (Campbell et al. 1982). In addition, after the year 2000, the production and importation of methyl bromide will be banned in the United States; by 2010, there will be further restriction on its use worldwide (EPA 1993).

For these reasons, genetic resistance is the most effective means for control of this disease. Genetic resistance has been identified in accessions of the wild tomato species *Lycopersicon hirsutum* and *L. peruvianum* (Hogenboom 1970). A single recessive gene (*py-1*) was shown to confer resistance to corky root rot and was introgressed into *L. esculentum* from *L. peruvianum* (Laterrot 1983). However, further efforts to transfer resistance into cultivated tomato have been hampered because direct screening for corky root rot resistance is very tedious and slow. In addition, field tests are required because greenhouse inoculation is unreliable (Jones et al. 1989).

Molecular markers [random amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs) etc.] are an alternative means of diagnosing and screening large populations for disease resistance. The identification of markers tightly linked to the *py-1* locus would allow marker-assisted selection

for introgression of this gene into cultivated tomato. In this paper, we describe the molecular mapping of *py-1* using RAPD and RFLP markers and the development of polymerase chain reaction (PCR)-based markers by converting RFLP clones into cleaved amplified polymorphism (CAP) markers.

Materials and methods

Plant material

A pair of near-isogenic lines (NILs) [Moboglan (R) and Monalbo (S); Laterrot (1987)] was used for RAPD isoline analysis to identify markers in the *py-1* region of the genome. To produce segregating populations for linkage analysis, we crossed two susceptible cultivars (*L. esculentum* CVs 'Peto 282' and CV 'Peto 76') to a single resistant cultivar (*L. esculentum* CV 'Moboglan') to produce F_1 hybrids. The derived F_3 populations (50 families each), segregating for *py-1* resistance, were screened under natural inoculation at two locations (Arroya Grande, Calif. and Latina, Italy). Bulked seed samples were collected from each F_3 family to produce the F_4 generation. Based on F_3 field screens, 12 resistant and eight susceptible F_4 lines, which were not segregating, were selected and used for linkage analysis (Fig. 1). DNA was extracted from 7–10 resistant or susceptible plants of each selected F_4 line, and DNA from lines was combined in equal concentrations to form resistant and susceptible DNA pools to verify linkage between RAPD markers and *py-1*.

Mapping population

Sixty-seven F_2 plants derived from the cross *L. esculentum* × *L. pennellii* (Tanksley et al. 1992) were used to map the polymorphic DNA fragments shown to be linked to *py-1* by RAPD isoline analysis. In addition, a F_2 population consisting of 100 plants derived from the cross *L. esculentum* CV 'E6203' (susceptible) × *L. esculentum* CV 'Moboglan' (resistant) was used for the fine mapping of *py-1*-linked markers.

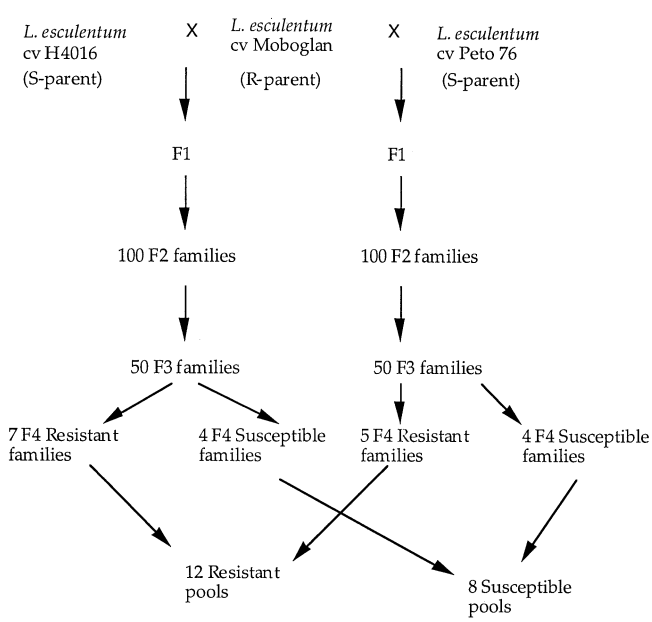

Fig. 1 Development of 12 resistant and eight susceptible DNA pools used for linkage analysis

Disease screening

Inoculum was produced by inoculating sterilized millet seed (100 g seed + 120 ml water) with twenty 10-mm^2 pieces of the fungus grown on V8 agar. The fungus was allowed to grow on the millet seed for 30 days. The millet seed was then uniformly mixed with soil (50% sand : 50% peat moss) at 1 : 20 dilution. The soil was placed in seedling trays, and the tomato seed was directly planted into this soil. At approximately the sixth true leaf stage, the seedlings were transplanted into a field site that was known to be uniformly infested with *Pyrenochaeta lycopersici*. The plants were watered using drip irrigation and no fertilizer was added. When approximately 15% of the fruits were mature red, the plants were dug and the root system was examined for disease symptoms. A visual rating scale of 1–5 was used:1 = no disease symptoms on the roots and 5 = extensive corking of the root system. Ratings of 1–2 were considered resistant and rating of 3 were classified as neither resistant nor susceptible.

RAPD analysis and mapping of *py-1*

A total of 575 random oligonucleotide primers (Operon Technologies, Calif.) was used to amplify DNA from the pair of resistant and susceptible NILs. DNA was isolated as described by Fulton et al. (1995). PCR reactions and amplification conditions were performed as described by Martin et al. (1991) using a MJ Research PTC100 Programmable Thermal Controller. Amplification products were analyzed by electrophoresis through a 2% agarose gel and ethidium bromide staining. The three oligonucleotide primers described in this paper and their nucleotide sequences are as follows: OPW-04, 5′d [CAGAAGCGGA]3′; OPC-02, 5′d[GTGAGGCGTC]3′; OPG-19, 5′d [GTCAGGGCAA]3′.

PCR fragments polymorphic between resistant and susceptible NILs and segregating with corky root rot resistance were cloned and mapped onto the tomato high-density map (Pillen et al. 1996) as RFLPs. The polymorphic fragments were excised from the agarose gel and isolated using the Geneclean Kit (Biolabs), ligated into PCR-II plasmid using the TA Cloning kit (Invitrogen) and transformed into the *E. coli* strain DH5α as recommended by the manufacturer (Stratagene). The purified PCR products were labelled with [^{32}P]-dCTP (Amersham International) using the random hexamer method (Feinberg and Vogeltesin 1983) and then used as probes on parental survey filters containing DNA of the mapping population parents (VF36-*Tm2*^{a} and *L. pennellii*) which had been digested with five restriction enzymes (*Eco*RI, *Dra*I, *Eco*RV, *Hae*III and *Hin*dIII). Southern hybridization conditions were the same as those described by Bernatzky and Tanksley (1986).

Linkage analysis

Association between the RAPD markers and the resistance gene was determined by a chi-square test for independence using the STATVIEW (Abacus 1992) statistical program for the MacIntosh. Linkage values and map distances were estimated using MAPMAKER software (Lander et al. 1987) and the Kosambi mapping function (Kosambi 1944).

Development of CAP markers

DNA sequences of selected RFLP clones (TG40, TG324, TG479) were determined, and three sets of specific PCR primers were

designed from these sequence data. The 100-ul amplification reaction mixture contained 100 ng genomic DNA, 10 m*M* TRIS-HCl (pH 8.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 µM of each dNTP, 100 n*M* of each primer and 1 unit of *Taq* DNA polymerase. Amplification was performed in a Perkin Elmer Cetus Thermocycler using the following profile: 94°C, 1 min; 55°C, 1 min; and 72°C, 2 min for 35 cycles followed by 72°C, 5 min and a hold at 4°C. The product was digested overnight with one of several enzymes (*Eco*RI, *Eco*RV, *Bst*NI, *Hin*dIII, *Hae*III, *Hin*fI, *Sca*I, *Taq*I, *Dra*I or *Xba*I) and separated by electrophoresis through a 2% agarose gel.

Results and discussion

RAPD marker analysis and mapping of *py-1*

Of the 575 primers screened on the *py-1* NILs, 530 primers produced a total of 3012 scorable bands, corresponding to an average of 5.7 fragments (1–16 bands) per amplification. Of the 530 primers, only 3 (OPW-04, OPG-19 and OPC-02) revealed polymorphism between NILs. The 3 polymorphic RAPD markers were then used to screen 12 resistant and eight susceptible bulked DNA pools selected from two F_4 populations in order to verify linkage to *py-1*. RAPD primer OPW-04 amplified a 510-bp product which was unique to the resistant NIL (Moboglan) and resistant bulks. Primers OPG-19 and OPC-02 yielded products of 1350 and 1420 bp, respectively, that were unique to the susceptible NIL (Monalbo) and susceptible bulks. These polymorphic markers were shown to be linked to the resistance gene using the 12 resistant and eight susceptible F_4 DNA pools ($P < 0.0001$, Fig. 2).

In order to identify on which chromosome *py-1* resides, we cloned the linked RAPD fragments and mapped them as RFLPs onto the tomato molecular linkage map (Pillen et al. 1996). The cloned and purified amplification products of the RAPD markers were first probed onto parental (*L. esculentum* × *L. pennelli*) genomic DNA digested with five different enzymes. Three presence/absence polymorphisms were found. OPW-04 hybridized to a 850-bp single-copy fragment present in *Eco*RV-digested *L. pennellii* DNA; OPC-02 hybridized to a 950-bp single-copy fragment present in *Hin*dIII-digested *L. esculentum* DNA; OPG-19 hybridized to a 750-bp single-copy fragment present in *Hae*III-digested *L. esculentum* DNA. DNA from the progeny of the mapping population was digested with *Eco*RV, *Hin*dIII and *Hae*III for RFLP mapping of OPW-04, OPC-02, and OPG-19, respectively. Two polymorphic RFLP fragments associated with the RAPD markers OPW-04 and OPC-02 mapped to the top of the short arm of chromosome 3 between the RFLP markers TG40 and CT31 (Fig. 3). The RFLP fragment associated with the RAPD marker OPG-19 mapped to chromosome 11. However, when 8 nearby RFLP loci from chromosome 11 were probed onto surveys of the resistant and susceptible NILs, no polymorphism was found suggesting that this was probably a spurious association.

In order to determine the size of the *py-1*-containing chromosome 3 segment introgressed from *L. peruvianum*, filters containing DNA (digested with six different enzymes) from *py-1* NILs and the 12 resistant and eight susceptible bulks were probed with 6 selected RFLP markers (TG40, TG324, TG479, CT31, TG114, TG581) from chromosome 3. Of the 6 selected RFLP loci, 4 (TG40, TG324, TG479 and CT31) detected polymorphisms. However, no polymorphism was detected with TG114 and TG581 for any of the enzymes tested. The polymorphic markers span an 8.8-cM interval

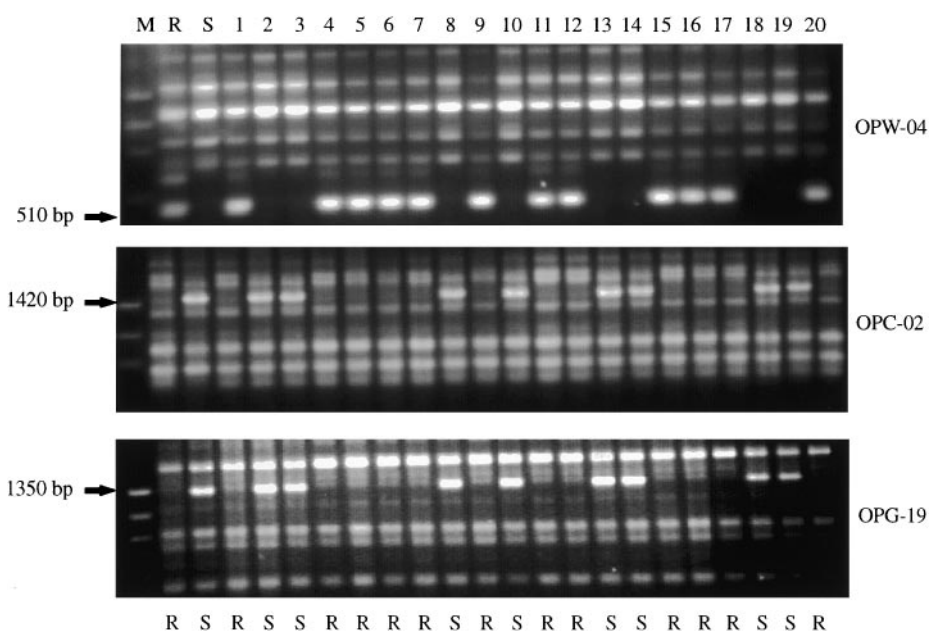

Fig. 2 RAPD markers that amplified polymorphic fragments in resistant and susceptible lines. The *arrows* indicate the polymorphic RAPD fragments linked to *py-1*. *M* Molecular-size standard, *R* resistant line (Moboglan), *S* susceptible line (Monalbo), *1-20* resistant and susceptible F_4 lines

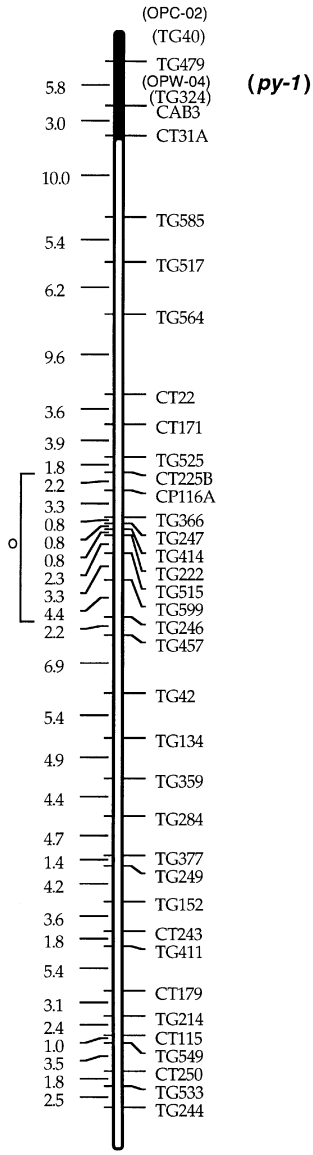

Fig. 3 Map of tomato chromosome 3 showing position of the *py-1* gene, the linked RAPD markers and the size of introgressed segment (*dark area*). *o* Centromeric region

between TG40 and CT31, which makes up 7% of chromosome 3 based on the published linkage map of tomato (Fig. 3, Pillen et al. 1996).

For finer mapping, 100 F_2 plants from the cross *L. esculentum* CV 'E6203' × *L. esculentum* CV 'Moboglan' were assayed with the 4 RFLP markers from the introgressed region in order to identify potential recombinants in the TG40-CT31 interval. All RFLP loci segregated in a ratio not significantly different than the expected 1 : 2 : 1 ratio. No recombinants were detected among any of the markers in the 100 F_2 plants. The 95% upper confidence limit for this interval is therefore estimated to be less than 1.5 cM. Since the same segment of chromosome 3 in the high-density tomato map corresponds to 8.8 cM, we concluded that recombination suppression is occurring in the introgressed segment (Fig. 3).

Development of CAP markers and marker-aided selection

Three sets of primers were developed from RFLP clone (TG40, TG324, and TG479) sequences. Table 1 shows primer designation, sequences, molecular weight and enzymes that cleave the PCR products. Primer sets TG479 R/F yielded a 1350-bp product in both parents; however, upon digestion with a battery of restriction enzymes, no polymorphism was detected between the PCR products. TG40 R/F gave a 1350-bp product in NILs. *Dra*I cleaved the product from both parents; the resistant parent had two fragments of approximately 500 and 450 bp while the susceptible parent had one fragment of 950 bp (Table 1). In addition, *Hin*dIII cleaved only the product from the susceptible parent and gave two fragments of 1000 and 350 bp. Primers TG324 R/F gave a 1350-bp product. When digested by *Dra*I, the resistant parent gave a 1300-bp product and the susceptible parent yielded a 1200-bp product. Heterozgous lines have both fragments (Fig. 4). Therefore, these primers (TG324 R/F) were chosen for the codominant CAP assay diagnosing *py-1* resistance.

Breeding for corky root rot resistance based on phenotypic selection has been difficult to date because of the slow progress of disease development and the lack of a reliable greenhouse screening procedure. However, a marker-aided selection approach could avoid this problem. One method is to convert mapped RFLP clones or RAPD markers to a cleaved amplified polymorphism (CAP) (Konieczny and Ausubel 1993). Because these markers are codominant, homozygous and heterozygous individuals can be easily

Table 1 The *py-1*-CAP assays. Primer designation, sequences, molecular size of PCR product and enzymes that cleave the PCR products

Primer designation	Primer sequences	PCR product size (bp)	Enzyme used to generate CAP
TG479 F/R	5′- GGTGATTATGGGTGATCCTATG-3′ 5′- CCAAGTGAGTACCAACAGTTCC-3′	1350	No enzyme found
TG40 R/F	5′- CGTTTAGGCAATTCACATCTAG-3′ 5′- AACAACAACTGTACCTCAGTCC-3′	1350	*Dra*I and *Hin*dIII
TG324 R/F	5′- CTTCTAGTAGTCCAACAGCAACTG-3′ 5′- CACTTGGTTGATGGATAGTG-3′	1350	*Dra*I

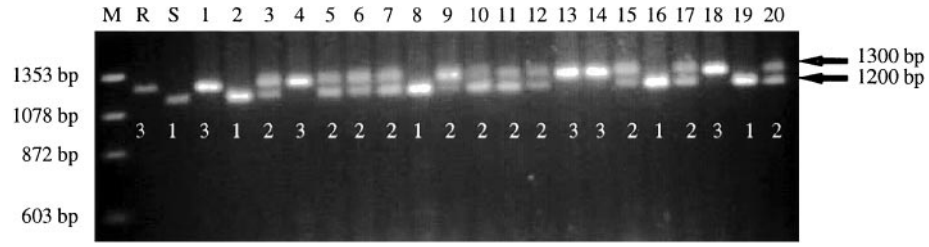

Fig. 4 Segregation of the codominant CAP marker TG324 F/R in 20 plants selected from the F_3 population derived from the cross *L. esculentum* cv 'Peto 76' and *L. esculentum* cv 'Moboglan'. When amplified DNA was digested with the restriction enzyme *Dra*I, the resistant parent (*R*) yielded a 1300-bp fragment, and the susceptible parent (*S*) produced a 1200-bp fragment. *M* Molecular-weight standard, *R* resistant parent (Moboglan), *S* susceptible parent (Monalbo), *1-20* F_3 segregating population. *3* hom R/R, *2* het R/S, *1* hom S/S

distinguished. The CAPs assay, based on the primer sets TG324R/F, is currently being used for the screening of disease resistance to aid in the introgression of the *py-1* gene into tomato cultivars.

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