Theor Appl Genet (1989) 78:489–494

An isozyme marker for resistance to race 3 of *Fusarium oxysporum* f. sp. *lycopersici* in tomato *

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Received April 17, 1989; Accepted May 19, 1989
Communicated by F. Salamini

Summary. The inheritance and the linkage relationship of resistance to race 3 of *Fusarium oxysporum* f. sp. *lycopersici* derived from *Lycopersicon pennellii* (LA 716) were analyzed in an interspecific backcross to *L. esculentum*. Progeny from each backcross (BC_1) individual were inoculated with race 3 and their response was measured according to a visual rating system; progeny responses were used to calculate a mean disease rating for each BC_1 individual. The frequency distribution of the disease ratings was bimodal, indicating that resistance to race 3 was controlled by one major locus. Linkage analysis of this locus proceeded in two steps. Initially, disease ratings were compared between homozygotes and heterozygotes at each of 17 segregating marker enzyme loci. Highly significant differences were detected for the chromosome segment marked by the *Got-2* locus on chromosome 7. This indicated that *Got-2* was linked to the race 3 resistance gene, designated *I-3;* this gene accounted for the observed bimodal distribution of BC_1 disease ratings. In the second step, the genotype of each BC_1 individual at the *I-3* locus was determined using cluster analysis of the disease ratings; a test for independent assortment between *I-3* and *Got-2* revealed strong linkage, with an estimated map distance of 2.5 centiMorgans. Additional evidence for this linkage was obtained from the analysis of five breeding lines previously selected for resistance to race 3 solely on the basis of inoculations; all five showed co-selection for *Got-2*. The *Got-2* locus is proposed as a selectable marker to expedite the transfer of race 3 resistance to commercial tomato cultivars.

Key words: Fusarium wilt – *Lycopersicon pennellii* – Isozyme marker – Tomato – Disease resistance

Introduction

Races 1 and 2 of *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen, which causes Fusarium wilt of tomato, are widespread in most production regions. Single dominant genes *I* and *I-2*, which control for resistances to races 1 and 2, respectively, have been derived from accessions of *Lycopersicon pimpinellifolium* (Bohn and Tucker 1939, 1940; Alexander and Hoover 1955; Stall and Walter 1965). These genes have been mapped to chromosome 11 (Paddock 1950; Latterot 1976) and have been incorporated into most cultivars.

Race 3, a new race with the ability to infect cultivars resistant to races 1 and 2, has been reported in both Australia and Florida (Grattidge and O'Brien 1982; Volin and Jones 1982), and more recently in California (Davis et al. 1988). The wild species *L. pennellii* represents a potential source of resistance for new cultivars. This species has been reported to carry monogenic dominant resistance to race 3 in accessions PI414773 (McGrath et al. 1987) and LA 716 (Scott and Jones 1989).

Tanksley and Rick (1980) proposed the use of isozymes as selectable markers to facilitate the efficient transfer of genes from a wild species into domesticated cultivars. Rick and Fobes (1974) were the first to report tight linkage between the enzyme locus, *Aps-1*, and *Mi*, a gene responsible for nematode resistance in tomato. Detection of this linkage has had a major impact on tomato breeding programs; the *Aps-1* locus is now widely used as a selectable marker for nematode resistance (Medina-Filho and Stevens 1980). In pea, the enzyme

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locus, *Pgm-p,* has been reported as a marker for *Mo,* a gene which controls for resistance to bean yellow mosaic virus (Weeden et al. 1984).

The objective of this project was to find a molecular marker for resistance to race 3 of *F. oxysporum* f. sp. *lycopersici* detected in *L. pennellii* (LA 716). Two independent approaches were taken. First, tests for independent assortment were conducted between race 3 resistance and each of 17 previously mapped enzyme loci in an interspecific backcross. Second, isozyme analysis was conducted on five breeding lines selected for race 3 resistance derived from LA 716 to test whether any LA 716 alleles had been co-selected.

Materials and methods

Plant materials

The following interspecific backcross was generated for segregation and linkage analyses: [*L. esculentum* × (*L.esculentum* × *L. pennellii,* LA 716)]. 'Bonny Best' was used as the *L. esculentum* parent due to its extreme susceptibility to race 3. In addition, five breeding lines that had been independently produced using conventional breeding techniques were analyzed [E427 (BC_1S_3), I3R-1 (BC_2S_2), 520 (BC_2S_2), 572 (BC_3S_1), and 604 (BC_1S_2)]. All lines included the initial cross 'Hayslip' × LA 716. With the exception of LA 716, all lines and cultivars included in the pedigrees of the breeding lines were race 3-susceptible. The cultivars 'Manapal' (resistant to race 1) and 'Hayslip' (resistant to races 1 and 2) were also used as controls in race 3 inoculations. In addition, five *L. esculentum* lines carrying *L. pennellii* (LA 716) chromosome substitutions for chromosome 2, 4, 6, 8, and 11 (Rick 1969) were inoculated.

Isozyme analysis

The starch gel electrophoresis and enzyme staining techniques employed have been described elsewhere (Rick et al. 1977; Tanksley and Rick 1980; Vallejos 1983; Bournival et al. 1988). The genotypes of 79 BC_1 individuals and the race 3-resistant breeding lines were determined at each of the following segregating enzyme loci: *Prx-1, Skdh-1, Bnag-1,* and *Dia-2,* on chromosome 1; *Est-7* and *Prx-2* on chromosome 2; *Prx-7* on chromosome 3; *Pgm-2* on chromosome 4; *Aps-1* on chromosome 6; *Got-2* on chromosome 7; *Aps-2* on chromosome 8; *Prx-4* on chromosome 10; *Sod-1* on chromosome 11; and *Est-4, 6Pgdh-2, Pgi-1,* and *Aco-1* on chromosome 12. The morphological marker, *Pn,* on chromosome 8 was also scored. Following isozyme analysis, the BC_1 individuals were raised to maturity and self-pollinated. The BC_1S_1 progenies, along with the breeding lines, were then inoculated with race 3.

Inoculations

Inoculum was prepared by incubating spores of race 3 of *F. oxysporum* f. sp. *lycopersici* (SC761 isolate, which is a race 3 strain isolated in 1982 by Dr. J. P. Jones at the University of Florida, Gulf Coast Research and Education Center at Bradenton) in sterilized 2.4% potato dextrose broth (Difco) at 28 °C with continuous shaking at 125 rpm for 3–4 days. After incubation, the culture was filtered through four layers of cheesecloth to remove mycelia and diluted to a concentration of $1.10–1.25 \times 10^8$ spores/ml; a hemacytometer was used to determine spore concentration. The phenotype of each backcross individual with respect to resistance to race 3 was determined through progeny tests; at least 20 BC_1S_1 progeny were tested per BC_1 analysis. Progeny tests were used to increase reliability of BC_1 scores and to allow for the eventual determination of BC_1 responses to races 1 and 2. 'Bonny Best', LA 716, and F_1 seedlings were inoculated as controls with every set of BC_1S_1 families. Also, another set of controls was incubated in distilled water only. Seedlings of 'Manapal' and 'Hayslip' were periodically inoculated to monitor the specificity of race 3 inoculum.

Seedlings at the 2–4 true leaf stage were inoculated by pruning roots and incubating in inoculum for 3–5 hours at 28 °C. They were then transplanted into a sterilized peat:sand:soil (1:1:1) media (pH 6.0) 4 cm apart and transferred to a growth chamber. Growth conditions were as follows: 28 °C constant temperature, 80% ± 20% humidity, and a 10-h photoperiod. The photon flux density (μmol quanta·m^{-2}·s^{-1}) in the chamber changed according to the following schedule: 90 for 2 h, 130 for 2 h, 200 for 2 h, 130 for 2 h, 90 for 2 h, and darkness for 14 h. Fertilizer (Peters 20-20-20) was applied approximately 1 week after inoculation. Seedlings were scored at least twice. The first score was carried out shortly after susceptible 'Bonny Best' plants began showing wilt symptoms, usually 10–14 days after inoculation. The purpose of this score was to determine the cause of death of dying plants. Stems of dying plants were cut and examined for vascular browning; living plants were judged as wilting, stunted, or healthy. A second score was carried out approximately 1 month after inoculation; for this score, all plants were dissected, and the degree of vascular browning was determined. Scores were assigned according to the following scale: 1 = no sign of browning, or browning only at root tip; 2 = at least some browning found above the root tip but not continuous from root to cotyledon; 3 = continuous browning from root to cotyledon in only one vascular tract; and 4 = continuous browning from root to cotyledon in more than one vascular tract. Dying plants showing wilt symptoms were given a rating of 5. A weighted mean disease rating was calculated for each backcross individual using the progeny scores. All statistical analyses were performed on a mainframe computer using the Statistical Analysis System (SAS Institute, SAS Circle, Box 8000, Cary, North Carolina).

Results and discussion

Analysis of monogenic segregation ratios of the marker loci in the BC_1 population showed that most conformed to the expected 1:1 ratio. However, a significant excess of heterozygotes was detected on chromosome 1 at *Prx-1* and *Dia-2,* on chromosome 8 at *Pn,* and at all tested loci on chromosome 12: *Est-4, 6Pgdh-2, Pgi-1,* and *Aco-1* (Table 1). Deviations from expected 1:1 ratios in a *L. pennellii*/*L. esculentum* backcross have been reported previously (Rick 1969; Tanksley et al. 1982); however, this is the first report of significant skewing on chromosome 12. All marker loci assorted as previously reported (Tanksley and Rick 1980; Bournival et al. 1988; Chetelat 1989), and no significant skewing was observed between unlinked loci.

In all inoculations, seedlings of *L. pennellii* (LA 716) and the interspecific F_1 consistently showed resistance to race 3, whereas seedlings of the *L. esculentum* cultivars, 'Bonny Best', 'Manapal', and 'Hayslip' showed susceptibility. Responses to race 3 for BC_1 individuals were deduced from mean disease ratings of their progenies.

Table 1. Analyses of monogenic segregations of marker loci and comparisons of BC_1 mean disease ratings using a one-way nonparametric analysis of variance. Mean disease ratings were calculated for BC_1 individuals based on response to race 3 of their progeny. To perform the one-way nonparametric analysis of variance, the Wilcoxon rank sum test was used. In this procedure, BC_1 individuals were ranked according to their mean disease rating, and homozygote and heterozygote mean disease rankings were compared at each marker locus. e/e *esculentum* homozygotes; e/p *pennellii* heterozygotes

Locus	Chromosome No.	No. plants		Monogenic χ^2	Mean disease rating[a]		Mean disease ranking		Wilcoxon rank sum test *Z* statistic
		e/e	e/p		e/e	e/p	e/e	e/p	
Prx-1	1	27	51	7.58**	3.23	3.24	38.8	39.9	NS
Skdh-1	1	31	44	NS	3.34	3.06	41.3	35.6	NS
Bnag-1	1	22	30	NS	3.35	3.08	28.5	25.0	NS
Dia-2	1	23	48	8.80**	3.33	3.14	34.7	38.6	NS
Est-7	2	41	38	NS	3.37	3.10	42.9	36.9	NS
Prx-2	2	37	34	NS	3.29	3.13	34.5	37.4	NS
Prx-7	3	34	39	NS	3.15	3.28	35.3	38.5	NS
Pgm-2	4	36	42	NS	2.92	3.52	32.1	45.8	2.66[b]
Aps-1	6	35	38	NS	3.09	3.29	38.8	35.1	NS
Got-2	7	47	32	NS	4.00	2.13	55.8	16.8	−7.40***
Aps-2	8	43	34	NS	3.40	3.00	43.1	33.8	−1.82*
Pn	8	14	53	22.70**	3.16	3.22	34.2	33.2	NS
Prx-4	10	42	36	NS	3.42	3.02	43.2	35.1	NS
Sod-1	11	23	33	NS	3.48	3.10	32.4	25.8	NS
Est-4	12	21	37	4.41*	3.50	3.15	32.7	27.7	NS
6Pgdh-2	12	20	55	16.33**	3.29	3.29	37.7	38.1	NS
Pgi-1	12	21	58	17.33**	3.16	3.27	38.3	40.6	NS
Aco-1	12	29	50	5.58*	3.08	3.34	36.4	42.1	NS

*, **, *** Significant at 0.05, 0.01, and 0.0001 levels, respectively

[a] BC_1 progeny were scored according to a visual rating system; 1 = resistant to 5 = susceptible

[b] Significant at 0.01 level using a two-tailed nonparametric analysis of variance with heterozygotes being more susceptible than homozygotes

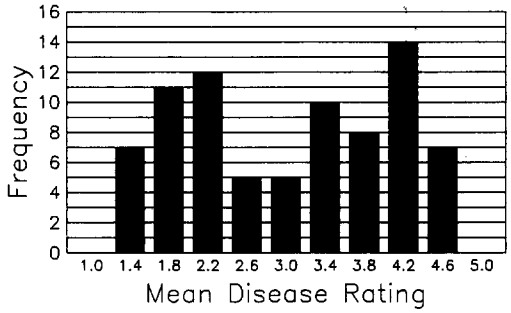

Fig. 1. Frequency distribution of backcross (BC_1) mean disease ratings. For each BC_1, at least 20 progeny (BC_1S_1) were inoculated with race 3 and scored for their level of vascular browning. Higher scores represented greater levels of vascular browning, and thus greater susceptibility. A mean disease rating for each BC_1 individual was calculated by taking a weighted mean of its progeny scores

Analysis of the frequency distribution of the mean disease ratings (Fig. 1) using the Kolomogorov *D* statistic (Kolomogorov 1933) indicated a significant deviation from normality ($D=0.128$, $\alpha<0.01$). Furthermore, the distribution proved to be bimodal according to the coefficient of bimodality ($b=0.586$, $\alpha<0.05$; $b=(m_3^2+1)/(m_4+3)$; m_3 = skewness, m_4 = kurtosis). This type of distribution indicated that race 3 resistance was a trait controlled by one major locus, which was designated *I-3* by Scott and Jones (1989).

The possible association between race 3 resistance and a segregating marker locus was analyzed using a one-tailed nonparametric analysis of variance – the Wilcoxon rank sum test (Wilcoxon 1945). This test is similar to the parametric two-sample *t*-test. The nonparametric analysis was implemented because the disease ratings had a limited range (1–5) and were based on a qualitative evaluation of disease symptoms. In this procedure, BC_1 individuals were first ranked according to their mean disease rating; then, the mean of the rankings of all homozygous plants was compared to that of the heterozygotes at each marker locus. Highly significant differences were detected for the chromosome segment marked by *Got-2*. BC_1 individuals carrying *L. pennellii* alleles for *Got-2* were significantly more resistant than those homozygous for *L. esculentum* (Table 1). These results clearly demonstrated linkage between *Got-2* and *I-3*. An additional significant effect was also detected for the chromosome segment marked by *Aps-2*. However, the effect of this locus was not as great as the one observed for *I-3* (Table 1). The presence of this minor factor was supported by the results obtained from inoculations

of five *L. pennellii* (LA 716) chromosome substitution lines developed by Rick (1969). The chromosome 8 line (carrying *Aps-2*) had a mean disease rating of only 3.00 compared to 4.62, 4.38, 4.85, and 4.84 for lines of chromosome 2, 4, 6, and 11, respectively. Other substitution lines were unavailable. Since the chromosome 8 substitution line showed tolerance to race 3, the locus linked to *Aps-2* is being designated *Tfw* for "tolerance to Fusarium wilt". It was also noted that heterozygotes at the *Pgm-2* locus were significantly more susceptible than homozygotes (Table 1). Such transgressive behavior has previously been reported in tomato (Tanksley et al. 1982; Vallejos and Tanksley 1983). Nevertheless, upon further investigation, this observation proved to be an artifact (see below).

The percent recombination between *Got-2* and *I-3* was estimated to evaluate the usefulness of *Got-2* as a marker for resistance. Although the bimodal distribution of mean disease ratings and the nonparametric analyses of variance indicated that race 3 resistance was controlled by one major locus, it was difficult to classify some BC_1 individuals for their *I-3* genotype. To overcome this difficulty, the average linkage clustering method (Sokal and Michener 1958) was used to classify all BC_1 individuals according to their mean disease ratings into two clusters: resistant and susceptible. This method sequentially clusters individuals, or groups of individuals, with the least distance until the desired number of clusters is attained – two in this case ($D_{KL}=(\bar{x}_K-\bar{x}_L)^2+s_K^2+s_L^2$. D_{KL} = distance between clusters K and L. $\bar{x}_K$, $\bar{x}_L$ and s_K^2, s_L^2 = means and variances of clusters K and L, respectively). The genotype of each BC_1 individual at the *I-3* locus was inferred according to its cluster classification: susceptible (*i-3/i-3;* $\bar{x}=4.01\pm0.63$) or resistant (*I-3/i-3;* $\bar{x}=2.11\pm0.32$). A test for independent assortment between *Got-2* and *I-3* using a two-way contingency table showed significant deviations from expected ratios; the recombination between the two loci was estimated at 2.5% (Table 2).

The evidence presented above indicated that race 3 resistance was controlled by one major gene, *I-3*. However, the variability observed within clusters (Fig. 1) suggested the presence of minor factors such as *Tfw*. To detect additional minor factors that may have been overshadowed by *I-3*, two-way factorial (2 × 2) analyses of variance were conducted for all *I-3*/marker locus pair combinations. The results of these analyses (Table 3) showed again that *Tfw*, linked to *Aps-2*, had a significant effect on the response to race 3. Furthermore, *Tfw* appeared to act independently from *I-3*, as suggested by the lack of a significant interaction (Table 3). Also, a significant interaction was detected between *I-3* and the chromosome segment marked by *Dia-2* (Table 3). However, one-way analyses of variance showed no significant differences between mean disease rankings of *Dia-2* homozygotes and heterozygotes within each *I-3* genotype (data not shown); thus, the *I-3*/*Dia-2* interaction appeared to be an artifact. Additional factors affecting resistance may have existed in parts of the genome not covered by the marker loci. The factorial analysis also

Table 2. Two-way contingency table between *Got-2*[a] and *I-3*. e/e *esculentum* homozygotes; e/p *pennellii* heterozygotes

		I-3		Totals
		e/e	e/p	
Got-2	e/e	46	1	47
	e/p	1	31	32
Totals		47	32	79

$\chi^2=70.92$
$P<0.001$
p (% recombination) = 2.5%
[a] Protocol for GOT detection: Electrophoresis (10%–11% starch, 15–20 V/cm); Gel buffer (15 m*M* TRIS, 4 m*M* Citrate pH 7.8 with T or C); Electrode buffer (0.3 *M* Borate pH 7.9 with NaOH); Activity stain: Solution A (0.1 *M* TRIS pH 8.5, Na Aspartate 2 mg/ml, Na α-ketoglutarate 1 mg/ml); B (Pyridoxal-5-phosphate 0.1 mg/ml, Fast Blue BB salt 1.5 mg/ml); mix A and B just before staining

Table 3. Detection of minor factors taking into account the effect of *I-3*. Two-way (*I-3* by each marker locus) factorial analyses of variance (2 × 2) comparing BC_1 mean disease rankings were utilized. Main effects were calculated according to Steel and Torrie (1960). ee *esculentum* homozygotes; ep *pennellii* heterozygotes

Loci	Mean disease ranking				Main effects		
	ee/ee	ee/ep	ep/ee	ep/ep	*I-3*	Marker	Interaction
I-3 Aps-2	58.15	53.00	19.72	13.28	−39.1**	5.80*	−0.64
I-3 Dia-2	50.78	58.52	21.57	15.39	−36.2**	0.78	−6.96*
I-3 Pgm-2[a]	56.09	56.42	13.58	21.38	−38.8**	4.06	3.74

*, ** Significant at the 0.05 and 0.0001 levels, respectively
[a] *Pgm-2* analysis included to illustrate that significance observed in the nonparametric analysis of variance (Table 1) was an artifact; no significance existed after accounting for *I-3*

indicated that the chromosome segment marked by-*Pgm-2* had no significant effect on resistance (Table 3). A two-way contingency table between *Pgm-2* and *I-3*, although not significant, showed a slight trend to skew in favor of recombinants (64%). This resulted in an excess of *Pgm-2* homozygotes in the *I-3/i-3* class, and an excess of heterozygotes in the *i-3/i-3* class. Thus, the cause of the significant difference in mean disease rankings observed at the *Pgm-2* locus (Table 1) could be attributed to the slight skewing. Finally, a two-way analysis of variance of mean disease rankings for all possible pair combinations between marker loci did not show any significant interactions.

Additional evidence for the presence of linkage between *Got-2* and *I-3* was obtained through an analysis of breeding lines previously selected for resistance to race 3. The genotype at all segregating marker loci, and the response to race 3 was determined for each line. Three lines, I3R-1, 520, and 604, were uniformly resistant to race 3 and homozygous for the *L. pennellii* allele at *Got-2*, whereas E427 and 572 were segregating for both resistance and *Got-2*. Thus, there had been no recombination between *Got-2* and *I-3* in any of the five lines. The lines were homozygous for *L. esculentum* alleles at the other marker loci with the following exceptions: E427 was segregating at *Prx-1, Aps-1, Prx-4,* and *Aco-1,* and 520 was homozygous for the *L. pennellii* allele at *Aco-1.* E427 is a breeding line known to be fixed for resistance to race 3. However, the E427 seed used in this study originated from a seed lot suspected of contamination by cross-pollination which occurred in the field during seed increase.

Conclusion

Currently, race 3 of Fusarium wilt is primarily confined to Florida, Australia, and parts of California; however, since races 1 and 2 have a worldwide distribution, it is probable that race 3 will eventually become a problem in other tomato growing regions. The results of this work demonstrate that *Got-2* can be used as a selectable marker to facilitate the transfer of *I-3* into commercial cultivars. Using the 2.5% recombination estimate, only two plants ($N=2$) would need to be selected, using *Got-2* as a marker to maintain a 99.9% chance of co-selecting *I-3* ($P=1-r^N$; $N=\ln(1-P)/\ln(r)$. $P=$probability, $r=$ percent recombination, $N=$no. of plants).

Work is currently being conducted to establish a more accurate map distance between *Got-2* and *I-3*. Minor factors affecting resistance that caused the variability observed within clusters in Fig. 1 make it very difficult to determine the *I-3* genotype of some BC_1 individuals without using progeny tests. To overcome this problem, near isogenic lines carrying *L. pennellii* alleles at *Got-2* and *I-3* are being developed to analyze the segregation of these loci in a uniform *L. esculentum* background.

It should be noted that only one isolate of race 3 was used as a source of inoculum in this study. However, breeding lines carrying *I-3* have also maintained resistance against the race 3 isolates form Australia and California (D.J. McGrath and M. Kuehn, personal communication).

Acknowledgements. This research was supported in part by a grant from the Florida Tomato Exchange. Our sincere gratitude is extended to Dr. D. Cantliffe for making his growth chambers available for this work, to Dr. H. Corby Kistler for use of his orbital shaker, and to Dr. J. P. Jones for providing the race 3 isolate. We are also indebted to Dr. C. M. Rick for supplying the *L. pennellii* chromosome substitution lines. We would also like to thank Dr. G. A. Moore, Dr. L. Curtis Hannah, and Dr. H. Corby Kistler for reviewing this manuscript.

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