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Localization of genes for bacterial canker resistance in *Lycopersicon peruvianum* using RFLPs

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Abstract A backcross population of the *L. peruvianum* accession LA 2157, which is resistant to bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis*, with the susceptible *L. peruvianum* accession LA 2172 was evaluated for the segregation of *C. michiganenis* resistance and of RFLP markers in order to map the loci involved in this resistance. The development of symptoms of the disease was scored using an ordinal scale. The mapping of the disease resistance was hampered by distorted segregation ratios of a large number of markers and unexpected quantitative inheritance of the resistance. By means of the Kruskal-Wallis rank-sum test, five regions on chromosomes 1, 6, 7, 8 and 10 were identified that may be involved in *C. michiganensis* resistance.

Key words *Clavibacter michiganensis* · Linkage study · Quantitative trait loci · Ordinal scale

Introduction

Bacterial canker in tomato (*Lycopersicon esculentum*) occurs mainly in (sub)tropical areas. The disease is caused by *Clavibacter michiganensis* ssp. *michiganensis* (Smith) Davies et al. (referred to as *Cm*), which is also known as *Corynebacterium michiganense* (Smith) Jensen. The first and most prevailing symptom after infection is the unilateral wilting of leaves. In severe cases this pathogen seriously affects fruit production by causing early plant death.

Several sources of resistance to *Cm* have been found in wild relatives of tomato (e.g., Thyr 1968, 1969; Stamova and Yordanov 1985; Laterrot et al. 1978; Vulkova and Sotirova 1993), with the highest level of resistance having been described to occur in *L. pimpinellifolium* and *L. hirsutum* (Thyr 1968; Berry et al. 1989). However, these sources have only partial levels of resistance. Lindhout and Purimahua (unpublished) screened 57 genotypes of *L. peruvianum* for resistance to *Cm*. A very high level of resistance was found in five accessions. Lindhout and Purimahua (1989) investigated the inheritance of the resistance of *L. peruvianum* accession LA 2157 using the F_2 of a cross between LA 2157 and the susceptible *L. peruvianum* accession LA 2172, as well as the backcrosses to both parents. They concluded that the resistance is inherited as a qualitative trait that is likely to be determined by two or three complementary, recessive genes.

The goal of the study presented here was to investigate the inheritance of *Cm* resistance in more detail using restriction fragment length polymorphism (RFLP) analysis. Various reports have shown that RFLPs can be used to map loci involved in the coding of both qualitative (Young et al. 1988; Van der Beek et al. 1992) and quantitative traits (Osborn et al. 1987; Paterson et al. 1988, 1990, 1991). The latter reports are concerned with mapping loci determining quantitative traits (QTLs) that behave according to a normal distribution. There exists, however, a major class of quantitative traits, among which are many disease resistances, that are best measured on an ordinal scale. This study reports the mapping of genes coding for such a trait.

Because the interspecific cross between *L. peruvianum* and *L. esculentum* is highly incompatible, the mapping study was performed on intraspecific *L. peruvianum* backcross populations of LA 2157 and LA 2172. Miller and Tanksley (1990) have shown that genomic DNA clones from *L. esculentum* can be used to detect polymorphisms in crosses involving other species of the

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genus *Lycopersicon* and for this reason, these DNA clones were employed in this experiment. Since Bonierbale et al. (1988) compared the RFLP linkage maps of potato and tomato and found them to be highly congruent (as was later confirmed by Tanksley et al. 1992), it was likely that the *L. peruvianum* map would not differ much from the map based on an F_2 of a cross between *L. esculentum* and *L. pennellii* (Tanksley and Mutschler 1990). For an efficient choice of the RFLP markers use was made of this RFLP linkage map. In the paper by Van Ooijen et al. (1994), it was shown that the maps of the *L. peruvianum* backcross and the *L. esculentum* × *L. pennellii* F_2 indeed are highly congruent.

Materials and methods

Plant material

Reciprocal F_1 crosses were made between two *L. peruvianum* accessions, the *Cm*-resistant LA 2157 (referred to as R) and the *Cm*-susceptible LA 2172 (referred to as S). Three reciprocal backcross populations were made between the F_1 and the resistant parent, **A**: (R × S) × R (280 plants), **B**: R × (R × S) (199 plants), and **C**: R × (S × R) (287 plants). Pollination was performed by mixing pollen from five male parent plants and applying it to the pistils of five female parents.

Prior to *Cm* inoculation a few leaves were harvested for RFLP analysis from all plants, and after completion of the *Cm* disease test redundant leaf material was harvested from resistant plants only.

Clavibacter michiganensis

The bacterial strain Cm 542 of *C. michiganensis* ssp. *michiganensis* (obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, UK; NCPPB 1064) was used to inoculate the plants. This strain is highly agressive (Van den Bulk et al. 1989). Bacteria were stored at 4 °C on an agar medium containing 1% special peptone (OXOID L72), 0.5% NaCl and 1.5% agar. Prior to use, bacteria were grown at 27 °C on yeast-peptone-glucose agar (YPGA), containing 0.5% yeast extract, 1% peptone, 0.5% D(+)-glucose and 1.5% agar. After 3 days, bacteria were collected and suspended in 0.85% NaCl at a concentration of 10^7 bacteria per milliliter ($OD_{400} = 0.3$).

Resistance screening

Plants were grown to the sixth leaf stage and injected with 10 μl bacterial suspension at the base of the stem. Additionally, the first true leaf of the plant was cut off with a scalpel dipped in the bacterial suspension (See Löffler et al. 1989). The development of symptoms of the plants was scored 19, 26, 33, 41, 48, and 59 days after inoculation using the 0–4 disease index scale presented in Table 1.

Table 1 Disease scale for symptom expression of *C. michiganensis* in tomato

Index	Symptoms
0	No symptoms
1	One leaflet is wilted
2	Two leaflets are wilted
3	Some leaves are partially wilted
4	Most leaves wilted, or plant dead

RFLP analysis

RFLP analysis was performed as described by Van Ooijen et al. (1994).

Linkage analysis of the *Cm* resistance genes

The construction of the RFLP linkage map of the present backcross populations is described by Van Ooijen et al. (1994). According to the model of Lindhout and Purimahua (1989), resistance was assumed to be determined by qualitative, complementary-acting, recessive genes. Consequently, resistant plants would be homozygous for all resistance alleles, whereas susceptible plants have at least one susceptibility allele at one of the loci. Hence, susceptible plants may be homozygous for resistance alleles at one of loci, rendering them less informative for mapping the resistance genes. Therefore, the experiment was initially designed to determine the markers in resistant plants only, which is a way of selective genotyping. The expected frequency of heterozygote marker genotypes within the class of resistant plants is low in the case a marker is linked to a resistance gene (0 in the case of complete linkage and smaller than 0.5 with partial linkage), and equal to 0.5 when they are unlinked. Plotting the heterozygote frequency of each marker against its map position provides evidence of the location of a resistance locus.

This procedure proved insufficient, because of an apparent quantitative inheritance of the resistance and distorted segregation ratios. Therefore, marker genotypes were determined in susceptible plants as well. Results obtained with both resistant and susceptible plants were analyzed with the Kruskal-Wallis rank-sum test (see e.g., Lehmann 1975). This test, which is the nonparametric equivalent of the analysis of variance, measures the association between marker genotype and disease index. Since it compares the disease index frequency distributions of the marker genotype classes, it is not affected by distorted segregation ratios. The Kruskal-Wallis test statistic has approximately a χ^2 distribution with the number of marker genotypes, minus one, degrees of freedom.

Results

Evaluation of *Cm* resistance

Symptom development on the plants of the three backcross populations was evaluated 19, 26, 33, 41, 48, and 59 days after inoculation with *Cm*. The symptoms could not be assessed reliably beyond the 59th day, as leaves were starting to show senescence. The results of the evaluation are given in Table 2, which shows that there was a large difference in disease development between LA 2157 and LA 2172. On the 59th day 18 out of 21 plants of the resistant LA 2157 showed no symptoms, or at most some wilting of only one leaflet (index 0 or 1), whereas all plants of the sucesptible LA 2172 showed severe symptoms (index 4). In the backcross populations the disease developed gradually. On day 19 most backcross plants were classified with index 0, 1, or 2, and during the development an important shift of the frequencies towards class 4 was observed.

Three plants of the resistant parent were classified with index 2, which had not been observed in earlier studies of Lindhout and Purimahua (1989 and unpublished). Furthermore, the fraction of backcross plants with index 1, 2, or 3 was much larger than that found in those earlier studies. This result implies that the genetic model proposed by Lindhout and Purimahua (1989),

Table 2 Evaluation of the development of the disease. Distribution of parental and backcross plants over disease index classes. The disease was evaluated at 19, 26, 33, 41, 48, and 59 days after inoculation with *Cm*. At the 59th day after inoculation 152 resistant backcross plants (index 0 or 1; for 1 plant of B, and 3 plants of C no DNA was available) were selected for RFLP analysis [156 = (A: 29 + 43) + (B: 12 + 21) + (C: 22 + 29)] [*R* LA 2157, *S* LA 2172, *A* (R × S) × R, *B* R × (R × S), *C* R × (S × R)]

Genotype	Disease index						
	0	1	2	3	4	Missing	Total
Day 19:							
R	15	6	1	0	0	3	25
S	5	4	10	5	1	0	25
A	150	87	42	1	0	0	280
B	94	65	38	2	0	0	199
C	133	92	59	3	0	1	288
Day 26:							
R	14	7	1	0	0	3	25
S	0	0	8	6	11	0	25
A	84	81	93	19	3	0	280
B	43	55	70	29	2	0	199
C	50	77	113	35	11	2	288
Day 33:							
R	10	10	1	0	0	4	25
S	0	0	1	4	20	0	25
A	67	63	95	41	14	0	280
B	27	48	73	37	14	0	199
C	36	54	102	54	40	2	288
Day 41:							
R	9	9	3	0	0	4	25
S	0	0	0	1	24	0	25
A	56	53	81	57	33	0	280
B	16	32	70	40	41	0	199
C	27	35	90	60	74	2	288
Day 48:							
R	9	9	3	0	0	4	25
S	0	0	0	0	25	0	25
A	32	53	75	52	68	0	280
B	12	25	57	37	68	0	199
C	24	27	61	60	114	2	288
Day 59:							
R	9	9	3	0	0	4	25
S	0	0	0	0	25	0	25
A	29	43	74	24	110	0	280
B	12	21	53	18	95	0	199
C	22	29	54	29	152	2	288

assuming two or three qualitative recessive genes, is not valid, and needs to be exchanged for a model with a quantitative inheritance.

Linkage analysis of the *Cm*-resistance genes: selective genotyping

If the resistance is inherited quantitatively, the backcross plants with index 0 on day 59 are likely to be homozygous for the resistance alleles, although a number of these plants may have susceptibility alleles. So, even with this quantitative model, the expected heterozygote frequency for plants with index 0 is low (<0.5) for linked markers, whereas for unlinked markers it is 0.5. Since 9 out of 21 plants of the resistant parent were classified with index 1, we decided to add backcross plants with disease index 1 at the last disease evaluation date to the class of the resistant plants. On the 59th day after inoculation 152 resistant backcross plants (index 0 or 1) were selected for RFLP analysis (Table 2). The critical value of the heterozygote frequency estimated from 152 plants at $P = 0.001$ (per marker; this corresponds to an overall error rate of approximately 5%) was 0.378 (calculated using the normal approximation of the binomial distribution).

Initially, 48 clones distributed evenly over the linkage map were used to analyze the segregating populations. If evidence for linkage was observed, additional clones mapped in the vicinity were used to obtain a better resolution. Finally, 72 clones yielding 73 segregating polymorphisms were used in this study. For these markers a linkage map was constructed (Table 3) (Van Ooijen et al. 1994).

The results of the (one-sided) selective genotyping with resistant plants are depicted in Figure 1. On chromosomes 3, 4, 5, 10, and 11 the heterozygote marker frequencies did not fall below the critical value, hence,

Table 3 Genetic linkage map of 73 RFLP markers, determined with backcross populations A, B, and C (Van Ooijen et al. 1994). The markers were determined in 152 resistant plants; markers indicated with an asterisk were determined additionally in 116 susceptible plants. In centiMorgan units with the Kosambi mapping function

Chromosome 1		Chromosome 2		Chromosome 3		Chromosome 4	
TG24	0	TG31	0	TG388	0	TG287	0
*TG310	33	TG293	13	TG166B	6	TG268	7
TG192	39	*TG165	15	TG288	25	TG272B	22
*TG59	55	TG227	25	TG311	64	TG65	30
TG376	59	*TG353	44	TG214	74	TG305	39
*TG326	66	*TG34	72	TG244	84	TG345	49
TG19	74						
TG142	79						
TG333	86						
TG158	105						
Chromosome 5		Chromosome 6		Chromosome 7		Chromosome 8	
TG358	0	*TG178	0	*TG20A	0	TG309	0
TG23	15	TG325	10	*TG128	18	*TG41	18
TG185	38	TG240	22	*TG170	23	TG16A	26
		TG162	59	*TG174	37	*TG307	29
		TG275	62	TG166A	42	*TG261	39
		TG193	73	*TG210A	43	TG201	60
		TG258	74	*TG61	47		
		TG221	82	TG342	76		
Chromosome 9		Chromosome 10		Chromosome 11		Chromosome 12	
*TG144	0	TG230	0	TG327	0	TG68	0
*TG35	14	*TG103	37	TG36	44	TG263A	17
*TG9	22	TG285	43			TG180	32
TG79	24	TG233	106			TG190	56
TG291	29					TG111	72
*TG223A	48					TG296	92
TG328	88					TG350	115

there was no evidence of resistance genes. For 1 marker on each of the chromosomes 6, 9, and 12 (TG178, TG223A and TG190, respectively) and for more markers on the other chromosomes (1, 2, 7, and 8) the heterozygote frequencies were below the critical value. When the heterozygote marker frequencies were studied for each backcross population separately, however, large differences were found between the populations for several chromosomes (Fig. 2). On chromosome 2, for instance, the heterozygote marker frequencies for populations B and C were below the critical value, whereas the frequencies for reciprocal population A were close to 0.5. Comparable results, although not as pronounced as for chromosome 2, were obtained for chromosomes 7, 8, and 9 (Fig. 2). Because of these differences between the reciprocal populations, it is likely that low heterozygote marker frequencies are caused by distorted segregation rather than by selective genotyping of resistant plants.

Linkage analysis of the *Cm*-resistance genes: Kruskal-Wallis test

Distorted segregation rendered the method of one-sided selective genotyping inadequate for detecting resistance loci. In order to discriminate between distorted segregation and resistance, susceptible plants were also genotyped and the Kruskal-Wallis test statistic was applied. For this purpose 116 plants (69, 27, and 20 plants of population A, B, and C, respectively) with a disease index 2, 3 or 4 were analyzed for 21 markers (indicated with an asterisk in Table 3) in regions of interest. Using the unabridged disease scale, we calculated the Kruskal-Wallis test statistics over the pooled data of populations A, B, and C for these 21 markers.

The simplicity of the Kruskal-Wallis test facilitates linkage analysis of all disease evaluation dates (Table 4). There was a significant effect on resistance at chromosome 1, with the highest significance for TG59 on the 59th day after inoculation. No significant effects were found at chromosome 2, although TG34 approached the $P = 0.005$ critical value on day 33, notably with the putative resistance allele coming from the susceptible parent. The effect of TG178 on chromosome 6 was significant at $P = 0.001$ on day 26. TG174, TG210A, and TG61 on chromosome 7 approached the $P = 0.005$ critical value mostly in the earlier stages of disease development. TG261 on chromosome 8 revealed a significant effect ($P < 0.001$) on day 48. On chromosome 9 none of the markers had a significant effect. TG103 on chromosome 10 showed an effect significant at $P = 0.005$ on days 48 and 59, with the putative resistance originating from the susceptible parent. No data of susceptible plants were obtained for TG190 on chromosome 12 due to lack of plant material. In conclusion, loci involved in

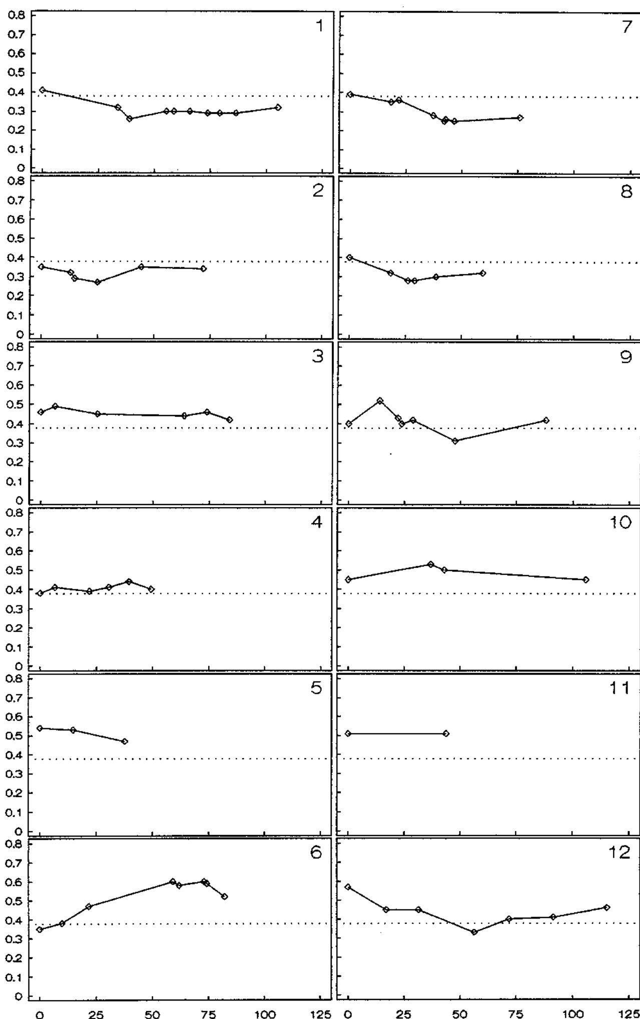

Fig. 1 Heterozygote marker frequency for the markers on all 12 chromosomes in the resistant plants of the backcross population; populations A, B and C pooled. The marker positions correspond to those given in Table 3. The *dotted horizontal line* indicates the significance threshold for 152 plants at $P = 0.001$

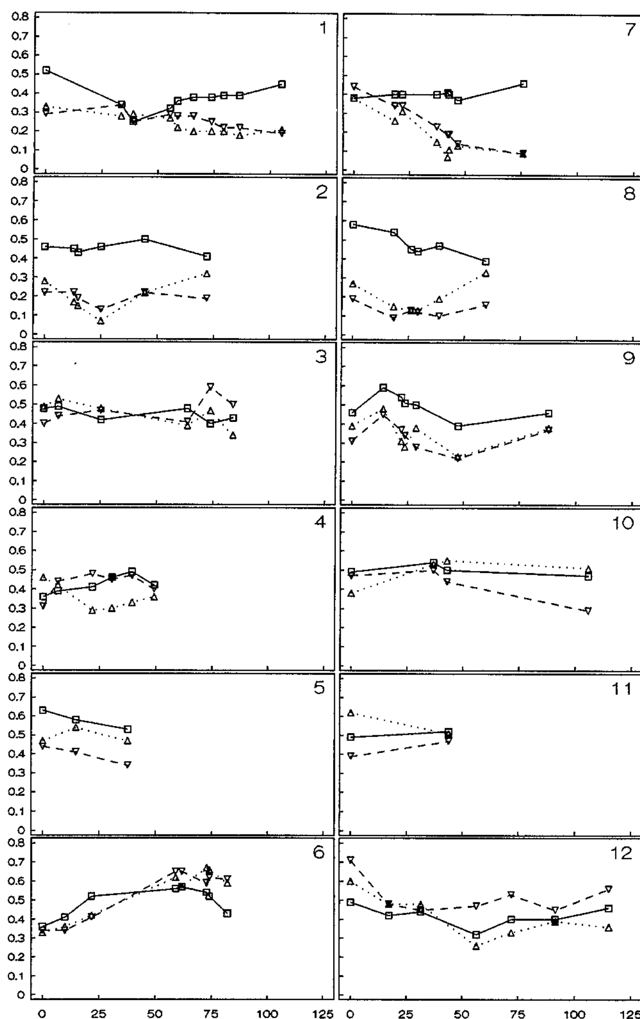

Fig. 2 Heterozygote marker frequency for the markers on all 12 chromosomes in the resistant plants of the three backcross populations: □ A, ∇ B, Δ C. The marker positions correspond to those given in Table 3

resistance are located on chromosomes 1, 6, 8, 10 and possibly on chromosome 7.

Discussion

Because the genetic study of Lindhout and Purimahua (1989) suggested that the *Cm* resistance is determined by two or three qualitative complementary recessive genes, the experiment was designed to map these genes in a backcross to the resistant parent within the species *L. peruvianum*. However, the results of the present study showed that the resistance was not inherited as a qualitative trait, but rather as a quantitative trait. In the earlier study (Lindhout and Purimahua 1989) the backcross and F_2 populations could be clearly separated on the ordinal disease index scale (Table 1) into two distinct groups, resistant and susceptible, but in the present backcross all disease index classes were frequently represented. Two aspects may explain the difference between the present results and the results of Lindhout and Purimahua (1989). First, the present experiment comprised a much larger population, therby reducing the effect of random variation. Second, the experimental conditions may have been different.

By artificially designating the plants with an index of 0 or 1 as resistant, and those with a higher index as susceptible, the planned selective genotyping strategy could still be applied. In this way several regions containing candidate resistance genes were detected. However, when these regions were studied for the reciprocal backcrosses separately, major differences in heterozygote marker frequencies were discovered (Fig. 2). Apparently, genetic factors other than resistance caused the low heterozygote marker frequencies. Obviously, low heterozygote marker frequencies due to gamete selec-

Table 4 The Kruskal-Wallis test statistic for the 21 markers, of which the genotype was determined in resistant as well as susceptible plants; applied on pooled data of populations A, B, and C at 19, 26, 33, 41, 48, and 59 days after inoculation. Critical values of the test statistic: 7.9 at $P = 0.005$, and 10.8 at $P = 0.001$

Marker	Chromosome number	Days after inoculation					
		19	26	33	41	48	59
TG310	1	10.2	13.4	10.9	11.7	12.1	10.5
TG59	1	5.0	11.8	17.9	17.6	20.4	21.1
TG326	1	0.7	1.3	5.7	5.6	4.8	4.4
TG165	2	2.7	0.1	1.0	1.1	1.8	1.8
TG353	2	0.5	0.3	0.1	0.1[a]	0.0	0.1
TG34	2	2.1[a]	6.7[a]	7.0[a]	6.2[a]	5.6[a]	3.9[a]
TG178	6	6.5	12.7	10.2	10.7	9.4	9.0
TG20A	7	1.0	0.3	0.1	0.3	0.2	0.2
TG170	7	4.5	3.0	1.7	3.4	2.8	2.7
TG128	7	5.3	1.7	1.4	2.8	3.0	2.3
TG174	7	7.6	3.1	3.3	4.4	2.2	2.6
TG210A	7	7.6	4.8	5.3	6.2	3.2	4.1
TG61	7	4.2	3.6	5.8	7.5	5.2	6.3
TG41	8	1.1[a]	1.0[a]	0.2[a]	0.1[a]	0.0	0.0
TG307	8	1.2	1.6	2.9	3.0	5.4	4.8
TG261	8	2.2	4.8	6.3	5.9	10.9	9.9
TG144	9	1.3	3.3	1.7	1.7	3.3	2.3
TG35	9	0.9	0.1	1.5[a]	1.3[a]	0.4[a]	0.5[a]
TG9	9	0.7	0.0	0.4[a]	0.4[a]	0.0	0.0
TG223A	9	3.3	3.0	2.7	3.0	3.8	5.1
TG103	10	2.1[a]	0.0[a]	0.6[a]	3.7[a]	9.1[a]	7.9[a]

[a] The association is such, that the putative resistance allele originates from LA 2172, otherwise it is from LA 2157

tion severely hampered the selective genotyping approach for mapping resistance genes.

Distorted segregation has been reported to occur in interspecific crosses within *Solanum* (Bonierbale et al. 1988; Gebhardt et al. 1991), *Lycopersicon* (Zamir and Tadmor 1986; Miller and Tanksley 1990), *Arabidopsis* (Patton et al. 1991) and *Oryza* (Lin et al. 1992), and also in intraspecific crosses (Zamir and Tadmor 1986; Lin et al. 1992). Distorted segregation ratios may be due to the effect of a self-incompatibility locus. Tanksley and Loaiza-Figueroa (1985) have mapped in *L. peruvianum* a gametophytic self-incompatibility locus on chromosome 1, distal of TG310, whereas TG59 lies on the other side of TG310 (Tanksley et al. 1992). Such a gametophytic self-incompatibility locus, however, would yield a preference for heterozygote genotypes of linked markers, which is the opposite of what was found. Moreover, Rick (1986) reported the resistant parent LA 2157 to be fully self-compatible, whereas LA 2172 was strongly self-incompatible. Therefore, gametophytic self-incompatibility can be ruled out as an explanation of the low heterozygote marker frequencies.

A more satisfying explanation can be given using the model for incongruity, as proposed by Hogenboom (1973). Due to reproductive isolation the two accessions LA 2157 and LA 2172 (Rick 1986) may have obtained a degree of evolutionary divergence, which revealed itself in the fertilization process, since low amounts of F_1 seed were produced. When the F_1 is used as a pollinator in a backcross, the pistil parent would show a preference for pollen with a genotype closest to the genotype of the pistil, resulting in a low frequency of heterozygotes. When the F_1 is used as a pistil parent in the backcross, there would be no segregation for fertilization capacity in the pollen of the pollinator, and hence no distorted segregation ratios would be found. This hypothesis is in agreement with the present results.

In conclusion, when qualitative loci are being mapped, one-sided selective genotyping is not hampered by distorted segregation (see Van Ooijen et al. 1994), but when quantitative loci are pursued, this approach is not sufficient.

To separate the effects of selection for resistance from the effect of distorted segregation, susceptible plants were genotyped in addition to resistant plants. The association of marker genotypes with quantitative disease score was determined by means of the Kruskal-Wallis rank-sum test. This test has the advantage of being unaffected by distorted segregation ratios, although it is likely that under extreme segregation distortion the test will have a low power due to the limited size of one of the marker genotype classes. Studying the test values over the six disease evaluation dates in Table 4, it is apparent that the test statistic is quite sensitive to moderate changes in the state of the disease.

The Kruskal-Wallis test revealed the presence of 3 loci with a significant effect for resistance, with the putative resistance allele originating from the resistant LA 2157: TG59, TG178 and TG261 on chromosomes 1, 6 and 8, respectively. Of the genotyped part of the backcross population 44 plants had all of the alleles of the resistant parent for these markers; of this group 35 had been classified as resistant (index 0 or 1) at the last disease evaluation date. When TG61 on chromosome 7, which was nearly significant on day 59, was included, 28 plants had all the putative resistance alleles. Of these, 25 plants had been classified as resistant, only 1 plant had a final score of 2, and only 2 plants a final score of 4.

The results indicate one marker with a significant effect with the putative resistance gene originating from

the susceptible parent: TG103 on chromosome 10. Two explanations can be given for this result. (1) There was a resistance allele present in LA 2172, which came only to expression in combination with alleles of genes present in LA 2157. (2) It was a statistical Type I error.

To confirm the present results we are currently performing a mapping study using a segregating population of an interspecific hybrid of LA 2157 with *L. esculentum* var 'Solentos', produced by embryo rescue.

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