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Introgression and DNA marker analysis of *Lycopersicon peruvianum*, a wild relative of the cultivated tomato, into *Lycopersicon esculentum*, followed through three successive backcross generations

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Abstract Segregation of the *Lycopersicon peruvianum* genome was followed through three generations of backcrossing to the cultivated tomato *L. esculentum* cv 'E6203' using molecular markers. Thirteen BC_1 plants were genotyped with 113 markers, 67 BC_2 plants with 84 markers, and finally 241 BC_3 plants were genotyped with 177 markers covering the entire genome and a BC_3 map constructed. Several segments of the genome, including parts of chromosomes 3, 4, 6, and 10, quickly became fixed for *esculentum* alleles, possibly due to sterility problems encountered in the BC_1. Observed overall heterozygosity and chromosome segment lengths at each generation were very near the expected theoretical values. Markers located near the top telomeric region of chromosome 9 showed segregation highly skewed towards the wild allele through all generations, suggesting the presence of a gamete promoter gene. One markers, TG9, mapped to a new position on chromosome 9, implying an intrachromosomal translocation event. Despite the great genetic distance between the two parents, overall recombination was only 25% less than that observed in a previous tomato cross, indicating that *L. peruvianum* genes may be more readily introgressed into cultivated germplasm than originally believed.

Key words Molecular markers · Chromosome segments · Segregation distortion

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Introduction

Crop improvement is highly dependent upon finding and using genetic variation. Years of cultivation and selection by both farmers and plant breeders have led to reduced levels of variation in elite germplasm; much of the variation left to be utilized is contained in the wild relatives of modern cultivars. This is especially true of self-pollinated crops whose genetic bases are apt to be narrower. For example, DNA polymorphism studies show that the cultivated inbred varieties of tomato contain less than 5% of the genetic variation that is available in cross-compatible landraces and wild species (Miller and Tanksley 1990). Thus, major improvements of the elite varieties are unlikely unless new genetic resources are exploited.

Efficient use of the genetic variation available in unadapted or wild relatives of modern cultivars is therefore essential to the continued improvement of crop varieties. de Vicente and Tanksley (1993) found that genes for improving many traits can be found in wild species, even if the phenotype of the unadapted donor line is inferior. Recent studies of interspecific crosses have proven that it is possible to identify and introgress genes from wild species which can improve a variety of traits, including those that are quantitatively inherited (Eshed and Zamir 1994; Tanksley et al. 1996). However, wild species have generally been used only as a last resort due to the inherent problems of interspecific crosses: hybridization and fertility issues (Rick 1986), non-Mendelian segregations (Stephens 1949; Zamir and Tadmor 1986), suppressed recombination and associated linkage drag (Rick 1969; Paterson 1990), and thus the perceived time commitment involved. Recently, an advanced backcross strategy has been used successfully to make practical use of this unexploited germplasm, even for quantitatively inherited traits (Tanksley and Nelson 1996; Tanksley et al. 1996). This strategy allows simultaneous quantitative

trait loci (QTL) detection and new variety development while mitigating problems typically associated with using wild germplasm, such as linkage drag and epistasis.

Lycopersicon peruvianum is a wild relative of the cultivated tomato, *L. esculentum*. Of all the species in the genus, *L. peruvianum* is one of the most distantly related to the cultivated tomato; embryo rescue is usually required to obtain viable F_1 hybrids (Miller and Tanksley 1990; Rick 1986). *L. peruvianum* is also one of the most variable of all the species, due in part to its allogamous nature, which is enforced by self-incompatibility. In DNA studies of eight *Lycopersicon* species, this species contains the highest amount of novel DNA and variation when compared to *L. esculentum* (Miller and Tanksley 1990). This makes it a likely source of new genes for crop improvement and, in fact, *L. peruvianum* has yielded many of the major resistance genes now critical for commercial tomato production, including resistance to root knot nematode (*Mi*) , tobacco mosaic virus (*Tm2a*), and tomato spotted wilt (*Sw-5*) (Kalloo 1991). In spite of this, little is known at the genetic level about the introgression of DNA from this species into the elite varieties. Information about segregation and recombination frequencies could expedite the utilization of this important germplasm resource. With the advent of high-density molecular maps comes the opportunity to follow genetic transmission and recombination through multiple generations.

Here we describe a series of backcrosses between a cultivated tomato variety, *Lycopersicon esculentum* cv 'E6203', and a wild relative *L. peruvianum*. To follow the genetic transmission of the introgressed segments of wild DNA, we used molecular markers to analyze the BC_1, BC_2, and BC_3 generations. We describe restriction fragment length polymorphism (RFLP) analysis, heterozygosity, segregation distortion, and the distribution of chromosome segment lengths at each generation. Also described is the construction of a *L. esculentum* × *L. peruvianum* map based on BC_3 segregation data.

Materials and methods

Population development

A single plant of the wild species *L. peruvianum* (LA1708) (hereafter referred to as PV) was hybridized as the pollen parent to the processing tomato inbred *L. esculentum* cv 'E6203' (E). A single F_1 plant was backcrossed to E using E as the pollen parent. One hundred fruit yielded 125 seeds of which 13 produced viable BC_1 plants. These were backcrossed to E again, in reciprocal directions. Of these 13 plants 8 would not set fruit after ten or more crosses in either direction and so were deemed sterile (Table 1).

Sixty-seven BC_2 plants, derived from the 5 fertile BC_1 plants, were grown in the greenhouse in Ithaca during the summer of 1993. The population was genotyped with the RFLP marker TG279 to select for homozygous *esculentum* alleles at the *sp* locus on chromosome 6. This selects for a determinate growth habit, a requirement for field evaluations. Seven plants heterozygous at this locus were discarded. The population was also genotyped with markers near the *S* locus on chromosome 1 to avoid self-incompatibility, but all plants were found to be already homozygous for the E allele at this locus. Additional RFLP markers throughout the genome were used to genotype this population, 84 markers in total, with 26 of those markers showing no PV alleles. The percent heterozygosity of each plant in the BC_2 population was calculated from the number of heterozygous markers out of the total markers scored for each plant. The 25 plants that contained PV alleles at the maximum number of loci were selected using HYPERGENE (Young and Tanksley 1989), a computer program for graphical genotyping, and STATVIEW (Abacus Concepts 1992). These 25 plants were backcrossed to E to generate the BC_3 generation (see Table 1).

For economy of labor and greenhouse space, the BC_3 population was grown in two sets of approximately 125 plants each, one in the winter of 1993/1994 and the other in the summer of 1994. A total of 241 BC_3 plants were backcrossed as females to E, and BC_4 seed was collected for later field testing of horticultural traits in the summer of 1995 (Fulton et al. 1997).

RFLP analysis

DNA was extracted from both the E and PV parents as well as the BC_1, BC_2, and BC_3 plants. Procedures for DNA extraction, restriction enzyme digests and Southern blotting were as described in Bernatzky and Tanksley (1986). Bulked DNA from 3 *L. peruvianum* LA1708 plants was used for surveys along with DNA from

Table 1 Development of the BC_1, BC_2, and BC_3 populations from a *L. esculentum* × *L. peruvianum* cross

BC_1 parent	M/F[a]	Number of BC_2 progeny	Number of BC_3 progeny/BC_2 parent[b]	% Total BC_3 progeny
92T259A-1	M	2	4, 12	7%
	F	6	12, 9, 11, 12, 13, 8	27%
92T259F-3	M	1	10	4%
92T259F-4	M	2	9, 7	7%
92T259F-5	M	2	9, 12	9%
	F	8	6, 12, 5, 10, 11, 10, 12, 8	30%
92T259F-7	M	4	9, 10, 11, 9	16%
8 plants	Sterile			
	Totals	25 BC_2	241 BC_3	100%

[a] M indicates plants used successfully as pollen parent, "F" indicates plants used successfully as pistillate parent, "S" indicates sterile (unsuccessful as either parent).
[b] Numbers separated by commas indicate the number of BC_3 progeny for each BC_2 plant

L. esculentum cv 'E6203' using the restriction enzymes *Eco*RI and *Hin*dIII. RFLP markers selected from a high-density tomato map (Tanksley et al. 1992) at 3-cM intervals were labeled by primer extension (Feinberg and Vogelstein 1983), hybridized, and washed to a stringency of $0.5 \times$ SSC at 65°C. Additional surveys were made with *Dra*I, *Eco*RV, *Xba*I and *Sca*I for areas of the map that did not show polymorphism with the original surveys.

Construction of the BC_3 map

The commands "group" (LOD 4.0), "near" (LOD 5.0) and "ripple" of MAPMAKER (Lander et al. 1987) were used to establish a linear order of markers in each linkage group of the BC_3.

Recombination (r) in the BC_3 generation was computed by solution of the likelihood equation:

$$\mathrm{AB}\quad (r^2 - 2r + 1)/(r^3 - 3r^2 + 3r - 7) + (\mathbf{Ab} + \mathbf{aB})(r^2 - 2r + 1)/(r^3 - 3r^2 + 3r) + \mathbf{ab}[1/(r-1)] = 0$$

where the AB terms represent the counts for BC lines having received the corresponding two-locus marker genotype from the donor parent. Recombination frequencies were converted to map distances in centiMorgans (cM) with the Kosambi mapping function (Kosambi 1944).

The variance of recombination estimates used is the reciprocal of the information I. The I for F_2 and BC_1 may be found in Allard (1956), and the others were calculated as

$$\text{for } BC_2\text{: } \frac{(2(2r^2 - 4r + 3)}{r(2-r)(3-2r+r^2)}$$

$$\text{for } BC_3\text{: } \frac{9(r-1)^4(7-4y)}{4y(7-y)(1-y)}$$

$$\text{where } y = r(r^2 - 3r + 3)$$

Chromosome segment length calculations

Chromosome segment lengths and expected segment lengths (from simulation) were calculated with QGene (Nelson 1997) as described in Tanksley and Nelson (1996).

Results and discussion

Polymorphism between PV and E

Of a total of 457 RFLP marker surveys, 65% showed polymorphism between PV and E using two restriction enzymes. Of these, 68% were identified by the restriction enzyme *Eco*RI, 57% by *Hin*dIII, and 25% were polymorphic with both enzymes. Fifty-five markers monomorphic with those two enzymes were resurveyed with four additional enzymes, and 43 (78%) were polymorphic with one or more of the additional enzymes.

Transmission of *L. peruvianum* alleles

BC_1 generation

One hundred and thirteen RFLP markers were scored on DNA blots from the 13 BC_1 plants. Of these marker loci 13 deviated significantly from the expected 1:1 allele frequency ($P < 0.01$) (Fig. 1). Of the skewed markers, 2 (15%) (CT182 on chromosome 11 and CT220 on chromosome 9) were skewed entirely towards E alleles, but 11 (85%) deviated toward the PV allele, consistent with segregation analyses on interspecific hybrids done by Zamir and Tadmor (1986) which also showed skewing in favor of the wild parent. On chromosome 9, 3 markers were entirely heterozygous, a bias which continued into the next two backcross generations. The average number of heterozygotes per locus was 62%, as compared to the expected 50% in a BC_1.

Only 5 of the 13 BC_1 plants set fruit in crosses as either the pollen or pistillate parent. Contingency tests showed only 1 marker to be significantly ($P = 0.07$) associated with this sterility, TG70 on chromosome 1, located near the self-incompatibility gene, *S*. Other sterility factors may be involved, but these could not be detected statistically with such a small population.

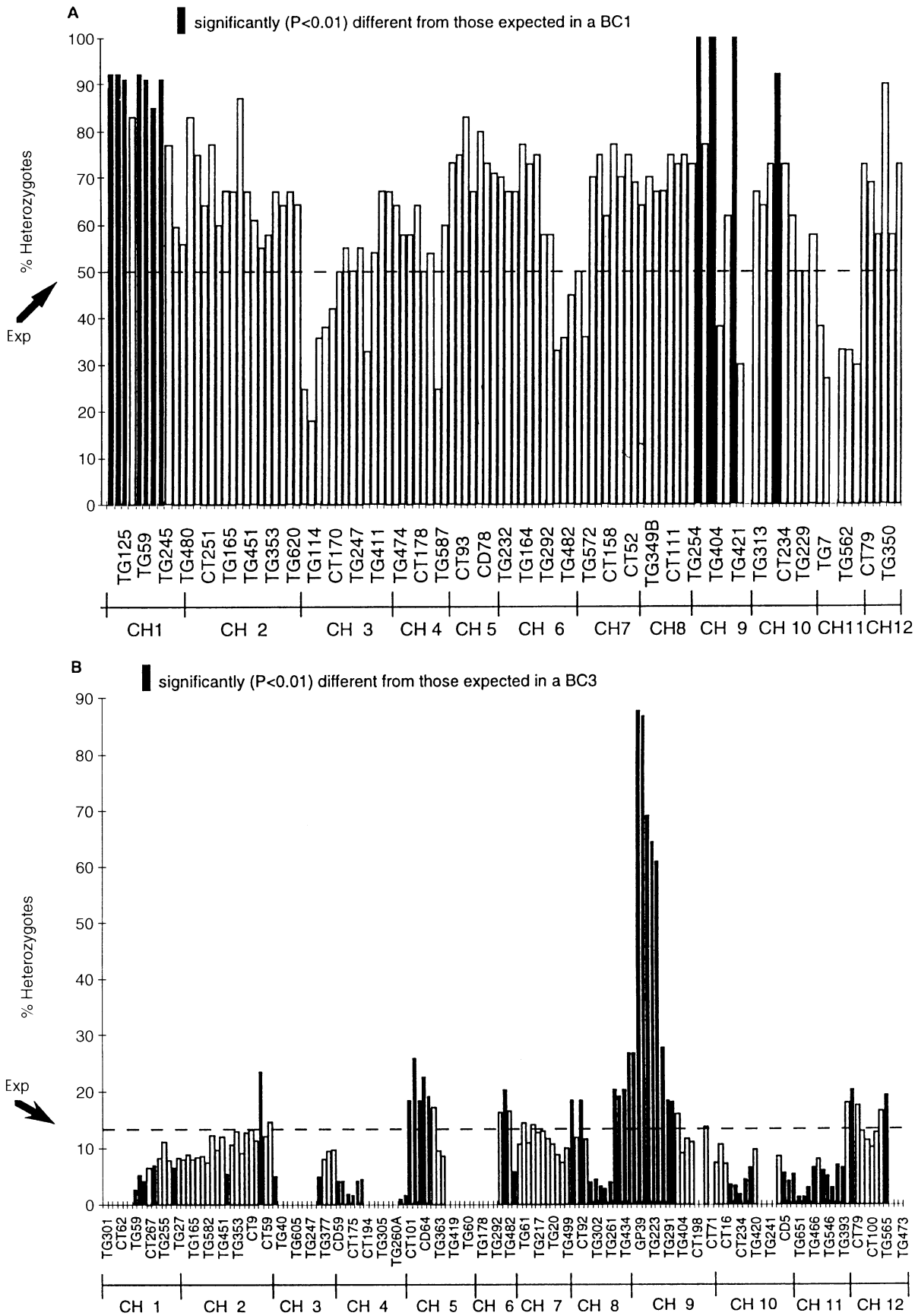
BC_2 generation

The BC_2 population was genotyped with 84 RFLP markers, 26 of which showed no transmission of PV alleles. Four of these markers were near the *S* (self-incompatibility) gene and 2 were near the *sp* (determinate growth habit) gene, as would be expected from the selection against the PV allele for these traits. The average proportion of heterozygous plants per locus was 20%, near the 25% expected for a BC_2 generation.

The mean heterozygosity of all the BC_2 plants was 24%. Selection of the 25 plants containing the most PV alleles increased the mean heterozygosity to 27% (Fig. 2). This decreased the number of plants to be backcrossed, minimizing the time and labor involved in producing the BC_3 population while maintaining the maximum number of PV alleles in the population.

BC_3 generation

The BC_3 population was analyzed with 171 RFLP markers and 3 polymerase chain reaction (PCR) markers. Since the area of chromosome 1 surrounding the *S* locus was fixed for *esculentum* alleles by the BC_2, no further markers were assayed in the BC_3 for this region of the genome. The 122 molecular markers still segregating for PV alleles in the BC_3 covered approximately 865 cM or 67% of the genome according to the high-density tomato map at an average spacing of 7 cM (Tanksley et al. 1992). The average number of


A
significantly (P<0.01) different from those expected in a BC1
% Heterozygotes
Exp
100
90
80
70
60
50
40
30
20
10
0
TG125 TG59 TG245 TG480 CT251 TG165 TG451 TG353 TG620 TG114 CT170 TG247 TG411 TG474 CT178 TG587 CT93 CD78 TG232 TG164 TG292 TG482 TG572 CT158 CT52 TG349B CT111 TG254 TG404 TG421 TG313 CT234 TG229 TG7 TG562 CT79 TG350
CH1 CH 2 CH 3 CH 4 CH 5 CH 6 CH7 CH8 CH 9 CH 10 CH11 CH12
B
significantly (P<0.01) different from those expected in a BC3
% Heterozygotes
Exp
90
80
70
60
50
40
30
20
10
0
TG301 CT62 TG59 CT267 TG255 TG27 TG165 TG582 TG451 TG353 CT9 CT59 TG40 TG605 TG247 TG377 CD59 CT175 CT194 TG305 TG260A CT101 CD64 TG363 TG419 TG60 TG178 TG292 TG482 TG61 TG217 TG20 TG499 CT92 TG302 TG261 TG434 GP39 TG223 TG291 TG404 CT198 CT71 CT16 CT234 TG420 TG241 CD5 TG651 TG466 TG546 TG393 CT79 CT100 TG565 TG473
CH 1 CH 2 CH 3 CH 4 CH 5 CH 6 CH 7 CH 8 CH 9 CH 10 CH 11 CH 12

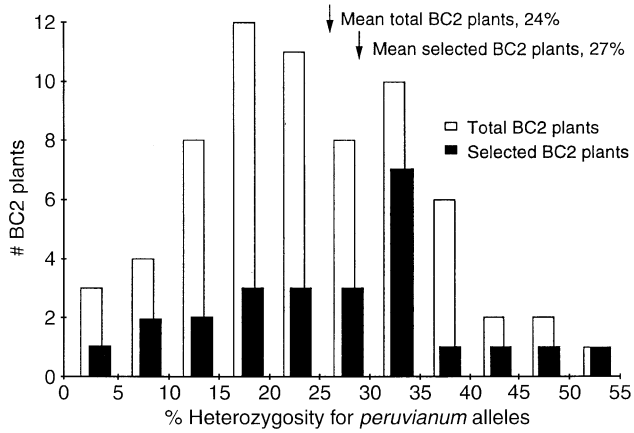


Fig. 2 Heterozygosity of the entire BC_2 population (*unshaded bars*) versus the 25 plants selected to produce the BC_3 population (*shaded bars*)

heterozygotes per locus was 12%, close to that expected for a BC_3 (12.5%). The percentage of heterozygotes for each marker is depicted in Fig. 1. In addition to areas of chromosome 1 and chromosome 6 which were fixed for the E allele as expected, areas of chromosomes 3, 5, and 10 were also fixed. Chromosome 4 had an unusually low number of heterozygotes for each marker. Some of this loss of heterozygosity may be due to the sterility problems of the original cross. Although the BC_2 population was not as thoroughly analyzed (only 84 markers were used), these regions of chromosomes 3, 5 and 10 were already fixed for E alleles at that time. Also, since the BC_1 population was very small (5 plants), parts of the genome would be expected to have been lost by chance. Chromosomes 5, 12, and especially 9 had higher than expected frequencies of heterozygotes.

Map construction with BC_3 data

Linkage analysis

The general order of markers corresponded to that of the high-density tomato map (Tanksley et al. 1992) with some exceptions (Fig. 3). Two markers mapped to a different location than expected. TG260, located on chromosome 4 of the high-density map, mapped to chromosome 1 in the E × PV cross, and CT145, on chromosome 5 of the high-density map, mapped to chromosome 1 in this study. Similar results were observed for these same probes in a *L. esculentum* × *L. hirsutum* cross, suggesting that these markers are multiple-copy and that the loci mapped in E × PV represent non-orthologous loci (D. Bernacchi, unpublished data). This inference can be made from looking at genomic blots where more than one fragment hybridized for most if not all restriction enzymes.

Chromosome 2 formed two distinct linkage groups, one containing the top 6 markers and another containing all markers from TG290 and below (see Fig. 3). While the markers within each group show linkage to each other, linkage was not detected between the markers in one group and the markers in the other. This area of chromosome 2 may represent a hot spot for recombination, a situation amplified by the genetic bottleneck imposed by the small size of the BC_1 population.

Evidence for intrachromosomal translocation

TG9 on chromosome 9 cosegregated with a marker near the centromere, rather than higher on the chromosome as expected. Genomic blots suggest that TG9 is not a multiple-copy marker, and segregation analysis with a different restriction enzyme gave the same mapping result. Marker analysis of NILs containing different chromosome 9 segments of the PV genome confirmed a new location, implying an intrachromosomal translocation of this locus. A previous cross using a different accession of *L. peruvianum* also suggested a possible rearrangement involving this marker (van Ooijen et al. 1994).

Estimating recombination

Recombination estimates in common mapping populations such as BC_1 and double haploid (DH) are simple ratios of the number of recombination events to the total number of meioses. After one or more additional meioses, as in an advanced backcross population, there have been more recombination events but they cannot be counted directly since the class of apparent non-recombinants now includes products of multiple recombinations in different generations. Conventional mapping software does not have recombination models for this design. Figure 3 depicts the maximum-likelihood recombination distances in the BC_3 population as compared to the high-density tomato map based on a *L. esculentum* × *L. pennellii* cross (Tanksley et al. 1992). However, it should be noted that such estimates are subject to considerable error. The 90% confidence interval allows about a 50% relative error in either direction.

Table 2 compares the lengths of the chromosomes in the BC_3 population to the lengths of the corresponding chromosomes of the high-density map (Tanksley et al.

Fig. 1A, B Percentage of heterozygotes for each locus in **A** the BC_1 population and **B** the BC_3 population. "*Exp*" indicated the expected number of heterozygotes in a population: 50% in a BC_1, 12.5% in a BC_3. *Solid bars* indicate those markers significantly ($p < 0.01$) deviating from the expected level of heterozygosity

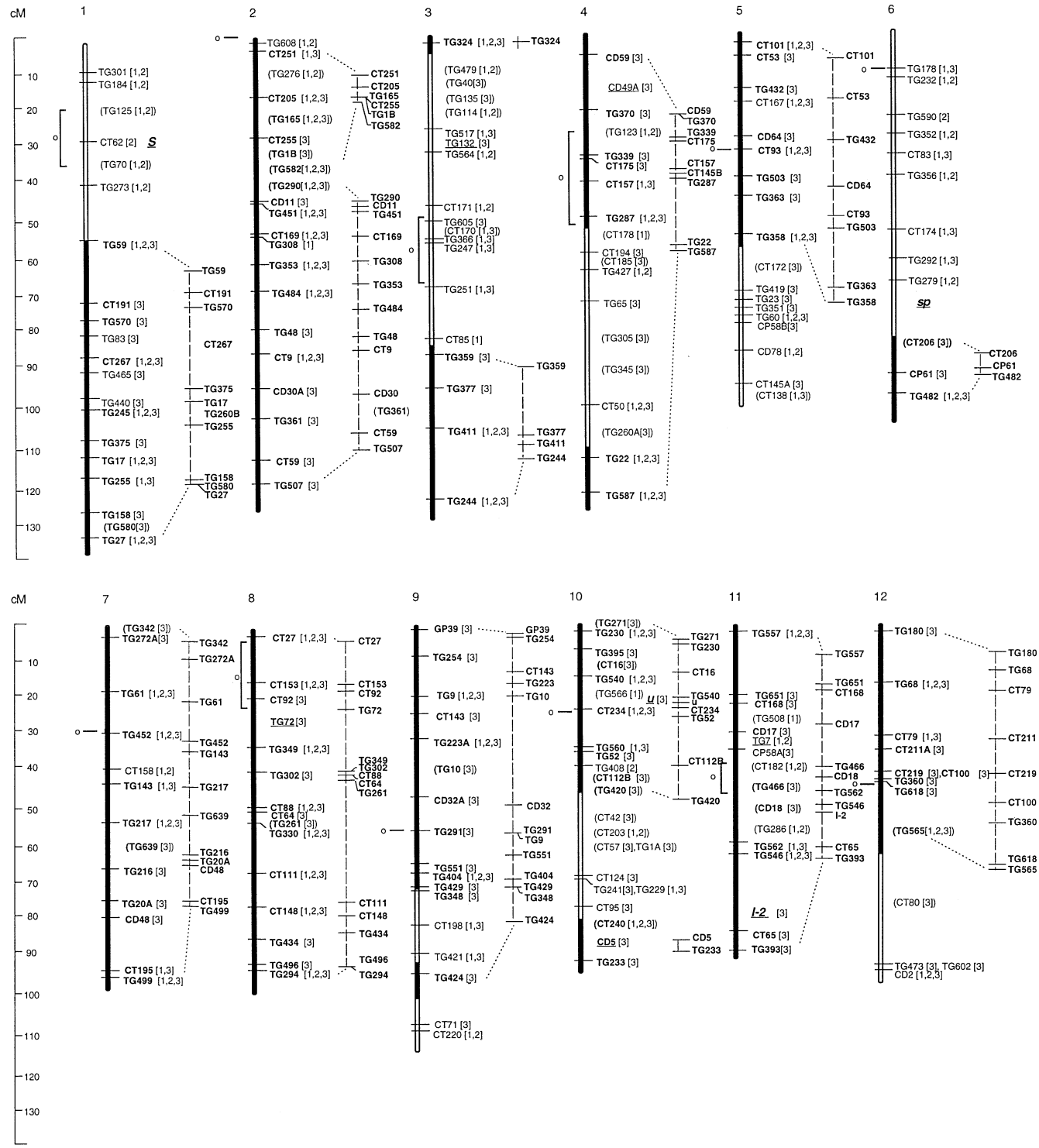


Fig. 3 Comparison of the BC_3 molecular linkage map (the *right* chromosome of each pair) to the high-density tomato map (the *left* chromosome of each pair) (Tanksley et al. 1992). CentiMorgan distances are indicated on the scale at the *left*. *Shaded sections* of chromosomes and corresponding *bold markers* indicate regions still showing segregation for PV alleles in the BC_3. *Numbers* in [ ] indicate for which generations each marker was analyzed: *1* BC_1; *2* BC_2; *3* BC_3

Table 2 Comparison of the lengths (in centiMorgans) of chromosome portions of the high-density map and those in the *L. peruvianum* BC_3. Only those portions of the tomato map still represented by PV alleles in the BC_3 were included in the calculation

Chromosome	E × PN F_2	E × PV BO_3	(BC_3/F_2) 100
Ch1	82	59	72%
Ch2	124	64	52%
Ch3	40	23	58%
Ch4	54	30	56%
Ch5	53	54	102%
Ch6	15	4	27%
Ch7	92	74	80%
Ch8	95	94	99%
Ch9	108	83	77%
Ch10	55	46	84%
Ch11	93	56	60%
Ch12	54	64	119%
Total cM	865	651	75% (ave)

1992). Segments of the tomato map not represented by PV alleles in the BC_3 have been omitted in these calculations. All but two of the chromosomes are reduced in size in the *L. peruvianum* cross compared with the high-density map. However, the overall total number of centiMorgans shows only a 25% decrease in recombination.

It should be noted that one-third of the *L. peruvianum* genome (those areas quickly fixed for *L. esculentum* alleles) remained inaccessible to genetic analysis, probably due to the sterility and possible pollen–stigma incompatibility problems suffered in the BC_1 (and possibly due to specific sterility or incompatibility genes located in those regions). Consequently, the distribution of recombination and the potential for breaking linkage blocks in those regions remain unknown. Future efforts of this kind could employ embryo rescue to obtain better representation of these regions.

Chromosome segment lengths

Figure 4 shows the lengths of chromosome segments represented by contiguous PV alleles in the BC_1, BC_2, and BC_3 populations, 47 cM, 31 cM, and 27 cM, respectively. Despite the loss of several chromosome segments, the mean lengths of segments for all three generations were near those predicted from simulation for an unselected population: 50 cM in a BC_1, 34 cM in a BC_2, and 27 cM in a BC_3. The simulation necessarily ignores transmission effects and genetic and physical interactions of chromosomes and so can be at best a rough guide in individual cases. However, in certain regions of the genome it does not appear difficult to obtain short introgressions in a *L. peruvianum* backcross series such as we have described.

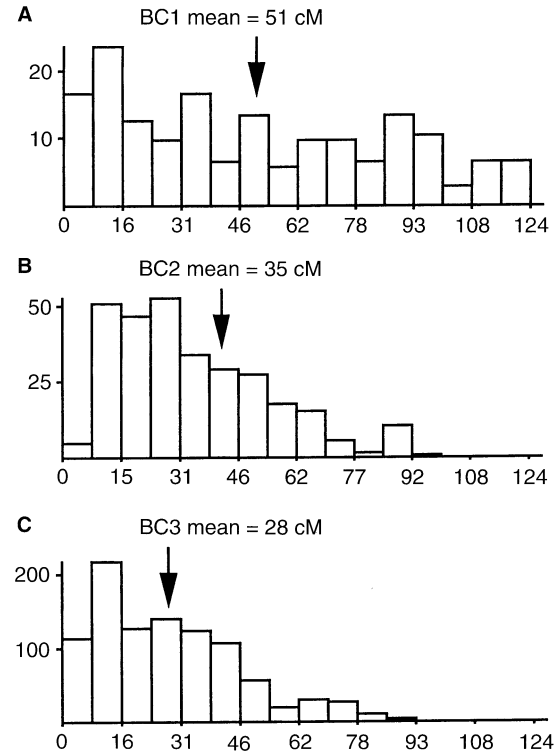


Fig. 4A–C Frequency and size of chromosome segments (in centiMorgans) containing PV alleles in the **A** BC_1, **B** BC_2, and **C** BC_3 populations

Gamete promoter locus maps to telomeric region of chromosome 9

The markers above the centromere on chromosome 9 contained up to 5 times the expected number of heterozygotes per locus, with the highest number (88%) at the most distal marker, GP39 (Fig. 1). An earlier study suggested that in some lines of *L. peruvianum* this area of chromosome 9 contains a gamete promoter (*Gp*) which is preferentially inherited over the E allele in segregating populations, causing skewed segregation (Pelham 1968). Inheritance of markers on other chromosomes or below the centromere on chromosome 9 was unaffected by the skewed inheritance of the markers at the top of chromosome 9.

Conclusions

Efficient use of the genetic variation available in the wild relatives of crop species depends on the ability to introgress desirable DNA segments from wild germplasm into cultivated varieties. Ample recombination must be available to allow the reduction of linkage drag. Results from this study indicate that relatively high levels of recombination occur between the *L.*

peruvianum and *L. esculentum* genomes. After three generations of backcrossing, the average lengths of heterozygous segments resemble those predicted from theory for an unselected population. While some regions of the PV genome are lost during backcrossing, most (67%) are faithfully transmitted to advanced backcross populations. Under the conditions of this cross, recombination suppression did not lead to an expanded mean introgression length. This study indicates the high fidelity with which segments of DNA from the *L. peruvianum* genome can be introgressed into the cultivated tomato genome, thus raising the possibility that the genetic variation found in this species can be harnessed for improving many traits in tomato, including those which are quantitatively inherited – a topic elaborated in a companion paper (Fulton et al. 1997).

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