Theor Appl Genet (1989) 77:353–359

RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding

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Received August 8, 1988; Accepted November 11, 1988
Communicated by F. Salamini

Summary. Genes introduced into cultivated plants by backcross breeding programs are flanked by introgressed segments of DNA derived from the donor parent. This phenomenon is known as linkage drag and is frequently thought to affect traits other than the one originally targeted. The *Tm-2* gene of *Lycopersicon peruvianum,* which confers resistance to tobacco mosaic virus, was introduced into several different tomato cultivars (*L. esculentum*) by repeated backcrossing. We have measured the sizes of the introgressed segments flanking the *Tm-2* locus in several of these cultivars using a high density map of restriction fragment length polymorphic (RFLP) markers. The smallest introgressed segment is estimated to be 4 cM in length, while the longest is over 51 cM in length and contains the entire short arm of chromosome 9. Additionally, RFLP analysis was performed on remnant seed from different intermediate generations corresponding to two different backcross breeding programs for TMV resistance. The results reveal that plants containing desirable recombination near the resistance gene were rarely selected during backcrossing and, as a result, the backcross breeding method was largely ineffective in reducing the size of linked DNA around the resistance gene. We propose that, by monitoring recombination around genes of interest with linked RFLP markers, one can quickly and efficiently reduce the amount of linkage drag associated with introgression. Using such a procedure, it is estimated that an introgressed segment can be obtained in two generations that is as small as that which would otherwise require 100 backcross generations without RFLP selection.

Key words: Introgression – Linkage drag – *Lycopersicon* – Restriction fragment length polymorphisms – Tobacco mosaic virus

Introduction

Many economically important traits have been introgressed into cultivated plant varieties by backcross breeding. In tomato, e.g., most of the genes for resistance to pathogens were introduced by backcross breeding from wild species, as have genes for stress tolerance and fruit quality (Rick 1974, 1982). In wheat, rust- and powdery mildew-resistance were introduced by introgression (Sharma and Gill 1983), and many other examples are known for most crop plants (Zeven and van Harten 1979). Backcross breeding is the method of choice for gene introduction when a cultivar possesses many desirable properties, but lacks a specific trait that is known to reside in a crossable relative. The process of introgression is conceptually straightforward. A hybrid is produced between the donor and the recipient (also called recurrent) lines. The hybrid is then backcrossed to the recurrent parent and the progeny screened with respect to the character being introduced. If the trait is dominant, scoring can be performed directly; if it is recessive, progeny tests must be performed. This process is repeated several times until a line is obtained that carries the target gene in a background which is nearly identical to that of the recurrent line (nearly isogenic lines). In practice, this requires that the breeding program continue for at least five backcross generations (Briggs and Knowles 1967), although most backcross programs proceed for eight or more.

Repeated backcrossing simultaneously accomplishes two essential goals. It allows segregation to remove donor parent chromosomes unlinked to the target gene and it allows recombination to remove donor parent segments which are linked to the target gene. Unlinked DNA is removed by a factor of two in each generation, so that by the eighth backcross generation, less than

0.2% of the unlinked donor genome is expected to persist.

By contrast, the removal of linked segments occurs in a complex fashion that was described first by Hanson (1959) and further elaborated by Stam and Zeven (1981). Their work showed that it takes many generations to remove the linked donor segments. For example, even after 20 backcross generations, a region of 10 cM flanking a target gene is expected to persist. In practice, this region may be larger or smaller than the expected value owing to the large variance associated with the expected value and because a breeder inevitably practices selection among the progeny. Not surprisingly, many examples of "linkage drag" are known in which undesirable traits that are closely linked to a target gene are carried along during the breeding program (Zeven et al. 1983).

In order to analyze the process of introgression in detail, we have examined several cultivars of tomato (*Lycopersicon esculentum*) derived from various breeding programs in which a disease resistance locus, known as *Tm-2*, was introduced from *L. peruvianum* by introgression (Alexander 1963). This analysis was made possible by the availability of a high resolution map of restriction fragment length polymorphisms (RFLPs) tightly linked to *Tm-2* (Young et al. 1988). Our results demonstrate that introgression, as it has traditionally been practiced, is only moderately successful at removing linked segments of donor DNA and leads to cultivars with a wide range of sizes of linked donor DNA. We go on to discuss how RFLP-assisted backcross breeding removes linked donor DNA more effectively, and in a much shorter time, than traditional backcross breeding alone.

Materials and methods

Plant varieties

Vendor, Vendor-*Tm-2*, Vendor-*Tm-2*^{2} (also known as *Tm-2a*), Vendor-VFT (Vendor with genes for resistance to *Verticillium* and *Fusarium*, as well as *Tm-2*^{2} resistance), New Yorker, New Yorker-*Tm-2*^{2}, lines 70-542N and 70-546N were provided by M. Mutschler, Cornell University, Ithaca/NY. Nova, Nova-*Tm-2*^{2}/*ah*, and New Yorker-*Tm-2-ah* were provided by R. Provvidenti, NY State Agricultural Experiment Station, Geneva/NY. Craigella and Craigella-*Tm-2*^{2} were provided by S. Bowes, Glasshouse Research Institute, Littlehampton, UK. Moneymaker and Mocimor were provided by H. H. Latterot, Institute Nationale du Recherche Agronomique, Montfavet, France. VFNT-Cherry and Ohio-MR-13 were provided by C. Rick, University of California, Davis/CA. Each of these tomato lines (with the exception of Vendor, Craigella, Nova, and Moneymaker) is the product of backcross breeding programs with *L. peruvianum*. There are two different alleles from *L. peruvianum* that have been identified at the *Tm-2* locus, known as *Tm-2* and *Tm-2*^{2}, and both confer resistance to tobacco mosaic virus (Schroeder et al. 1967). *Tm-2* is derived from accession P.I. 126926 and *Tm-2*^{2} is derived from accession P.I. 128650. Both alleles have been used in breeding programs and both are represented in the lines described here. Details of these lines are shown in Table 1.

Table 1. Lines of tomato with introgressed *Tm-2* and *Tm-2*^{2} segments

Name	Source	Genotype	No. backcrosses[a]
Craigella-*Tm-2*^{2}	Bowes	*Tm-2*^{2}/+	11
Mocimor	Latterot	*Tm-2*^{2}/*Tm-2*^{2}	21
Vendor-*Tm-2*	Mutschler	*Tm-2*/*Tm-2*	12
Vendor-*Tm-2*^{2}	Mutschler	*Tm-2*^{2}/*Tm-2*^{2}	19
Vendor-VFT	Mutschler	*Tm-2*^{2}/*Tm-2*^{2}	20
New Yorker-*Tm-2*^{2}	Mutschler	*Tm-2*^{2}/*Tm-2*^{2}	12
70-546-N	Mutschler	*Tm-2*/+	4
70-542-N	Mutschler	*Tm-2*^{2}/+	8
Nova-*Tm2*^{2}/*ah*	Provvidenti	*Tm-2*^{2}/*Tm-2*^{2}	–[b]
New Yorker-*Tm-2*/*ah*	Provvidenti	*Tm-2*/*Tm-2*	5
Ohio-MR13	Rick	*Tm-2*^{2}/*Tm-2*^{2}	–[b]
VFNT-Cherry	Rick	*Tm-2*^{2}/*Tm2*^{2}	–[b]

[a] In addition to the number of backcross generations indicated, one or more selfings, followed by selection, were also performed in some of these breeding programs

[b] Information on number of backcrosses not available

RFLP Analysis

Nine DNA markers on chromosome 9 of tomato that have recently been mapped with respect to the *Tm-2* locus (Young et al. 1988) were used in this study. Three additional markers located more distally on the long arm of chromosome 9 were also analyzed (Tanksley et al. 1988). A genetic map of this region, showing the DNA markers used in this study and their position relative to *Tm-2*, is shown in Fig. 1.

The genotype at each marker locus was determined by hybridization of radio-labeled RFLP clones to restriction enzyme digested genomic DNA. Enriched nuclear DNA was isolated from plants of each line as described in Murray and Thompson (1980), as modified in Bernatzky and Tanksley (1986). The isolated DNA was digested with six different restriction enzymes (*Dra*I, *Hind*III, *Rsa*I, *Sst*I, *Tag*I, *Xba*I, Bethesda Research Laboratory, Gaithersburg/MD) in separate reactions, separated by 1.0% agaorse gel electrophoresis, and Southern blotted (Southern 1975) onto GeneScreen Plus (New England Nuclear, Boston/MA. Several restriction enzymes were employed because FRLP markers frequently uncovered polymorphisms with only one or a few enzymes. The Southern blots were probed with miniprep DNA (Willimzig 1985) that had been radio-labeled with alpha-^{32}P-dCTP (New England Nuclear) by the random hexamer method (Feinberg and Vogelstein 1983).

Results

Survey of Tm-2^2 *region in various introgressed lines*

The *Tm-2*^{2} allele has been introgressed into cultivars of tomato on many different occasions and by many different workers (Table 1). In order to determine the sizes of the segments derived from *L. peruvianum* that persist in various introgression lines, DNA markers flanking the *Tm-2*^{2} gene were analyzed by RFLP analysis. Because of the high level of sequence divergence between *L. esculentum* and *L. peruvianum* (Miller, personal communication)

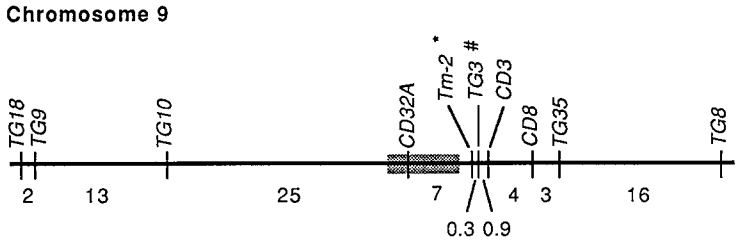
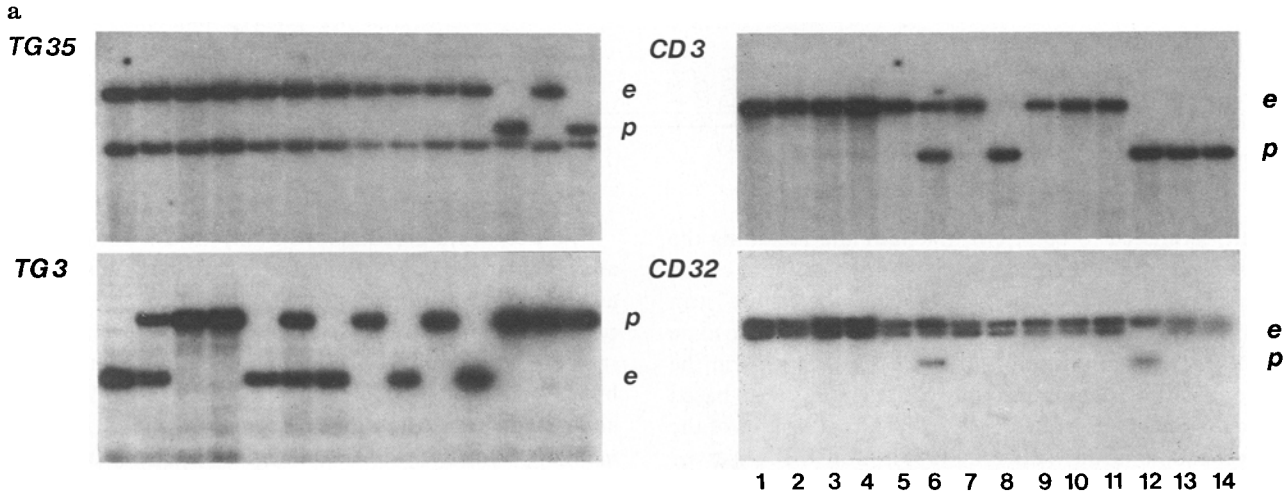


Fig. 1. Genetic map of chromosome 9 showing RFLP markers used in this study (Tanksley et al. 1988; Young et al. 1988). Distances are given in centimorgans. * – *TG79* and *TG101* map to the *Tm-2* locus. # – *PC6* maps to the same locus as *TG3*. The approximate location of the centromere is indicated as a shaded region. The "short" arm of chromosome 9 is on the left in this figure and the "long" arm is on the right



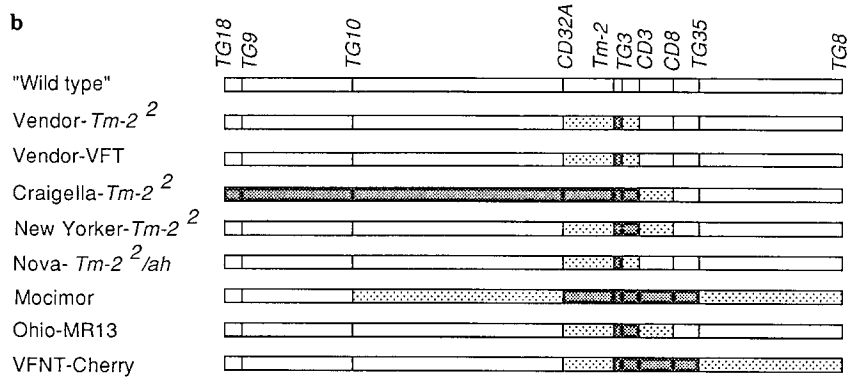


Fig. 2. **a** Southern blot analysis of four of the RFLP markers used to analyze the size of the introgressed segment derived from *L. peruvianum* around the *Tm-2* locus in several lines of tomato. *e* – *L. esculentum* allele, *p* – *L. peruvianum* allele. *TG35* and *CD32* both contain an additional band that is identical in both *L. peruvianum* and *L. esculentum*. *1* – Vendor; *2* – 70-542-N; *3* – Vendor-*Tm-2*^{2}; *4* – Vendor-VFT; *5* – Craigella; *6* – Craigella-*Tm-2*^{2}; *7* – New Yorker; *8* – New Yorker-*Tm-2*^{2}; *9* – Nova; *10* – Nova-*Tm-2*^{2}/*ah*; *11* – Moneymaker; *12* – Mocimor; *13* – Ohio-MR-13; *14* – VFNT-Cherry. **b** Graphical genotypes of tomato lines showing the estimated sizes of the introgressed segments around *Tm2*^{2}. Hybridization results, such as those shown **a**, were analyzed and converted into graphical genotypes, as described in Young and Tanksley (1988). "Wild type" shows the graphical genotype for recurrent parents, Vendor, Craigella, New Yorker, and Nova. White regions indicate *L. esculentum*-derived segments, darkly stippled regions indicate *L. peruvianum*-derived segments and lightly stippled regions indicate segments in which crossovers occurred

RFLPs could be identified for each of the markers analyzed. The size of the introgressed segment for a given line could then be inferred by determining whether the line contained the allele from *L. peruvianum* or from *L. esculentum* for each of the RFLP markers. It was only possible to estimate the size for each introgressed segments because the RFLP data only indicate the interval in which recombination occurred, not the precise point at which crossing-over took place. The results of the RFLP analysis for seven different *Tm-2*^{2}-containing tomato cultivars are shown in Fig. 2a and the corresponding "graphical genotypes" (Young and Tanksley 1988) are shown in Fig. 2b.

These results show that there is a wide variation in the size of the introgressed segment around *Tm-2*^{2} in different cultivars. Craigella-*Tm-2*^{2} contains the largest seg-

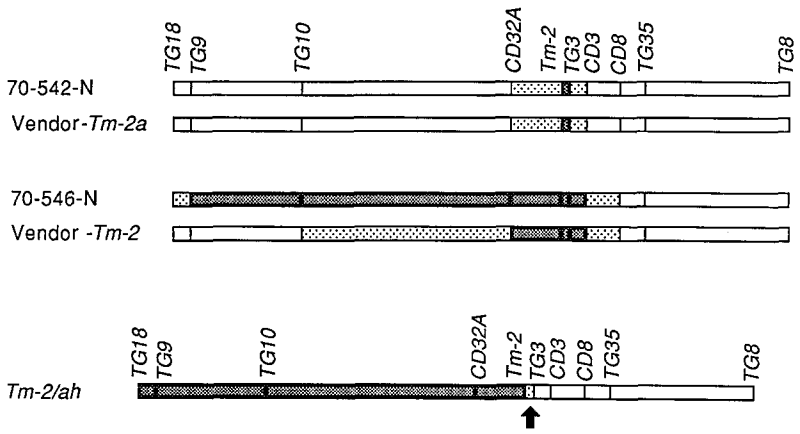


Fig. 3. Graphical genotypes of tomato lines in the Vendor-*Tm-2* and Vendor-*Tm2*^{2} backcross breeding programs. Symbols are the same as in Fig. 2b

Fig. 4. Graphical genotype of a tomato line produced by selecting for recombinants between *Tm-2* and the nearby visible marker, *ah*. Symbols are the same as in Fig. 2b. *Arrow* indicates interval containing selected crossover event

ment, estimated to be 51 cM in length and including the entire short arm of chromosome 9, but very little of the long arm (Young et al. 1988). (Note that the "short" arm of chromosome 9 is the longer of the two chromosome arms in terms of centimorgans, and the "long" arm the shorter. The designations "long" and "short" were based on cytological observations of the apparent lengths of the chromosome arms (Burton 1950), not the observed recombination frequency). In contrast, Mocimor, which also contains a long segment estimated to be 37 cM in length, extends from the middle of the long arm of chromosome 9 to the middle of the short arm. The shortest introgressed segments were observed in Nova-*Tm-2*^{2}/*ah* and Vendor-*Tm-2*^{2}. In both these lines, the length of the *L. peruvianum* fragment was estimated to be 4 cM.

The Vendor backcross breeding program

Remnant seed was available from various generations of two different backcross breeding programs, one in which the *Tm-2* allele was introduced into Vendor and the other in which the *Tm-2*^{2} allele was introduced into Vendor. Thus, it was possible to determine when and where crossover events removing *L. peruvianum* DNA occurred during these programs.

The first material available in the program leading to Vendor-*Tm-2*^{2} was line 70-542N. Ten backcrosses to *L. esculentum* (six by L. J. Alexander, OARDC, Wooster/OH, and four by R. Wilkenson, Cornell University, Ithaca/NY) had already taken place to produce this material. As shown in Fig. 3, the size of the *L. peruvianum* segment had already been reduced to approximately 4 cM in length. Line 70-542N was used by Dr. H. Munger, Cornell University, Ithaca/NY, as a source for a breeding program that consisted of eight additional backcrosses to Vendor to produce Vendor-*Tm-2*^{2}. RFLP analysis of this material demonstrated that during this program, spanning several years, no additional reduction was made in the size of the *L. peruvianum* segment (Fig. 3).

In a separate breeding program, the *Tm-2* allele was also introgressed into Vendor. The first remnant seed available in this program was 70-546N. This seed was the product of four backcross generations to *L. esculentum* (twice by P. Pecault, INRA, Monfavet, France, and twice by R. Wilkenson, Cornell University, Ithaca/NY). RFLP analysis demonstrated that a segment from *L. peruvianum*, approximately 50 cM in length and including most of the short arm of chromosome 9, remained in line 70-546N (Fig. 3). This material was used by Dr. Munger as a source for a breeding program of seven additional backcrosses to produce Vendor-*Tm-2*. RFLP analysis of Vendor-*Tm-2* demonstrated that the size of the *L. peruvianum* segment was reduced significantly during these seven backcrosses. RFLP markers, *TG9* and *TG10*, which had both been derived from *L. peruvianum* in line 70-546N, were found to be of *L. esculentum* origin in Vendor-*Tm-2*. Thus, the size of the *L. peruvianum* segment in Vendor-*Tm-2* had been reduced to an estimated size of 26 cM during this breeding program. This result indicates that a crossover event removing a large segment of *L. peruvianum* DNA occurred during these intervening backcross generations.

Recombinants around Tm-2 selected with the visible marker ah

A visible marker, known as *ah*, is located approximately 2 cM away from *Tm-2* on the long arm of chromosome 9 (Robinson et al. 1970). The phenotype of *ah* is the absence of a characteristic purple color in the hypocotyl of young plants due to the lack of synthesis of anthocyanin. In a backcross breeding program introducing *Tm-2* into a tomato line carrying *ah*, Dr. R. Provvidenti used this visible marker to select for recombinants carrying both *Tm-2* and *ah*. The result of this marker-aided selection program was to produce a tomato line with *Tm-2*, but with an exceptionally small segment of *L. peruvianum* DNA on the long arm of chromosome 9. As shown in Fig. 4, the segment derived from *L. peruvianum* extends

less than 0.3 cM in length in this direction, shorter than any other *Tm-2-* or *Tm-2²*-containing line examined. Since no selection was practiced to removed *L. peruvianum* DNA from the other side of *Tm-2*, this portion of the introgressed segment is quite large, extending all the way to the end of the short arm of chromosome 9 (Fig. 4).

Discussion

Size of introgressed segments

RFLP analysis of the region around the *Tm-2* locus in several tomato cultivars has demonstrated that the size of introgressed segments can be rapidly and accurately estimated using a high density map of RFLP markers. Since many plant genes of interest have been introgressed into cultivated lines (Zeven and van Harten 1979) and since RFLP maps are now being developed for most cultivated plant species (Helentjaris et al. 1986; Landry et al. 1987; Tanksley et al. 1988; Apuya et al. 1988; McCouch et al. 1988), it should soon be possible to carry out an extensive survey of introgression around a large number of important plant genes.

RFLP analysis of introgression around *Tm-2* has also demonstrated that backcross breeding is only moderately effective in reducing linkage drag around gene targets. The estimated sizes of the introgressed fragments observed in these experiments ranged all the way from 4 cM to as great as 51 cM. An interesting observation is the fact that some breeding programs included large numbers of generations (>20), yet achieved relatively little reduction in linkage drag, while other programs achieved significant reduction within only a few generations. This discrepancy is clearly due to the inability to select for desirable recombinants around *Tm-2* (except by visual examination for recurrent parent-like characteristics). Our data also shows that the breeding program leading to Vendor-*Tm-2²*, in which a small introgressed segment was obtained in an early generation, continued for many additional years with no additional progress in reducing linkage drag. Again, this situation stemmed from the inability to detect recombinants near the *Tm-2* locus. In the one breeding program in which a visible genetic marker (*ah*) was used to identify recombinants near *Tm-2*, a cross-over located less than 0.3 cM away from *Tm-2* was obtained.

Comparison of linked and unlinked donor segments

In addition to linkage drag, unlinked DNA from the donor parent must also be removed during a backcross breeding program. The RFLP analysis described in this study does not give any information on how much, if any, unlinked DNA from *L. peruvianum* persists in cultivars carrying *Tm-2*. However, in order to obtain a better idea of the relative importance of linked versus unlinked donor segments in backcross breeding, we have developed a simple curve [derived from the works of Hanson (1959) and Stam and Zeven (1981)] comparing the amount of foreign DNA due to these two sources as a function of the number of backcross generations (Fig. 5). The results of this analysis demonstrate that for a hypothetical genome of 10 chromosomes of 100 cM each, the proportion of unlinked DNA derived from the donor genome is greater than that of remaining linked DNA only in the first four backcross generations. After this time, the proportion of donor DNA due to linkage drag far exceeds that from unlinked regions. In the tenth backcross generation, linked donor DNA is expected to exceed unlinked DNA by a factor of 50 and in the 20th backcross generation, linked donor DNA exceeds unlinked by a factor of more than 10^5. This simple analysis clearly emphasizes the importance of linkage drag as the preeminent problem in backcross breeding programs.

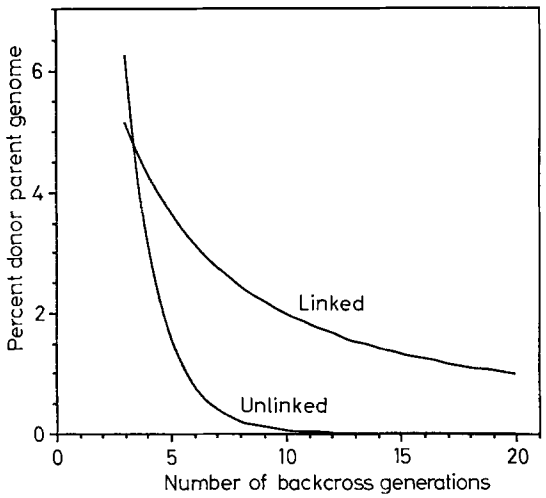


Fig. 5. Graph comparing the predicted percentage of DNA derived from the donor parent due to linked versus unlinked segments in a backcross breeding program. Values are calculated for a hypothetical genome of 10 chromosomes of 100 cM each using equations developed by Hanson (1959) and Stam and Zeven (1981)

RFLP-assisted backcross breeding

RFLP analysis is obviously a very powerful tool for determining the extent of linkage drag during backcross breeding. More importantly, RFLP analysis also provides the tool to dramatically increase the effectiveness of backcross breeding by its ability to select for desirable recombinants in the regions flanking a target gene. Unlike visible phenotypic markers, such as the *ah* marker described above, RFLPs can almost always be identified in any pair of donor and recurrent parents. And, of course, RFLPs produce no visible effect on the performance of a plant.

The mechanics of RFLP-assisted backcross breeding are quite simple. First, RFLP markers tightly linked to the target gene must be identified. Previously developed nearly isogenic lines (if available) can be extremely useful in this step and, in fact, were the means by which several of the RFLPs used in this study were uncovered (Young et al. 1988). Alternatively, a complete RFLP map can be used to analyze a population segregating for the gene of interest in order to determine its precise map location.

Once nearby RFLPs are obtained, individuals in a segregating backcross population can be scored for the target phenotype along with flanking RFLP markers. In this manner, individuals that have retained the target gene, yet also have a crossover event near the gene, can be rapidly identified. For example, a backcross population of only a few hundred individuals could be analyzed with an RFLP marker located 1 cM on one side of the target gene and the individuals with crossovers between the target gene and RFLP marker identified. Selected individuals could then be backcrossed to the recurrent parent and the progeny analyzed with another RFLP marker located 1 cM on the other side of the target gene. Thus, in only two generations, the size of the introgressed segment flanking a target gene could be reduced to only 2 cM. By comparison, traditional backcross breeding would be expected to require 100 generations to obtain such a small segment of flanking DNA.

Recently we have begun to utilize RFLP-assisted backcross breeding to rapidly develop tomato cultivars with small introgressed segments around *Tm-2* (Young et al. 1988). Recombinants on the *CD32A* (Fig. 1) side of *Tm-2* have been selected from selfed progeny of the tomato line, Craigella-*Tm-2*^{2}. In only one generation, we reduced the size of the *L. peruvianum* segment on this side of the *Tm-2* locus from more than 47 cM to less than 7. We are now backcrossing this individual to Craigella to isolate individuals with a cross over on the other side of the *Tm-2* locus.

Significantly RFLPs, as well as other neutral genetic markers, can also be used to select for individuals with the least amount of unlinked donor DNA during backcross breeding programs (Tanksley and Rick 1980). This fact could be used in conjunction with selection for crossovers near a target gene. Thus, if large backcross populations are generated and several individuals with crossovers near the target gene obtained, the recombinant individual which is best with respect to percent recurrent parent genome could simultaneously be selected.

It is likely that the most important application of RFLP-assisted backcross breeding will be in introducing desirable traits from unadapted germplasm. Although wild material often offers the greatest potential for variation and improvement of certain characters (e.g., disease resistance, stress tolerance), breeders have generally been unwilling to access this valuable resource. In general this reluctance has been due to the severe effects on phenotype that are carried along with the selected gene by linkage drag. Now that linkage drag can be minimized by the application of RFLP analysis, backcross breeding with wild germplasm may be much more feasible.

Acknowledgements. We would like to thank H. Munger, M. Mutschler, J. Prince, R. Provvidenti and M. Sorrells for their comments on the manuscript. We also wish to thank R. Messeguer for his technical assistance. This research was supported by grants United States Department of Agriculture (85-CRCR-1-1609), National Science Foundation (DCB-8517773), and by a grant from the Cornell Biotechnology Program.

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