Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2336–2340, March 1991
Genetics

Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines

(plant breeding/polymerase chain reaction/*Pto* gene/chromosome walking/linkage mapping)

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Communicated by Luis Sequeira, December 19, 1990

ABSTRACT **An approach to isolating DNA sequences that are linked to important plant genes is described. The strategy is based upon a recent modification of the polymerase chain reaction in which synthetic primers are used to amplify random sequences from genomic DNA. This technique, used in conjunction with near-isogenic lines (which differ only by the presence or absence of the target gene and a small region of surrounding DNA), leads to the rapid identification of sequences linked to the gene of interest. The feasibility of this method has been demonstrated by analyzing a pair of tomato near-isogenic lines that differ for a region on chromosome 5 that contains a gene (*Pto*) conferring resistance to *Pseudomonas syringae* pv. tomato. One hundred forty-four random primers were screened on these lines, and seven amplified products were identified that were present in one but not the other line. Of four products that were further investigated, three were confirmed by segregation analysis to be tightly linked to the *Pto* gene. Linked sequences identified by this method are useful for detecting the presence of the target gene in plant populations (e.g., in plant breeding) and, if very tightly linked, as starting points for a chromosome walk to isolate the gene. Since near-isogenic lines are a typical product of plant breeding and classical genetic studies, this method is applicable to a wide variety of species.**

Generally established techniques for gene isolation presuppose a knowledge of the transcript or the protein product of the gene (1). However, for most genes this information is not available. Knowledge of the location of a gene on a genetic linkage map offers an alternative method of gene isolation. Map-based cloning consists of three general steps. First, markers are identified that show tight genetic linkage to and that flank the target gene. Second, a "walk" to the gene is undertaken by using various genomic libraries constructed in, for example, λ or yeast artificial chromosome vectors (1, 2). Finally, confirmation requires the comparison of the isolated gene with a wild-type allele or, in the case of plants, complementation of the recessive phenotype by transformation (3). Recent successes in isolating human disease genes have validated the map-based cloning approach (2, 4).

In this paper we describe a rapid method for identifying DNA sequences that are linked to known plant genes. The approach relies on the availability of pairs of near-isogenic lines (NILs), which have been developed in many crop species by introgression (5). Introgression is accomplished by repeatedly backcrossing a line carrying a gene of interest (donor parent) to a cultivated line having otherwise desirable properties (recurrent parent). After each cross, progeny are selected that possess the phenotype of the target gene. This process results in a line that carries a small segment of the donor parent in a genetic background almost exclusively that of the recurrent parent. If the genomes of the donor parent and the recurrent parent are sufficiently divergent, it is possible to detect polymorphisms between the pair of NILs. Markers that detect such polymorphisms will likely be linked to the target gene. In fact, studies that have screened random genomic clones on pairs of NILs have been successful in identifying markers linked to two disease resistance genes in tomato (6, 7).

Recently Williams *et al.* (8) have developed a method that employs random primers in a PCR to rapidly generate polymorphic markers that can be used to create genetic linkage maps. These polymorphic markers appear as DNA segments that are amplified from one parent but not the other and are inherited in a Mendelian fashion.

We present the application of this technique to the analysis of a pair of tomato NILs. The NILs chosen differ for a region on chromosome 5 that contains a bacterial disease resistance gene (*Pto*) that was introgressed into a *Lycopersicon esculentum* cultivar from a wild species, *Lycopersicon pimpinellifolium* (9). Our objective was to identify DNA sequences that originated from the introgressed region containing the *Pto* gene. We show that three markers that were generated by random primers are polymorphic on the NILs and are, in fact, linked to the *Pto* gene. This strategy is applicable to many species for which NILs exist and allows the rapid identification of markers that may be used in plant breeding programs, for high-resolution linkage mapping, and potentially as starting points for chromosome walking.

MATERIALS AND METHODS

Plant Material and Segregation Analysis. A pair of *L. esculentum* NILs was used in this study: Rio Grande and Rio Grande-PtoR (J. Watterson, Peto Seed, Woodland, CA). Rio Grande-PtoR derives its *Pto* allele from *L. pimpinellifolium* and has undergone six backcrosses to Rio Grande and a final selfing generation. Two F_2 populations segregating for the *Pto* gene were used for linkage mapping. One population consisted of 12 F_2 individuals derived from a cross between Rio Grande and Rio Grande-PtoR. The second population, 88FW2137, consisted of 35 F_2 plants derived from a cross between a breeding line carrying the *Pto* allele and a susceptible line (J. Watterson, Peto Seed). A third population, 86T64, consisting of 80 plants that were derived from a cross between *L. esculentum* (VF36) × *Lycopersicon pennellii* (TA56), was used for high-resolution mapping of markers on chromosome 5. *Pto* was not segregating in this population.

Markers were placed on the linkage maps by using the program MAPMAKER (10). Standard errors were calculated by the maximum likelihood method (11).

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Abbreviations: NIL, near-isogenic line; RFLP, restriction fragment length polymorphism; nt, nucleotide(s); cM, centimorgans.
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Scoring Plant Reaction to *Pseudomonas syringae* pv. tomato. Seeds were sown in the greenhouse (20–25°C) in flats containing a 1:2:1 (vol/vol) mixture of peat, loam, and perlite. One week after sowing they were transferred to 1.5-liter clay pots containing the same mixture. *Pseudomonas syringae* pv. tomato strain Bakersfield (J. Watterson, Peto Seed) was grown for 48 hr at 30°C on King B agar medium and harvested with a sterile bent-glass rod into sterile water. Cell concentration was determined by a standard curve calibrated by dilution plating on King B agar medium and spectrophotometry at 590 nm. The final inoculum concentration was adjusted to 10^8 colony-forming units per ml. Plants were inoculated with the bacterial suspension at the three- to four-leaf stage by using sterile cotton swabs. Sterile water was inoculated onto a separate leaf as a control. Reaction to the pathogen was scored independently by two individuals after 6 days as either susceptible—indicated by numerous necrotic specks surrounded by chlorotic halos—or as resistant—indicated by the absence of necrotic specks on the inoculated leaf.

DNA Isolation. DNA was prepared from fresh leaves as in Murray and Thompson (12) as modified by Bernatzky and Tanksley (13).

Primers. A total of 144 primers was surveyed. The primer length in nucleotides and the number tested of each length were as follows: 18 nucleotides (nt), 2 primers; 16 nt, 10 primers; 11 nt, 3 primers; 10 nt, 41 primers; 9 nt, 82 primers; 6, 7, 8, 12, and 14 nt, 1 primer each. Primers were synthesized by standard phosphoramidite chemistry on a Du Pont Coder 300 automated DNA synthesizer. The three primers described in detail in this paper and their nucleotide sequences are as follows (5' → 3'): D47, CTCTTGCTAC; D110, CAACCACGA; D120, ATCCGCGTG.

PCR and Analysis. Amplification reactions were in volumes of 25 μl containing 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.9 mM $MgCl_2$, 0.001% gelatin, dATP, dCTP, dGTP, and dTTP (each at 0.1 mM; Pharmacia), 0.2 μM primer, 20 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Perkin–Elmer/Cetus). Amplification was performed in either a Perkin–Elmer/Cetus DNA thermal cycler or a Coy Tempcycler (model 50) programmed for 45 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C, followed by 7 min at 72°C. Reaction products were resolved by electrophoresis (1.2 V/cm) for 15 hr in a gel composed of 1% Nusieve GTG agarose (FMC) and 1% ultrapure agarose.

Hybridization. Agarose gels were blotted to Hybond-N+ (Amersham). PCR products were isolated from agarose by using DEAE membranes (1) and were labeled by the random-hexamer method (1). Hybridization conditions have been described (13).

RESULTS

Screening NILs with Random Primers. Approximately 625 discrete products, ranging from 0.3 to 4.0 kilobase pairs, were amplified by the 144 primers tested (average of 4.3 products per primer). The majority of the products were identical in both Rio Grande and Rio Grande-PtoR (data not shown). However, 7 primers produced fragments that appeared in one NIL but not the other (data not shown). We arbitrarily chose four of these primers, designated D47, D94, D110, and D120, to use in further experiments. Later experiments with D94 revealed that the polymorphic product generated by this primer mapped to a region unlinked to *Pto* on chromosome 4 [4.4 centimorgans (cM) from TG182 and 8.1 cM from TG-287 (14)], and it was not pursued further. Primers D47 and D110 each generated one product that was polymorphic between the NILs [1.8 kilobases (kb) and 2.1 kb, respectively; Fig. 1]. On the basis of the origin of the polymorphic band, these products were designated S.47 (PCR product amplified from susceptible line, Rio Grande) and R.110 (product amplified from resistant line, Rio Grande-PtoR). Primer D120 produced two polymorphic products at 1.2 kb (R.120) and 1.4 kb (S.120).

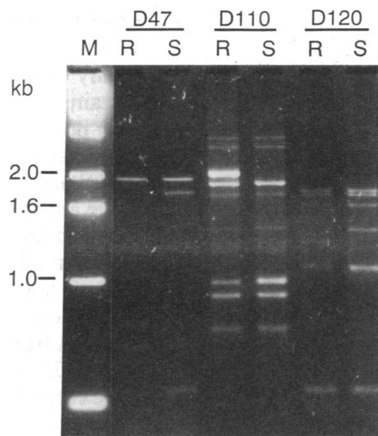

FIG. 1. Amplification of genomic DNA from the *Pto* NILs using primers D47, D110, and D120. Twenty nanograms of total genomic DNA from Rio Grande-PtoR (R, resistant to *P. syringae* pv. tomato) or Rio Grande (S, susceptible) was amplified by using the primer indicated above each pair of lanes. Lane M, molecular size markers.

Confirmation That Polymorphic PCR Products Originate from Introgressed Regions. To confirm that the products S.47, R.110, and R/S.120 originated from DNA introgressed from *L. pimpinellifolium* (or *L. esculentum* DNA that is colinear to this region) and to convert them to restriction fragment length polymorphisms (RFLPs) for mapping, we radiolabeled the polymorphic PCR products and hybridized them to filters containing DNA from the NILs digested with six restriction enzymes (Fig. 2). On the basis of the hybridization results, R.110 was found to be a single-copy sequence that detects an RFLP between the NILs in three of the restriction digests (*Bst*NI, *Hin*dIII, and *Eco*RI; Fig. 2).

The observation that D120 generates two different sized PCR products between the NILs raised the possibility that these fragments would map to the same locus (i.e., that they are allelic). This was confirmed by the fact that R.120 and S.120 hybridized to identically sized restriction fragments and detected the same RFLPs (with *Hae* III and *Eco*RI; data not shown). Moreover, subsequent segregation analysis showed that both products map to the same locus (see below). Thus, in this case, the polymorphism apparently

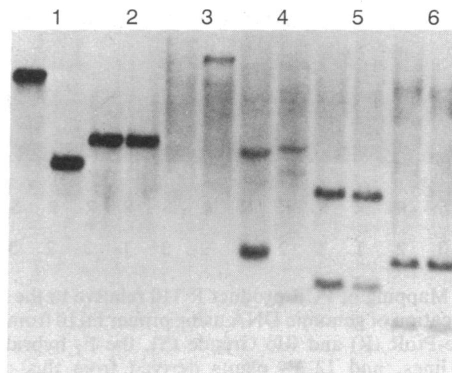

FIG. 2. Hybridization of radiolabeled PCR product R.110 to DNA from Rio Grande and Rio Grande-PtoR. The survey consists of pairs of DNA samples from Rio Grande-PtoR (left lane in each pair) and Rio Grande (right lane). Three micrograms of DNA was digested with one of six restriction enzymes, separated on a 1.0% agarose gel, and blotted onto Hybond-N+. Lanes: 1, *Bst*NI; 2, *Hae* III; 3, *Hin*dIII; 4, *Eco*RI; 5, *Eco*RV; and 6, *Xba* I.

results from an insertion or deletion event between the two primer annealing sites.

The S.47 product hybridized to many sequences in the tomato genome, indicating that it contains a repetitive element, and we were unable to detect any polymorphism even after a higher stringency wash (0.01× standard saline citrate).

Linkage to *Pto*. To confirm putative linkage between S.47, R.110, and R/S.120 and the *Pto* gene, we analyzed two small F_2 populations (a total of 47 plants) segregating for *Pseudomonas* resistance. The first population derived from a cross between Rio Grande and Rio Grande-PtoR and contained 12 plants that had been scored for their reaction to *P. syringae* pv. tomato Bakersfield strain (Fig. 3). DNA from the parents of this population, an F_1 plant, and the 12 F_2 plants was analyzed by both Southern blotting (except for S.47) and PCR. All three PCR-derived markers were found to be cosegregating, and all showed significant linkage to *Pto* (χ^2 = 4.79, $P < 0.05$). Data for R.110 are presented in Fig. 3. The recombinational distance between the three markers and *Pto*, based upon this small population, was estimated to be 19.7 ± 13.2 cM.

A second *Pto*-segregating F_2 population (88FW2137) consisting of 35 plants was also examined (data not shown). In this population R.110 was determined to be 11.1 ± 7.7 cM from *Pto*. We also used this population to analyze the linkage of another RFLP marker, TG96, which we had previously mapped to chromosome 5 (15) and which had shown linkage to *Pto* in other populations. TG96 mapped on the same side of *Pto* as R.110 and was estimated to be 6.8 ± 17.4 cM from the disease resistance gene. These data support earlier classical mapping, which had placed *Pto* on chromosome 5 (9).

Placement of PCR-Derived Markers on a High-Resolution RFLP Map of Chromosome 5. Because our two F_2 populations segregating for *Pto* contained relatively few individuals (47 plants) and the resulting genetic distances had large standard errors, we wished to more accurately place these markers in the tomato genome. For this purpose, we used an F_2 population consisting of 80 individuals on which more than 800 RFLP markers now have been placed with an average distance between markers of 2 cM (S.D.T., unpublished results). Results from this analysis placed markers R.110, R.120, and S.120 (all of which cosegregated) on chromosome 5, 1.1 ± 1.3 cM from TG96 in a region flanked by CD41 and TG358 (Fig. 4). The two markers CD41 and TG358 have been found to delimit the introgressed segment from *L. pimpinellifolium* in the NIL Rio Grande-PtoR (Fig. 4; G.B.M., unpublished results). S.47 could not be placed on the high-resolution map due to the repetitive nature of this sequence in the tomato genome.

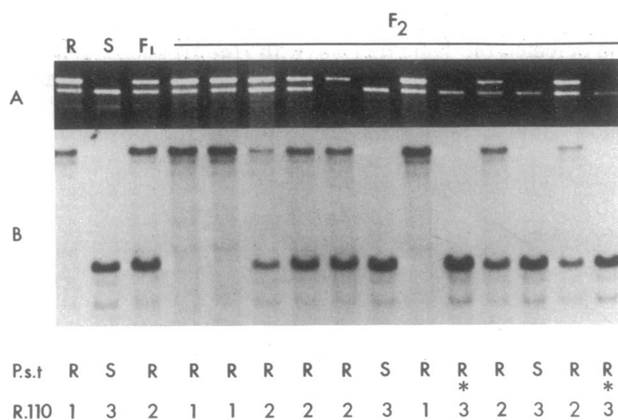

FIG. 3. Mapping of PCR product R.110 relative to the *Pto* gene. (*A*) Amplification of genomic DNA using primer D110 from the NILs Rio Grande-PtoR (R) and Rio Grande (S), the F_1 hybrid between these two lines, and 12 F_2 plants derived from this cross. (*B*) Hybridization of the PCR product R.110 (upper band in *A*) to a Southern blot containing DNA from the same plants as in *A* that had been digested with *Bst*NI. The reaction of each plant to *P. syringae* pv. tomato (P.s.t.) inoculation is shown (R, resistant; S, susceptible) as is the genotype for R.110 of the individual plants (1, *L. pimpinellifolium*/*L. pimpinellifolium*; 2, *L. pimpinellifolium*/*L. esculentum*; 3, *L. esculentum*/*L. esculentum*). Recombinant individuals are denoted by an asterisk.

DISCUSSION

To reliably detect a target gene by RFLP analysis in plant populations (e.g., in plant breeding) or for high-resolution linkage mapping in preparation for chromosome walking, it is necessary to identify linked markers. Finding such linked markers can require screening many clones. We describe a strategy for rapid identification of markers linked to important genes that is based upon the amplification of random sequences from genomic DNA (8). An application of this strategy to a pair of tomato NILs has succeeded in identifying 3 additional markers that are linked to a gene conferring resistance to the pathogen *P. syringae* pv. tomato. Considering that we surveyed 144 primers, these three "hits" represent a 2% success rate. We have tried other methods of identifying markers linked to the *Pto* gene, including hybridizing random genomic clones onto NIL pairs (6) and following up RFLP markers that have been placed to chromosome 5 through our ongoing mapping efforts. The random clone approach has yielded 3 *Pto*-linked markers from a total pool of over 600 clones (0.5% success). Five *Pto*-linked markers have been confirmed from the general mapping program, which has placed over 800 markers in the tomato genome (0.6% success). These efforts have taken approximately 2

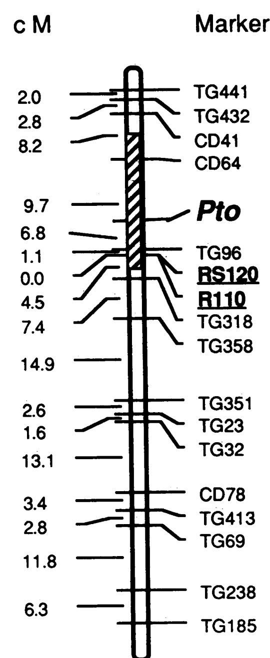

FIG. 4. RFLP map of chromosome 5 of tomato showing the location of the *Pto* gene and linked markers. A recombinational linkage map of chromosome 5 was developed by segregation analysis of RFLPs of PCR products R.110, R/S.120, TG96, and other genomic clones (TG) and cDNA clones (CD). All distances are given as cM and were derived from the VF36 × TA56 cross (14), except for the distance between TG96 and *Pto*, which was calculated from the cross 88FW2137 (see *Materials and Methods*). The hatched region represents the chromosomal segment derived from *L. pimpinellifolium* that is present in the NIL Rio Grande-PtoR; the white regions represent segments from *L. esculentum*. Analysis of RFLP markers flanking TG96 provided the information on the size of the introgressed segment around the *Pto* gene.

years. In contrast, the identification and confirmation of the 3 markers presented here has taken about 1 month—considerably less effort than other strategies. Moreover, an almost limitless number of additional random primers can be synthesized and tested on these NILs to find more markers.

In the experiments described in this paper, we screened an arbitrary number of random primers and then determined how many PCR products could be identified that are linked to the target gene. However, it is also possible to estimate, *a priori*, the number of primers that would need to be screened on average in order to have a high probability of finding at least one marker within a specified distance from the target gene. Fig. 5 depicts the average expected distance between the target gene and the closest PCR-detected marker for various numbers of screened primers. If each primer generates four products (as observed in these experiments), originating from independent genomic sites, we estimate that 100 primers would yield a marker within an expected distance of 1.9 cM from any target gene in tomato. For this same number of primers, the upper 95% confidence limit is 5.6 cM. If the number of primers is increased to 500, the values drop to 0.4 cM and 1.1 cM, respectively. Although there is likely to be heterogeneity in the relationship between map units and physical distance in different regions of the genome, in tomato these genetic distances correspond to an average of 190 kb and 525 kb, respectively, based on a *C* value of 700 megabases (16). These distances are within the insert size range of yeast artificial chromosome vectors that may be used for chromosome walking (1).

The probability of obtaining a marker within a specified distance of a targeted gene in NILs depends not only on the number of primers screened but also on the genome size in map units and the degree of DNA sequence divergence between the NILs in the region surrounding the targeted gene. If the sequences are very similar, a primer is less likely to detect PCR polymorphism, even if the priming event takes place near the targeted gene. The plot in Fig. 5 assumes that 100% of the priming events in the vicinity of the target gene in the NILs will result in a detectable PCR polymorphism. The expected and 95% confidence distance obtained with a given number of primers increases proportionally as the percentage of detectable polymorphisms decreases. An increase in the genome size will also result in proportional increases in the estimated distances.

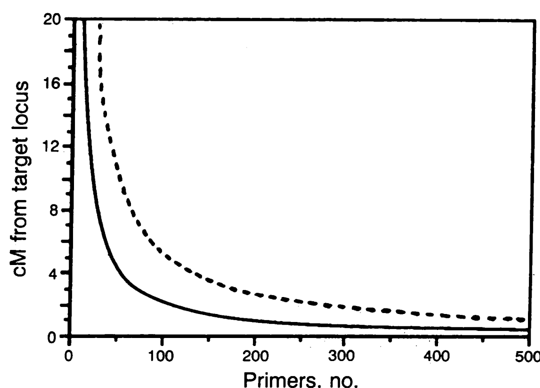

FIG. 5. Number of random primers versus distance from the target locus to closest PCR-derived marker. Expected minimum distance (solid line) = $c/2(nx + 1)$, where c = genome size in cM (1500 for tomato), n = number of primers, x = average number of PCR products per primer (four, based on results presented in this paper). The distance at a 95% confidence level (dashed line) = $(c/2)(1 - 0.05^{1/nx})$. Calculations assume the target locus is not at the very end of a chromosome and a random distribution of PCR-derived sequences. See the *Appendix* for derivation of formulas.

In the case of the *Pto* NILs, the tightest confirmed linkage was ≈8 cM (Fig. 4), which is greater than the expected (1.3 cM) or the 95% confidence estimates (3.9 cM). This difference between the expected and observed distances can probably be attributed to the existence of less than 100% detectable polymorphism between the NILs in the introgressed region since *L. esculentum* and *L. pimpinellifolium* DNA share a relatively high degree of sequence similarity (17). There is also a possibility that one of the remaining three polymorphic PCR products that appeared in one NIL but not the other will be more tightly linked to *Pto*.

It is important to note that NILs vary greatly in the degree to which both linked and nonlinked donor DNA has been removed by backcrossing. The expected rate of recovery of the recurrent parent is given by the function $1 - (1/2)^t$, where t is the number of backcross generations plus the final selfing generation. Thus after six backcrosses and selfing (as have occurred with the *Pto* NILs), there theoretically exists just 0.8% donor parent DNA. However, in the few cases examined, the actual proportion of donor DNA remaining has been found to vary considerably from this prediction (18). The line Rio Grande-PtoR that we have used still carries donor DNA segments on chromosome 11 (G.B.M., unpublished results), and this study found that another region of donor DNA still exists on chromosome 4 (detected by primer D94). On the basis of these observations, it is clear that segregation analysis is necessary in order to confirm linkage to the target gene.

Because of the costs associated with conducting PCR on large numbers of individuals, it may make sense to rely on PCR only for the initial surveying of random primers. Once a polymorphic PCR product is identified, it can be isolated (or cloned) and then used as a standard RFLP marker. In addition to reducing costs, this would permit using the marker on new populations segregating for the target gene, which may, however, not carry the same primer-annealing sequences as the line from which the product was originally amplified.

NILs have been developed by plant breeders in many crop species (5). In tomato a set of over 130 NILs exists, which differ for genes involved in fruit color, plant habit, and disease resistance (19). Similar extensive genetic stocks are available in soybean (20) and barley (21). In addition, NILs have been developed in other organisms such as mouse (22) and various fungi (23). This strategy may therefore be applicable to many species for which map-based cloning techniques are being developed.

APPENDIX

Derivation of Formulas for Expected Value and 95th Percentile of Distance from Target Locus to Closest PCR-Derived Marker. Let the random variable X denote the distance from a randomly located point to the target locus, and let Y denote the distance from the closest of nx of these random points to the target locus, where n = number of primers and x = average number of PCR products per primer. Then X is uniformly distributed on the interval $(0, c/2)$, where c is the genome size in cM, and

$$P(Y > d) = P(\text{all } nx \ X \text{ values exceed } d) = \left(1 - \frac{2d}{c}\right)^{nx}$$

for any value d between 0 and $c/2$. The cumulative distribution function (*cdf*) of Y is then

$$F_Y(d) = P(Y \leq d) = \left(1 - \frac{2d}{c}\right)^{nx}$$

for any value d between 0 and $c/2$, so the probability density function (pdf) of Y is

$$f_Y(d) = F_{Y'}(d) = (2nx/c)\left(1 - \frac{2d}{c}\right)^{nx-1}$$

for all d between 0 and $c/2$.

The expected value of Y is the integral

$$\int_0^{c/2} y f_Y(y)dy,$$

which is easily transformed to a beta function (see ref. 24), giving

$$E(Y) = (nxc/2)B(2, nx) = \frac{c}{2(nx+1)}.$$

The 95th percentile of Y is the value w that satisfies the equation

$$0.95 = \int_0^{w} f_Y(y)dy = \left[-\left(1 - \frac{2y}{c}\right)^{nx}\right]_0^w = 1 - \left(1 - \frac{2w}{c}\right)^{nx}.$$

Solving for w gives $w = (c/2)(1 - 0.05^{1/nx})$.

We thank Dr. Jon Watterson and Dr. Scott Tingey for their generous assistance during this project, Dr. Steven Schwager for deriving the formulas used in Fig. 5 and given in *Appendix* 1, and Jim Prince and Dr. Gillian Turgeon for comments on the manuscript. This work was supported by grants from U.S. Department of Agriculture Competitive Grant 88-37262-3921 and U.S./Israel Binational Agricultural Research and Development Fund Grant US-1388-87 to S.D.T. G.B.M. was supported by a National Science Foundation Postdoctoral Research Fellowship in Plant Biology.

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