

# Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*

(genetic map/Brassicaceae)

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**ABSTRACT** We have constructed a restriction fragment length polymorphism linkage map for the nuclear genome of the flowering plant *Arabidopsis thaliana*. The map, containing 90 randomly distributed molecular markers, is physically very dense; >50% of the genome is within 1.9 centimorgans, or ≈270 kilobase pairs, of the mapped DNA fragments. The map was based on the meiotic segregation of markers in two different crosses. The restriction fragment length polymorphism linkage groups were integrated with the five classically mapped linkage groups by virtue of mapped mutations included in these crosses. Markers consist of both cloned *Arabidopsis* genes and random low-copy-number genomic DNA clones that are able to detect polymorphisms with the restriction enzymes *EcoRI*, *Bgl* II, and/or *Xba* I. These cloned markers can serve as starting points for chromosome walking, allowing for the isolation of *Arabidopsis* genes of known map location. The restriction fragment length polymorphism map also can associate clones of unknown gene function with mutant phenotypes, and vice versa.

Cloning of genes known only by mutant phenotype and genetic map position can be accomplished by successively isolating overlapping clones from a DNA library until the desired locus is reached (1, 2). This approach, known as "chromosome walking," is facilitated by having starting clones in close proximity to the endpoints of interest. To provide such starting points in the flowering plant *Arabidopsis thaliana*, we have constructed a genetic linkage map of the *Arabidopsis* nuclear genome consisting of 90 molecular markers that are distinguished on the basis of restriction fragment length polymorphisms (RFLPs). RFLPs are codominant, typically neutral, genetic markers. They are provided in abundance by differences between allelic DNA sequences that result from nucleotide substitution, rearrangement, insertion, or deletion. Accordingly, they can be used to construct detailed genetic linkage maps, follow inheritance of genetic diseases and other heritable traits, and examine variation between and within populations (3-5). This RFLP linkage map consequently enhances the utility of *Arabidopsis* for experiments in plant molecular genetics.

Previously recognized attributes of *Arabidopsis* include an extremely small genome, rapid generation time, fecundity, ease of growth, and an extensive background of classical genetic analysis (6). Chromosome walking in *Arabidopsis* should be facilitated by the small genome and virtual absence of dispersed repetitive DNA sequences [an average spacing of 125 kilobases (kb) between repeats as compared to 1.4 kb in tobacco and 0.3 kb in pea (6)]. The end of a chromosomal walk in *Arabidopsis* can be determined by complementation

of the mutant phenotype through transformation (7). Complementation has been demonstrated in an *Arabidopsis* alcohol dehydrogenase null mutant transformed with the *Arabidopsis* alcohol dehydrogenase gene (C.C. and E.M.M., unpublished work). Numerous mutations affecting a range of developmental processes, various enzyme activities, and hormone synthesis and response have been isolated in *Arabidopsis*, and approximately 80 of these mutations have been ordered into a genetic linkage map (8, 9). The RFLP map is consistent with this map, and the two have been partly integrated.

Over half of the genome is within 1.9 centimorgans (cM) of the RFLP markers on the map. On average, 1 cM in the *Arabidopsis* genome represents 140 kb, since the genome consists of 501 cM and ≈70,000 kb (10).

## MATERIALS AND METHODS

**Cloned *Arabidopsis* Genes.** Cloned *Arabidopsis* genes used as RFLP markers were kindly provided by the following individuals: chalcone synthase ( $\lambda$ CHS2) by R. Feinbaum and F. Ausubel (Massachusetts General Hospital); nitrate reductase ( $\lambda$ At-24) (11) by N. Crawford (University of California, San Diego); actin (pAtc4) by R. Ferl (University of Florida); phytochrome (Aph.Ara.1) by R. Sharrock, C. Gatz, and P. Quail (U.S. Department of Agriculture, Albany, CA); and acetolactate synthase (pGH1) (12) by G. Haughn and C. Somerville (Michigan State University). The following cloned genes were from this laboratory:  $\lambda$ At3012 (alcohol dehydrogenase) (13), sAt2105 (12S seed storage protein), and nAt1511 (small seed RNA-coding) (P. Pang, R. Pruitt, and E.M.M., unpublished results). The remaining mapped fragments were chosen from a genomic DNA library.

***Arabidopsis* Strains.** Ecotypes Niederzenz (Nd-0) (14), Landsberg (La-0) (15), and Columbia (Col-0) (14), and mutations, *an* (16), *gl-1* (16), *tz* (17), *er* (18), *ap-2* (19), and *clv-1* (7), have been described. To perform crosses, flowers of the female recipient were hand-emasculated and anthers from the donor were applied to the stigmatic surface of the recipient.

**Library Screening.** The *Arabidopsis* (Col-0) genomic  $\lambda$  library was screened by standard techniques (20).

**Isolation of DNA.** Rapid  $\lambda$  DNA preparations were as described in ref. 21, except that the clear lysate was incubated with 1  $\mu$ l of DNase I (1 mg/ml) and 1  $\mu$ l of RNase A (1 mg/ml) for 20 min at 37°C and then incubated with 5  $\mu$ l of diethylpyrocarbonate for 5 min at room temperature, prior to treatment with NaDodSO<sub>4</sub>.

Whole-plant DNA was extracted from 3- to 6-week-old plants by either of two methods described in refs. 22 and 23.

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Abbreviations: RFLP, restriction fragment length polymorphism; cM, centimorgan(s).

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The procedure in ref. 23 was modified as follows. Stirring of the lysis mixture and the first phenol/chloroform extraction were omitted. Instead, after adding the NaDodSO<sub>4</sub>, the mixture was Vortex mixed and incubated at 65°C for 10 min. Two extractions were performed using a 2:1 phenol/chloroform molar ratio. The DNA was precipitated in 1 vol of 1 M KOAc/isopropanol at room temperature. The DNA was centrifuged ( $2.55 \times 10^5 \times g$  for 20 hr) in a CsCl gradient and the CsCl was removed by ethanol precipitation of the DNA.

**Genome Blot Hybridizations.** Whole-plant DNA (0.5–2.0  $\mu$ g) was digested with 10–20 units of restriction enzyme (*EcoRI*, *Xba* I, or *Bgl* II) and 1 mM spermidine (Sigma) for 2–4 hr and then subjected to gel electrophoresis in 0.8% agarose gels at 0.5–2 V/cm until the orange G (Sigma) loading dye migrated  $\approx$ 13 cm. (The digested DNA was loaded into  $5 \times 1.5 \times 5$  mm wells.) Gels were blotted and the DNA was cross-linked to Hybond-N filters (Amersham) according to the manufacturer's instructions. <sup>32</sup>P-labeled DNA probes ( $2-3 \times 10^8$  cpm/ $\mu$ g) were produced by nick-translation (24) of  $\lambda$  clone DNA (21). Filters were prehybridized for 1–10 hr and hybridized ( $10^6-10^7$  cpm/ml) for 18–48 hr at 65°C in a solution containing  $5 \times$  SSPE (900 mM NaCl/50 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O/40 mM NaOH/5 mM Na<sub>2</sub>EDTA),  $5 \times$  Denhardt's solution (1 $\times$  Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), 0.5% NaDodSO<sub>4</sub>, and denatured salmon sperm DNA (20  $\mu$ g/ml). Filters were washed at 65°C in  $2 \times$  SSPE for 30 min, in  $2 \times$  SSPE with 0.1% NaDodSO<sub>4</sub> for 30 min, and in  $0.1 \times$  SSPE for 15 min. Preflashed Kodak XAR-5 film was exposed to filters for 1–7 days at  $-70^\circ\text{C}$  with an intensifying screen. Filters were re-used up to 30 times after removing the previous probe by submerging filters in boiling 0.1% NaDodSO<sub>4</sub>.

Most of the detected RFLPs were distinctly resolved codominant alleles. However, 12% of all RFLPs were the dominant/null allele type in which there are new (or absent) fragment(s) in one of the lines with no apparent alterations in any of the other fragment lengths. These polymorphisms probably did not result from sequence duplications since polymorphisms were not revealed by all three enzymes. Most likely, they are caused by the presence of a common restriction site within and a polymorphic restriction site outside of the probe sequence; if the polymorphic site is far enough from the probe sequence to produce a restriction fragment that is larger than the mean size of the isolated DNA, then it will not be detected. These RFLPs were not mapped or included in the calculations of RFLP frequencies given in the results.

**Linkage Analysis.** Multipoint linkage analysis was performed by using the MAPMAKER computer program (25, 26). Initially two-point linkage analysis was performed to determine the maximum likelihood recombination fraction and the associated lod score for each pair of loci. The lod score reflects the degree of support for linkage (27) and is defined as the  $\log_{10}$  of the ratio of the probability that the data would arise if the loci were actually linked at a given recombination fraction divided by the probability that the data would arise if the loci were unlinked. Pairs of loci were considered linked if the lod score exceeded 3.0. (The traditional threshold for declaring linkage in human genetics is a lod score of 3.0. Since the *Arabidopsis* genome is roughly seven times smaller in genetic length than the human genome, the use of a lod score threshold of 3.0 in this study is quite conservative.) Once the linkage groups were assembled, three-point and subsequently *n*-point linkage analysis was performed on each linkage group.

## RESULTS

Construction of the *Arabidopsis* RFLP map involved the following three steps: (i) collecting and identifying potential

RFLP markers, (ii) performing the genetic crosses, and (iii) determining the linear order of RFLP loci and the map distances between them by examining segregation of the RFLPs.

**Obtaining RFLP Markers.** Cloned inserts for the first step should be large and have a low copy number in the genome to increase the probability of detecting polymorphisms while at the same time avoiding RFLPs at multiple loci. Both these criteria are met by large genomic clones of *Arabidopsis* due to the low amount of dispersed repetitive sequences. Our primary source of RFLP clone candidates was a collection of random low-copy-number clones obtained by screening (20) an *Arabidopsis* total genome  $\lambda$  library (22) (estimated average insert size of 12.5 kb). The probe was total *Arabidopsis* DNA, and an internal  $\lambda$  vector DNA sequence (to detect nonrecombinant phage), that had been <sup>32</sup>P-labeled by nick-translation (24). In agreement with the proportion predicted by genome studies (10, 22), 60% of the screened recombinant clones gave either a weak or undetectable signal, presumably because they represent sequences of low copy number in the nuclear genome. These clones were isolated and rescreened by either plaque screening or clone DNA blotting (20), resulting in the isolation of 280 low-copy-number clones. We also used 18 single-copy *Arabidopsis* clones (isolated from the same DNA library (22)), and 8 cloned *Arabidopsis* genes.

To screen plant DNA for the presence of RFLPs, the clones were used as <sup>32</sup>P-labeled probes in hybridizations to gel blots containing genomic DNA of three *Arabidopsis* strains, digested separately with one of three restriction enzymes (Fig. 1). The following strains were used: (i) Niederzenz (Nd-0); (ii) a triply marked line (which we designate as C) in a background that is largely Columbia (Col-0) carrying the recessive visible mutations *angustifolia* (*an*), *glabra-1* (*gl-1*), and thiazole requiring (*tz*); and (iii) Landsberg (*La-0*) carrying the recessive visible mutation *erecta* (*er*). [Nearly all the available and mapped mutations in *Arabidopsis* have been induced in Landsberg *erecta* (8).] By using 202 clones, we screened all three lines for RFLPs that

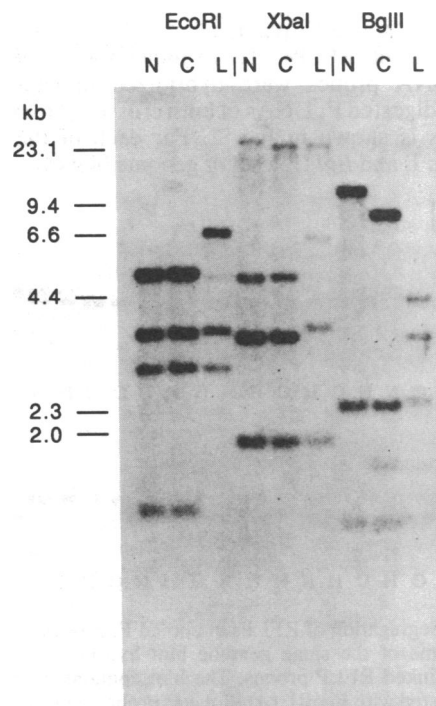


FIG. 1. Autoradiogram of a typical genomic blot to screen for RFLPs between parental lines. The figure shows several RFLPs detected by clone 305 hybridized to Nd-0 (N), C (C), and *La-0* (*er*) DNA digested with *EcoRI*, *Xba* I, or *Bgl* II.

are revealed by at least one of the three enzymes *EcoRI*, *Xba* I, and *Bgl* II. Nd-0 and C were screened with 23 additional clones, and C and La-0 *er* were screened with 3 additional clones. The overall frequency of clones that detected RFLPs with at least one of the three enzymes for at least two pairs of lines was 54%; the frequency was 37% between Nd-0 and C, 46% between Nd-0 and La-0 *er*, and 39% between C and La-0 *er*. The frequency of the clones detecting fragments of different lengths in all three strains was 15%. The three restriction enzymes were found to be equally useful for RFLP detection.

A survey of all RFLPs (including dominant/null types) indicated that Nd-0 and La-0 *er* differ in  $\approx 1.4\%$  of the nucleotides in their low-copy-number genomic DNA, C and Nd-0 in  $\approx 1.3\%$  in their low-copy-number genomic DNA, and C and La-0 *er* in  $\approx 1.1\%$  of their low-copy-number genomic DNA. In this calculation we assumed that (i) there is one nucleotide substitution per RFLP, (ii) there are four restriction sites detected by a probe per enzyme, and (iii) all sites are nonoverlapping. The above results suggest that a cross between any two of the three strains is suitable for RFLP mapping.

**Genetic Crosses.** Our RFLP map is based on two crosses, each including a set of visible mutations for the purpose of positioning RFLP linkage groups relative to the five linkage groups established by mapping morphological and biochemical mutations. One of the two crosses was between Nd-0 and the triple mutant line C with mutations on chromosomes 1, 3, and 5. The other cross was between Nd-0 and La-0 with the recessive visible mutations *clavata-1* (*clv-1*), *er*, and *apetala-2* (*ap-2*) on chromosomes 1, 2, and 4, respectively. For each cross, the resulting  $F_1$  plants were allowed to self-pollinate to produce  $F_2$  individuals.  $F_2$  plants were allowed to self-pollinate to each produce a pool of  $F_3$  plants. Although segregation of RFLPs is present in  $F_2$  individuals, the  $F_3$  plants enabled us to score the genotypes of  $F_2$  plants for the recessive visible markers. DNA was prepared from pools of 15 or more  $F_3$  plants per  $F_2$  individual, providing a greater amount of tissue, and thus of DNA, than would have been provided by a single  $F_2$  plant.

**Segregation of RFLPs.** To examine the segregation of RFLPs, DNA probes were hybridized to genome blots containing digested  $F_3$  DNAs of both crosses. An example for two probes is shown in Fig. 2. For each of the enzymes *EcoRI*, *Xba* I, and *Bgl* II, a set of genome blots was prepared

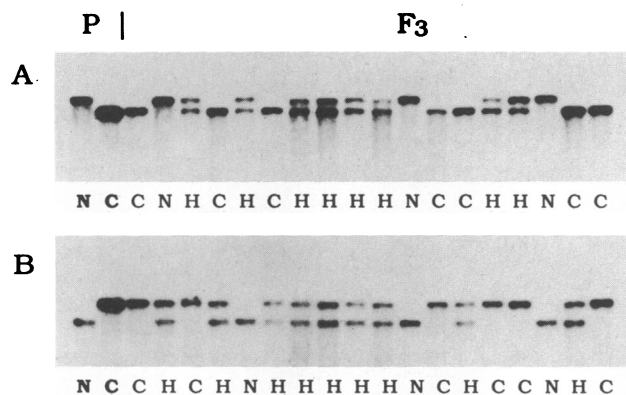


FIG. 2. Segregation of RFLPs in pooled  $F_3$  progeny is shown in autoradiograms of the same genome blot hybridized sequentially with two unlinked RFLP probes. The blot contains genomic DNA samples digested with *EcoRI*. (A) Blot was probed with clone 322. (B) Blot was probed with clone 214. The first and second lanes contain DNA of the parental lines Nd-0 (N) and C (C), respectively. The 18 lanes to the right contain DNA of pooled  $F_3$  progeny derived from the cross between Nd-0 and C, scored as either N, C, or H (heterozygous).

by using the  $F_3$  DNAs of each cross. RFLP probes were hybridized sequentially to filters of the appropriate digest and cross. Each  $F_3$  pool was scored with respect to the parental DNA restriction patterns as being homozygous for one of the parental types, heterozygous, or unscorable for technical reasons. By using the Nd-0  $\times$  C cross alone, for which 106  $F_3$  pools were scored, 41 RFLPs were mapped. By using the Nd-0  $\times$  La-0 cross alone, for which 119  $F_3$  pools were scored, 25 RFLPs were mapped. To align linkage groups of clones mapped in the separate crosses, 24 additional markers were mapped in both crosses. The resolution afforded by 225  $F_3$  pools is 0.22 cM—i.e., one recombinant divided by 450 chromatids, which represents on average 30 kb in the *Ara-bidopsis* genome.

All but two of the clones appeared to detect single loci. Clone nAt1511 (which contains a gene encoding an abundant seed-specific mRNA) hybridized to two pairs of segregating RFLP alleles; one RFLP was within the cloned copy and the other was within an uncloned homologous sequence whose existence was indicated by a comparison between genome blots and the restriction map of the clone. We mapped both RFLPs and found them to be closely linked. Probe 281 hybridized to two polymorphic fragments that mapped to different chromosomes. This probe may hybridize to nonadjacent members of a gene family or may contain two unrelated sequences that were ligated together during the library construction. When such markers are used to initiate a chromosome walk, a clone specific to the RFLP locus of interest must be obtained.

**Linkage Analysis.** The genetic map was constructed by using the MAPMAKER computer program (25, 26) to analyze the combined data from the two crosses. Two-point linkage analysis was first performed to determine the maximum likelihood recombination fraction and the associated lod score (27) for each of the 4560 pairs of the 96 loci. Pairs of loci were considered linked when the lod score exceeded 3.0. The 96 loci fell into five linkage groups that were assigned to the five chromosomes by virtue of the visible markers with previously assigned genetic positions.

Three-point linkage analysis was next used to narrow down possible genetic orders for the loci (a-b-c, a-c-b, b-a-c). For each order, we computed the likelihood that the data would have arisen given the maximum likelihood three-point map for that order. A particular three-point order was ruled out if some alternative order for the loci was at least 100,000 times more likely to have given rise to the data. For each linkage group, we determined all genetic orders that contained no suborders that had been ruled out by the three-point analysis. Typically, some 20–50 genetic orders for the linkage group met this criterion.

Finally, full  $n$ -point linkage analysis was performed for each remaining genetic order to compute the most likely recombination fractions and the chance that the map would have given rise to the data. Genetic orders were discarded that were at least 1000 times less likely to give rise to the data.

A single genetic order emerged for each chromosome, which was unique up to the possible uncertainty in the order of certain pairs of loci separated by  $<4$  cM. In the final map, shown in Fig. 3, map distances have been corrected for the discrepancy between actual crossovers and the observed recombination frequencies by using the Kosambi (28) function. Any order that is supported by odds of  $<1000:1$  is indicated. As a confirmation, the entire process was repeated with just the data for the Nd-0  $\times$  C cross alone and the Nd-0  $\times$  La-0 cross alone; the resulting genetic orders and maps were consistent with the map shown in Fig. 3.

The orientation of RFLP chromosomes 1 and 5 with respect to the standard map has been determined by using two visible markers on each chromosome: *an* and *clv-1* on chromosome 1, and a recessive visible mutation *pistillata*

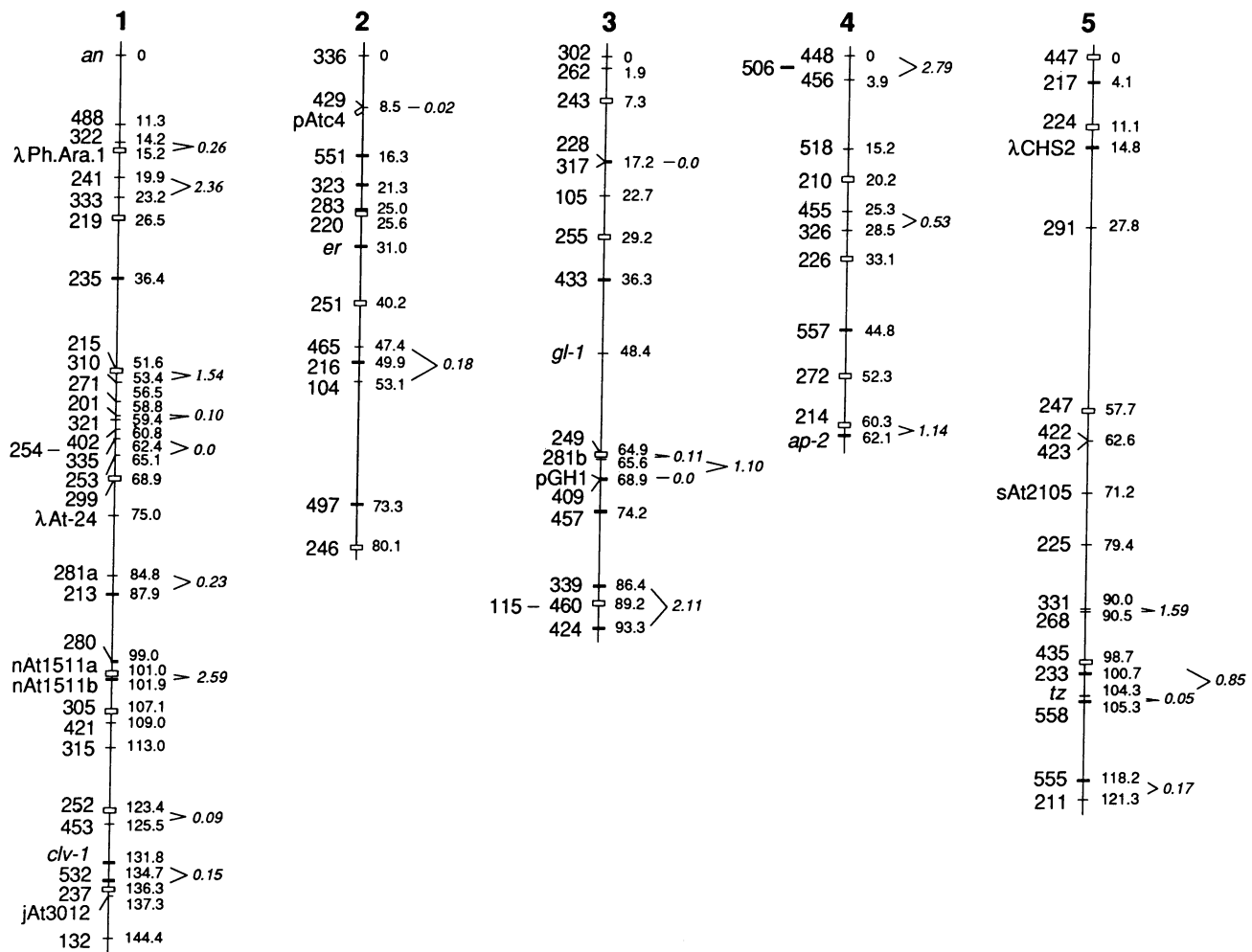


FIG. 3. An RFLP linkage map of the five chromosomes of the *Arabidopsis thaliana* genome. Map distances are shown in centimorgans. (We arbitrarily assigned a position of zero to the top-most marker on each chromosome.) Markers are designated by a clone number for the random low-copy-number clones and by a clone name for the known genes. The letter a or b after the marker name indicates that the clone detects more than one map locus. Markers scored in both crosses are indicated by an open box. Markers scored only in the cross between Nd-0 and La-0 are indicated by a solid box. The remaining markers were scored only in the cross between Nd-0 and C. Markers, placed in an order that is <1000 times more likely to have given rise to the data than any alternative order, are bracketed to the right of the map distances; the numbers by the brackets are the  $\log_{10}$  of likelihood ratio of the order shown to the next most likely alternative order. In three separate cases (115, 254, and 506), the order of markers could not be resolved—i.e., the likelihood for any of the possible intervals was virtually the same. The approximate positions of these markers are indicated to the left of the other markers.

from a third cross (J.L.B. and E.M.M., unpublished results) and *tz* on chromosome 5. Chromosomes 3 and 4 are shown in the orientation most likely to agree with the standard map based on the map position of an acetolactate synthase (ALS) mutation, chlorsulfuron resistance, on chromosome 3 (ref. 29; G. W. Haughn and C. Somerville, personal communication) for which we have mapped the ALS gene clone and on the distal position of *ap-2* on chromosome 4. The orientation of chromosome 2 relative to the standard map is not known.

### DISCUSSION

We have constructed an RFLP linkage map of 90 molecular markers that can be integrated with the standard linkage map of approximately 80 mutation markers. The RFLP map contains linkage groups of approximately 144, 80, 93, 62, and 121 cM for a total of 501 cM, which corresponds well with the standard genetic map showing linkage groups of 126, 51, 91, 69, and 100 cM for a total of 437 cM (9). The positions of the visible markers that are common to both maps are also in essential agreement. On the standard mutation map, *an* and *clv-1* are on chromosome 1 at positions 0 and 114.6 cM, respectively, *er* is on chromosome 2 at 15.9 cM, *gl-1* is on

chromosome 3 at 40.9 cM, *ap-2* is on chromosome 4 at 58.6 cM, and *tz* is on chromosome 5 at 82.4 cM. The RFLP map is also consistent with the cytological karyotype (30, 31). The positions of centromeres in the RFLP map might be determined by the use of existing telotrisomic lines (8). One reason this is of interest is that the C-banded heterochromatin associated with the centromeres is likely to present a barrier to chromosome walks across centromeres.

RFLP mapping of cloned genes (which have been obtained by other means) may associate the genes with mapped loci that are responsible for mutant phenotypes. For example, the RFLP map position of the chalcone synthase gene clone suggests a correlation between this gene and the pigment mutation transparent testa-4 (9); and the map position of the nitrate reductase gene clone suggests a correlation between this gene and the mutation chlorate resistance-3 (9) in which nitrate reductase activity is reduced. Thus the combined maps have the potential to provide information on clones having unknown gene function or to associate mutant phenotypes with gene products.

The RFLP map should facilitate cloning of genes known only by mutant phenotype by providing starting points for

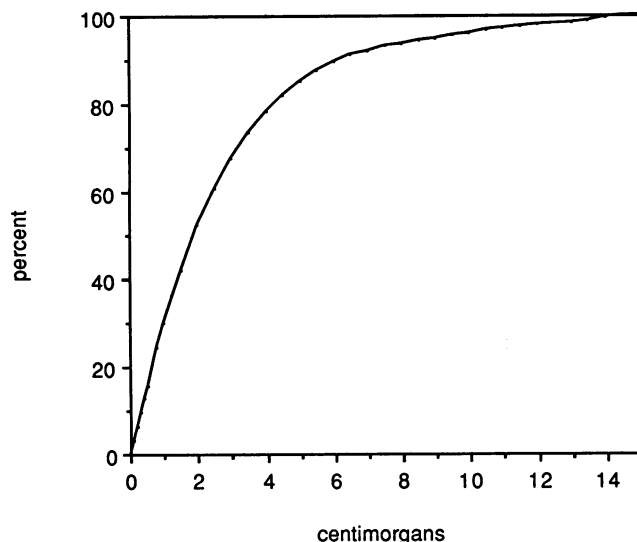


FIG. 4. A graph depicting the completeness of the RFLP linkage map. For given genetic distances ( $N$ ) in centimorgans, the percent of the genome within  $N$  cM of the RFLP markers was calculated. The results of these calculations are depicted for all values of  $N$  from 0 to 15 cM. To avoid assuming lengths for the ends of the chromosomes, the genome is considered here to be the sum of the RFLP chromosome lengths, where the two ends of each chromosome are defined by the outermost RFLP markers. All (100%) of the genome is within 15 cM of one or more of the RFLP markers; >50% is within 1.9 cM. On average, 1 cM is equal to  $\approx$ 140 kilobase pairs.

chromosome walking. The labor involved in chromosome walking depends upon the physical distance between the starting clone and the desired gene. Although an actual correlation of physical distance and genetic distance has yet to be established for specific regions of the genome, a general measure is 140 kb per cM (the genome size in kilobases divided by the RFLP map genome size in centimorgans). In terms of averages, the mean distance between RFLP markers is 780 kb, and the mean walking distance to reach an arbitrary gene, therefore, is 195 kb. A more descriptive measure of the map's usefulness than the mean spacing between markers is to examine the percent of the genome (the genome being defined by the RFLP map) within a given distance from any RFLP marker (Fig. 4). This calculation shows that more than half of the genome lies within only 1.9 cM ( $\approx$ 270 kb) of an RFLP marker, and if one is willing to walk about three times that distance, then one has access to 90% of the genome. The required walking distances can be reduced by increasing the density of RFLP markers on the map. The distances between consecutive loci closely fit the negative exponential distribution that would be expected from randomly picking points along linear chromosomes, which indicates that current gaps in the map are likely to be filled.

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