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Restriction Fragment Length Polymorphism Linkage Map of *Arabidopsis thaliana*

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We have constructed a restriction fragment length polymorphism (RFLP) linkage map of the nuclear genome of the small flowering plant *Arabidopsis thaliana*. The map is based on the meiotic segregation of both RFLP and morphological genetic markers from five independent crosses. The morphological markers on each of the five chromosomes were included in the crosses to allow alignment of the RFLP map with the established genetic map. The map contains 94 new randomly distributed molecular markers (nine identified cloned *Arabidopsis* genes and 85 genomic cosmid clones) that detect polymorphisms between the Landsberg erecta and Columbia races. In addition, 17 markers from an independently constructed RFLP map of the *Arabidopsis* genome [Chang, C., Bowman, J.L., DeJohn, A.W., Lander, E.S., and Meyerowitz, E.M. (1988). Proc. Natl. Acad. Sci. USA 85, 6856–6860] have been included to permit integration of the two RFLP maps.

INTRODUCTION

Arabidopsis thaliana is a small flowering plant with many features that make it useful as an experimental organism. Its short life cycle, small size, and large seed output make it well suited for classical genetic analysis (reviewed in Meyerowitz, 1987). Mutations have been described affecting a wide range of fundamental developmental and metabolic processes (reviewed in Estelle and Somerville, 1986). Approximately 80 of these mutations have been ordered into a genetic linkage map (Koornneef, 1987), including numerous morphological mutations that are useful markers for mapping. For molecular biological studies, *Arabidopsis* offers the additional advantages of having a very small genome (70,000 kb) and a remarkably low content of interspersed repetitive DNA (Pruitt and Meyerowitz, 1986). Both of these features are highly unusual among higher plants, and should facilitate the cloning of genes by techniques such as genome walking. *Arabidopsis*, therefore, offers the potential for the identification and isolation of genes involved in fundamental physiological processes based solely on their mutant phenotype and genetic map location.

In an effort to develop *Arabidopsis* further as a model system for gene isolation and genetic studies, several laboratories are engaged in constructing a detailed restriction fragment length polymorphism (RFLP) linkage map of its nuclear genome. An RFLP map containing 90 molecular markers has recently been published (Chang et al., 1988); we describe here the construction of a similar map containing 94 new molecular markers. We have also incorporated 17 markers from the RFLP map of Chang et al. (1988) to provide contact points between the two maps. The two RFLP maps appear to be complementary in that each of them partially fills the gaps present in the other; their combination thus provides an improved coverage of the genome with a higher density of markers.

Alignment of the RFLP map with the established genetic map is based on the segregation of genetic markers from each of the five *Arabidopsis* chromosomes. The RFLP markers thus constitute convenient starting points for chromosome walks toward loci of interest. The characteristics of the *Arabidopsis* genome indicate that chromosome walking should be feasible; however, this technique is both tedious and limited in scope. As an alternative approach, this laboratory is engaged in constructing a physical map of the *Arabidopsis* genome (B.M. Hauge and H.M. Goodman, unpublished results) that will ultimately consist of a set of overlapping cloned DNA fragments encompassing the five linkage groups. Most of the probes used to identify RFLPs in the present study are genomic cosmid clones that have been incorporated into the physical map. Therefore, these probes establish contact points

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between the physical, the RFLP, and the classical genetic maps. The combined RFLP/physical maps should provide immediate access to any region of the genome that can be genetically identified, i.e., genes for which the locus but not the product of the gene is known.

RESULTS

Selection of RFLP Probes

The majority of the probes used to detect RFLPs in this study are random clones from a genomic cosmid library (having a mean insert size of 40 kb) that is currently being used to construct a physical map of the *Arabidopsis* (Col-0) genome (B.M. Hauge and H.M. Goodman, unpublished results). Candidate clones for the RFLP map were subjected to fingerprint analysis (clones are digested with HindIII, end-labeled, and then digested with Sau3A), and the resultant fragments resolved on a denaturing 4% polyacrylamide gel and visualized by autoradiography (Coulson et al., 1986). Two classes of highly repeated sequences can be identified by examination of the banding patterns: a family of tandemly repeated sequences, having a unit length of ~180 bp, that is thought to be associated with heterochromatic regions of chromosomes (Martinez-Zapater, Estelle, and Somerville, 1986) and the ribosomal DNA (rDNA). These sequences represent 1.5% and 4.5%, respectively, of the *Arabidopsis* genome (Martinez-Zapater, Estelle, and Somerville, 1986; Pruitt and Meyerowitz, 1986). Clones harboring the small tandem repeats containing a HindIII site are readily detected by the appearance of intensely labeled bands in the fingerprint and were excluded from further analysis. Similarly, clones with an unusually low number of HindIII sites, a characteristic of the rDNA cluster in *Arabidopsis* (Pruitt and Meyerowitz, 1986; B.M. Hauge, unpublished observations), were also excluded. No additional selections were imposed, and all remaining clones were considered candidate probes for the RFLP mapping. It should be noted that the middle repetitive fraction in *Arabidopsis* is composed largely of chloroplast DNA (Pruitt and Meyerowitz, 1986). Since our genomic cosmid libraries were constructed from DNA prepared from purified nuclei, clones that hybridize to middle repetitive sequences have been largely excluded.

Candidate clones were labeled with ^{32}P and used as probes for hybridization to genomic blots of DNA prepared from the Columbia (Col-0) and Landsberg erecta (La) races. The DNA was digested with one of the following restriction enzymes: BclI, ClaI, DraI, EcoRI, HindIII, or XbaI (Figure 1). Each of these enzymes has a 6-bp recognition sequence containing four A+Ts. Since *Arabidopsis* has an A+T content of 58% (Leutwiler, Hough-Evans, and Meyerowitz, 1984), the enzymes were chosen in an attempt to maximize the number of recognition sequences and, therefore, the detection of RFLPs. At this stage, cosmid clones hybridizing to repetitive DNA were occasionally found and were excluded from further consideration. Under our screening conditions, approximately 20% of the remaining cosmid clones detect an RFLP with a single enzyme (HindIII), whereas 65% detect an RFLP with at least one of the six enzymes tested.

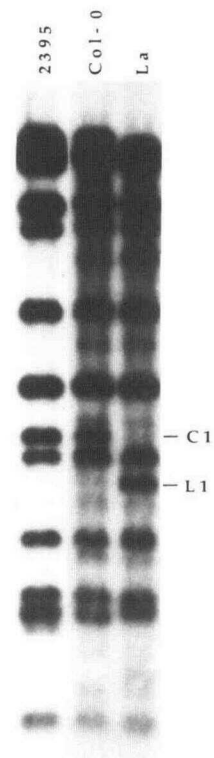

Figure 1. Autoradiogram of a DNA Gel Blot Comparing the Restriction Pattern and the Genomic Hybridization Patterns of an RFLP Probe.

The cosmid clone 2395 was used to probe a DNA gel blot carrying HindIII digests of Columbia (Col-0) and Landsberg erecta (La) genomic DNA and of the corresponding cosmid (2395). The polymorphic bands are indicated. The cosmid restriction digest contains a band that co-migrates with the Columbia polymorphic band (C1); the detected RFLP locus is, therefore, in all likelihood contained within the cloned insert.

For each of the probes that detect an RFLP, the restriction pattern of the cosmid clone was compared with the corresponding hybridization pattern on the genomic blot (Figure 1). This enabled us to determine which of the polymorphic bands detected on the genomic blot are physically represented in the insert of the clone and which might potentially correspond to cross-hybridizing DNA sequences. However, since the cosmid clones were propagated in a *Dam*$^+$ host (DK1), this comparison could not be

performed with probes that detect an RFLP using the restriction enzyme BclI. (Cleavage with BclI is blocked by *dam* methylation.)

In addition to random cosmid clones, nine of the probes used to construct the map were derived from characterized *Arabidopsis* genes (see Methods). Seventeen additional clones, which have been previously mapped by Chang et al. (1988), were also included. These latter clones were incorporated to facilitate the alignment of the two independently constructed RFLP maps.

Crosses

Two races of *Arabidopsis*, Landsberg erecta (La) and Columbia (Col-0), were used as parents for segregation analysis of the RFLP markers. These races were chosen because most of the well-characterized mutations have been isolated in the La background, whereas the Col-0 strain was used as the source of DNA for constructing the genomic cosmid library.

Alignment of the RFLP map with the established *Arabidopsis* genetic map was facilitated by the inclusion of morphological genetic markers on each of the five *Arabidopsis* linkage groups. Crosses were performed with five La marker lines with recessive mutations on each of the five chromosomes: chlorina-1 (ch-1), apetala-1 (ap-1), and glabra-2 (gl-2) on chromosome 1; compacta-2 (cp-2), asymmetric leaves (as), and eceriferum-8 (cer-8) on chromosome 2; long hypocotyl-2 (hy-2) and glabra-1 (gl-1) on chromosome 3; brevipedicellus (bp), eceriferum-2 (cer-2), and apetala-2 (ap-2) on chromosome 4; and transparent testa glabra (ttg) and yellow inflorescence (yi) on chromosome 5. In addition, each line carries the erecta (er) mutation on chromosome 2.

For each cross, the F1 plants were allowed to self-pollinate, as were the resultant F2 progeny. F3 plants were grown from seeds of individual F2 plants and pooled into groups of 100 to 1000. Each pool was used to score the genotype of the corresponding F2 plant with respect to the recessive morphological markers and to purify DNA for subsequent blot hybridization analysis.

A total of 118 F2 plants were used to analyze the segregation of the RFLPs. (In this context, F2 refers to the pooled F3 plants, which were derived from individual F2 plants.) Twenty-five of the progeny were derived from a cross containing markers on chromosome 1, 27 from chromosome 2, 22 from chromosome 3, 23 from chromosome 4, and 21 from chromosome 5.

Segregation of the RFLPs

For segregation analysis of the RFLPs, DNA probes were hybridized to a set of genomic blots containing DNAs prepared from the F2 progeny that had been digested with the appropriate enzyme (Figure 2). Each lane was then scored for the presence or absence of the polymorphic parental bands (or declared unscorable for technical reasons). The data were then analyzed to determine whether the polymorphic bands segregate as Mendelian alleles and therefore constitute a usable RFLP marker. First, individual bands are expected to segregate 3:1 (presence:absence). Second, allelic RFLPs must show the 1:2:1 (Col-0:Col-0/La:La) segregation ratio predicted for co-dominant genetic markers. A χ^2 test was used to ensure that the observed segregation was statistically significant (i.e., clones with $P < 0.05$ were rejected). Only clones fulfilling both of these criteria (approximately 90% of the random cosmid clones) were retained and used to construct the linkage map.

All but two of the cosmid clones detect a single locus. For 70% of the cosmid clones, the segregating Col-0 band co-migrates with a band contained within the cloned insert; the RFLP locus is, therefore, in all likelihood contained within the cloned insert. For the remaining clones, further analysis will be required to determine whether the RFLP locus is within the insert, but resides at the vector-insert junction, or is the result of a cross-hybridizing sequence. For the clones that hybridize to two loci, one locus is always represented in the cloned insert (see legend to Figure 3).

Construction of the Linkage Map

The RFLP map is based on the meiotic segregation of both the RFLP and the morphological genetic markers that were included in the crosses. Segregation data were ana-

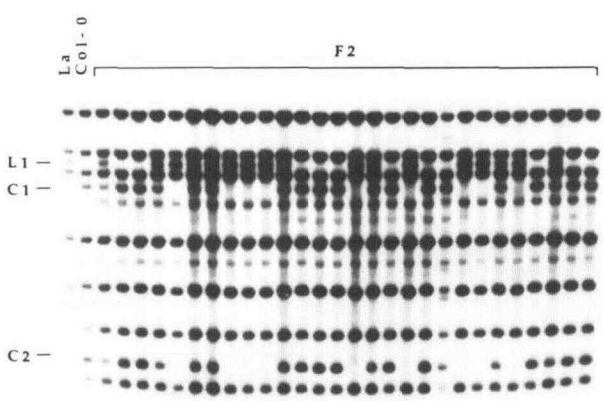

Figure 2. Autoradiogram of a DNA Gel Genomic Blot Used for Segregation Analysis of RFLPs.

Cosmid clone 3791 was used to probe a blot containing genomic DNAs digested with HindIII. The first two lanes contain DNA of the parental lines, and the others contain DNA of pools of F3 plants derived from 28 individual F2 progeny. The polymorphic bands are indicated.

lyzed with the MAPMAKER computer program (Lander and Green, 1987; Lander et al., 1987). First, the markers were placed into putative linkage groups based on the lod scores generated by pairwise 2-point analysis. Lod scores are determined by comparing the maximum likelihood recombination fractions for two markers based on the assumption that the markers are either: (1) linked or (2) unlinked. The lod score (Ott, 1985) is expressed as the $\log_{10}$ of the ratio of the probability that the markers are linked divided by the probability that the markers are unlinked, and is a measure of the deviation from nonlinkage (i.e., a lod score of 3.0 indicates that the probability that unlinked markers would generate the observed data is 0.001). We consider two markers linked if the lod score is greater than 3.0 and the recombination fraction is less than 0.40. Following this analysis, the loci fall into five linkage groups, which can be assigned to the five chromosomes based on the segregation of the genetic markers within each group.

The order of markers within each linkage group was determined by performing 3-point, followed by *n*-point, analysis. A given order is established only if the difference between the log-likelihoods is greater than 3.0. In certain cases, clusters of tightly linked markers cannot be unambiguously ordered by this criterion; these clusters are indicated on the map (Figure 3). The *n*-point analysis also

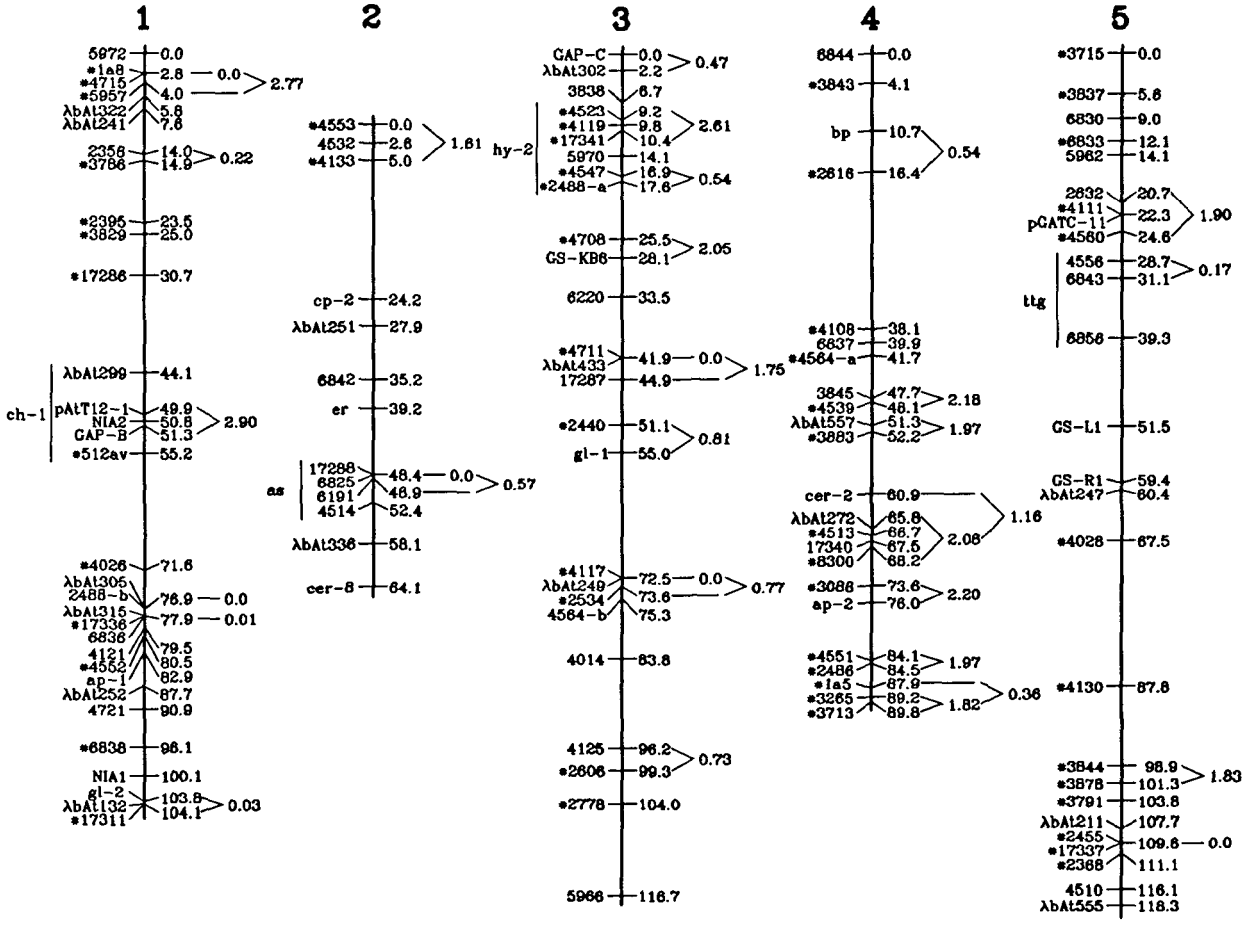

Figure 3. An RFLP Linkage Map of the Five Chromosomes of the *A. thaliana* Nuclear Genome.

The five linkage groups are numbered and oriented with respect to the genetic map (Koornneef, 1987). Clones 2488 and 4564 detect two loci, indicated by a and b in each case. Asterisks indicate the cosmid clones for which the restriction pattern of the probe contains a band(s) co-migrating with the Col-0 polymorphic band(s). Map distances are in centiMorgans; for each chromosome, position zero was assigned to the top-most marker in this map and thus differs from other maps. Clusters of tightly linked markers that cannot be unambiguously ordered (likelihood of this order divided by an alternative order is $<10^3$) are indicated by brackets; the numbers to the right of the brackets are the $\log_{10}$ of the likelihood ratio of the order shown to the next most likely order. The position of some morphological genetic markers (ch-1, as, hy-2, and ttg) is very uncertain; these markers are shown to the left of the other markers, and the vertical bars delineate the regions of their possible position.

generates the maximum likelihood recombination fractions for each pair of adjacent markers, which can then be transformed with the Kosambi function (Kosambi, 1944) into centiMorgan distances. The orientation of the markers with respect to the classical genetic map is based on the segregation of the morphological genetic markers that were included in the crosses. For chromosome 5, where only one genetic marker was scored (ttg), RFLP markers common to our map and the map of Chang et al. (1988) were used to help establish the orientation.

DISCUSSION

We have constructed an RFLP linkage map of the *A. thaliana* genome containing 94 new markers: 85 random genomic cosmid clones and nine cloned *Arabidopsis* genes. In addition, the map contains 17 markers from an independently constructed RFLP map (Chang et al., 1988). Alignment of the RFLP map with the classical genetic map is based on the meiotic segregation of 13 morphological genetic markers that were included in the crosses. The current RFLP map encompasses a total of 493 cM, a number slightly in excess of the genetic map (Koornneef, 1987), which spans 437 cM. The approximate sizes of the linkage groups of the RFLP map (104 cM, 64 cM, 117 cM, 90 cM, and 118 cM) are in rough agreement with the genetic map, which has linkage groups of 126 cM, 51 cM, 91 cM, 69 cM, and 100 cM for chromosomes 1 to 5, respectively. Furthermore, the distances between the genetic markers shared by both maps agree reasonably well, considering the limited number of progeny. (Each genetic marker was carried by the progeny of only one of the five crosses.)

The genomic cosmid clones used as probes readily detect RFLPs. Generally, genomic clones harboring large inserts are avoided when constructing RFLP maps since they are more likely to contain repetitive DNA sequences that hybridize to multiple loci, thereby complicating segregation analysis. This problem was not encountered during this study for two reasons: first, the *Arabidopsis* genome contains a very low amount of interspersed repetitive DNA (Pruitt and Meyerowitz, 1986) and second, the cosmid clones that were used as probes were pre-selected to eliminate clones containing highly repeated sequences.

The *Arabidopsis* RFLP map described in this paper can be aligned with the previously published RFLP map (Chang et al., 1988) by using the 17 markers shared by the two maps. This number of contact points is too low to ensure an accurate alignment of the maps over the entire length of the five linkage groups. However, to a first approximation, the two maps complement one another and, therefore, integration of the two maps partially fills the gaps in the individual maps. For example, the present data partially fill the major gap on chromosome 5, which is present in the map of Chang et al. (1988), whereas the converse is true for chromosome 1. Therefore, the two maps provide improved coverage of the genome with a higher density of markers.

RFLP mapping of cloned genes to genetically defined loci with known mutant phenotypes can be used, at least in part, to ascertain the biological function of the gene based on linkage. For example, the observed linkage of a nitrate reductase clone (NIA2) to the *chl-3* locus (Cheng et al., 1988) is consistent with previous data, which suggest that *chl-3* encodes an NR structural gene (Braaksma and Feenstra, 1982). In addition, RFLP mapping provides a tool for studying the chromosomal location of sequences that are refractory to classical genetic analysis. For instance, Richards and co-workers (E. Richards, W. Loos, J. Giraudat, H.M. Goodman, and F.M. Ausubel, unpublished data) have used this RFLP map to localize a clone containing putative centromeric sequences to the genetically defined centromere region of chromosome 1.

RFLP maps provide a potentially powerful tool for isolating genes that are defined only by their mutant phenotype and genetic position. The RFLP markers can be used as genetically defined starting points for the isolation of neighboring loci. This can be achieved by chromosome walking or, eventually, by utilization of the physical map of the *Arabidopsis* genome currently under construction in this laboratory.

The ease with which a given locus can be isolated depends on the resolution of the RFLP map. Theoretically, the analysis of 118 F2 progeny should permit resolution of markers that are separated by at least 0.42 cM (one map unit divided by 236 chromatids). However, the actual position of a marker cannot be determined with such precision. This is clearly illustrated in Figure 3 for the clusters of closely linked markers that cannot be unambiguously ordered. We estimate that the resolution of the current map is on the order of 2 cM. In addition, it is not possible to assign with confidence a precise position on the genetic map to a given RFLP, since only one to four morphological genetic markers per chromosome were used to align the two maps. Therefore, for each gene to be cloned, it is first necessary to map more accurately the locus with respect to the RFLP map. Nonetheless, the map presented in this paper, together with the RFLP map of Chang et al. (1988), provides an important tool for the isolation of genes where only the locus and not the product of the gene is known. Clearly, as more loci become integrated into the RFLP map and the density of markers increases, the general utility of the RFLP map will be enhanced.

METHODS

Arabidopsis Strains

The Columbia (Col-0) and Landsberg erecta (La) lines were obtained from F. Ausubel and M., Koornneef, respectively. For the

crosses, the Landsberg marker lines were used as female parents and the wild-type Columbia line was used as pollen donor.

Cloned Genes

The following cloned *Arabidopsis* genes were used as RFLP markers: glutamine synthetases Atgsr1, Atgskb6, and Atgsl1 (K. Peterman and H.M. Goodman, unpublished results); nitrate reductase gNR1 and gNR2 (Cheng et al., 1988), which correspond to the NIA1 and NIA2 loci, respectively; glyceraldehyde-3-phosphate dehydrogenase GapB and GapC (Shih, Heinrich, and Goodman, 1988); and the middle repetitive element flanking the *Arabidopsis* telomeric probe pAtT12-1 (E. Richards, W. Loos, J. Giraudat, H.M. Goodman, and F.M. Ausubel, unpublished data); pGATC-11 is a dark-inducible cDNA (D. Inze and M. Van Montagu, personal communication).

A set of *Arabidopsis* (Col-0) λ genomic clones (λbAt###) were mapped to provide contact points with the RFLP map of Chang et al. (1988).

The remaining RFLP probes correspond to random clones from a genomic cosmid library.

Genomic Cosmid Library

Cosmid clones were constructed by ligation of mechanically sheared nuclear DNA from the Columbia Col-0 strain of *Arabidopsis thaliana* into the BamHI site of a derivative of the vector Lorist B (Cross and Little, 1986). Nuclear DNA was prepared essentially as described by Hamilton, Kunsch, and Temperli (1972). Cosmid clones were propagated in DK1: *(srl-recA)Δ306,hsdr,araD-139,(ara-leu)Δ7697,(lac)ΔX74,mcrA, mcrB,rpsL-20*.

Nucleic Acid Extractions

Total *Arabidopsis* genomic DNA was extracted from whole plant tissue according to Watson and Thompson (1986).

Cosmid and plasmid DNA were isolated by alkaline extraction (Birnboim and Doly, 1979) and purified by centrifugation in a CsCl gradient (Maniatis, Fritsch, and Sambrook, 1982). λ DNA was prepared from either plate lysates as described by Reddy, Tuwabara, and Sherman (1988) or from liquid lysates (Maniatis, Fritsch, and Sambrook, 1982).

Genomic Blots

Total *Arabidopsis* genomic DNA (1 μg to 2.5 μg) was incubated overnight with a fivefold excess of a restriction enzyme. The digested DNA was size-fractionated by electrophoresis in either 0.5%, 0.8%, or 1.2% agarose gels for 640 V-hr. After electrophoresis, the gels were treated with 0.25 N HCl for 15 min, denatured with 0.5 N NaOH, 1.5 M NaCl for 20 min, and finally neutralized with three washes in 25 mM $NaPO_4$, pH 6.5, for 20 min each. DNA was transferred to nylon filters (Biotrans from ICN Biomedicals, Inc.) by capillary action with 25 mM $NaPO_4$ buffer, pH 6.5. After transfer, the DNA was cross-linked to the filters by UV irradiation. ^{32}P-DNA probes (1 to 5 × 10^8 cpm/μg) were generated by nick translation (Rigby et al., 1977) or random primer extension (Feinberg and Vogelstein, 1983). Filters were hybridized (10^6 to 10^7 cpm/mL) for 16 hr to 24 hr according to Church and Gilbert (1984). Filters were washed three times at 45°C in 0.1 × SSC, 0.1% SDS for 20 min each. Filters were stripped for subsequent rehybridization by submerging the filters in boiling 5 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 0.05% SDS for 2 min.

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