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Random-amplified-polymorphic DNA markers in sorghum

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Abstract Conditions have been identified that allow reproducible amplification of RAPD markers in sorghum. High resolution of RAPD markers was accomplished by radiolabeling PCR-amplified DNAs followed by separation on denaturing 5% polyacrylamide gels. Reaction parameters including $MgCl_2$ concentration and temperature significantly influenced yield and the type of amplification products synthesized. Unexplained amplified DNAs increased when more than 35 cycles of PCR amplification were used. Under standard conditions, approximately 80% of the primers tested amplified DNA, and most revealed 1–5 polymorphisms between BTx 623 and IS 3620C. Primers were used to amplify RAPDs in 32 genotypes of sorghum. In addition, 8 primers detected RAPDs in a population previously used to create an RFLP map for sorghum. These RAPDs were mapped successfully using a population of 50 F_2 plants.

Key words Sorghum (*Sorghum bicolor*)
RAPD markers

Introduction

Sorghum is a highly diverse genus consisting of cultivated and wild species, many of which are interfertile. *Sorghum bicolor* ssp *bicolor* (2N=20) is the most important taxon agronomically in that it includes the cultivated grain races, it is highly self-pollinated and it possesses considerable diversity both morphologically and in agronomic traits (Doggett 1986). Drought tolerance is one of sorghum's most important traits, allowing it to be grown in harsh environments. Genotypes differing in pre-flowering and post-flowering drought tolerance have been selected of from over 20,000 accessions available in the sorghum germplasm collection (Rosenow 1987). Genotypes varying in post-flowering drought tolerance (B 35, Tx 7000) have been crossed, and F_6 recombinant inbred lines are being analyzed with restriction fragment length polymorphism (RFLP) markers to determine the number and location of genes controlling post-flowering drought tolerance. This research has been facilitated by the existence of a bank of RFLP markers used to create a genetic map of sorghum (Xu et al. 1994). Subsequent analysis and map-based cloning of genes in sorghum controlling drought tolerance and other traits will require the identification of markers closely linked to genes of interest. The combination of polymerase chain reaction (PCR)-amplifiable markers (Williams et al. 1990) and bulk segregant analysis (Michelmore et al. 1991; Martin et al. 1991) offers one approach to this problem.

In 1990, molecular markers generated using Arbitrarily Primed PCR (AP-PCR) (Welsh et al. 1990; Williams et al. 1990) were used to identify random-amplified-polymorphic DNAs (RAPDs) in several organisms, including plants. This approach to the production of genetic markers has been widely tested in the subsequent 3-year period (i.e. Williams et al. 1991; Klein-Lankhorst et al. 1991; Tulsieram et al. 1992; He et al. 1992; Waugh et al. 1992; Dweikat et al. 1992; Devos and Gale, 1992; Reiter et al. 1992; for review, Tingey and del Tufo 1993). In sorghum, RAPDs have been used to analyze the amount of polymorphism available in grain sorghum (Tao et al. 1993). The benefits of PCR-based marker analysis include a low requirement for input DNA, high sensitivity, automatability and ease of generating primers. The limitations of this technique have been reported to include reproducibility (Ellsworth et al. 1993; Bell and DeMarini 1991; Giovannoni et al. 1991;

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Riedy et al. 1992), cost of *Taq* polymerase and an inability to distinguish heterozygotes from homozygotes when a RAPD is present. Modifications of the original method such as analysis of bulk segregants (Michelmore et al. 1991; Martin et al. 1991) and interval mapping (Giovannoni et al. 1991) have further extended the importance of this technique, especially as a step in map-based cloning (Doggett 1986) and fingerprinting (Dweikat et al. 1992; Fukuoka et al. 1992; Caetano-Anollés et al. 1991; Welsh et al. 1991a,b).

Because of the potential benefits of AP-PCR/RAPD analysis for our study of sorghum, we have investigated the parameters that influence RAPD amplification and detection. A standard set of conditions was found that allowed RAPDs to be used for genotype identification and in the construction of genetic maps.

Materials and methods

Plant varieties and segregation analysis

Sorghum genotypes BTx 623 and IS 3620C were crossed to produce F_1, F_2 and F_3 populations. Young leaves from 15-day-old seedlings were harvested, frozen in liquid nitrogen and stored at –80 °C prior to DNA extraction. RAPDs were scored as dominant markers in an F_2 population of 50 individuals. Linkage analysis was done using a Macintosh implementation of the MapMaker program (Lander et al. 1987) provided by S.V. Tingey. The F_2 population has been previously used to create an RFLP map (Xu et al. 1994). Linkage analysis preformed on this data used 2-point analysis with LOD=3.0, followed by multipoint analysis (LOD=3.5) with a maximum of six markers by using the "compare" command to determine an acceptable order for the markers. RAPD markers were placed into the framework using the "try" command (Lander et al. 1987).

DNA extraction and electrophoresis

Genomic DNA was isolated using the procedure of Gustincich et al. (1991), with modifications. Tissue was ground in liquid nitrogen, and freshly prepared 8% DTAB extraction buffer (1.5 *M* NaCl, 1 m*M* EDTA, 0.1 *M* TRIS-HCl (pH 8.6), 20 m*M* DTT, 1% PVP, 0.05% ascorbic acid and 1% SDS) was added at a ratio of 1:3 (w/v). Tubes were incubated at 68 °C for 15 min and extracted with chloroform. DNA was precipitated by the addition of 2 vol of ethanol and 0.1 vol of 5 *M* ammonium acetate. DNA pellets were washed with 70% ETOH, and resuspended in 1 × TE, (10 m*M* TRIS-HCl (pH 8.0), 1 m*M* EDTA). DNA samples were further treated with RNase A (50 µg/ml) for 15 min at 37 °C followed by proteinase K (50 µg/ml) for 15 min. Samples were then extracted two to four times with phenol:chloroform:isoamyl alcohol (25:24:1) and DNA precipitated using ETOH as described above. DNA was resuspended in 1 × TE, and DNA concentration was determined using Hoechst dye 33258 (Brunk et al. 1979) and a TKO-100 mini fluorometer (Hoefer Scientific Instruments). DNA quality was checked on a 0.6% agarose gel, and samples were diluted to a concentration of 4 ng/µl in water. PCR-amplified DNA (1.5 µl) was mixed with 3.5 µl of formamide dye solution and denatured at 85°C for 4 min. Samples (3.3 µl) were loaded onto a 5% polyacrylamide gel containing 8 *M* urea (0.4 mm thickness). Electrophoresis was performed using a BioRad sequencing unit. After approximately 2.5 h of electrophoresis time, gels were dried and exposed to Kodak X-omat film for 12 h at 23°C. Genomic DNA extracted from field samples required an altered extraction procedure to remove phenolics. For these samples, the 8% DTAB extraction buffer described above was modified by increasing the PVP to 2%, ascorbic acid to 0.1% and DTT to 0.2%, and by adding diethyldithiocarbamic acid (DIECA) at 0.1% (w/v) with the buffer adjusted to pH 8.0 (Loomis 1974)

PCR amplification conditions

Amplification parameters including DNA, primer, $MgCl_2$, temperature and number of cycles were varied to determine the optimal conditions. Standard 15-µl reaction mixtures contained 9.6 ng of template DNA, 3 µM of primer (10 mers obtained from Operon Technology Inc), 0.38 units of *Taq* polymerase, 2.5 m*M* $MgCl_2$, 11.1×10^4 Bqi of [^{32}P]-dCTP and a final concentration of 100 µ*M* dATP, dGTP, dTTP and 6 µ*M* dCTP. In some experiments (Figs. 2D, 3 and 4) the Amplitaq DNA polymerase Stoffel fragment (Perkin Elmer Cetus) was used because it gave a more reproducible amplification of lower-molecular-weight DNAs. Amplifications were performed using a PCR-9600 Thermocycler (Perkin Elmer Cetus) and the following protocol; denaturation at 92 °C for 1 min (after cycle 1, 40-s denaturations were used), annealing at 48 °C for 40 s, extension at 72 °C for 1.15 min with a ramp of 15 s and a final extension at 72 °C for 15 min. A total of 30 cycles were used under standard conditions.

Results

Resolution and reproducibility

The analysis of AP-PCR products is often accomplished by the separation of amplified DNAs on agarose gels. In other cases, DNAs have been separated on polyacrylamide gels with variation in denaturation conditions (Welsh and McClelland 1990). In our early experiments, PCR-amplified DNAs were separated on 1% agarose gels and detected using ethidium bromide and fluorometry. While many DNAs could be clearly identified, there often were DNAs that co-migrated, making analysis difficult. Eventually it was determined that the best resolution could be obtained on sequencing gels (8 *M* Urea, 5% polyacrylamide). While these gels provided excellent resolution DNA could not be easily detected after ethidium bromide staining. Therefore, [^{32}P]-dCTP was added to the amplification reactions so that the amplified DNAs could be detected by autoradiography or by using a Betascope. A typical result using this approach to label and detect PCR-amplified DNAs is shown in Fig. 1. In this example, DNAs from BTx 623 and IS 3620C were amplified in ten separate reactions using primer K16. The autoradiogram in Fig. 1 shows that the amplified DNAs which range from 50 to 1,300 bp in length are reproducibly amplified and readily detected by this approach. Minor bands could be detected with longer exposures. Similar results were obtained when DNA extracted from 10 different plants was used for amplification. Reactions which lacked DNA, primers or *Taq* polymerase showed no amplification.

PCR amplification conditions

The results in Fig. 1 demonstrated that it was possible to reproducibly amplify a specific set of DNAs from a given genotype. However, based on the lack of reproducibility reported for this technique (Ellsworth et al. 1993; Bell and DeMarini 1991; Eckert and Kunkel 1991; Riedy et al. 1992), we decided to examine conditions that may alter the results of PCR amplifications in order to anticipate sources of error in future experiments. Using common DNA stocks

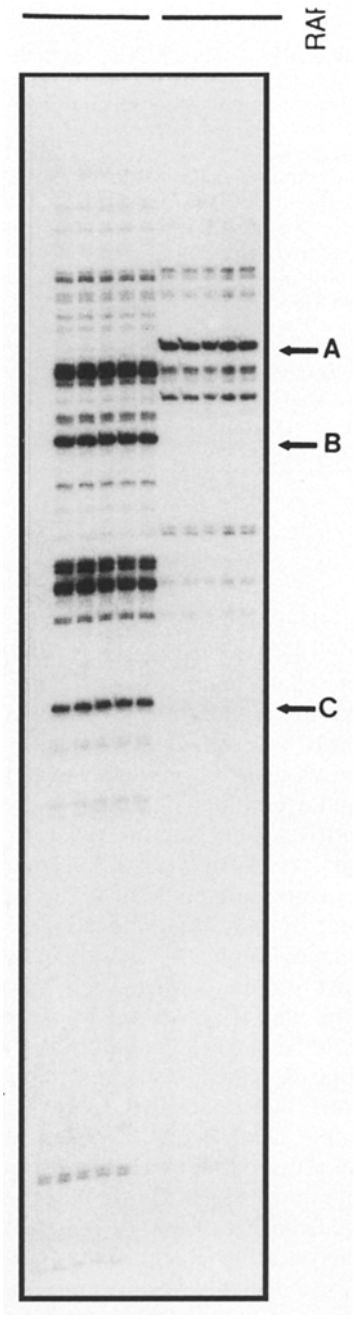


Fig. 1 Testing the reproducibility and resolution of RAPDs. DNAs were amplified by the PCR using primer K16 and genomic DNA from BTx 623 and IS 3620C. Five separate reactions were carried out with each genomic DNA in the presence of [^{32}P]-dCTP. The resulting products were electrophoretically separated on a denaturing 5% polyacrylamide gel, the gel dried and an autoradiogram obtained. Molecular weight markers are shown on the *left* of the figure and representative RAPDs that distinguish the genotypes are marked on the *right* of the figure

(BTx 623, IS 3620C) and a single primer (K16), we tested variation due to primer, DNA, *Taq* polymerase, $MgCl_2$ concentration, number of cycles and temperature.

Figure 2A lanes 1–5 show the influence of variation in primer concentration on amplified DNA products. Not surprisingly, low levels of primer decreased the yield of amplified product. Similarly, at very high concentrations of primer, the yield of amplified products was also reduced. Variation in primer concentration altered the profile of PCR-amplified products to only a small extent, and optimal concentrations (3–6 μ*M*) were similar to those used by others, which ranged from 0.2 to 10 μ*M* (i.e. Williams et al. 1990; Welsh et al. 1991). Lanes 6–10 showed the influence of variation in genomic DNA on PCR-amplified products. Except at very high DNA concentrations, the yield and profile of PCR-amplified products was not greatly altered by variation in DNA level. We also examined the influence of *Taq* polymerase concentration on PCR-amplified DNAs in part because of the role of the polymerase in stabilizing primer/DNA interactions and in part because of the expense of *Taq* polymerase. The results in lanes 11–15 show that variation of *Taq* polymerase from 0.19 units to 3.04 units influenced the results only at limiting polymerase levels (less than 0.38 units per 15-μl reaction). In a separate set of experiments, amplification products produced by *Taq* polymerase were compared to those observed when amplification reactions were carried out with the Amplitaq DNA polymerase Stoffel fragment. Both polymerases amplified similar-sized DNAs. However, the Amplitaq polymerase amplified lower-molecular-weight DNAs to a greater extent than *Taq* polymerase. Amplitaq was used to generate the data shown in Figs. 2D, 3 and 4.

Figure 2B shows the influence of annealing temperature and $MgCl_2$ on PCR-amplification products using primer K16. A strong temperature dependence was observed, with little amplification observed at 36 °C, an optimum at 48 °C and with significant reduction observed above 48 °C. While temperature had a strong effect on the accumulation of product, only small effects were seen on the type of products amplified. Many previous studies of PCR-amplified markers used 36 °C as a standard annealing temperature (Williams et al. 1990). This indicates that it is likely that not all primers show the type of temperature dependence observed for K16. An analysis of amplification using 27 primers showed that 22 primers amplified DNA when reactions were carried out at 48 °C, five of these primers amplified DNA at 48 °C but not at 36 °C; an additional 5 primers of the 27 tested amplified DNA at 36 °C but not at 48 °C.

$MgCl_2$ concentration has been previously found to alter the amount and type of product produced in PCR reactions (Ellsworth et al. 1993). The results in Fig. 2B document the extent of this effect under our reaction conditions. Low and high levels of $MgCl_2$ decreased the production of amplified DNA and of the concentrations tested; 2.5 m*M* allowed the greatest amplification.

The final reaction parameter analyzed was the number of PCR cycles. Products resulting from 25, 30 or 35 cycles are shown in Fig. 2 C. These results show that product is accumulating rapidly between 25 and 30 cycles, with a somewhat lower increase occurring between 30 and 35 cycles. In addition, spurious products begin to appear at the 35-cycle stage and by 45 cycles, numerous additional

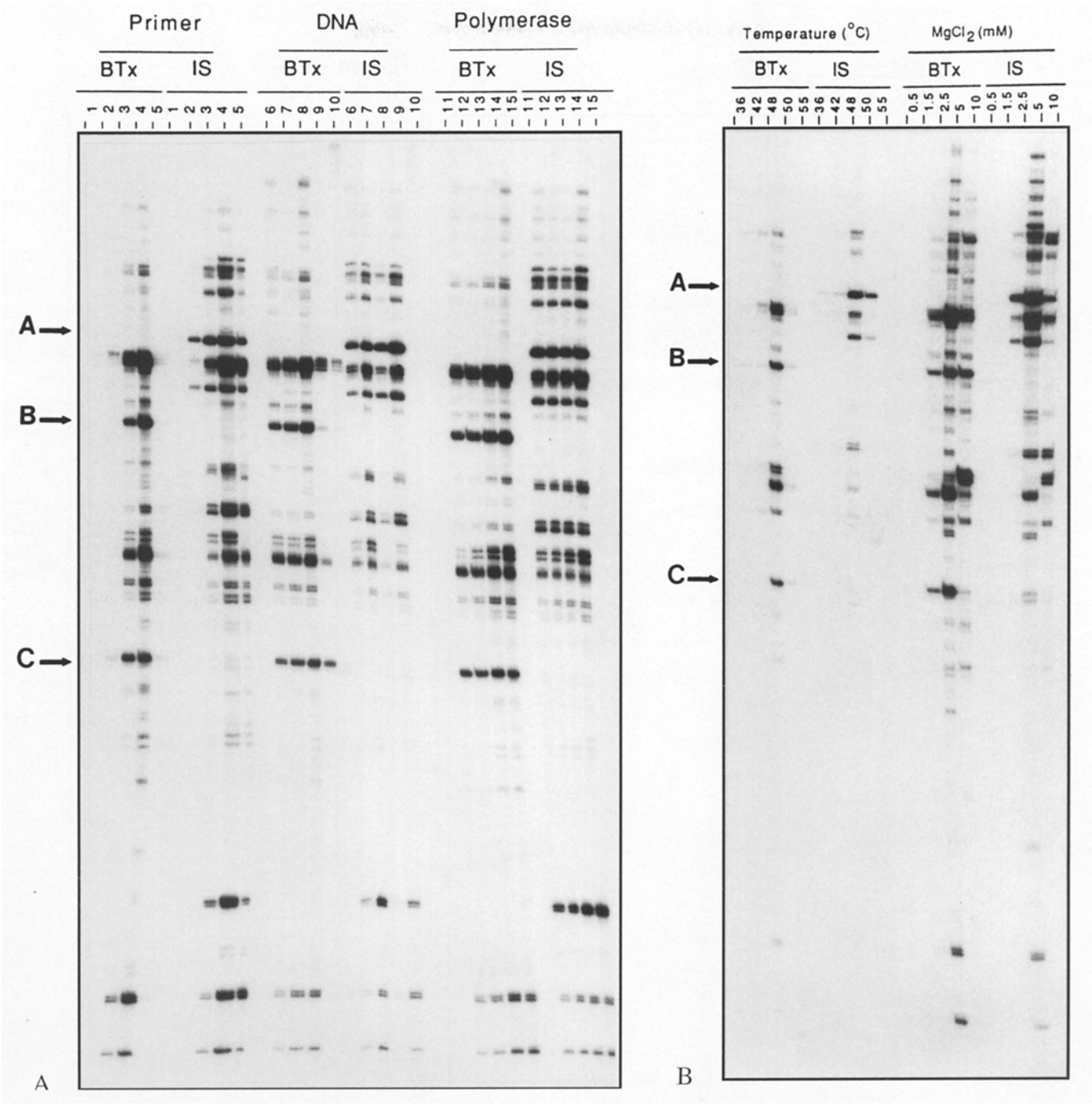
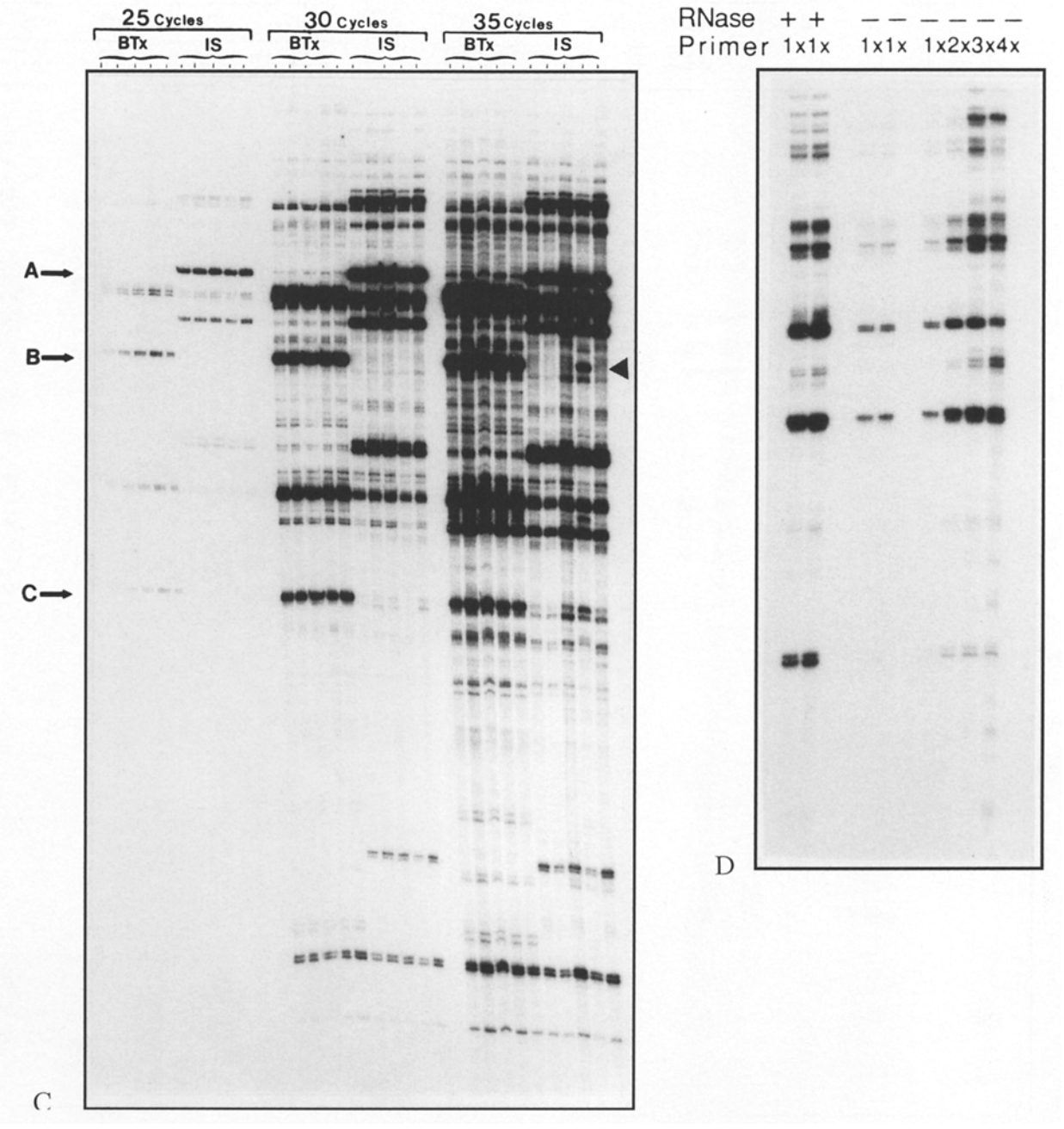


Fig. 2A–D Optimizing PCR-amplification conditions for RAPD analysis. The influence of primer, genomic DNA, *Taq* polymerase, $MgCl_2$ concentration and temperature on the production of PCR-amplified DNAs was tested. Primer K16 and genomic DNA from BTx 623 and IS 3620C were used in all reactions. **A** Influence of primer, genomic DNA and *Taq* polymerase concentration. PCR-amplification products in *lanes 1–5* were derived using primer concentrations of 0.75, 1.5, 3, 6 and 12 μ*M*, respectively, at constant genomic DNA concentration (9.6 ng/15 μl) and *Taq* polymerase (0.38 units). In *lanes 6–10*, PCR reactions were carried out at constant primer (3 μ*M*) and *Taq* polymerase (0.38 units) concentrations but with increasing amounts of genomic DNA (2.4, 4.8, 9.6, 19.2, 38.4 ng/15 μl, respectively). PCR-amplification products in *lanes 11–15* were derived at constant primer (3 μ*M*) and genomic DNA (9.6 ng/15 μl) concentrations but different *Taq* polymerase concentration (0.19, 0.38, 0.76, 1.52, 3.04 units, respectively). **B** Influence of temperature and $MgCl_2$ concentration. Variation in PCR-amplification products as a function of annealing temperature (36°–55 °C) is shown in the *first 10 lanes*. The influence of variation of $MgCl_2$ (0.5–10 m*M*) concentration on PCR-amplification products is shown in the *10 lanes* on the *right* of the figure. The highest incorporation occurred at 48 °C and 2.5 m*M* $MgCl_2$. **C** Influence of amplification cycle number. PCR-amplification products observed after 25, 30 and 35 cycles of amplification are shown. Genomic DNA from each genotype was amplified in 5 separate reactions at 48 °C. Variation in some reactions were observed after 35 cycles and in most lanes after 45 cycles. **D** Influence of RNase treatment. Genomic DNA from BTx 623 was isolated and a portion treated with RNase (*lanes* marked with a +). With a standard amount of primer (1×), DNA samples treated with RNase amplified more effectively than those not treated with RNase. This difference in amplification could be overcome by adding increasing amounts of primer (lanes marked 2×, 3× and 4×)



bands are observed (data not shown). Because of the variation observed as PCR cycle number increased beyond 30 cycles, we chose 30 cycles for all subsequent standard reactions.

Figure 2D shows the influence of RNase treatment on amplification. Under standard conditions, RNase-treated samples amplified products more efficiently than samples that had not been treated with RNase. This difference could be overcome by increasing the amount of primer in the reactions, indicating that in the samples not treated with RNase, primer hybridization to RNA was reducing primer concentration below optimum levels.

Primers

Once standard conditions were established, we tested whether these conditions were applicable for primers other than K16. To test this, 54 primers (10 mers) were chosen at random from over 300 Operon primers and used to amplify BTx 623 and IS 3620C DNA. Of the 54 primers tested, 45 amplified DNA. The DNAs amplified by 15 of these primers are shown in Fig. 3. All of the primers amplify multiple DNAs and all detected RAPDs that distinguish the two genotypes used in this experiment. Occasionally, amplified products were observed with one genotype but

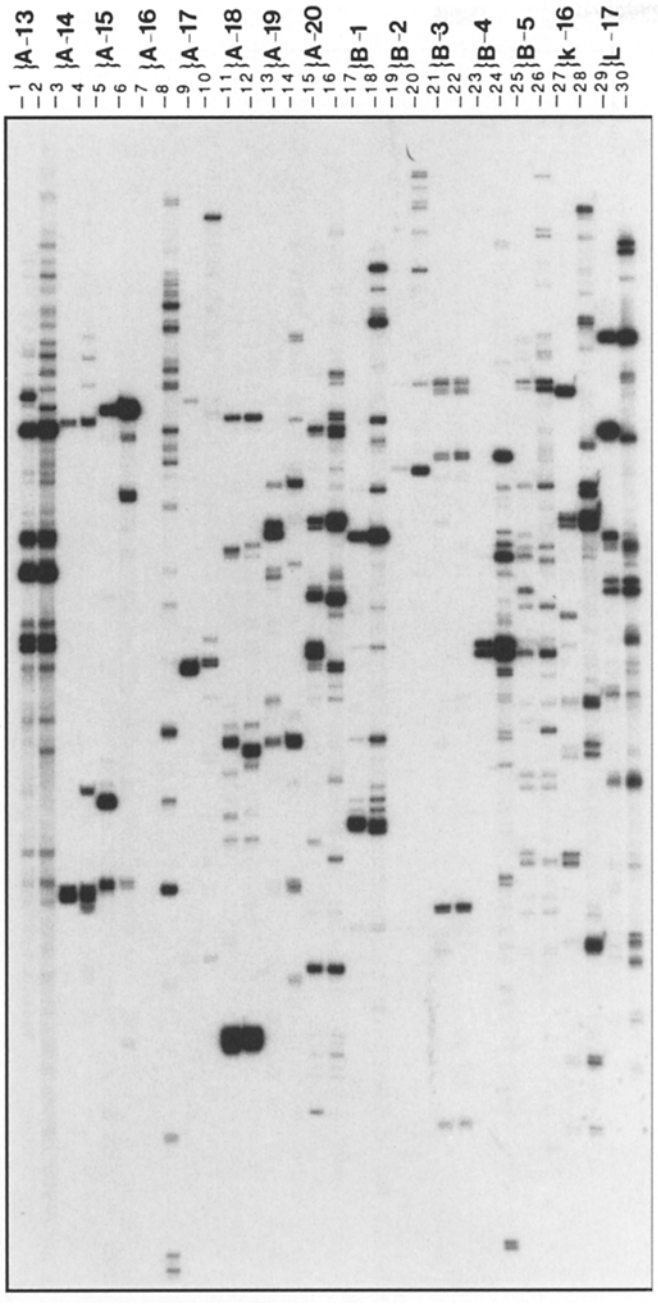


Fig. 3 Primer utility for RAPD analysis. Fifteen primers were used to amplify DNA from BTx 623 (*odd-numbered lanes*) and IS 3620C (*even-numbered lanes*). Most primers revealed RAPDs that distinguish the two genotypes

not the other (lanes 7 vs. 8; 19 vs. 20). This was due to an incorrect reaction mixture set-up because a repeat of these reactions allowed DNA from both genotypes to be amplified.

Genotypes

AP-PCR has been shown to be useful in generating RAPDs for genotype fingerprinting (Welsh and McClelland 1990). We wanted to test our standard amplification conditions on a series of genotypes to determine if these conditions would be useful for genotype analysis. The results in Fig. 4 show PCR-amplification products generated using primer K16 on DNA isolated from 32 different genotypes. The genotypes represent a collection of material differing in maturity, height, rust resistance, headbug and greenbug resistance, drought tolerance and male sterility. The results indicate that the standard PCR conditions allowed amplification of DNA from all of the genotypes and that some amplified DNAs were common to all genotypes while the presence of other amplified DNAs could be used to distinguish among genotypes. The greatest variation was observed in high-molecular-weight amplified DNAs. The intensity of these bands varied considerably from experiment to experiment, making them unreliable for analysis.

Analysis of F_2 progeny

RAPDs have been used to generate data for genetic maps although RAPD markers are less informative for this purpose than co-dominant RFLP markers in F_2 populations. Xu et al. (1993) have generated a 200-point RFLP map of the sorghum genome using BTx 623 and IS 3620C as parents. In order to test our ability to map RAPDs we isolated DNA from 50 F_2 progeny of BTx 623 and IS 3620C which were used to generate the RFLP map. Eight primers which revealed RAPDs in this population were selected, and segregation of the RAPDs in the F_2 population was analyzed. One example of this analysis is shown in Fig. 5. In this example, primer L17 revealed 3 RAPDs which segregated in the cross described above. Using MapMaker for Macintosh, the data was analyzed and the location of mapped RAPDs is shown in Fig. 6 in relation to RFLP markers established in a separate study (Xu et al. 1993). All of the RAPDs mapped showed normal segregation except for OP-K-16(5) which showed distorted segregation at the 1% level (an overabundance of IS3620C alleles).

Discussion

Standard conditions have been established that allow the reproducible detection of PCR-amplified molecular markers (RAPDs) in sorghum. Reaction parameters such as $MgCl_2$ and primer annealing temperature significantly influenced the yield and type of amplification products synthesized. Therefore, these parameters were kept as constant as possible in all reactions. In addition, the abundance of unexplained amplified DNAs increased when the number of PCR cycles exceeded 35 in our experiments. Sensitivity to cycle number has been previously reported (Bell and DeMarini 1991) but may have been caused in part to our use of low dCTP concentrations (0.016 *M*). The dCTP concentration was kept low in order to increase radiolabel incorporation into PCR-amplified products. Radiolabeling in conjunction with separation of PCR products on dena-

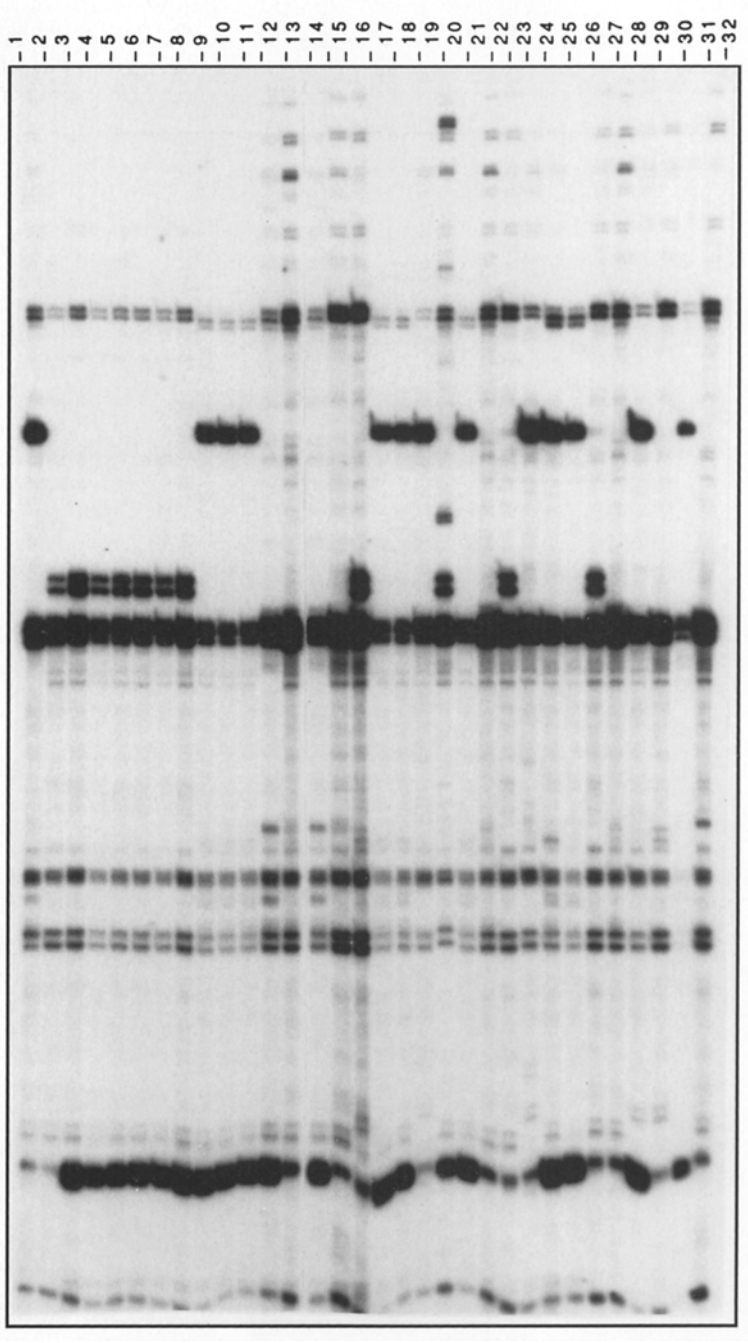


Fig. 4 Genotype analysis. The ability of primer K16 to amplify DNA and identify RAPDs among a group of 32 genotypes was tested. Genomic DNA used for amplification reactions was derived from 'Hegari' (*lane 1*), 38 M (*lane 2*), 60 M (*lane 3*), 58 M (*lane 4*), 90 M (*lane 5*), 100 M (*lane 6*), 'Standard Yellow Milo' (*lane 7*), SA 403 (*lane 8*), Tx 7078 (*lane 9*), Tx 3197 (*lane 10*), Tx 616 (*lane 11*), Tx 406 (*lane 12*), 'Combine Shallu' (*lane 13*), Tx 635 (*lane 14*), Tx 09 (*lane 15*), Tx 430 (*lane 16*), Tx 7000 (*lane 17*), Tx 2785 (*lane 18*), BTx 623 (*lane 19*), IS 3620C (*lane 20*), SC5614E (*lane 21*), SC1598C (*lane 22*), Tx 2817 (*lane 23*), ATx 623 (*lane 24*), ATx 3197 (*lane 25*), ATx 399 (*lane 26*), '3-Dwarf W. Sooner Milo' (*lane 27*), RTx 432 (*lane 28*), RTx 1720 (*lane 29*), RTx 2536 (*lane 30*), SC 103 (*lane 31*) and B 35 (*lane 32*)

turing 5% polyacrylamide gels allowed excellent resolution and easy detection of amplified products. Incorporation of [^{32}P]-dCTP in the amplification reactions resulted in greater labeling of the larger amplified products. Uniform labeling per mole of amplified DNA fragment could be obtained using end-labeled primers. In addition, fluorescently tagged primers could be used although the initial cost of synthesizing this type of primer prevented us from examining this possibility. Finally, only 1.5 µl of the amplified reaction was required for autoradiography indicating that PCR reaction volumes could be reduced, thereby lowering the cost of *Taq* polymerase per reaction. Our standard sequencing gel system allows the separation of 60 samples per gel, and each run requires approximately 2.5 h. It is possible that shorter gels could be run, thus reducing electrophoresis time.

The standard set of PCR amplification conditions determined in this study were useful for approximately 80% of the Operon primers. Amplification was successful on a wide range of genotypes, and RAPDs which distinguish the genotypes examined were observed. This indicates that RAPDs will be useful for analysis of the genotypes in which we are most interested. It should also be possible to readily establish the genetic relationship among sorghum genotypes on a wider scale once the steps in this process are automated. Finally, we examined whether or not

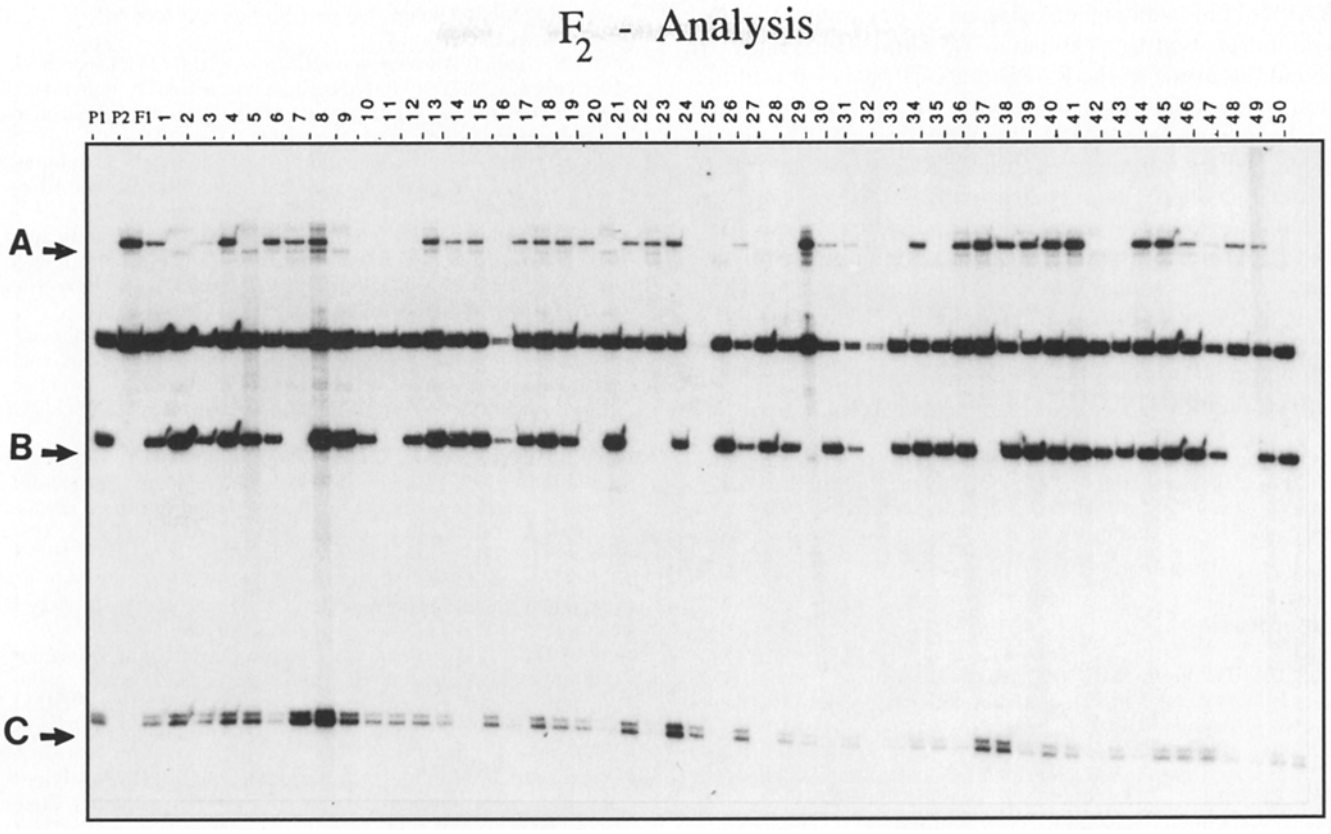


Fig. 5 Segregation of RAPDs in F_2 populations. Primer L17 revealed 3 RAPDs that distinguish BTx 623 and IS 3620C (marked as *A, B, C*). Analysis of the segregation of these RAPDs in an F_2 population from a cross of BTx 623 and IS 3620C is shown and provided information for assigning the map location of the RAPDs

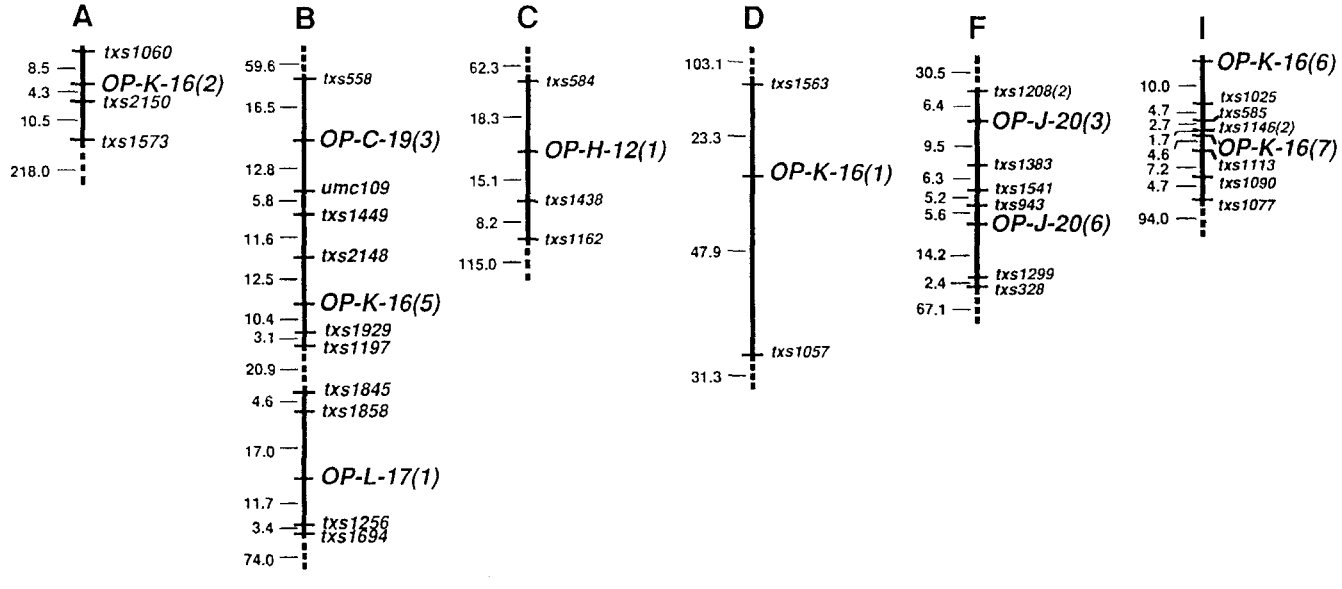


Fig. 6 Portions of the sorghum genetic map showing the location of RAPD markers. Linkage map positions of RAPDs that were detected by primers obtained from Operon Technology Inc. Segregation data for RFLPs and RAPDs were analyzed and map positions of the loci determined using Mapmaker Macintosh V1.0. *Numbers* on the *left sides of vertical lines* are map distances in centiMorgans. Loci designated *txs* were identified with sorghum DNA probes isolated at Texas A&M University, and loci designated *umc* with maize probes isolated at the University of Missouri. The *prefix X*, which designates RFLP loci detected with anonymous probes, is omitted from the *txs* and *umc* loci on these maps. RAPDs are designated in *upper-case letters* and *numbers*

RAPDs could be mapped using an F_2 population that was previously used to establish a 200-point RFLP map. We found that some of the RAPD markers provided information that enabled map position to be established by this analysis. For other RAPDs, map position could not be assigned with confidence. Similarly, Reiter et al. (1992) reported that approximately 20% of the RAPD markers identified in *Arabidopsis* could not be assigned a map position. While the creation of genetic maps will not be the primary use of RAPDs in our studies, establishing the map position of RAPDs provides useful information. In the near future 150 recombinant inbred lines derived from BTx 623 and IS 3620C will be available for analysis. This population will be used in subsequent studies to map RAPDs.

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