Theor Appl Genet (1998) 97: 337–344

Z. Jeffrey Chen · Ronald L. Phillips · Howard W. Rines

Maize DNA enrichment by representational difference analysis in a maize chromosome addition line of oat

Received: 18 November 1997 / Accepted: 12 March 1998

Abstract The recent recovery of maize (*Zea mays* L.) single-chromosome addition lines of oat (*Avena sativa* L.) from oat x maize crosses has provided novel source materials for the potential isolation of maize chromosome-specific sequences for use in genetic mapping and gene cloning. We report here the application of a technique, known as representational difference analysis (RDA), to selectively isolate maize sequences from a maize chromosome-3 addition line of oat. DNA fragments from the addition line and from the oat parent were prepared using *Bam*HI digestion and primer ligation followed by PCR amplification. A subtractive hybridization technique using an excess of the oat parental DNA was employed to reduce the availability for amplification of DNA fragments from the addition lines that were in common with the ones from the oat parental line. After three rounds of hybridization and amplification, the resulting DNA fragments were cloned into a plasmid vector. A DNA library containing 400 clones was constructed by this method. In a test of 18 clones selected at random from this library, four (22%) detected maize-specific repetitive DNA sequences and nine (50%) showed strong hybridization to genomic DNA of maize but weak hybridization to genomic DNA of oat. Among these latter nine clones, three detected low-copy DNA sequences and two of them detected DNA sequences specific to chromosome 3 of maize, the chromosome retained in the source maize addition line of oat. The other eight out of the 13 clones that had strong hybridization to maize DNA detected repetitive DNA sequences or high-copy number sequences present on most or all maize chromosomes. We estimate that the maize DNA sequences were enriched from about 1.8% to over 72% of the total DNA by this procedure. Most of the isolated DNA fragments detected multiple or repeated DNA sequences in maize, indicating that the major part of the maize genome consists of repetitive DNA sequences that do not cross-hybridize to oat genomic sequences.

Key words Representational difference analysis (RDA) · Maize · Oat · Addition line · Repetitive DNA sequence

This is joint contribution of the Minnesota Agricultural Experiment Station and USDA-ARS, Scientific Journal Series Paper No. 97113004 of the Minnesota Agricultural Experiment Station, St. Paul, MN, USA

Communicated by M. A. Saghai Maroof

Z. J. Chen (✉)[1] · R. L. Phillips
Department of Agronomy and Plant Genetics,
University of Minnesota, St. Paul, MN 55108, USA

H.W. Rines
Plant Science Research Unit, U.S. Department of Agriculture,
Agriculture Research Service and Department of Agronomy and
Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA

Present address:
[1] Department of Biology, Campus Box 1137, One Brookings Drive,
Washington University, St. Louis, MO 63130, USA
Tel.: +1 314 935-8529
Fax: +1 314 935-4432
E-mail: chen_j@biodec.wustl.edu

Introduction

RFLP (restriction fragment length polymorphism)-based genetic maps are now available in most crop species (Phillips and Vasil 1994). These maps have provided new insights into the genome structure and organization of plant species (Hulbert et al. 1990; Whitkus et al. 1992; Ahn et al. 1993), as well as providing tools for plant breeding (Paterson et al. 1988; Tanksley and Hewitt 1988; Tanksley et al. 1989) and novel practices for the positional cloning of genes (Davies 1991; Jonsson and Weissman 1995; Tanksley et al. 1995). Comparative mapping indicates that the Gramineae family can be considered as a single genetic system (Appels et al. 1989; Bennetzen and Freeling 1993). Such

a similarity in genome structure may facilitate identifying and cloning genes across boundaries of species and families (Bennetzen and Freeling 1993).

Many types of DNA markers are available, including RAPDs (random amplified polymorphic DNAs) (Caetano-Anolles 1993; Williams et al. 1990), simple sequence repeats (Ostrander et al. 1992; Weber and May 1989) and AFLPs (amplified fragment length polymorphisms) (Konieczny and Ausubel 1993), all of which facilitate genetic mapping. There are two types of DNA probes, cDNA and random-genomic DNA. cDNA probes detect DNA sequences that are transcribed but do not represent the entire genome. The use of randomly selected genomic DNA probes can increase the coverage of the linkages being mapped and reduce the number of unlinked groups. Genomic DNA probes are useful in mapping species with a low frequency of polymorphisms, such as in wheat (Chao et al. 1989; Chen et al. 1994). The common procedure to prepare genomic DNA probes involves several hybridization steps to eliminate clones that contain repetitive DNA sequences and to select ones that detect single or low-copy number polymorphic sequences. However, this technique is relatively inefficient and laborious. Therefore, there is an increasing demand to develop new techniques for isolating and screening the DNA sequences that are present in one genome but not in the other (Jonsson and Weissman 1995). Wigler and his colleagues have developed two such techniques, genomic subtraction (Wieland et al. 1990) and representational difference analysis (RDA) (Lisitsyn et al. 1993). There are two major steps involved in the RDA technique, first to reduce genome complexity by producing amplicons using restriction digestion and adapter ligation followed by PCR amplification, and second to apply selective hybridization and then amplify the target DNA sequences. Repeating the second procedure will result in high enrichment of the restriction DNA fragments present in one genome (tester or target) but not in the other genome (driver or non-target). This technique relies on the occurrence of either missing restriction sites or genome rearrangements, including deletions, insertions, duplications, and translocations. Rosenberg et al. (1994) have developed a similar technique called RFLP subtraction. They used gel purification for DNA-size fractionation and hybridized the target DNA with biotinylated non-target DNA. The hybridized non-target DNA was then removed by avidin binding. After three rounds of subtractive hybridization, the remaining target DNA was amplified by PCR. They demonstrated that 21 out of 22 clones isolated by this RFLP subtraction technique detected sequences present in the target strain of mouse, but not in the non-target strain, and only one clone detected repetitive sequences in both of the mouse strains.

The production of genetically stable maize chromosome-addition lines of oat has broken down the sexual boundary between the genera *Zea* and *Avena*, which belong to two different subfamilies of the Gramineae. Studies involving wheat (*Triticum aestivum* L.) × maize hybridizations, another combination across subfamilies, show that F_1 haploid plants have only wheat chromosomes, whereas the maize chromosomes are completely eliminated (Laurie and Bennett 1986, 1989; Laurie et al. 1990) . In progeny of oat x maize crosses, several haploid lines were characterized that contained a complete set of oat chromosomes plus an additional 1–4 maize chromosomes (Riera-Lizarazu et al. 1996). Selfing of lines containing one (or two) maize chromosomes in addition to the 21-chromosome haploid complement of oat produced disomic maize addition lines of oat through a meiotic restitution process (Riera-Lizarazu et al. 1996). The maize chromosome-addition oat lines are valuable not only for studying gene transfer from maize to oat and maize gene expression in the oat genetic background, but also for developing maize chromosome-specific libraries. We report here progress toward the construction of a maize chromosome-specific library. We first prepared two DNA fragment samples by size selection (Rosenberg et al. 1994) and then performed three rounds of subtractive hybridizations coupled with PCR amplification (Lisitsyn et al. 1993). Using this modified RDA method, we have reduced the oat DNA and enriched the maize DNA in a DNA fragment preparation from the maize chromosome-3 addition line of oat. The DNA fragment population isolated after RDA was enriched for maize DNA sequences in that about two-thirds of the recovered clones hybridized strongly to maize DNA. In a test of 18 individual clones, two of the three clones that detected single- or low-copy sequences also detected DNA fragments specific to maize chromosome 3, the only maize chromosome in the source maize addition line of oat. The distribution of clones that detected repetitive versus low-copy sequences is consistent with the notion that the maize genome consists of 60–80% repetitive DNA sequences (Bennett and Smith 1976; Hake and Walbot 1980; Springer et al. 1994). Only two clones tested detected DNA sequences specific to oat. Our results indicate that the proportion of maize DNA was enriched by the RDA technique. Whereas many single- or low-copy sequences share homology between maize and oat, most repetitive DNA sequences are genome-specific.

Materials and methods

Plant materials and DNA preparation

Maize chromosome-addition lines of oat were produced as previously described (Rines and Dahleen 1990; Riera-Lizarazu et al. 1996). The plants were grown in a growth chamber with a 12/12 h and a 20/15°C temperature (day/night) cycle for 6–8 weeks and a 14/8 h (day/night) cycle thereafter to induce flowering. DNA was isolated from 3 to 4-week-old leaves as described previously (Saghai-Maroof et al. 1984; Chen et al. 1994). Chromosome preparation and

observation were adopted from a previous method (Chen and Yu 1989).

Preparation of the driver and tester

SunII-1 (normal oat line) was used as driver and Sn # 3/2 (the maize chromosome-3 addition oat line that contains a pair of maize chromosomes 3 from Seneca 60 in a SunII-1 genetic background) as tester in the genomic subtraction. DNA was purified and digested to completion with *Bam*HI. Three sets of *Bam*HI oligo adaptors (primers) were designed as published (Lisitsyn et al. 1993) and provided by Dr. John Doebley of the Plant Biology Department, University of Minnesota. The sequences are: set 1, 5′-AGCACTCTCCAGCCTCTCACCGAG-3′ and 5′-GATCCTCGGTGA-3′; set 2, 5′-ACCGACGTCGACTATCCATGAACG-3′ and 5′-GATCCGTTCATG-3′; and set 3, 5′-AGGCAACTGTGCTATCCGAGGGAG-3′ and 5′-GATCCTCCCTCG-3′. DNA was ligated to primer set 1 in a molar ratio of 1 : 2 (DNA/oligo) and the ligated DNA was electrophoresed in 1% agarose gel. The gel slices containing DNA fragments (150–5500 bp for driver and 250–3500 bp for tester) were cut out and the DNA fragments were recovered by freezing the gel and collecting the DNA solution after centrifugation in a filter column (Midwest Scientific). Following phenol/chloroform-extraction and ethanol-precipitation, the DNA was recovered and dissolved in water. One hundred microliters of PCR reaction contained 50 ng of template DNA, 25 ng of each primer, 200 μM of each dNTP, and 5 units of *Taq* DNA polymerase in a 1 × buffer [67 mM Tris/Cl (pH 8.9), 4 mM $MgCl_2$, 16 mM $(NH4)_2SO_4$, 10 mM β-mercaptoethanol, and 0.1 mg/ml BSA]. The reaction was incubated at 72°C for 5 min to fill in 5′-protruding ends of the ligated adaptors. PCR amplification was performed in a Perkin Elmer Cetus DNA thermocycler at 95°C for 1 min, 65°C for 30 s, and 72°C for 2 min (6 min for the last cycle) for 25 cycles. The PCR-amplified DNA was passed through a Sephacryl S-300HR (Phamacia Biotech) spin-column, ethanol-precipitated, and dissolved in water. To remove the adaptors, the DNA was digested with *Bam*HI and purified using the spin-column. The *Bam*HI-digested tester DNA was then ligated to primer set 2 and diluted in water plus 10 μg/ml of yeast tRNA.

Hybridization

A solution containing 100 ng of tester and 10 μg of driver DNA in 100 μl of TE buffer was mixed in a 0.5-ml microcentrifuge tube, extracted with phenol/chloroform, and ethanol-precipitated. The DNA pellet was re-suspended in 4 μl of a buffer solution containing 3 mM EDTA and 30 mM Na-EPPS [*N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid), pH 8.0], overlaid with mineral oil, and denatured for 5 min at 100°C. After adding 1 μl of 5 M NaCl, the DNA mixture was incubated at 65°C for 18 h. The sample was diluted by adding 100 μl of water and extracted with chloroform to remove oil. A 100-μl PCR reaction was set up by adding 10 μl of a hybridized DNA sample and the appropriate PCR mixture as above, except that the primer was omitted at this time. The reaction mixture was then incubated at 72°C for 5 min to fill-in the adaptor ends. After adding the 24-mer primer (set 2), PCR amplification was performed as above for 15 cycles. The amplified DNA was extracted with phenol/chloroform, ethanol-precipitated, and dissolved in 100 μl of water. An aliquot of 10 μl was mixed with 10 μl of 2 × mung bean nuclease (MBN) buffer and 10 units of MBN to remove unhybridized single-stranded DNA. After 30 min at 37°C, the reaction was inactivated by adding 80 μl of 50 mM Tris-Cl (pH 8.9) followed by incubating at 100°C for 5 min. The MBN-treated product was used in five separate PCR reactions in the same cycler for 20 cycles. The amplified DNA was extracted with phenol/chloroform, ethanol-precipitated, and dissolved in water. An aliquot of 1 μg of the amplified DNA product was digested with *Bam*HI to remove the adaptors and ligated to adaptor 3 (primer set 3). The second and third rounds of hybridization were performed as described above.

Cloning and analyzing the subtraction/amplification enrichment products

The amplified subtraction products were digested with *Bam*HI and ligated to *Bam*HI-digested de-phosphorylated pUC18. After transformation, single colonies were selected and boiled and the inserts amplified using M13 forward and reverse sequence primers. DNA transfer, hybridization and washing conditions were adopted from Chen et al. (1994) . Under the same stringent conditions for hybridization and washing at 65°C, the DNA blots were exposed to X-ray films for 3–5 days for single- or low-copy probes and 1–12 h for repetitive DNA sequences in order to reveal the hybridization signals at an analyzable level.

Results

Maize chromosome-3 addition line of oat

The maize chromosome-3 addition line of oat used in this study was produced as previously described (Rines and Dahleen 1990; Riera-Lizarazu et al. 1996). The disomic addition lines ($2n = 42 + 2'$) tend to be quite stable. The added pair of maize chromosomes appear stably transmitted in the oat background based on two to three generations of selfing (data not shown). A karyotype prepared in a root-tip cell of the addition line is shown in Fig. 1. The two chromosomes indicated by arrows were identified as maize chromosomes, which were verified by genomic in-situ hybridization using maize genomic DNA randomly labeled with

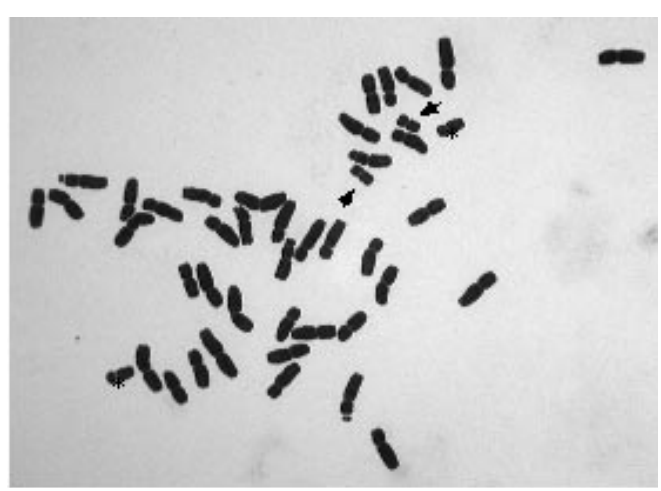

Fig. 1 Chromosome preparation of a maize chromosome-3 addition line of oat in a root-tip cell. A pair of maize chromosomes showing a sub-median arm ratio is indicated by the *arrows*. The two smallest oat chromosomes with a sub-telomeric arm ratio are indicated by *asterisks*

digoxigenin-dUTP (data not shown). Only one pair of chromosomes was hybridized with the dig-dUTP labeled-maize DNA probe. In addition, DNA probes specific to maize chromosome 3 detected maize-specific RFLPs in this line (Riera-Lizarazu et al. 1996), confirming that the extra pair of chromosomes present in this line represents maize chromosome 3. The phenotypes of this addition line include a distorted panicle, branches in the lower internodes, and liguleless leaves (Riera-Lizarazu et al. 1996).

Genomic subtraction

The genomic subtraction procedures were adopted from those of Lisitsyn et al. (1993) and Rosenberg et al. (1994). We used DNA from a maize chromosome-3 addition line of oat, Sn3#2–4, as a tester, and DNA of SunII-1, the oat parent of this line, as a driver. Total genomic DNA of each sample was extracted, purified and ligated to *Bam*HI oligo adaptors. The ligated DNA was then gel-purified as described in Materials and methods and amplified by PCR (Fig. 2 A, lanes 2 and 3). The size of DNA fragments amplified ranged from 250 bp to 6 kb. Two limitations of the procedure are the low efficiency in DNA ligation and random amplification in the PCR reaction. We therefore used a ligation and amplification control. The control DNA was a plasmid vector (2.7 kb) containing a 3.8-kb chloroplast DNA insert (Chen et al. 1993). Upon *Bam*HI-digestion, the circular DNA was cut into two fragments, a 1-kb chloroplast DNA fragment and a 5.5-kb fragment containing the vector and part of the insert DNA. The two DNA fragments were then ligated to the second set of *Bam*HI adaptors, column purified, and mixed into the genomic DNA samples (ligated with set-1 primers) at a single- or low-copy level, i.e. about 10 pg in 10 μg of a total DNA mixture. As expected, the two fragments were amplified only by the second set of primers (Fig. 2 A, lane 4) and not by the first set (Fig. 2 A, lane 5). The 1-kb fragment was amplified more than the 5.5-kb fragment, reflecting the fact that PCR amplification favors smaller fragments. In addition, the very low level of background amplification (lane 4) indicated that the two primers did not cross-amplify under these conditions.

We then performed subtractive hybridization using SunII-1 (driver) and Sn3#2 (tester) DNA in a ratio of 100:1. After the first hybridization, DNA fragments ranging from 400 to 1500 bp in size were amplified dramatically (Fig. 2 B, lane 7), whereas other DNA fragments were not amplified after subtractive hybridization. In the sequential two rounds of hybridization and amplification (lanes 8 and 9), product profiles were very similar to each other, indicating that most of the poorly hybridized fragments and fragments without appropriate adaptors were not amplified and enriched (Lisitsyn et al. 1993; Rosenberg et al. 1994). The proportion of DNA fragments in PCR products that were homologous to maize and oat genomic DNA after each round of subtractive hybridization was monitored by dot-blot analysis using the blots containing oat and maize genomic DNA hybridized with the PCR products as probes (data not shown).

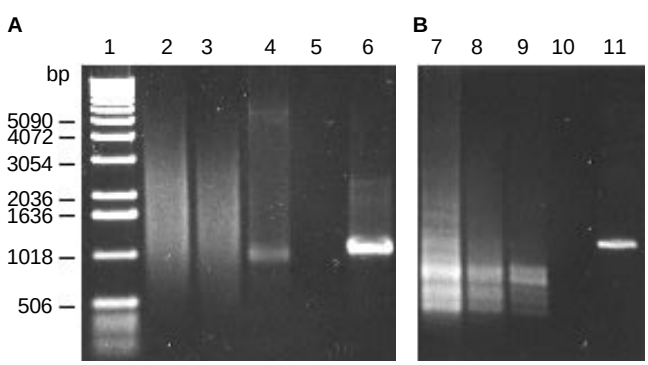

Fig. 2A, B Electrophoresis of DNA products from PCR amplification and subtractive hybridization in a 1.5% agarose gel stained with ethidum bromide. **A**. DNA size marker (*lane 1*), driver (*lane 2*), tester (*lane 3*), *Bam*HI-adaptor ligated plasmid DNA for ligation and amplification control (*lane 4*, see text), negative PCR control (*lane 5*, same reaction as in *lane 4* except that set-1 primers were used), and positive PCR control with a plasmid DNA containing a known DNA fragment (*lane 6*). **B** the amplified products from subtractive hybridizations (rounds one to three correspond to *lanes 7 to 9*), negative PCR control with template DNA omitted (*lane 10*) and positive PCR control as in lane 6 (*lane 11*)

Characterization of DNA clones from the subtractive library

Some of the products after the third round of subtraction and amplification were purified and cloned into a pUC18 plasmid vector to produce a library of about 400 clones. We amplified the inserts from 18 colonies selected at random from this library (Fig. 3 A). Each clone contained an insert. These inserts ranged from 300 to 1000 bp in size, matching the sizes of DNA profiles observed during subtractive hybridization. The nomenclature for UMN DNA clones is that adopted at the 7th International Wheat Genetics Symposium (Hart and Gale 1988; Fig. 3). Clone pAZUMN14 had two fragments amplified by PCR. The smaller fragment could be a PCR artifact or another insert. We did not further characterize it because it hybridized poorly to both maize and oat genomic DNA. A weakly amplified insert was shown in clone pAZUMN1 here, but it amplified more strongly in later analysis (data not shown). When the DNA-blot was probed with labeled maize genomic DNA, most insert DNA fragments (lanes 2 to 8, 11 to 14-1, 16, and 18) showed strong hybridization to maize DNA (Fig. 3 B). Among them, four (Fig. 3 B, lanes 5, 11, 12, and 14-1) showed very strong signals as would be expected with repetitive

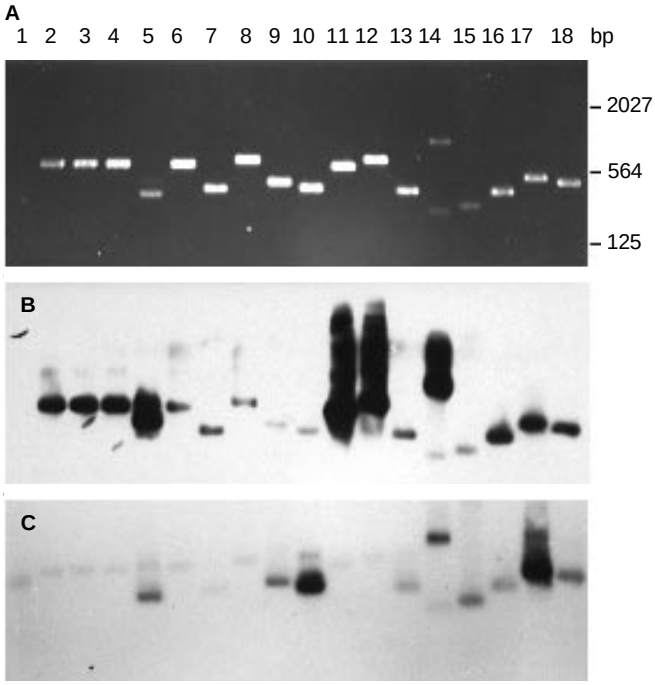

Fig. 3A–C DNA-blot analysis shows 18 clones containing DNA fragments that hybridized to maize and oat genomic DNA. **A** PCR-amplified insert DNA in each of the 18 individual clones resolved in a 1.2% agarose gel stained with ethidum bromide. **B** Autoradiogram of a blot containing the same DNA as shown in **A** that was hybridized with labeled maize genomic DNA. **C** Autoradiogram of the same blot as **B** with the maize probe washed off and hybridized with labeled oat genomic DNA. The blots were exposed to X-ray films for 24 h. The nomenclature for UMN DNA clones is that adopted at the 7th International Wheat Genetics Symposium (Hart and Gale 1988). Each of the 18 clones was designated as pAZUMN followed by a number from 1 to 18. The larger fragment in clone 14 designated as pAZUMN14-1, was used in this study. The smaller fragment in the clone 14, designated as pAZUMN14-2, was not further characterized since it hybridized very weakly to both maize and oat DNA

DNA sequences. When the same blot was stripped of the maize probe and hybridized with labeled oat DNA (Fig. 3 C), only two clones (lanes 10 and 17) showed strong signals, indicating that the DNA fragments cloned after three rounds of subtractive hybridization and amplification contain more sequences homologous to maize than to oat. Except for the insert of clone pAZUMN17, the insert fragments that hybridized strongly to maize DNA had low homology to oat DNA. Clone pAZUMN17 contained a fragment showing strong hybridization with both oat and maize. It is notable that clones pAZUMN5, 11, 12 and 14-1 showed very strong hybridization to the maize DNA probe, but weak hybridization to oat DNA. These four DNA clones indeed contained repetitive DNA sequences highly specific to maize (see below). pAZUMN11 and 12 appear to be more maize-specific than pAZUMN5 and 14-1.

We further analyzed the clones containing DNA fragments that showed strong hybridization to maize DNA. DNA blots prepared from genomic DNA of maize (Seneca 60 and A188), oat (SunII-1, Starter-1, and GAF/Park), and maize addition lines of oat containing maize chromosomes 2, 3, 4, 4/7, 5, 6, 7, 8 and 9, respectively, were used to hybridize the individual DNA fragments isolated from each of the clones. Two clones (Fig. 4 A and 4 B) each detected a DNA fragment only in the maize chromosome-3 addition line of oat, the original line used for subtraction, in addition to an oat DNA fragment. The low level of hybridization detected in the chromosome-3 addition line relative to the maize lines is probably due to the relatively low maize-DNA content in each of the addition lines, because the gels for the DNA-blots shown were loaded with nearly equal amounts of DNA for each source. On the assumption that the DNA content of the haploid genome of oat and maize is 13.5 pg and 2.5 pg, respectively (Bennett and Smith 1976). About 5–6-times as much DNA from each addition line should be loaded to yield an equal signal if the sequences detected in maize are chromosome-specific (i.e., only on one maize chromosome). The hybridization signal detected in the addition line is indeed less than those in the two maize lines. If the sequences detected are highly dispersed and nearly equally distributed among the maize chromosomes ($n = 10$), about 50-fold more DNA in the addition line should be loaded to achieve equal hybridization between the maize DNA in an addition line and the maize control. The different mobility of the fragments detected across the lanes in Fig. 4 B was probably due to variable salt conditions in the DNA samples subjected to electrophoresis. Four clones, pAZUMN5, 11, 12 and 14-1, detected repetitive DNA sequences in maize including eight maize addition lines of oat, but had little or no hybridization to oat DNA (Fig. 5 A and B). Clones pAZUMN5 and 14-1 showed relatively higher levels of hybridization to oat than pAZUMN11 and 12 (Fig. 3 B and C and data not shown). As just mentioned, the low signals observed in the addition lines are because of the relatively low amounts of maize DNA. It is notable that pAZUMN12 contained a fragment that failed to detect repetitive sequences in chromosome 4 of maize (Fig. 5 B). The data obtained in the DNA-blot analysis (Fig. 3 B and C) matched fairly well to the Southern-blot data analyzed with the individually labeled-fragment as a probe, except for pAZUMN6. DNA-blots (Fig. 3 B and C) indicate that pAZUMN6 hybridized more strongly with maize than with oat, but this was not evident in the Southern analysis (Fig. 4 A) (see Discussion).

Discussion

We applied a genomic subtraction method to two large-genome species in the grass family, maize (*Zea mays* L.) and oat (*Avena sativa* L.). Using oat DNA as

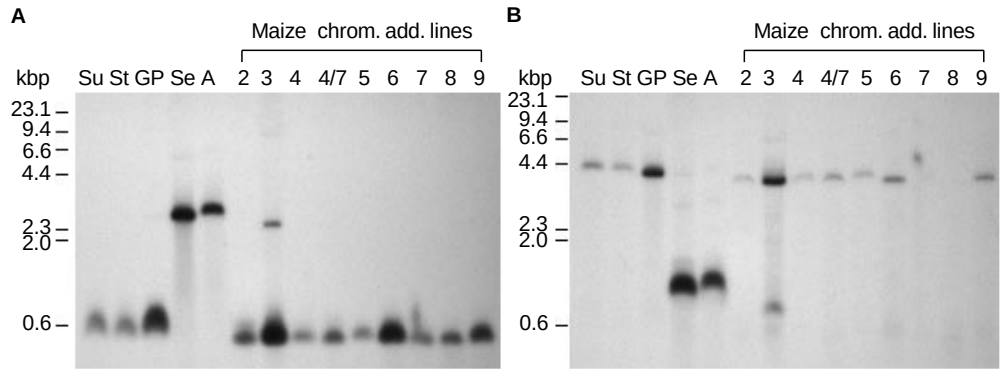

Fig. 4 DNA-blot analysis indicated that clone pAZUMN6 (in **A**) and pAZUMN4 (in **B**) each detected a fragment from maize chromosome 3, the original target chromosome used for subtractive hybridization. The blots contained *Bam*HI-digested genomic DNA from oat lines [SunII-1 (*Su*), Starter-1 (*St*), and GAF/Park (*GP*)], maize lines [Seneca 60 (*Se*) and A188 (*A*)], and maize chromosome addition lines as indicated. One line labeled as "4/7" contained two maize chromosomes, namely, 4 and 7, in the oat (Starter-1) genetic background. The blots were exposed to the X-ray films for 2 days (**A**) and 1 day (**B**), respectively

a driver and DNA from an addition line of oat containing a single maize chromosome as a tester, we have removed most of the DNA sequences in common in the two DNA pools and obtained a subtraction library containing over 400 clones. Eighteen of these clones have been characterized using DNA-blot analysis. About two-thirds of the clones tested, including three clones that detected low-copy sequences, contained fragments that hybridized strongly to maize but weakly to oat. Two clones tested detected DNA sequences specific to chromosome 3 of maize, the chromosome retained in the source maize chromosome-addition line of oat. The others with stronger hybridization to maize than oat hybridized to multiple maize chromosomes indicating that they contained dispersed repetitive sequences. Only one clone had insert DNA that did not hybridize to maize or to oat in two separate DNA-blot analyses. It has been calculated that the average maize genome is 2.5 pg per haploid (n = 10) while that of oat is 13.5 pg per haploid (n = 21) (Bennett and Smith 1976; Hake and Walbot 1980). Therefore, we estimate that by employing the modified RDA method with the addition-line source DNA, we increased the chances from about 1.8% to over 72% that a cloned DNA fragment was of maize origin. That the large majority of the clones are either maize-specific, or else show stronger hybridization to maize than oat, demonstrates that enrichment occurred from starting material that was about 1.8% maize DNA.

The data obtained by DNA-blot analysis using individual clones (except for pAZUMN6) as hybridization probes to total oat and maize genomic DNA matched the results obtained from the blot containing PCR-amplified inserts hybridized with maize and oat genomic DNA as probes. This indicates the possibility that we could use DNA-blot analysis of PCR products amplified from the clones to quickly screen the library for homology (Rosenberg et al. 1994) to maize and oat DNA. The PCR-amplified insert DNA of pAZUMN6 showed strong hybridization to maize and weak hybridization to oat total-genomic DNA (lane 6 of Fig. 3 B and C), whereas the fragment cut from the plasmid detected nearly equal signals to both maize and oat (Fig. 4 A). The reason is that using oat total-genomic DNA as a probe is less sensitive to detect signals than using total maize DNA as a probe due to the smaller genome size of maize. The equal signals detected in the oat and maize lines by the insert fragment used as probe imply that the pAZUMN6 insert actually showed stronger hybridization to maize than oat considering the relatively low amount of maize DNA present in each of the maize addition lines.

Repetitive DNA sequences

Of the 18 clones tested, 12 had DNA fragments that detected repetitive DNA sequences either in maize or oat. Our method to determine repetitive DNA sequences is based on a relative estimation. We define the DNA fragments that detected strong or analyzable hybridization signals after exposure to the X-ray film for only 1–12 h as repetitive DNA sequences, whereas the low- or single-copy DNA fragments, under the same high stringent conditions for hybridization and washing, require 3–5 days exposure to reveal similar or lower-level signals. Previous studies have estimated that about 60–80% of the total maize DNA is comprised of repetitive (including medium and highly repetitive) sequences (Bennett and Smith 1976; Hake and Walbot 1980; Springer et al. 1994). In a YAC clone containing 280 kb surrounding the *Adh1* locus of maize, Springer et al. (1994) concluded that only 18% of the DNA is present as low-copy number sequences. About 75% of the YAC ends screened in a maize YAC

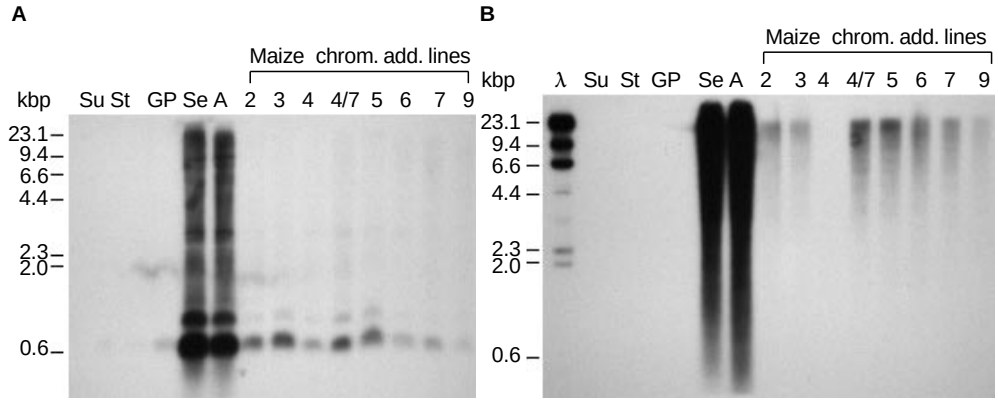

Fig. 5 DNA-blot analysis showed that two clones detected repetitive sequences in maize but not in oat. **A** The probe pAZUMN11 was hybridized to DNA fragments from oat, maize, and the addition lines (see legend in Fig. 4) digested with *Bam*HI. **B** The probe pAZUMN12 detected repetitive DNA sequences from the maize and addition lines digested with *Hin*dIII. The DNA-blots were exposed to the X-ray films for 1–5 h

library were repetitive. The arrangement of repetitive DNA sequences in the maize genome has been proposed to exist in two modes; repetitive sequences are either interspersed in a random fashion and spatially separate from single-copy sequences (Gupta et al. 1984) or else they are interspersed with single-copy sequences (Springer et al. 1994). Some repetitive DNA sequences were found to be species-specific in the Gramineae family (Peacock et al. 1981; Dennis and Peacock 1984). Most single-copy DNA sequences cross-hybridize among different species of the grass family (Hulbert et al. 1990; Whitkus et al. 1992; Ahn et al. 1993), suggesting that single- or low-copy number sequences share homology beyond species boundaries (Bennetzen and Freeling 1993) .

It is not surprising that in our subtractive library most of the DNA fragments isolated contained repetitive DNA sequences. Using a different approach, Ananiev et al. (1997) demonstrated that 95% of the clones from a maize genomic cosmid library could be detected by a mixture of highly repetitive DNA sequences used as a multiprobe. Similarly, much of the large oat genome is composed of repetitive sequences highly specific to oat relative to maize (Ananiev et al., personal communication). Common DNA sequences including single- or low-copy ones present in both oat and maize genomes would be selected against by the enrichment techniques (Lisitsyn et al. 1993; Rosenberg et al. 1994). Because the initial target DNA from the oat-maize chromosome-3 addition line presumably had a large proportion of oat-specific repetitive DNA fragments, whereas the final library had only a few (2 of 18) clones of this type, the RDA procedure using oat genomic DNA as driver was highly successful in reducing the relative amount of oat repetitive DNA. Thus, we might predict that if one were to include some source of maize repetitive DNA in the driver, such as DNA from an addition line for a maize chromosome other than the target chromosome, then the subtractive procedure would also remove maize chromosomally dispersed repetitive DNAs resulting in an increased proportion of maize chromosome 3-specific sequences in the final library.

Applications

The novel maize addition lines of oat are valuable for mapping DNA markers (especially non-polymorphic ones) onto an individual maize chromosome. The RDA technique employed in this study has the potential to use cDNA as a starting material in order to reduce the large amount of repetitive DNA sequences present in higher plants. The maize chromosome-3 addition line is particularly interesting, because this line has some novel phenotypes. It produces liguleless leaves, increased leaf-sheath pubescence, semi-aborted panicles (Riera-Lizarazu et al. 1996), broad leaves, air-roots and premature branches in the first 3–4 internodes above ground. One would expect that these unusual phenotypes are caused by the expression of maize genes in chromosome 3, since most of the other addition lines have normal oat phenotypes. The application of RDA with cDNA sources will provide an opportunity for isolating maize and oat cDNA sequences that are differentially expressed (McClelland et al. 1995) due to the influence of the added maize chromosome.

It will also be interesting to examine the organization of the repetitive DNA sequences isolated in our library within the maize genome (SanMiguel et al. 1996). In this respect the clone that detected repetitive DNA sequences in all tested maize chromosomes except maize chromosome 4 is of particular interest.

Acknowledgments We thank Dr. John Doebley for providing primers, Dr. David Somers for reviewing the manuscript, and Oscar Riera-Lizarazu, Lisa Gulbranson, and Suzanne Livingston for their helpful suggestions and discussions. We are grateful for financial support from the Quaker Oats Company and the Midwest Plant Biotechnology Consortium (USDA Prime/Purdue University sub. # 593-0120-13).

References

Ahn S, Anderson JA, Sorrells ME, Tanksley SD (1993) Homoeologous relationships of rice, wheat and maize chromosomes. Mol Gen Genet 241 : 483–490

Ananiev EV, Riera-Lizarazu O, Rines HW, Phillips RL (1997) Oat-maize chromosome addition lines: a new system for mapping the maize genome. Proc Natl Acad Sci USA 94 : 3524–3529

Appels R, Reddy P, McIntyre CL, Moran LB, Frankel OH, Clarke BC (1989) The molecular-cytogenetic analysis of grasses and its application to studying relationships among species of the Triticeae. Genome 31 : 122–133

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Phil Trans R Soc Lond B: Biol Sci 274 : 227–274

Bennetzen JL, Freeling M (1993) Grasses as a single genetic system: genome composition, collinearity and compatibility. Trends Genet 9 : 259–261

Caetano-Anolles G (1993) Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl 3 : 85–94

Chao S, Sharp PJ, Gale MD (1989) RFLP-based genetic maps of wheat homoeologous group-7 chromosomes. Theor Appl Genet 78 : 495–504

Chen Z, Yu H (1989) Comparative studies of C-banded and N-banded heterochromatin of *Triticum timopheevi* Zhuk. Chinese J Genet 16 : 103–109

Chen Z, Muthukrishnan S, Liang GH, Schertz KF, Hart GE (1993) A chloroplast DNA deletion located in RNA polymerase gene *rpoC2* in CMS lines of sorghum. Mol Gen Genet 236 : 251–259

Chen Z, Devey M, Tuleen NA, Hart GE (1994) Use of recombinant substitution lines in construction of RFLP-based genetic maps of chromosomes 6A and 6B of tetraploid wheat (*Triticum turgidum* L.). Theor Appl Genet 89 : 703–712

Davies K (1991) Mapping the way forward. Nature 353 : 798–799

Dennis ES, Peacock WJ (1984) Knob heterochromatin homology in maize and its relatives. J Mol Evol 20 : 341–350

Gupta M, Shepherd NS, Bertram I, Saedler H (1984) Repetitive sequences and their organization on genomic clones of *Zea mays*. EMBO J 3 : 133–139

Hake S, Walbot V (1980) The genome of *Z. mays*, its organization and homology to related grasses. Chromosoma 79 : 251–270

Hart GE, Gale MD (1988) Guidelines for nomenclature of biochemical/molecular loci in wheat and related species. In: Miller TE, Koebner RMD (eds) Proc 7th Int Wheat Genet Symp, Bath Press, Bath, UK, pp 1215–1218

Hulbert SH, Richter TE, Axtel JD, Bennetzen JL (1990) Genetic mapping and characterization of sorghum and related crops by means of maize DNA probes. Proc Natl Acad Sci USA 87 : 4251–4255

Jonsson JJ, Weissman SM (1995) From mutation mapping to phenotype cloning. Proc Natl Acad Sci USA 92 : 83–85

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J: 4403–410

Laurie DA, Bennett MD (1986) Wheat × maize hybridization. Can J Genet Cytol 28 : 313–316

Laurie DA, Bennett MD (1989) The timing of chromosome elimination in hexaploid wheat × maize crosses. Genome 32 : 953–961

Laurie DA, O'Donoughue LS, Bennett MD (1990) Wheat × maize and other wide sexual hybrids: their potential for genetic manipulation and crop improvement. In: Gustafson JP (ed) Gene manipulation in plant improvement. Plenum Press, New York, pp 95–126

Lisitsyn N, Lisitsyn N, Wigler M (1993) Cloning the differences between two complex genomes. Science 259 : 946–951

McClelland M, Mathieu-Daude F, Welsh J (1995) RNA fingerprinting and differential display using arbitrarily primed PCR. Trends Genet 11 : 242–246

Ostrander EA, Jong PM, Rine J, Duyk G (1992) Construction of small-insert genomic DNA libraries highly enriched for microsatellite repeat sequences. Proc Natl Acad Sci USA 89 : 3419–3423

Paterson AH, Damon S, Hewitt JD, Peterson S, Lincoln SE, Tanksley SD (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment polymorphisms. Nature 335 : 721–726

Peacock WJ, Dennis ES, Rhoades MM, Pryor AJ (1981) Highly repeated DNA sequence limited to knob heterochromatin in maize. Proc Natl Acad Sci USA 78 : 4490–4494

Phillips RL, Vasil IK (1994) DNA-based markers in plants. Kluwer Academic Publishers, Dordrecht, The Netherlands

Riera-Lizarazu O, Rines HW, Phillips RL (1996) Cytological and molecular characterization of oat × maize partial hybrids. Theor Appl Genet 93 : 123–135

Rines HW, Dahleen LS (1990) Haploid oat plants produced by application of maize pollen to emasculated oat florets. Crop Sci 30 : 1073–1078

Rosenberg M, Przybylska M, Straus D (1994) "RFLP subtraction": a method for making libraries of polymorphic markers. Proc Natl Acad Sci USA 91 : 6113–6117

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81 : 8014–8018

SanMiguel P, Tikhonov A, Jin Y-K, Motchoulakaia N, Zakharov D, Melake-Berhan A, Springer P, Edwards KJ, Lee M, Avramova Z, Bennetzen JL (1996) Nested retrotransposons in the intergenic regions of the maize genome. Science 274 : 765–768

Springer PS, Edwards KJ, Bennetzen JL (1994) DNA class organization of maize *Adh1* yeast artificial chromosomes. Proc Natl Acad Sci USA 91 : 863–867

Tanksley SD, Hewitt J (1988) Use of molecular markers in breeding for soluble solids content in tomato, a re-examination. Theor Appl Genet. 75 : 811–823

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7 : 257–264

Tanksley SD, Ganal M, Martin GB (1995) Chromosome landing: a paradigm for map-based gene cloning in plants with large genomes. Trends Genet 11 : 63–68

Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44 : 388–396

Whitkus R, Doebley J, Lee M (1992) Comparative genome mapping of sorghum and maize. Genetics 132 : 1119–1130

Wieland I, Bolger G, Asouline G, Wigler M (1990) A method for difference cloning: gene amplification following subtractive hybridization. Proc Natl Acad Sci USA 87 : 2720–2724

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18 : 7213–7218