Journal of Biochemistry and Molecular Biology, Vol. 36, No. 5, September 2003, pp. 427-432

JOURNAL OF
Biochemistry
and
Molecular Biology

Distinction between Cold-sensitive and -tolerant Jute by DNA Polymorphisms

Mohammad Belayat Hossain, Aleya Awal, Mohammad Aminur Rahman, Samiul Haque† and Haseena Khan*

Department of Biochemistry & Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh

†Physiology Department, Bangladesh Jute Research Institute, Dhaka, Bangladesh

Received 31 December 2002, Accepted 10 March 2003

Jute is the principal coarse fiber for commercial production and use in Bangladesh. Therefore, the development of a high-yielding and environmental-stress tolerant jute variety would be beneficial for the agro economy of Bangladesh. Two molecular fingerprinting techniques, random-amplified polymorphic DNA (RAPD) and amplified-fragment length polymorphism (AFLP) were applied on six jute samples. Two of them were cold-sensitive varieties and the remaining four were cold-tolerant accessions. RAPD and AFLP fingerprints were employed to generate polymorphism between the cold-sensitive varieties and cold-tolerant accessions because of their simplicity, and also because there is no available sequence information on jute. RAPD data were obtained by using 30 arbitrary oligonucleotide primers. Five primers were found to give polymorphism between the varieties that were tested. AFLP fingerprints were generated using 25 combinations of selective-amplification primers. Eight primer combinations gave the best results with 93 polymorphic fragments, and they were able to discriminate the two cold-sensitive and four cold-tolerant jute populations. A cluster analysis, based on the RAPD and AFLP fingerprint data, showed the population-specific grouping of individuals. This information could be useful later in marker-aided selection between the cold-sensitive varieties and cold-tolerant jute accessions.

Keywords: AFLP, Genetic homology, RAPD

Introduction

Jute is a major fiber of Bangladesh. It is classified in the Magnoliophyta division, Magnolopsida class, Malvales order and *Tiliacea* family. Jute adapts well to loamy soil in any hot and humid region. The fiber strands are 6 to 10 ft. long (2-3 m) and are separated from the woody stalk. Because of its low cost and ease of dyeing and spinning, jute is the main fiber for commercial production and use. It is also used for twine, rope, carpet, linoleum backing, insulation, and paper manufacturing. Temperature and light are important factors that control the growth and development of jute (Janick *et al.*, 1974). Most of the biological reactions that take place in jute are controlled by temperature. *Corchorus olitorius* jute varieties, O-4 and O-9897, grow well in high temperatures, early April to July. Jute could be grown more profitably in Bangladesh if an intense cropping pattern could be developed, where jute would be cultivated from late February to early March. For this purpose, new varieties that are tolerant to low temperatures will be required; therefore, it is essential to develop an efficient way of detecting a genetic marker that is linked to the cold-tolerant trait of jute. Molecular markers provide a reliable and cost-effective alternative. They have been successfully used for assessing the parentage, examining genetic relationships among and within species, and for marker-assisted breeding. DNA fingerprinting by AFLP and RAPD was undertaken to find the polymorphism between cold-sensitive jute varieties and cold-tolerant jute accessions. This polymorphism would be used to identify or distinguish between cold-tolerant and cold-sensitive jute samples.

RAPD is a fast, PCR-based method of genetic typing that is based on genomic polymorphisms. Random-amplified polymorphic DNA (RAPD) markers (Williams *et al*., 1990) have been successfully used for cultivar analysis in a number of plant species. These include the following: broccoli and cauliflower (Hu and Quiros 1991; Kresovich *et al.*, 1992), cocoa (Wilde *et al*., 1992), banana (Kaemmer *et al*., 1992; Howell *et al*., 1994), papaya (Stiles *et al*., 1993), apple (Koller

*To whom correspondence should be addressed.
Tel: 88-2-966-1920-59 (6102); Fax: 88-2-861-5583
E-mail: haseena@bangla.net

et al., 1993), celery (Yang and Quiros *et al*., 1993), onion (Wilkie *et al*., 1993), cranberry (Novy *et al*., 1994), sunflower (Lawson *et al*., 1994), rice (Takeuchi 1994), maize (Yazaki *et al*., 1994), *Brassica juncea* (Fujushiro *et al*., 1994), soybean (Lin, 1996; Powel, 1996), rose (Denbener *et al*., 1996), kenaf (Zhou *et al*., 1998; Cheng *et al*., 2000), roselle (Hanboonsong *et al*., 2000), and jute (Belayat *et al*., 2002).

AFLP (Vos *et al*., 1995; Henry *et al*., 1997) is a highly informative assay for evaluating plant genomes since it can be used to generate fingerprints for any DNA, regardless of complexity. AFLP relies on PCR amplification of an arbitrary set of restriction fragments that are randomly distributed throughout the genome, producing a large number of high quality markers without the need for prior sequence knowledge. AFLP has been used to examine the genetic relationships among other crop species. These include potato (van Eck *et al*., 1995), lettuce (Hill *et al*., 1996), rice (Cho *et al*., 1996), barley (Powell *et al*., 1997), soybean (Keim *et al*., 1997), maize (Vuylsteke *et al*., 1999), eggplant (Mace *et al*., 1999), and sunflower (Quagliaro *et al*., 2001). Previous studies showed that an AFLP analysis has greater discriminatory power than a RAPD analysis and other genomic fingerprinting methods. Neither an AFLP nor RAPD analysis requires previous detailed knowledge of the DNA in order to be analyzed, and both have been shown to be reliable methods of distinguishing small genomic differences.

The objective of this study was to find the polymorphism between the cold-sensitive jute varieties and cold-tolerant jute accessions through DNA fingerprinting (RAPD and AFLP) and to use this polymorphism to identify or distinguish between the cold-tolerant and cold-sensitive jute populations.

Materials and Methods

Plant material Sampling of the selective varieties was conducted in different locations (Table 1). Sample plants were collected from the Physiology Department, Bangladesh Jute Research Institute (BJRI, Dhaka, Bangladesh). The leaves and seeds were used for DNA extraction.

DNA extraction DNA was extracted using the CTAB procedure that was modified from Doyle and Doyle's method (1990). Next, 1.0-1.5 g of tissue was homogenized by grinding in the presence of liquid nitrogen, then a 5.0 ml CTAB extraction buffer was added and incubated at 60°C for 30 min. One volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1) was added. The mixture was mixed and centrifuged at 4,000 rpm for 10 min at room temperature. The supernatant was transferred to a new tube. The DNA was precipitated with ice-cold isopropanol. The DNA pellet was washed in 70% ethanol (ice-cold), dried, dissolved in a TE buffer (10 mM Tris-HCl, 1 mM EDTA). After the RNase treatment, the DNA was treated with one volume of phenol : chloroform : isoamylalcohol (25 : 24 : 1). The mixture was then centrifuged at 14,000 rpm for 10 min at 4°C. The DNA was precipitated with 1/10 volume of 3 M Na-acetate and a double volume of ice-cold 99% ethanol. After washing with 70% ethanol, the DNA was dried, dissolved in a TE buffer and stored at −20°C.

RAPD analysis DNA from an individual plant of each jute variety was screened with 30 random primers from Operon Technologies Inc. Alameda, USA. The PCR reaction (25 µl) contained the following: 1x reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.2 mM dNTPs, 2 mM $MgCl_2$, 60 ng primer, 1.0 unit of Taq DNA polymerase, and 25-40 ng genomic DNA. The DNA was amplified in a thermal cycler (Eppendorf Mastercycler Gradient) that was programmed as follows: preheating for 5 min at 94°C; 45 cycles of 45 s at 94°C (denaturation), 60 s at 40°C (annealing), and 90 s at 72°C (extension); and a final extension at 72°C for 7 min, followed by cooling to 4°C. The amplified-DNA samples were analyzed by electrophoresis on 2% agarose gel in a 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gels were stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Verification of restriction enzyme digestion AFLP requires restriction digestion of the DNA by rare and common cutting restriction enzymes. Here, *Eco*RI (6 bp cutter) and *Mse*I (4 bp cutter) were used as the rare and common cutting enzymes, respectively. The *Eco*RI/*Mse*I mixture contained 2.0 µl of the enzyme mixture (1.25 U/µl, Gibco BRL), 15.0 µl sample DNA (15 ng/µl), 5.0 µl of 5x reaction buffer (AFLP Core Reagent Kit, Gibco BRL), and were made to a final volume of 25 µl with ultra-pure PCR water. The reaction mixtures were prepared on ice prior to incubation for 2 h at 37°C. The digested DNA was separated on a 0.8% agarose gel, and run in a 1x TAE buffer. The DNA was stained with ethidium bromide (0.5 µg/ml) and visualized under UV illumination.

AFLP analysis AFLP fragments were generated using the Life Technologies AFLP Analysis System II and an AFLP Core

Table 1. The jute samples used in this study

No.	Species	Populations	Source
1	*C. olitorius*	Var. O-4	Bangladesh
2	*C. olitorius*	Var. O-9897	Hybridization between var. O-5 and Brazilian var. BZ-5
3	*C. olitorius*	Acc. No.1540	India
4	*C. olitorius*	Acc. No.1805	Egypt
5	*C. olitorius*	Acc. No.1852	Bangladesh
6	*C. olitorius*	Acc. No.2015	India

Table 2. Distribution of RAPD fragments in the jute populations

Primer Name	Number of RAPD fragments								
	Var. O-4	Var. O-9897	Acc. No. 1540	Acc. No. 1805	Acc. No. 1852	Acc. No. 2015	Total	Polymorphic bands	Polymorphic (%)
OPAB-16	10	10	9	9	9	9	56	2	3.6
OPAB-18	5	6	7	7	7	7	39	9	23.1
OPG-05	8	8	8	8	8	8	48	11	23.0
OPH-04	12	12	13	13	13	13	76	4	5.3
OPH-12	13	13	14	14	14	14	82	4	4.9

Table 3. Distribution of AFLP fragments in the jute populations

Primer Combinations	Number of AFLP fragments								
	Var.O-4	Var. O-9897	Acc. No. 1540	Acc. No. 1805	Acc. No. 1852	Acc. No. 2015	Total	Polymorphic bands	Polymorphic (%)
M-CTG/E-TC	25	24	25	26	25	27	152	20	13.2
M-CTG/E-AT	36	36	37	37	37	37	220	4	2.0
M-CTG/E-TT	26	25	26	25	26	25	153	3	2.0
M-CAG/E-TC	34	34	34	34	34	34	204	6	3.0
M-CAC/E-TT	45	42	41	43	40	40	251	10	4.0
M-CAA/E-TT	18	18	17	17	17	17	104	2	2.0
M-CAG/E-AG	30	31	28	30	28	28	177	8	4.5
M-CAC/E-AT	73	74	70	71	71	71	430	26	6.0

Reagent Kit (Gibco BRL). DNA (250 ng) was digested with *Eco*RI/*Mse*I and ligated to double-stranded *Eco*RI/*Mse*I adapters, as described in the AFLP System II Manual. After ligation, the PCR products were diluted 10x with a TE buffer.

The pre-amplification reactions contained 5.0 µl of the diluted DNA, 40.0 µl of the pre-amplification primer mix, 1.0 unit of Taq DNA polymerase, 2.0 ml of 1x reaction buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), and 2 mM $MgCl_2$. Next, 20 cycles (Eppendorf Mastercycler Gradient) were performed at 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. The soak temperature was 4°C.

For selective amplification, a 1 : 50 dilution was performed. Each AFLP amplification was assembled by combining 5.0 µl of the diluted-template DNA, 5.0 µl of Mix 1 (primers/dNTPs), and 10 µl Mix 2 (Taq DNA polymerase, 1x reaction buffer, 2 mM $MgCl_2$). Touchdown PCR was performed in the following manner: one cycle performed at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The annealing temperature for each cycle was lowered 0.7°C during 12 cycles. This gave a touchdown phase of 13 cycles. Next, 30 cycles were performed at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s.

The AFLP products were denatured for 2-5 min at 95°C and then separated on 6% (w/v) polyacrylamide gels at 1,300 V at 45°C in a 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA) on a Model S2001 gel electrophoresis system. After electrophoresis, the gel was silver stained and dried. The following silver-staining protocol (Gresshoff *et al.*, 1994) was used: the gel was kept in a fixer solution (10% acetic acid) for 30 min, washed three times with distilled, deionized water for 2 min each. The silver solution (1.5 g/L $AgNO_3$, 1.5 ml of 37% formaldehyde) was then added for 25 min, and the gel was rinsed with water for 10 s. The image was developed with a developer solution (30 g/L Na_2CO_3, 1.5 ml of 37% formaldehyde, 300 µl of 10 mg/ml sodium thiosulfate) for 2-5 min, followed by the addition of a fixer solution.

The DNA fragments that were amplified by a given primer were scored as present '1' or absent '0' for all of the samples that were studied. A cluster analysis for the RAPD and AFLP data was accomplished using the software STATISTICA: Cluster Analysis (StatSoft 1994).

Results

Initially, all of the jute samples were tested for polymorphism using the RAPD technique. When RAPD was used, 25 out of 30 primers gave no polymorphism. The remaining 5 primers gave significant polymorphism within and between the cold-sensitive and cold-tolerant jute samples. The five primers are OPAB-16, OPAB-18, OPG-05, OPH-04, and OPH-12. A limited number of RAPD bands (<15 per lane) were observed on the gels. These five primers gave a total of 301 scorable DNA fragments of which 30 (10%) were polymorphic among the jute samples (Table 2). In RAPD, the amplification levels varied between the reactions, resulting in the loss of fainter bands and reproducibility, thus further complicating the potential interpretation of the gels.

In order to circumvent the typing problems with reproducibility in the RAPD procedure, we used AFLP as an alternative method to obtain molecular fingerprinting for jute. The average number of bands that were generated per primer pair was 211, which were three to four fold greater than the number of bands resulting from RAPDs.

We employed 25 combinations of selective-amplification

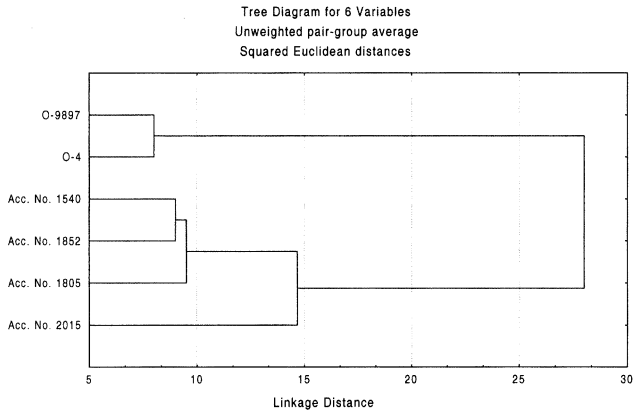

Fig. 1. Dendrogram of the jute populations obtained from the RAPD data.

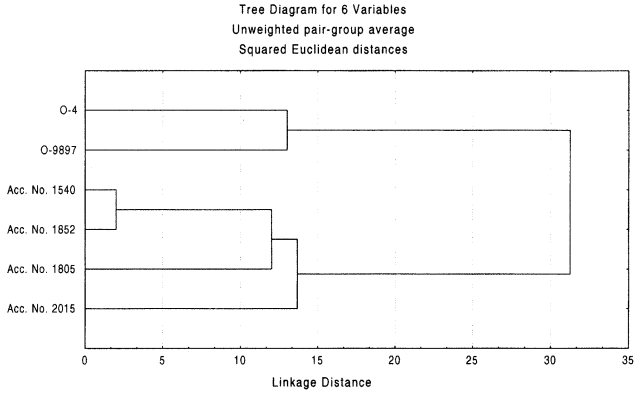

Fig. 2. Dendrogram of the jute populations obtained from AFLP data.

primers, 8 combinations of which generated polymorphic bands. These 8 combinations are as follows: M-CTG/E-TC, M-CTG/E-AT, M-CTG/E-TT, M-CAG/E-TC, M-CAC/E-TT, M-CAA/E-TT, M-CAG/E-AG and M-CAC/E-AT, where M-5'-GACGATGAGTCCTGAGTAA-3' and E-5'-CTCGTAGA CTGCGTACCAATTC-3'. Amplification of the 8 selected AFLP primer combinations resulted in a total of 1691 AFLP fragments of which 93 (6%) were polymorphic among the jute samples (Table 3).

For the different jute samples, the differences in the number of bands that were obtained by the RAPD and AFLP primers were used in the construction of the dendrogram. Both dendrograms (for RAPD and AFLP) showed the same results; two cold-sensitive jute varieties were in the one cluster and four cold-tolerant jute accessions were in another cluster (Figs. 1 and 2).

Discussion

In this study, we compared two typing techniques in order to obtain molecular fingerprints from six jute populations. Our immediate objective was to determine whether polymorphism was sufficient to distinguish between the cold-sensitive varieties and cold-tolerant accessions, and to assess the patterns of genetic diversity among the varieties.

All of the jute samples that were used in this study (collected from three different locations) were analyzed using both techniques in order to generate polymorphism between the samples. Initial analyses were based on 30 different RAPD primers, scored in 2 cold-sensitive varieties and 4 cold-tolerant jute accessions to see whether they could generate suitable polymorphism among different jute samples. It was found that among the 30 primers, 5 primers were able to generate substantial differences between the cold-sensitive and cold-tolerant jute.

From the AFLP results, we see that the lines that were studied (cold-sensitive and cold-tolerant) fell into two distinct groups. However, because of the small number of sample sizes we cannot conclude that the lines were representative of broad categories of jute. All eight primer combinations gave significant polymorphism. After an analysis of both the RAPD and AFLP data, similar results were obtained. A cluster analysis from the RAPD and AFLP data using the STATISTICA: Cluster Analysis (StatSoft 1994), based on the genetic distance matrix (data not shown), showed that cold-sensitive jute varieties and cold-tolerant jute accessions clustered in different groups.

The derived dendrogram for both the RAPD and AFLP data showed that Acc. No. 1540 and Acc. No. 1852 clustered in close proximity, even though the origin was different for the two. The other two accessions [one is an Indian (Acc. No. 2015) and the other an Egyptian (Acc. No. 1805)] were quite distant on the dendrogram. This implies that the genomic sequence of Acc. No. 1540 and Acc. No. 1852 had more homology at the genetic level.

Because of the relatively long primers (~21 bases), amplification of polymorphism by an AFLP analysis was much more stringent and reproducible than that by a RAPD analysis. Although a RAPD analysis was quicker and less labor intensive, the smaller, random primers were more permissive. Also, more time was required to achieve optimization by the RAPD analysis than by the AFLP analysis. In addition, although the RAPD analysis is reliable and the results are reproducible in the same laboratory, difficulties have been reported when the results from different laboratories were compared (Ellsworth *et al.*, 1993; Skroch *et al.*, 1995; Olivier *et al.*, 1999). In summary, the AFLP markers appeared to be more reliable than the RAPD markers. A RAPD analysis was quicker and less technical, but an AFLP analysis was more consistent and easier to optimize. The genetic difference between the varieties that were observed with the AFLP fingerprinting confirmed the differences that were found with that of the RAPD fingerprinting.

Acknowledgments We would like to thank Professor Zeba Islam Seraj (Department of Biochemistry and Molecular

Biology), Professor Rakha Hari Sarker (Department of Botany, University of Dhaka, Bangladesh), and Dr. Selina Begum (Principal Scientific Officer, Physiology Department, Bangladesh Jute Research Institute) for supplying the valuable information related to this study. We are grateful to the U.S. Department of Agriculture (USDA) for the financial support (Grant No. BG ARS-104), without which this work could not have been carried out.

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