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COTTON IMPROVEMENT

Effect of Lyophilization of Cotton Tissue on Quality of Extractable DNA, RNA, and Protein

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INTERPRETIVE SUMMARY

Molecular genetics involves DNA, RNA, and protein analyses. Fresh tissue is usually used for molecular analyses in cotton. If tissue could be freeze dried and stored for future use it would be helpful. This study compared the use of nonfreeze-dried tissue with freeze-dried leaf and root tissue of cotton as sources for isolation of DNA, RNA, and protein. The freeze-dried leaf and root tissue was suitable for DNA and protein isolation, but not for RNA isolation. The ability to freeze dry and store leaf and root tissue of cotton for DNA and protein analyses should be of great benefit to laboratories involved in cotton molecular genetics.

ABSTRACT

Molecular genetic analysis in cotton (*Gossypium hirsutum* L.) is often limited by the availability of fresh tissue and the time necessary to extract deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein from it. An alternative would be the use of freeze-dried tissue. We compared nonfreeze-dried and freeze-dried leaf and root tissue as sources for DNA, RNA, and protein isolation. Our results showed that freeze-dried leaf tissue from either greenhouse-cultured or field-grown cotton yielded high molecular weight genomic DNA. The DNA was suitable for restriction-enzyme digestion and as a template for polymerase chain reaction (PCR) amplification. In contrast, freeze-drying led to complete degradation of RNA in leaf and root tissue. Total proteins of leaves and roots were unaffected by freeze-drying based on comparison of polypeptide profiles by denaturing polyacrylamide electrophoresis. While these results rule out freeze-dried tissue as a source for RNA isolation, the ability to freeze-dry, powder, and efficiently store voluminous tissue samples for later use in DNA and protein isolation could be of great benefit to laboratories involved in cotton molecular genetics.

High quality DNA, RNA, and protein are required for meaningful molecular biological studies. Cotton tissue, however, is notoriously recalcitrant to many otherwise common extraction methods because of high levels of polyphenolic compounds. When cells are disrupted during sample grinding, phenolic compounds interact with protein and nucleic acids, leading to their oxidation and degradation (Dabo et al., 1993). Several extraction methods have recently been developed for DNA, RNA, and protein extraction in cotton (Baker et al., 1990; Callahan and Mehta, 1991; Dabo et al., 1993; Paterson et al., 1993). However, many of these techniques require either fresh tissue or tissue stored and strictly maintained at ultralow temperatures (-50 to -70°C). Generally, only a limited number of freshly collected tissue samples can be extracted at any given time, while ultralow freezer space for storage of bulky tissue samples is usually at a premium in most laboratories. In this report, we tested whether lyophilized (freeze-dried) cotton tissues were suitable as a source for DNA, RNA, and protein isolation using some standardized extraction techniques. In theory, freeze-drying should limit or slow degradation of cellular components by inactivation of proteolytic enzymes and nucleases. In addition, freeze-dried tissue can be ground as dry powder for efficient storage in limited freezer space.

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Abbreviations: PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; mRNA, messenger RNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; RFLP, restriction fragment length polymorphism.

MATERIALS AND METHODS

Plant Material

Several commercial cultivars and breeding lines (Shepherd, 1987) of upland cotton were grown in the greenhouse as described previously (Creech et al., 1995). At 25 d after germination, seedlings in individual clay pots were washed free of soil with low pressure water spray. The youngest fully expanded leaves and the adventitious roots were collected and immediately plunged into liquid N. Frozen tissue samples were either placed in sealed bags and stored at -50°C (i.e., nonfreeze-dried), or freeze-dried before use in the various extraction protocols. Shelf temperatures for the freeze-drier (Genesis 25LL, Virtis Co., Gardiner, NY)[1] were -40°C for the first 48 h, 0°C for 24 h, and finally 20°C for 4 h. Freeze-dried tissues were placed in sealed bags and stored at -50°C until ready for use. Storage times for tissues used in this study were from 7 to 28 d. For DNA extraction, additional samples consisting of the youngest fully expanded leaves of 30 to 40 d-old field-grown plants were collected on ice, brought back to the lab, then quick frozen in liquid N. Frozen leaves of field plants were then either stored at -50°C or freeze-dried as described above prior to DNA extraction.

DNA Extraction

The overall protocol, with slight modifications, was adapted from Paterson et al. (1993). Leaf samples (1 g nonfreeze-dried leaves vs. 0.25 g of freeze-dried tissue) were ground to a powder in liquid N using a mortar and pestle. The powdered tissue was transferred to a 50-mL centrifuge tube containing 10 mL of DNA extraction buffer (Paterson et al., 1993), gently mixed by vortexing, then centrifuged at 3000 X *g* for 20 min at 4°C. The resulting pellet, including nuclei, was resuspended in 4 mL lysis buffer (Paterson et al., 1993) then incubated for 20 to 30 min at 65°C. Five milliliters of chloroform - isoamyl alcohol (24:1 v/v) were added and mixed by gentle swirling. Samples were centrifuged in a swing-out rotor (Sorvall HB-4) at 3000 X *g* for 10 min to accelerate phase separation. The upper (aqueous) phase was transferred to a clean 50-mL tube. If necessary, the phase partition was repeated to clarify the aqueous phase. The DNA was precipitated by addition of 0.6 volumes cold isopropanol and gentle mixing. After approximately 10 min, the DNA was pelleted at 12 000 X *g* for 10 min at 4°C, washed in 1 mL of 70% (v/v) ethanol then repelleted by centrifugation. The air-dried pellet was resuspended in 0.5 mL of Tris-EDTA Buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) containing RNase (40 $\mu g mL^{-1}$) and incubated at 65°C for 30 min. After centrifugation at 12 000 X *g* for 15 min, the DNA in the resulting supernatant was re-precipitated by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and two volumes of chilled absolute ethanol. After 30 min at -20°C, the DNA was pelleted at 12 000 X *g* for 5 min. The air-dried pellet was resuspended in 100 to 200 µL Tris-EDTA Buffer. The DNA concentrations in the final samples were estimated by optical density at 260 nm. Aliquots were taken for digestion with Hind III restriction endonuclease per supplier's protocol (Promega Inc., Madison, WI) and for polymerase chain reaction (PCR) amplification of the gene for ACC synthase (Yu et al., 1979). The primers employed were CTCATTCCCTCCCCGTACTA and CTCTAAAACCAGGAAGTCCC, complimentary to the 5' and 3' ends, respectively. Thermal cycling conditions were 40 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 120 s followed by one cycle at 72°C for 10 min (Feng, 1996) in a Perkin-Elmer Cetus Thermo Cycler.

The uncut DNA, restriction-digested DNA, and PCR-amplified products were resolved by agarose gel electrophoresis (Sambrook et al., 1989).

RNA Extraction

Total RNA of nonfreeze-dried and freeze-dried leaf and root tissue was isolated using the Promega Total RNA Isolation Kit as per instructions in Technical Bulletin 087 (Promega Inc., Madison, WI). Tissue samples (100 mg of nonfreeze-dried vs. 25 mg of freeze-dried samples) were ground to a powder in liquid N using a mortar and pestle. While still frozen, 600 µL of the Denaturing Solution

[1]Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

(Promega Inc., Madison, WI) was added to the mortar with subsequent grinding. Once thawed, the homogenate was carried through the protocol as described. Final RNA concentrations were estimated by optical density at 260 nm (Sambrook et al., 1989). The integrity of the RNA was determined by denaturing (formaldehyde) agarose gel electrophoresis (Sambrook et al., 1989). Poly(A)$^+$RNA was isolated from total RNA by affinity chromatography on oligo(dT)-cellulose as per supplier's protocol (Collaborative Research Inc., Bedford, MA). *In vitro* translation of poly(A)$^+$RNA was performed using a rabbit reticulocyte lysate translation system as per kit instructions (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD), with ^{35}S-methionine as the radioactive amino acid. Translation products were resolved by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on a 10 to 20% polyacrylamide gradient gel (Hoefer Scientific Instr., San Francisco, CA). The gel was treated with the EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR film for fluorographic detection of newly synthesized proteins.

Protein Extraction

Tissue samples (100 mg of nonfreeze-dried vs. 25 mg of freeze-dried samples) were ground to a powder in liquid N using a mortar and pestle. Just as the liquid N evaporated, 0.1 mL SDS sample buffer (Laemmli, 1970) was added and the tissues were further ground. After thawing, the homogenates were heated at 95 °C for 5 min and then briefly centrifuged at 12 000 X *g* to pellet the cellular debris. The resulting supernatants (total protein extracts) were stored at -50 °C until analyzed. Prior to electrophoresis, protein concentrations in the samples were estimated from 1 µL aliquots (Marder et al., 1986) and then equalized with the addition of SDS sample buffer to approximately 500 µg protein mL^{-1}. One-dimensional SDS-PAGE was run with 4.5% (w/v) stacking and 15% (w/v) resolving gel bis-acrylamide concentrations using the buffer system of Laemmli (1970) in a mini-gel electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA). Ten µg of protein were loaded per lane. Constant voltage of 50 V was applied for 1 h followed by 150 V until the tracking dye (bromophenol blue) reached the bottom of the gel.

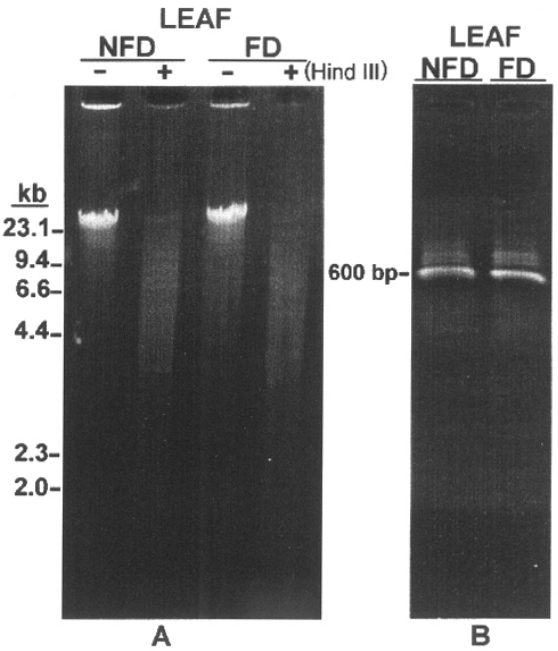

Fig. 1. Quality of genomic deoxyribonucleic acid (DNA) extracted from freeze-dried (FD) vs. nonfreeze-dried (NFD) leaf tissue of cotton. *A*. Agarose gel of DNA isolated from the respective tissues. Also shown is the sensitivity of the DNA preparations to digestion with the restriction endonuclease Hind III (-, no enzyme; +, plus enzyme). The DNA was visualized by ethidium bromide staining. The positions of DNA markers (lambda DNA Hind III digests) of known size (kb) are indicated. *B*. Agarose gel of polymerase chain reaction amplification product (600 bp) from DNA of freeze-dried (FD) vs. nonfreeze-dried (NFD) leaf tissue. Polymerase chain reaction conditions for amplification with primers complimentary to the ACC synthase gene are described in Materials and Methods.

RESULTS AND DISCUSSION

DNA

Our results showed that both freeze-dried and nonfreeze-dried leaf tissue yielded high molecular weight DNA that was readily digestible with the restriction enzyme Hind III (Fig. 1A) and served as a template for PCR amplification (Fig. 1B). Typical yields ranged from 300 to 1200 μg DNA from either freeze-dried or nonfreeze-dried tissues. The DNA from either source served similarly as template for PCR amplification of a 600 bp fragment corresponding to the ACC synthase gene (Fig. 1B). A similar size fragment was amplified from other cotton lines using these same primers and was used

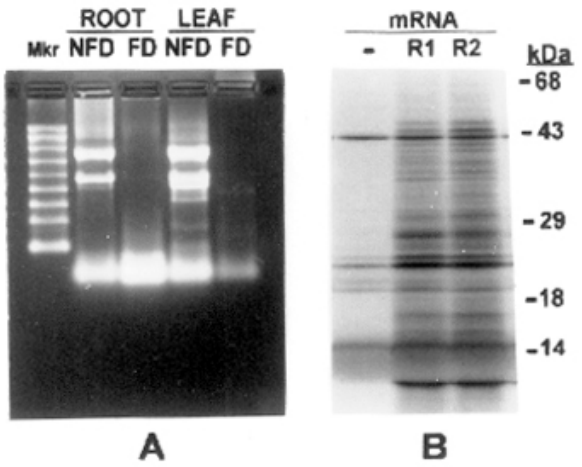

Fig. 2. Quality of ribonucleic acid (RNA) extracted from freeze-dried (FD) vs. nonfreeze-dried (NFD) root and leaf tissue of cotton. *A*. Denaturing (formaldehyde) agarose gel of total RNA isolated from the respective tissues. The resolved RNA was visualized by ethidium bromide staining. The RNA markers (Mkr) are shown on the left and represent from top to bottom the following sizes in kilobases: 6.6, 5.0, 3.7, 2.6, 1.9, 1.4, 1.0, 0.6, 0.3 (Promega Corp., Madison, WI). Note that the total RNA from freeze-dried tissues is completely degraded. *B*. Fluorograph of *in vitro* translation products obtained with mRNA of nonfreeze-dried root tissue. R1 and R2 represent mRNA of two different root samples added to the rabbit reticulocyte lysate translation system (Materials and Methods). A negative control (-) indicates the endogenous background labeling where no mRNA was added to the reaction. The positions of ^{14}C-labeled protein standards (BRL Life Technologies) of known molecular weight (kDa) are indicated.

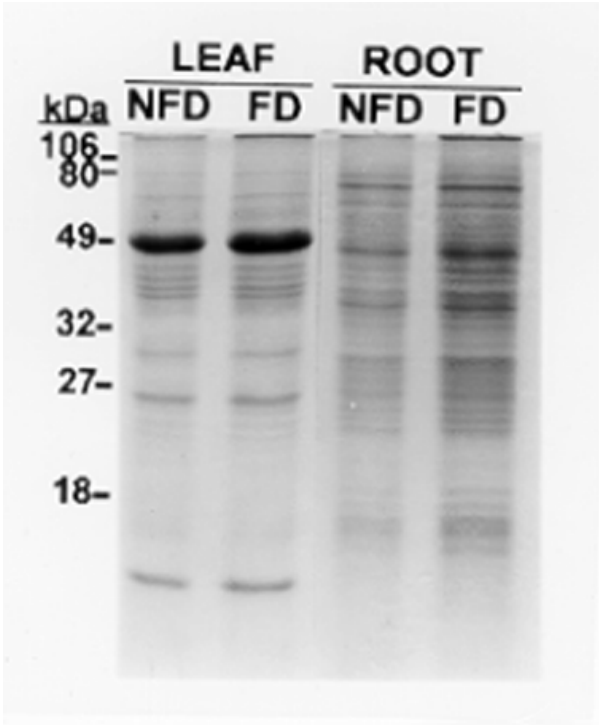

Fig. 3. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total protein extracted from freeze-dried (FD) vs. nonfreeze-dried (NFD) leaf and root tissue of cotton. Following electrophoresis the proteins were visualized by Coomassie staining. The positions of prestained protein standards (BioRad Labs., Hercules, CA) of known molecular weight (kDa) are indicated.

in an restriction fragment length polymorphism (RFLP) mapping program of cotton (Feng, 1996). Leaves collected from field-grown cotton plants yielded DNA preparations of equivalent quality (data not shown).

RNA

In contrast to the results for DNA isolation, we found that freeze-drying led to complete degradation of RNA (Fig. 2A). The two prominent bands from nonfreeze-dried leaf and root tissue represent the 28S and 18S subunits of ribosomal RNA, typical of normal total RNA preparations. These bands are completely lost in the preparations from freeze-dried tissue (Fig. 2A). Longer freeze-drying regimes limited to the lowest possible shelf temperature (-40°C) did not prevent this degradation. These results clearly indicate that RNA isolations should be limited to nonfreeze-dried tissues. Additionally, we could not isolate usable RNA from field-grown plants (data not shown), possibly due to drought conditions in the field. Baker et al. (1990) reported similar problems in recovering good quality RNA from plants grown under drought stress.

In order to confirm that the RNA isolated from nonfreeze-dried tissue was biologically active, we isolated mRNA from the total RNA preparation using affinity chromatography. The mRNA fraction was then assayed for the ability to promote protein synthesis in an *in vitro* translation system. The fluorograph showed that a range of polypeptides was synthesized in the rabbit reticulocyte system with mRNA from two different root tissue samples (R1 and R2, Fig. 2B). The negative control lacking added mRNA (-, Fig. 2B) indicates the minor endogenous activity associated with the lysate system. The simplified RNA isolation method that we describe here therefore, does provide high-quality RNA from nonfreeze-dried cotton tissue.

Protein

Comparison of total proteins extracted from freeze-dried and nonfreeze-dried leaf and root tissues indicated that they are unaffected by freeze-drying (Fig. 3). The SDS-PAGE gel shows equivalent polypeptide profiles from the respective tissues regardless of freeze-drying. The prominent band at approximately 49 kDa in leaf samples (Fig. 3) is characteristic of leaf protein fractions of many plant species and represents the large subunit of ribulose-1,5-bisphosphate carboxylase.

CONCLUSIONS

Our results showed that freeze-drying cotton tissue is an acceptable storage method for genomic DNA and protein extractions. The freeze-drying of tissue for RNA investigations was unacceptable due to complete degradation of the RNA. The ability to handle and store bulk samples for genomic DNA and protein extractions is greatly aided by freeze-drying, thereby freeing precious freezer space and preserving samples for later use.

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