Genomic DNA extraction from seed leaves of *Gossypium barbadense L.* for radioinduced mutations studies

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Resumen

Se ha desarrollado un método rápido, simple y seguro para la extracción de ADN en mutantes radioinducidos de *Gossypium barbadense*. Este método se basa en el bromuro de cetyltrimetil amonio y extrae ADN de hojas primordiales procesadas en tubos de 1.5 mL con 0.7 mL de buffer de extracción; es altamente eficiente para tejidos de algodón y mucho más económico en términos de tiempo, uso de químicos y labores. El protocolo descrito presenta consistente rendimiento y buena calidad de ADN para varios análisis moleculares (AFLP, SSR, etc.).

Abstract

A fast, simple and reliable method for the extraction of DNA from Gossypium barbadense radioinduced mutants has been developed. This method is cetyltrimethylammonium bromide (cTAB) based, and it extracts DNA from seed leaves processed in a 1.5 mL tube with 0.7 mL of extraction buffer; is highly efficient to cotton tissue and much cheaper in terms of time, chemical use and labor input. The described protocol presents consistent yield and good quality of DNA for several molecular analyses (AFLP, SSR, etc.).

1. Introduction

Cotton (*Gossypium* sp) has large amounts of polysaccharides and polyphenols in its tissues; these compounds make it difficult extraction of genomic DNA obtaining a low yield and quality. When cells are disrupted during sample grinding, phenolic compounds interact with protein and nuclei acids, leading to their oxidation and degradation [1]. This irreversible binding between phenolics compounds with proteins and nucleic acids, produces a gelatinous material, which is hard to separate from organelles, and which contains DNA unsuitable for PCR or restriction enzyme digestion [2,3]. Several extraction methods have recently been developed for DNA extraction in cotton [4,5,6]. Previously reported protocols and commercial kits for cotton DNA extraction are expensive, time consuming, and require ultracentrifugation in CsCl [7].

At the moment, the more used method for cotton DNA extraction is based on the employment of CTAB [8]. However, this method has some variations of the original protocol (Krizman et al., 2006), for example, the addition of PVP-10 binds phenolic compounds [9], NaCl to remove polysaccharides maintain free DNA solution [10], and 2-mercaptoethanol used as antioxidant [11,12]. On the other hand, the use of primordial leaves allows minimizing the quantity of polysaccharides and polyphenols facilitating the extraction of DNA.

Studies of genotype identification at molecular level in radio-induced mutants of cultivated plants require an optimal method of DNA extraction, thus, hundreds of individual plants need to be evaluated at the DNA level using molecular markers by a fast, simple, and especially reliable DNA extraction method. A rapid DNA extraction method should be not only fast and simple, but also should produce high quality and quantity DNA and use small amounts of tissue and extractions solutions.

This paper describes the method developed by the laboratory to carry out genomic DNA extraction from seed leaves for studies of molecular biology in radioinduced mutants of Peruvian cotton (*Gossypium barbadense L.).*

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2. Material and Methods

2.1. Plant material

Cotton seeds were cultivated during 15 days under conditions of laboratory. Seed leaves were collected of the plants cultivated and heavy in analytical balance before the extraction of the DNA.

2.2. Reagents and chemicals

- Extraction buffer: 0.1M Tris-HCl (pH 8), 2.0 M NaCl, 0.02M EDTA (pH 8), 2 % (w/v) CTAB, 2% (w/v) PVP (PVP K10, MW 10.000), 1mM 1,10-fenantroline.
- TE buffer: 1M Tris-HCl (pH 8), 0.5M EDTA (pH 8).

2.3. Protocol

- Freeze 0.2 g of leaves in liquid nitrogen and grind the leaves to a fine powder in a pre-cooled mortar.
- Transfer 1/3 of powder to a 1.5 mL eppendorf tube (pre-cooled with liquid nitrogen).
- Add 0.7 mL Extraction Buffer, homogenize and pre-incubate at 65°C and add 0.05 mL 2-mercaptoethanol.
- Mixture and produce an emulsion by gently inverting the tube four times; incubate at 65° for 4 h inverting the tube every 10 m.
- Centrifuge at 12000 rpm for 1 min.
- Transfer the supernatant to a second 1.5 mL eppendorf tube and add 0.7 mL of CIA.
- Mix gently 100 times by inversion and centrifuge by 13000 rpm for 15 min.
- Transfer the supernatant to a new 1.5 mL eppendorf tube using a Pasteur micropipette and repeat the previous step. (Fig.1).

Figure 1: DNA extraction.

- Transfer the supernatant to a new 1.5 mL eppendorf tube and add 1.4 mL of cooled absolute ethanol until the nucleic acid precipitates (whitish fiber).
- Centrifuge at 12000 rpm for 10 min and discard the supernatant.
- Resuspend and wash the pellet in 1.4 mL of 70% ethanol, centrifuge by 6500 rpm for 5 min.
- Resuspend and wash the pellet in 1.4 mL of absolute ethanol, centrifuge by 6500 rpm for 5 min.
- Dry the nucleic acid for 1 day at room temperature until evaporation of alcohol.
- Suspend the pellet in 0.1 mL TE buffer.
- Add 0.02 mg RNAse and incubate at 37°C for 1 h.
- Add 0.1 mL Buffer TE to suspend the DNA and incubate with 0.02 mg RNAse at 37°C for 1 h.
- Extracted DNA can be stored to 4°C for later studies.
- The measurement of the DNA concentrations in the tubes was realized in the Nanodrop® Spectrophotometer calibrated with several concentrations of Hind III (Fig.2).

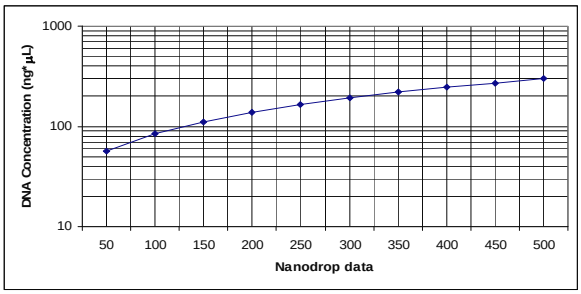

Figure 2: Calibration curve of Nanodrop®

3. Results and Discussion

3.1. DNA yield

Our method was based on the one of Zhang & Stewart [8], with some modifications. DNA from 17 *G. barbadense* seed leaves was extracted. After the cleaning procedure, the DNA concentration obtained from 180 to 270 mg of leaf tissue averaged 165 ng/µL in a total of 300 µL of DNA solution. The total DNA yield was 153 µg of DNA per g of leaf sample and was greater than that obtained from the CTAB macro method (10 µg of DNA per g of fresh leaf tissue) but smaller that the method of Zhang & Stewart (232 µg of DNA per g of fresh leaf tissue).

3.2. Statistical considerations

Table 1 show the total DNA yield expressed in ng per mL. In order to evaluate the normal distribution of the obtained results the test of Kolmogorov-Smirnov was realized using SPSS; the test demonstrated that the obtained results follow a normal distribution (Fig. 3).

One-Sample Kolmogorov-Smirnov Test

		TOT_DATA
N		46
Normal Parameters[a,b]	Mean	164.8785
	Std. Deviation	66.2582
Most Extreme Differences	Absolute	.249
	Positive	.249
	Negative	-.143
Kolmogorov-Smirnov Z		1.686
Asymp. Sig. (2-tailed)		.007

a. Test distribution is Normal.

b. Calculated from data.

Figure 3: Kolmogorov-Smirnov Test.

Table 1: DNA yield of Cotton seed leaves.

Code	*Average* ($ng^*\mu L^{-1}$)	*Standard deviation*	*Variability (%)*
BG 109	274.06	1.69	0.62%
BG 113	154.26	1.18	0.76%
BG 119	132.73	0.96	0.72%
BG 128	139.53	1.95	1.40%
BG 169	123.09	1.18	0.96%
BG 178	166.49	1.55	0.93%
BG 183	119.29	1.36	1.14%
BG 193	102.75	0.24	0.23%
BG 196	116.50	0.87	0.74%
BG 201	169.59	1.32	0.78%
BG 216	186.73	3.40	1.82%
BG 230	408.03	2.64	0.65%
BG 268	236.20	5.93	2.51%
BG 296	162.07	2.79	1.72%
BG 298	122.01	1.39	1.14%
BG 307	158.06	1.02	0.65%
BG 325	145.98	0.83	0.57%
BG 402	153.61	3.51	2.28%
BG 405	93.21	0.79	0.85%
BG 407	166.19	1.28	0.77%
BG 410	144.73	1.78	1.23%
BG 474	192.28	1.81	0.94%
BG 496	124.79	0.60	0.48%

With respect to the method of Zhang & Stewart, where the degree of dispersion is great (Table 2), our method presents very small coefficients of variation (0.48% to 2.51%).

Table 2: Comparisons of DNA yield.

Method	*Average* ($ng^*\mu L^{-1}$)	*Standard deviation*	*Variability (%)*
Macro-prep*	126.6	124.1	79.3%
Micro-prep*	200.6	117.9	62.5%
IPEN	164.9	1.7	1.04%

*Reference: Zhang & Stewart [8].

In order to study the repeatability degree, our method was applied by two independent analysts and the results were evaluated by means of the variance analysis (Table 3).

Table 3: Variance Analysis between analysts.

	Sum of squares	*d.f.*	*Mean Square*	*F*	$F_{crit.}$
Between Analysts	9.0345	1	9.0345	0.0049	4.35
Within Analysts	36906.9	20	1845.35		
Total	36915.9	21			

The result of the variance analysis indicates that the method provides similar results with different analysts.

4. Conclusion

The laboratory of molecular radiobiology has established a method of DNA extraction in cotton that is not affected by the presence of polysaccharides and polyphenols. This method will allow realizing the molecular genotype identification of radio-induced mutants produced by programs of genetic improvement of cotton.

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6. References

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