IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 2, Issue 7 (Nov. – Dec. 2016), PP 49-53
www.iosrjournals.org

High Resolution Melting Curve Analysis: an Efficient Method for Genetic Purity Analysis of Cotton (*Gossypium hirsutum*) Hybrid and their Parental Lines

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Abstract: *Cotton (Gossypium hirsutun) is one of the most important crops in India. It is essential to develop an efficient method for genetic purity analysis of hybrid cotton and their parental lines. Use of morphological differences, between true hybrids and off types in grow out test (GOT) for genetic purity analysis, are not always apparent and cannot be recognised easily. Further, morphological traits are costly, tedious to score and environment sensitive. Alternatively, it is suggested that recent breakthrough in molecular markers can be employed in genetic purity analysis. Gossypium hirsutum Fı hybrid AJ-999 and it's parents P402 as male and T28-II as female were studied for identification and genetic purity testing with high resolution melt analysis and agarose gel electrophoresis. There are 30 cotton microsatellite loci i.e. SSR primers were used for the analysis. Microsatellite loci, JESPR-151 were found to be heteroallelic for parents. JESPR-151 identified male specific 155bp repeat and female specific 170bp repeat. However, hybrid (AJ-999) exhibited the alleles of both parents, confirming the heterozygosity of the hybrid with the presence of two bands at 155bp and 170bp. High resolution melt analysis shows three different melting curves for male, female and hybrid with different melting peak values. The result suggested that agarose gel electrophoresis and high resolution melting curve analysis could be used as potential, efficient and valuable methods for genetic purity analysis of cotton hybrid and parental lines, and high resolution melting curve analysis should be given priority compared with agarose gel electrophoresis for its high accuracy and high efficiency. Since, these techniques are simple to use, more accurate and not affected by environment when compared with GOT.*

Keywords: *Agarose gel electrophoresis; Cotton; Genetic purity; Gossypium hirsutun; High resolution melting curve analysis*

Abbreviation: ***CTAB****-Hexadecyl trimethyl ammonium Bromide;* ***HRM****-High resolution melting curve analysis;* ***ISSR****-inter-simple sequence repeat;* ***SSR****-simple sequence repeat*

I. Introduction

Cotton is an important commercial crop in India and contributes a major share to the national economy. Increasing lint production and fiber quality has long been a major breeding objective to meet out the demands of growing population and modernized textile mills[1]. India ranks second in cotton production (22% of the global production) with the average productivity of 526 kgha-1. Cotton is cultivated in ~ 9 million hectares and 70% of this area is occupied by hybrids (Mehetre *et al*., 2007)[2]. The success of hybrid cotton technology depends on the timely production and adequate supply of genetically pure hybrid seeds to the farmers. The genetic purity of a variety/hybrid refers to the absence of seeds of other genotypes than the specific one. Therefore, seed testing or genotype identification is of prime importance for assuring good quality seed. It is estimated that for every 1% impurity in the hybrid seed, the yield reduction is 100 kg per ha². Thus, it is of critical importance to evaluate the genetic purity in seed production and trade[3].Genetic purity of hybrid seeds is assayed conventionally by the 'grow-out test' (GOT), which involves growing plants to maturity and assessing several morphological characteristics that distinguish the hybrids. The environmental influences on morphological characters and time factor make it difficult to collect the morphological data, besides other limitations in unambiguous differentiation of genotypes. Molecular markers can be used because they offer a faster and reliable technique for precisely assessing the genotype of a plant. Among the different molecular markers such as RAPD, ISSRs, Microsatellites or SSRs, RFLP, AFLP and isozymes markers. Microsatellites have been successfully employed in many genetic diversity studies and are useful for a variety of applications in plant genetics and breeding because of their reproducibility, multiallelic nature, codominant inheritance, relative abundance and good genome coverage[4-6]. High resolution melting(HRM) curve analysis is a novel, closed-tube, post-PCR technique invented in 2003 (Wittweret al., 2003) to detect DNA variation, which has been used in clinical chemistry, epidemical analysis, microorganism typing and molecular biology (Wu et al., 2008; Hofingeret al., 2009; Ganopouloset al., 2011; Thomsen et al., 2012)[7-8]. In HRM experiments, the target sequence is amplified by PCR in the presence of a saturating fluorescent dye (e.g. EvaGreen). HRM dye fluoresces strongly only when bound

to dsDNA. This change of fluorescence during an experiment can be used both to measure the increase in DNA concentration during PCR amplification and, subsequently, to measure temperature-induced DNA dissociation during high resolution melting. After PCR in the presence of the dsDNA-binding fluorescent dye, amplifications are briefly denatured and then rapidly reannealed. If the DNA sample is heterozygous, perfectly matched hybrids (homoduplexes) and mismatched hybrids (heteroduplexes) are formed. When the temperature is slowly increased again, the dsDNA begins to melt, and the shapes of melting curves are significantly different based on the G-C content, length and sequence of the fragments (Herrmannet al., 2006)[9]. In fact, the HRM technique is so sensitive that it can even detect single base variations between homozygous samples. SSR-HRM method has the advantages of high efficiency and high accuracy[10]. The objective of the present study to identify SSR based molecular markers for genetic purity analysis of commercial hybrid Cotton AJ-999 and their parental lines using agarose gel electrophoresis, and high resolution melting curve analysis, and to develop a more efficient method for genetic purity analysis of cotton hybrid and parental lines

II. Materials and methods

2.1 Plant material

Seeds of commercial hybrid F1 cotton and their parental lines (Table 1) used in this study were acquired from Marathawada region of Maharashtra. Fresh young leaves from all plants were collected for DNA extraction.

2.2 DNA extraction

Genomic DNA was isolated according to a modified CTAB method (Zhu et al., 2010)[11]. The concentration and quality of the obtained genomic DNA samples were estimated by measuring O.D. at 260/280 nm in UV spectrophotometer. Finally, all the genomic DNA samples were diluted to a final concentration of 40ng/µl with 1X TE buffer (10mM Tris-HCL; pH 8.0; 1mM EDTA). Intactness of genomic DNA was checked by agarose gel electrophoresis. DNA samples were stored at -20ºC for further use.

2.3 SSR-PCR amplification and agarose gel electrophoresis analysis

SSR amplification was conducted in a 20µl volume containing 40 ng of genomic DNA, 10X buffer,1 U *Taq* DNA polymerase, 1.5mM $MgCl_2$, 2.5mM dNTPs, 6pmol forward and reverse primer. The PCR protocol consisted of an initial denaturation at 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 sec, annealing for 30 sec at 57ºC, 72ºC for 1 min and final extension step of 72ºC for 10 min. The amplification reaction was carried out in thermo cycler (Applied Biosystems). The PCR products were analysed on 3% agarose gel along with 100bp molecular weight marker and photographed under UV light using Bio-Rad gel documentation system.

2.4 SSR-HRM (Simple sequence repeat-high resolution melting curve) analysis

SSR-HRM amplification was conducted in a 20µl volume containing 40 ng of genomic DNA, 2X Melt Doctor™ HRM master mix (Invitrogen), 6pmol forward and reverse primer and made up to 20µl with deionized water. The HRM amplification reaction procedure and melting analysis were performed as follows: 10 min initial denaturation at 95ºC followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 57ºC for 30 sec and extension at 60ºC for 30 sec. The amplification procedure was immediately followed by the high resolution melting steps: 90ºC for 2 min and then the temperature was raised to 72ºC to 82ºC, raised by 0.2º each step and wait for 2 sec for each step afterwards. Fluorescent acquisitions were selected at 70ºC. The HRM curve was acquired and analysed in 36-well rotor of the Qiagen real time PCR system. After the verification of amplification curves and the presence of a specific melting peak for the microsatellite amplification, the melting curve stage was analysed.

III. Results

3.1 SSR fragments analysis by agarose gel electrophoresis

Microsatellite markers were used for identification of cotton F1 hybrid AJ-999 (*G.hirsutum*) and it's parents P402 (male) and T28-II (female) shown in Table 1. Out of 30 JESPR microsatellite primers, 7 pairs i.e. JESPR-7,-56,-58,-101,-127,-151and-152, were scorable on agarose gel and showed polymorphism in parents. Out of these JESPR-151 [Forward5' *CGAGAAGATGAGATTGGAGGAG*3' Reverse5 *GGTTTTCCATT CTCTTTCA TTT*3'] were used to identify the hybrid AJ-999 and it's parents. Primer JESPR-151 amplified two repeats of different lengths, out of which a repeat of 155bp was P402 specific and a repeat of 170bp was T28-II specific, were amplified in hybrid AJ-999 (fig.1). Microsatellite analysis has been successfully employed for parentage verification, hybrid identification, cultivar characterization and purity testing in other crop plants[12-14]. In the present study, Microsatellite primer, JESPR-151 strongly support the hybridity.

3.2 SSR fragments analysis by high resolution melting curve analysis (SSR-HRM)

Based on the results of agarose gel electrophoresis primer JESPR-151 were selected for high resolution melting curve analysis of F_1 hybrid (AJ-999) and it's parental lines. Materials having different amplification fragments were able to be identified by the shape of melting curves, which is the principle of HRM analysis. Significantly different curves were achieved based on the different amplifications of F_1 hybrid and it's parental lines. F_1 hybrid (AJ-999) had two amplification fragments, and the sizes of them were 155bp and 170bp. However, it's male line (P402) had one amplification fragment (155bp), and female line (T28-II) also had only one amplification fragment (170bp). As the results, F_1 hybrid (AJ-999) and it's parental lines had significantly different curves, which could be used for hybrid purity. The present study indicates that Microsatellites banding and curve patterns of the parents compared with it's hybrid is able to clearly recognize the true hybrid and it's profile.

IV. Discussion

Agarose gel electrophoresis was traditional method to analyze SSR fragments. In this study agarose gel electrophoresis was selected for initial analysis of the amplifications because of it's simple and affordable technology. The agarose gel electrophoresis results showed that SSR molecular markers were valuable for genetic analysis of cotton because of it's high polymorphism. Based on the agarose gel electrophoresis results SSR primer, JESPR-151 which produce stable, distinct and polymorphic amplification was selected for high resolution melting curve analysis to compare their efficiency in seed purity analysis. However, high resolution melting curve analysis technique showed a potential application for plant genotyping. Compared with agarose gel electrophoresis, high resolution melting curve analysis have the advantages of high accuracy, high-throughout, high efficiency, and no-touch of toxic reagents. High resolution melting curve analysis was able to distinct the differences in G-C content. The result of high resolution melting curve analysis was showed with significantly different curves based on different amplifications.

V. Figures and Tables

Table 1. Name of cotton hybrid and their parental lines used in this study

Hybrid	Female line	Male line
AJ-999	T28-II	P402

Fig 1

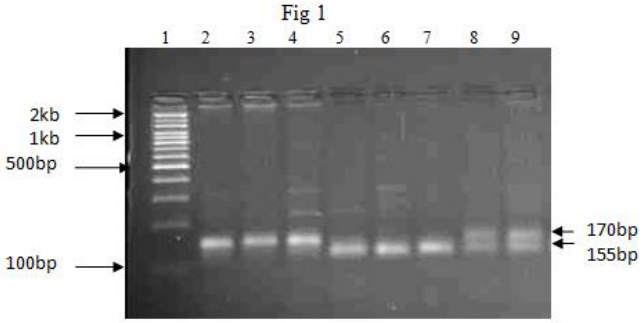
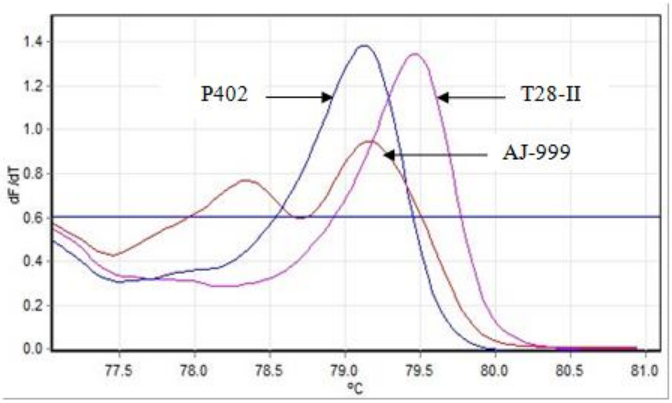


Fig 1. Amplification results of primer JESPR-151 from cotton hybrid AJ-999 and their parental lines. Lane 1-100bp ladder (100bp-2kb), Lanes 2-4-represent female line (T28-II), Lanes 5-7- represent male line (P402), Lanes 8-9- represent hybrid (AJ-999).

Fig 2



No.	Colour	Name	Peak 1	Peak 2
1		T28-II	79.47	
2		P402	79.13	
3		AJ-999	78.35	79.15

Fig 2.HRM analysis of amplification from AJ-999 and their parental lines by JESPR-151. Temperature-shift melting curves for the identification of AJ-999 (Red curves, amplification sizes 155:170 bp), T28-II (Pink curves, amplification size 170 bp) and P402 (Blue curves, amplification size 155bp). All curves had also different peak values. P402 (79.13), T28-II (79.47) and AJ-999 (78.35 and 79.15), at SSR locus JESPR-151.

VI. Conclusion

It is concluded from this study that it is possible to differentiate cotton hybrid more accurately and efficiently from their parental lines using molecular markers. DNA markers are more accurate for determining hybrid seed purity. Hybrid purity was studied by agarose gel electrophoresis as well as high resolution melting curve analysis. Based on the result of this study, high resolution melting curve analysis is an efficient and potential method for genetic purity analysis of hybrid cotton and their parental lines. Marker analysis will also result in considerable savings for the seed industry, as this technique may avoid the cost of storage for an entire season.

Acknowledgments

I would like to thanks Dr. Indrani Chandra for providing me an opportunity to work in the Department of Biotechnology, The University of Burdwan and her technical suggestion during the preparation of the manuscript is gratefully acknowledged. I am also thankful to my Parents and all of them who helped me during this project.

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