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Optimization of PCR Conditions to Amplify Microsatellite Loci in Cotton (*Gossypium hirsutum* L.) Genomic DNA

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ABSTRACT

A total of five different primer pairs were optimized for polymerase chain reaction (PCR) to amplify microsatellite loci in total genomic DNA of cotton (*Gossypium hirsutum* L.). Different concentrations of $MgCl_2$, DNA and different regimes of annealing temperature were optimized. For all the primer pairs, 2mM $MgCl_2$ concentration was found optimum. For DNA concentration, 37.5ng in the final reaction volume was suitable for good amplification. Annealing temperature 48EC and 61EC were found optimum for the primer pairs CM-27 and CM-162, respectively. However, the primer pairs CM-42, CM-43 and CM-56 gave the best amplification at annealing temperature of 55EC. The other reagents used in PCR and temperature regimes (denaturation and extension temperature) were kept constant. These loci will be used to detect linkage between different traits such as cotton leaf curl virus disease (CLCD), leaf color and hairiness.

Key Words: Cotton; PCR; Microsatellite; DNA

INTRODUCTION

In recent years, yield of cotton has become static rather it is declining due to the infestation of insect pests and diseases (Anonymous, 2001). One of the major factors of low yield in Pakistan is the infestation of cotton leaf curl virus disease (CLCD), as an epidemic of the disease has been occurred in early nineties. During the last five years (1992-1996), the disease has resulted in a heavy loss of 7.4 million bales of cotton with an estimated value of 5 billion US$. Secondly, insect pests are responsible for reduction in yield. The crop is handicapped due to 96 insect and mite pests (Younus *et al.,* 1980). Among these, cotton bollworms and sucking pests such as whitefly (*Bemisia tabaci* Genn.), cotton jassid (*Amrasca devastans* Dist.), thrips (*Thrips tabaci* Lind.) and aphid (*Aphis gossyii* Glov.) are very serious (Satpute *et al.,* 1988).

Plant breeders have exploited germplasm resources for the evolution of cotton cultivars resistant to the insect pests and diseases. However, the manipulation of genetic material using conventional breeding ways is often difficult, time consuming and sometimes ineffective. The resistant cultivars have often-poor yielding potential and are very difficult to improve because of linkage drag and phyletic barriers. Moreover, it is often difficult to estimate the effect of minor genes or cannot be detected in the presence of major genes. It is also not feasible to measure the precise effect of individual minor genes and their number. However, with molecular markers their individual loci contributing to durable resistance can be identified and mapped. Within the last few years, restriction fragment length polymorphism (RFLP) technology has been applied to several cotton species to study evolution, population genetics, phylogenetic relationships and genome mapping (Shappley *et al.,* 1996; Yu *et al.,* 1997), but it produces low variation in cotton compared to other plant taxa (Brubaker & Wendel, 1994). Another disadvantage and limitation to use the RFLP in *Gossypium* is the difficult task of isolating good quality pure DNA due to the presence of polysaccharides and polyphenolic compounds. In addition, the development of RFLP markers requires radioactivity, large amount of DNA, more time and energy.

To overcome the limitation of RFLP, a new PCR based diagnostic marker assays were developed independently by three different laboratories (Williams *et al.,* 1990). These techniques were named as arbitrary primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF). These techniques were based on arbitrary /random sequence primers, having more than 50% GC content.

The identification of specific, polymorphic loci circumvents the problem of irreproducibility common to random-primer fingerprinting methods. However, unlike random primer techniques, locus-specific methods often require a substantial initial effort to identify and characterize suitable loci. One category of site-specific polymorphisms exploits microsatellite DNA. Microsatellite regions are PCR-amplified using primers either based on adjacent, conserved sequences or consisting of a short, repeated sequence known to be present in the test organism from probe hybridization data (Morgante & Olivieri, 1993). It has been suggested that slippage mechanism during replication of the DNA is a major source of polymorphisms in microsatellites (Tautz & Renz, 1984; Levinson & Gutman,

1987). Their polymorphism proved useful in genome mapping studies.

For genome mapping, it is a prerequisite to optimize PCR conditions for given primer pairs of microsatellite loci. Once the PCR conditions will be optimized, then it will be easy to study DNA polymorphisms for genome mapping purposes.

MATERIALS AND METHODS

The plant material used in the study consisted of three cotton genotypes *viz.*, S-12, CP-15/2 and LRA-5166. The cotton genotypes were mostly collected from their center of origin.

DNA extraction. DNA was extracted from the leaves of selected cotton genotypes by a method proposed by Iqbal *et al.* (1997). For DNA extraction, approximately 3 g of young leaves (bulked from 4-6 different plants) were ground to a very fine powder in liquid nitrogen and transferred to 50 mL centrifuge tube. The 15 mL of hot (at 65 ^{o}C) 2XCTAB [2% CTAB, 1.4 M NaCl, 20mM EDTA (pH 8), 0.1 M Tris HCl (pH 8.0), 1% polyvenyl pyrrolidone (PVP), 1% mercapto-ethanol] was added and incubated for 30 min at 65°C with occasional swirling. The mixture was emulsified with an equal volume of chloroform: isoamyl alcohol (24:1) and mixed gently by inverting the tube. The centrifuge tubes were spun at 4000 rpm for 10 min. The supernatant was collected into another tube and DNA was precipitated with 0.6 volume of isopropanol. DNA was pelleted at 4000 rpm for 4 min and supernatant was discarded. After adding 10 mL of 70% ethanol to the DNA pellet, it was kept on slow shaker for 20 min and then centrifuged for 2 min at 4000 rpm. The above wash step was repeated and then the sample was spun at 4000 rpm for 10 min. The pellet was air dried (20 minutes) and resuspended in 0.5 mL 0.1 X TE buffer. Then 7 µL of RNase was added and supernatant was collected. The DNA was precipitated with absolute ethanol (2 volumes) and centrifuged at 13000 rpm for 10 min. The supernatant was discarded and washed with 70% ethanol mixed gently followed by incubation for one hour at 37^{o}C followed by the addition of equal volume of (equal to the volume of the suspension + 7 µL RNase) of chloroform/isoamylalcohol. The samples were spun for 10 min at 13000 rpm and ethanol. Then the pellet was air dried and resuspended in 0.1 X T.E buffer.

Concentration of DNA. Concentration of the DNA was measured by spectrophotometer. Absorbance ratio of the extracted genomic DNA at 260 nm and 280 nm was 1.76. Quality of DNA was observed by running 50 ng DNA in 0.8% agarose gel. The DNA samples giving smear in the gel were rejected. Moreover, the quantity of DNA was also confirmed by comparing with Quantification Standards Phage λ DNA (Gibco BRL) in 0.8% gel.

Primer sequence of five SSRs was sent by Crop Biotechnology Centre, Taxas A & M University, USA. The method of Connell *et al.* (1998) was followed for genomic library construction of *Gossypium hirsutum* var. Coker to isolate SSR loci. These primer pairs were custom-synthesized by GIBCOBRL (Life Technologies, USA). The sequence of the primers and annealing temperature is given below.

Cotton micro-sat.# Primer Sequence (5' to 3')

CM-162	F=CCATTCTCTTTCATTTTCTCC
	R=GGAGCAACGAAACTTAGCAGC
CM-27	F=GAATTGTGTAAAAGACACTTC
	R=TTCTTGGAGAATGCTCT
CM-42	F=GTCTTAAAGACTGACATGCAGC
	R=GAGTATAGGATAGGGGCTAAAC
CM-43	F=GCGCAGATATTATTATCACAGC
	R=TATATAAATTTGCATCAGTTGGC
CM-56	F=CAATCATAGACCGCCACATC
	R=ACATTGCTAGATATTATAAAAGAAG

The PCR condition for amplification was same for all the 5 SSR primer pairs. The dNTPs, 10 X buffer, $MgCl_2$ and Taq polymerase were supplied by MBI Fermentas.

PCR profile was optimized as:

One cycle of Denaturation temperature 94°C for 5 min

35 cycles of

1) Denaturation temperature 94°C for 30 sec
2) Annealing temperature 30 sec
3) Extension Temperature 72°C for 1 min

One cycle of Extension temperature 72°C for 4 min

PCR condition optimisation. For optimisation, different concentrations of $MgCl_2$, Taq DNA polymerase and annealing temperature were optimised for the five SSRs. Amplification was performed in thermacycler (Perkin Elmer,USA) for 35 cycles. However, the amount of genomic DNA and concentration of dNTPs was kept constant. After amplification, the concentration of amplicons was determined on 1.2% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

Microsatellite are preferred in genome mapping studies because of higher heterozygosity values than isozymes (Trujillo *et al*., 1995; Belaj, 1998), restriction fragment length polymorphism (RFLP) (Wu & Tanksley, 1993) and random amplified polymorphic DNA (RAPD) markers (Dayanandan *et al*., 1998). Moreover, development of microsatellite markers may provide a new approach to marker-assisted selection (MAS) in cotton breeding practice. To be suitable for use in plant breeding practice markers should be easy to use, have low cost and highly informative (Mba *et al*., 2001). Based on these criteria, microsatellite markers are preferable to RFLP markers. Furthermore, microsatellite DNA markers are preferred over

RAPDs because of high reproducibility (Jones *et al*., 1997).

The microsatellite markers are PCR based that makes them attractive for use in genome mapping studies which requires only tens of the nanograms of DNA or even crude DNA extracts. Template DNA is easy to obtain. Moreover, presence of easily scorable, unique alleles and/or allele combinations, make them an ideal system for cultivar identification (Rallo *et al*., 2000). Before conducting any genome mapping study, optimization of concentration of different reagents and temperature regimes used in polymerase chain reaction (PCR) is necessary. However, annealing temperature and concentration of $MgCl_2$ are important parameters, which needs optimization. The genomic DNA of S-12, CP-15/2 and LRA-5166 was used to optimize the PCR conditions. Amount of template DNA strongly influences the outcome of the reaction. More than 30 ng / 25 µL give the premium amplification (Henegariu *et al*., 1997). However in the present studies, 37.5 ng / 20 µL was found optimum. Optimization of $MgCl_2$ is an important factor for precise amplification. In this experimental studies, 2 mM of $MgCl_2$ was found optimum in 20 µL final volume. The Mg ions binds tightly to the phosphate sugar backbone of nucleotides and nucleic acids, and variation in the $MgCl_2$ concentration has strong effects on nucleic acid interactions. Variations in $MgCl_2$ concentration below 4 mM can improve performance of PCR by affecting specificity (higher concentration lower the specificity, lower concentrations raise specificity) (Blanchard *et al*., 1993). Moreover, concentration of dNTPs in reaction mixture is also strongly correlated to the Mg ions concentration due to the interaction between mononucleotides and the Mg^{2+}. A higher concentration of Mg^{2+} allows amplification with a higher concentration of dNTPs, that is not seen at lower Mg^{2+} concentrations (Blanchard *et al*., 1993).

In PCR, 2-2.5 units of Taq Polymerase is normally used in 100 µl final volume. Higher Taq Polymerase concentration (above 4 units/100 µL) can generate non-specific products and may reduce the yield of the desired product (Saiki, 1989). However, in the present study, 1 unit/20 µL reaction was used to amplify the loci without non-specific products. Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 36-75°C. The annealing temperature 55°C was found optimum to amplify with primer pairs CM-42, CM-43 and CM-56. Similarly, 48°C and 61°C were found optimum for primer pair CM-27 and CM-162, respectively. The optimized conditions for PCR and reagents would be used to amplify genomic DNA of segregating populations developed by crossing the cotton genotypes for genome mapping purposes.

REFERENCES

Anonymous, 2001. *Cotton: Review of the World Situation.* Monogram by International Cotton Advisory committee. Washinhton, D.C.

Belaj, A., 1998. Identification y classification de variedades de olive (*Olea europaea* L.) del Banco de Germoplasma de Cordoba con marcadores RAPDs. *MSc*. Universidad de Cordoba, Spain.

Blanchard, M.M., P. Tailon-Miller, P. Nowotny and V. Nowotny, 1993. PCR buffer optimization with a uniform temperature regimen to facilitate automation. *PCR Methods Applic.*, 2: 234–40.

Brubaker, C.L. and J.F. Wendel, 1994. Re-evaluating the origin of domesticated cotton using nuclear restriction fragment length polymorphism. *American J. Bot.*, 81: 1309–26.

Connell, J.P., S. Pammi, M.J. Iqbal, T. Huizinga and A.S. Reddy, 1998. A high through-put procedure for capturing microsatellites from complex plant genomes. *Plant Mol. Biol. Rep.*, 16: 341–49.

Dayanandan, S., O.P. Rajoraand K.S. Bawa, 1998. Isolation and characterization of microsatellites in trembling aspen (*Populus trenuloides*). *Theor. Appl. Genet.*, 96: 950–56.

Henegariu, O., N.A. Heerema, S.R. Dlouhy, G.H. Vance and P.H. Vogt, 1997. Multiplex PCR: Critical parameters and step-by-step protocol. *Biotechniques*, 23: 504–11.

Iqbal, M. J., N. Aziz, N. A. Saeed, Y. Zafar and K. A. Malik, 1997. Genetic diversity evaluation of some elite cotton varieties by RAPD analysis. *Theor. Appl. Genet.*, 94: 139–44.

Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. van de Wiel, G. Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, n. Marmiroli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez and A. Karp, 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Mol. Breed*., 3: 381–90.

Levinson, G. and G.A. Gutman, 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol*., 4: 203–21.

Mba, R.E.C., P. Stephenson, K. Edwards, S. Melzer, J. Nkumbira, U. Gullberg, K. Apel, M. Gale, J. Tohme and M. Fregene, 2001. Simple sequence repeat (SSR) markers survey of the cassava (*Manihot esculenta* Crantz) genome: towards an SSR-based molecular genetic map of cassava. *Theor. Appl. Genet.*, 102: 21–31.

Morgante, M. and A. M. Olivieri, 1993. PCR- amplification microsatellite as markers in plant genetics. *Plant J*., 1: 175–82.

Rallo, P., G. Dorado and A. Martin, 2000. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). *Theor. Appl. Genet.*, 101: 984–89.

Saiki, R.K., 1989. The design and optimization of the PCR. *In:* Erlich, H.A. (Eds.), *PCR Technology - Principles and Applications for DNA Amplification*, pp: 7–16. Stockton Press, New York

Satpute, U.S., D.N. Sarnaik and P.D. Bhalerao, 1988. Assessment of avoidable field losses in cotton yield due to sucking pests and bollworms. *Indian J. Pl. Prot.*, 16: 37–9.

Shappley, Z.W., J.N. Jenkins, J.C.E. Watson, A.L. Khaler, W.R. Meredith, 1996. Establishment of molecular markers and linkage groups in two F2 populations of upland cotton. *Theor. Appl. Genet.*, 92: 915–19.

Tautz, D. and M. Renz, 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucl. Acids Res.*, 12: 4127–38.

Trujillo, I., L. Rallo and P. Arus, 1995. Identifying olive cultivars by isozyme analysis. *J. Am. Soc. Hort. Sci.*, 120: 318–24.

Williams, J.G., K.A.R. Kubelik, K.J. Levak, J.A. Rafalski, S.V. Tingey, 1990. DNA polymorphism amplification by arbitrary primes are used as genetic markers. *Nucl. Acids Res.*, 18: 6531–5.

Wu, K.S. and S.D. Tanksley, 1993. Abundance, polymorphism and genetic mapping of microsatellite in rice. *Mol. Gen. Genet.*, 241: 225–35.

Younus, M., M. Yousaf and G. Jilani, 1980. Insect and spider mite pests of cotton in Pakistan. Monogr. *PL-480,* Deptt. Entomol. Univ. Agric., Faisalabad, Pakistan, pp. 256.

Yu, Y.G., M.A.S. Maroof and G.R. Buss, 1997. Divergence and allelomorphic relationship of a soybean virus resistance gene based on tightly linked DNA microsatellite and RFLP markers. *Theor. Appl. Genet.*, 92: 64–9.

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