OPTIMISING DNA AMPLIFICATION PARAMETERS FOR RAPD ANALYSIS IN COCOA (*Theobroma cacao L.*)*

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Conventionally Cocoa (*Theobroma cacao L.*) accessions have been characterized by morphological and agronomic traits. But these are subject to environmental and physiological influences. Also descriptors of this kind only afford limited information of the relatedness of accessions. Therefore, interest is directed on the use of molecular marker techniques. Such genetic markers are believed to be unaffected by external parameters and also provide a measure of genetic relatedness. The development of a PCR-based arbitrarily primed genetic assay called RAPD (Random Amplified Polymorphic DNA, (Williams *et al.*, 1990) or AP-PCR (Arbitrarily Primed PCR, Welsh and McClelland, 1990) has greatly changed the prospects for application of molecular markers to study populations and to accelerate breeding. Of the several molecular markers used, RAPD is the simplest and particularly popular in the characterization of plant genetic resources. The present investigation was carried out to standardize the optimum PCR parameters for template DNA amplification for RAPD analysis.

DNA Extraction

Total genomic DNA extraction was standardized using just mature green leaves by modified CTAB protocol. DNA concentration was measured by running the aliquots (5µl) on 0.8% agarose gel and taking the absorbance at 260 nm in UV Spectrophotometer

Amplification

Protocol for the polymerase chain reaction was optimized by varying the concentration of template DNA, Mg^{++}salt, Taq DNA polymerase, dNTP concentration, primer concentration and annealing temperature. PCR was carried out in DNA Thermal cycler (Eppendorf mastercycler gradient, Germany) with 25µL reaction mixture. PCR chemicals including Taq polymerase were procured from Bangalore Genei and primers were obtained from Operon Technologies, Alameda, USA. Reaction products were mixed with loading dye (0.25% bromophenol blue and 40% sucrose w/v) and spun briefly before loading onto gel. The amplification products were separated on 1.2% agarose gel at 80 V followed by staining with ethidium bromide. Molecular weight of bands was estimated by 100 bp and 500bp ladder (Bangalore Genei).

Standardization of template DNA

DNA concentration is the first and most important parameter to be optimized as it influences the reproducibility of RAPD patterns (Williams *et al.*, 1993). Varying

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the concentration of template DNA from 20 to 50 ng revealed that 30 ng DNA gave maximum number of reproducible bands and thus considered ideal for RAPD analysis. Template DNA from 20-50 ng in 25 µL reaction mixture gave good amplification. However at 20 ng and 50 ng DNA template concentration, some bands were missing compared to other concentrations. 30 ng templates DNA gave uniform bright bands. Hence 30 ng was taken as optimum for PCR amplification (Fig1.).

Standardization of $MgCl_2$

$MgCl_2$ is an important variable that affects intensity of bands. In addition to Mg++ ions bound by the template DNA, the nucleotides (dNTPs) and the primers, Taq DNA polymerase also requires free $Mg2^+$ ions. Their concentration has an influence on primer annealing, the melting temperature of the PCR product and product specificity. A titration of different concentrations of $MgCl_2$ from 0, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM and 3.5 mM in 25 µL reaction mixture showed that 1.0 mM and 1.5 mM was optimum for PCR. This is in addition to the 0.6mM of $MgCl_2$ present in the PCR assay buffer. An excessively high concentration lead to a reduction in stringency, i.e. reaction specificity.

Standardization of dNTPs and Taq Polymerase

A final concentration of 0.4 µM of dNTP and 0.9 U of Taq polymerase in the reaction mix were found to be optimum for RAPD analysis. Taq Polymerase is active over a broad range of temperatures, primer extension will occur at the specific temperatures of annealing.

Standardization of primer concentration

The primer is an oligonucleotide of 10 bp length. The primer concentration from 0, 0.2 µM, 0.4 µM, 0.6 µM, 0.8 µM and 1 µM showed that 0.8 µM was optimum for amplification of Cocoa DNA. Primers from kit OPC were used for standardizing primer concentration. The G+C composition of primers varied from 60-70%. GC content of a primer is the best indicator of whether it will result in amplification (Fritsch *et al*, 1993). The higher the G:C content, the higher the likelihood of amplification. The primer sequences that resulted in good polymorphic bands are recorded in the Table 1. The choice of primer and nucleotide concentration has significant influence on PCR. A high primer concentration increases the probability of spurious priming and leads to the generation of nonspecific products.

Standardization of Annealing temperature

Gradient of annealing temperature from 35°C to 55°C in the thermal profile showed that 45°C was optimum for obtaining reproducible RAPD patterns. The PCR thermal profile is as follows. Initial denaturation at 94°C for 2 min followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min and elongation at 72°C for 1 min with a final extension at 72°C for 5 min.

Annealing temperature along with $MgCl_2$ influences relative intensities of amplified bands. In fact, high-temperature annealing should result in enhanced specificity, because the hybridization of the primer to the template DNA occurs under more stringent conditions. If the temperature is too low, non-specific priming will

occur. Very long annealing times normally do not improve yield, but rather produce an increase in spurious priming and, thus, greater amounts of nonspecific PCR products.

Primer Survey

Initially 40 primers from primer kits OPB and OPC were screened using three accessions namely Jarangau Red Axil, Na –33 and EET-272 in order to identify the suitable ones for analysis. Most of the primers we tested produced good number of amplified fragments that were detected by gel electrophoresis and staining with ethidium bromide. Some primers produced polymorphisms detected by amplification of DNA fragments that varied in size depending on the accession used. Out of 40 primers screened, 8 primers (B4, B8, B16, B17, B18, B19, C3 and C20) did not produce any amplification. This may be due to the lack of homology between the primer sequence and the genome sequence of accessions. Ten primers viz., B5, B11, B7, B9, B14, B15, B2 and B20, C15 and C16 resulted in inconsistent or sub optimal products while C1, C14, C17 and C19 gave very similar amplified products (monomorphic bands). Hence these were discarded. Eighteen primers were found to be polymorphic between the accessions tested. Table 2 shows the nucleotide sequences of the 10 primers used for RAPD analysis to distinguish cocoa accessions.

PCR was carried out using standardized PCR parameters for different cocoa accessions resulted in good amplification. Hence 30 ng template DNA, 1.0 mM $MgCl_2$, 0.4 μM of dNTP and 0.04 U of Taq polymerase and 0.8 μM primer concentration were found optimum for carrying out RAPD analysis in cocoa

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Table1. Standard RAPD protocol for DNA amplification

Concentration	Volume (µL)	Final concentration
Double distilled water	10.17	
PCR Buffer 10X	2.50	1x
$MgCl_2$, 25 mM	1.00	1mM
DNTP, 10 mM	4.00	400µM
Primer, 5µM	4.00	0.8 µM
Taq DNA Polymerase 3U/ µL	0.33	0.9 U
Template DNA, 10ng/ µL	3.00	30 ng/ µL

Table 1a.Thermal profile for DNA amplification

Initial denaturation	94°C	2 min	
Denaturation	94°C	1 min	
Primer annealing	45°C	1 min	45 cycles
Polymerization	72°C	1 min	
Final extension	72°C	5 min	

Fig.1.Optimisation of DNA template concentration.