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OPTIMIZATION OF PCR CONDITIONS FOR AMPLIFY MCROSATELLITE LOCI IN COTTON (*Gossypium hirsutum*) DNA

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Abstract

Optimization of polymerase chain Reactian (PCR) condition has been performed to amplification of genomic DNA for cotton *Gossypium hirsutum.* The optimization using 5 SSR primers pairs from JESPR series, that is JESPR 247, JESPR 280, JESPR 292, JESPR 299, and JESPR 303. The aim of optimization is to obtain the right conditions for amplification through PCR reaction. The course Optimization includes, annealing temperature, the amount of DNA template, and the amount of PCR mix. The results showed that the amount of DNA template 2.5 µl of total volume of 22 µl , and the volume of 12.5 µl PCR mix, an optimal volume to deliver a good amplification product. Annealing temperature of 48^0C was the optimum temperature for the primary JESPR 280, JESPR 292, JESPR 299, and JESPR 303, while for the JESPR 247 primary optimum temperature is 50^0C. .

Key words: optimization , PCR , SSRs primary, amplification

INTRODUCTION

Cotton plants *(Gossypium hirsutum)* is a fiber-producing plants are required as a raw material in the textile industry. National cotton production can only meet the needs of 0.5% of the total requirement of cotton, while the remaining 99.5% met from imported cotton (Shakir, 2010).

Effort in breeding cotton plants should continue to be done in order to obtain improved varieties both in production, fiber quality, and disease resistance. For this purpose the identification of molecular genetic approach is needed. For cotton germplasm characterization needs to be intensified, this process should be done by combining the techniques of molecular, physiological analysis, which allow the identification of genes for improved varieties (Sulityowati and Hasnam, 2007). It is also presented by Xiao et all (2009) that combines molecular breeding techniques with phenotypic selection can improve usability important traits in cotton.

The process of plant breeding today increasingly helped by the discovery of molecular markers such as microsatellite. Microsatellite markers are often referred to as Simple Sequence Repeat (SSRs), is one of the molecular genetic markers based on a short sequence of nitrogenous bases in DNA, usually consisting of 2 to 5 bases are repeated without interrupted. Microsatellite is a marker that is based on PCR (polymerase chain reaction) is a technique to amplify DNA in vitro, with the help of taq polymerase enzyme.

PCR reactions consisted of three stages: denaturation, annealing, and elongation. Denaturation is fisrt stage where the DNA double strand will separate and each strand would be the template during the amplification, this process takes place at high temperatures.The next stage is annealing, at this stage of the primary will be attached to the DNA template, and the last stage is the elongation, which is the primary polymerization to form a new strand of DNA. The amplification process is sensitive to a variety of conditions, such as the quality and quantity of DNA template, primer, the concentration of $MgCl_2$, Taq polymerase, and the annealing temperature. Therefore, in order DNA amplification process can take place properly so that the resulting amplification product is good, it is necessary to optimization of PCR reaction conditions.

Formulation of the problem in this study are: How optimal conditions (volume of template DNA, PCR mix, and annealing temperature) for the amplification of cotton DNA through PCR reactions using 5 SSRs primer pairs. The purpose of the study is: Obtaining optimal conditions for DNA amplification of cotton through the course of the PCR reaction using 5 SSRs primer pairs. The benefit of this research is to obtain the right conditions on the volume of template DNA, PCR mix, and the annealing temperature, which is necessary for the ongoing cotton DNA amplification using 5 SSRs primer pair. This is particularly important considering the PCR reaction is one part of the DNA molecular analysis techniques that can be applied to the field of plant breeding cotton.

RESEARCH METHOD

Materials used are: (1) young leaf of cotton, (2) CTAB buffer (Glucose 1 M, PVP, 2 M Tris HCl, 0.5M EDTA, dH_2O, Na bisulfite), (3) lysis buffer (5% CTAB, PVP, 2 M Tris HCl, 0.5 M EDTA, 5 M NaCl, dH2O) , (4) Chloroform (5) isoamyl alcohol (6) absolute ethanol (7) sodium acetate (8) TE buffer (9) liquid nitrogen, (10) bromophenol blue, (11) ethidium bromide, (12) parafilm, (13) agarose, (14) 5 microsatellite (SSRs) primer, from JESPR (247, 280, 292, 299, 303), PCR mix (dNTPs, taq polymerase, buffer), dH_20, Temed, APS, 10x TBE, ACRYLAMID 30%, NH_4OH, NaOH, $AgNO_3$, Sodium carbonate, formaldehyde, acetic acid, glycerol.

The tools used in the study are: gloves, mortar and pastle, eppendorf, centrifuges, vortex, incubator, analytical balance, micropipette, horizontal electrophoresis, vertical electrophoresis, tip size of 100-1000 µl, gel tray, shaker, document gels, PCR tubes, PCR machine,

DNA Isolation

The study was conducted in the laboratory of Biotechnology Muhammadiyah University of Malang in April-August 2011. DNA isolation performed using the CTAB method (Nuraida, 2013), the isolation procedure is as follows: 1 gram of young leave were ground into powder with the addition of liquid nitrogen. The powder is then put into the tube already containing 80 µl buffer extract with pH 7.5. Samples were subsequently incubated in the refrigerator for 5 minutes and then centrifuged at 12,000 rpm at 4^0C for 10 minutes. Supernatant was discarded, the precipitate was added with 500 ml lysis buffer at pH 8, and then homogenized at vortek. Further samples were incubated at 65^0 C for 30 minutes. Samples were added with chloroform and isoamyl (24:1) of 500 µl, then centrifuged at 12,000 rpm at 4 ° C for 10 minutes. The supernatant was transferred into another tube and added 800 µl of absolute ethanol and 80 µl of sodium acetate and then inverted back. Samples were centrifuged at 12,000 rpm at 4^0 C for 10 minutes. Supernatant was discarded, the precipitate added with 800 µl of 70% ethanol and then centrifuged. Supernatant was discarded, the pellet was air dried for 24 hours, and then

added 30 µl TE. The quality of the isolated DNA was observed by 0.8% agarose gel electrophoresis (Ali *et al*, 2009; Rahman *et al*, 2002). To visualize DNA bands, the results of electrophoresis staining with ethidium bromide, and observed and photographed using a gel documentation system.

Optimization of PCR conditions

DNA obtained from the isolation further amplified in the PCR machine. In this process used 5 SSRs primer pairs from JESPR series, that is JESPR 247, JESPR 280, 292 JESPR, JESPR 299, and 303 JESPR. The primer chosen because it has a high PIC value (Azmat and Khan, 2010). Amplification process begins by mixing DNA isolation, PCR mix, microsatellite primer Forward and Reverse, and dH_2O, into the PCR tube until the mixture is homogeneous.

Optimization is done by trying various volumes of template DNA, PCR mix, as well as the annealing temperature to obtain the right conditions for amplification which can produce a good product. Volume of template DNA in this study is 2-3 µl, PCR mix 11-13 mL, and forward and reverse primer each as much as 2 mL. To perform amplification, composition is set placed in the PCR machine, with predenaturasi at 94° C for 5 min, followed by 35 sikulus with each cycle programmed for denaturation 94° C for 30 seconds, annealing for 30 seconds at various temperatures were tested, elongation at temperature of 72° C for 1 min, followed by a final cycle of post-elongation at 72°C for 10 min.

After amplifikasi, then performed DNA separation based on the size of the base, using a 10% PAGE (Poly ACRYLAMID Gell Electrophoresis), and to visualize DNA bands generated performed using silver nitrate staining. In addition, the separation was also carried out with 1.5% agarose with ethidium bromide staining.

RESULT AND DISCUSSION

Amplification conditions were not optimal will result in amplification products that are less well characterized with DNA bands Smir or unclear, it is a frequent problem encountered in the amplification process. Many factors will affect the success of the amplification process, that is: the concentration of template DNA, primer match with the template DNA, Mg^{2+} concentration, Taq DNA polymerase, and dNTPs. Except the template DNA, the components in this study are all already present in the PCR mix. Therefore the volume of PCR mix should be optimized to obtain the proper volume.

One of the components contained in the PCR mix is Mg^{2+}, the ions act as cofactors of the polymerase enzyme that acts to bind nucleotide at the time of the formation of a new strand of DNA (Rahman, et al., 2002).The precision of the concentration of Mg^{2+} is very important, the low concentration causing no amplification so it does not appear the DNA band, whereas too high a concentration of the primary causes is not bound to a specific place.

There was an interaction between $Mg^{2+}$and dNTPs, dNTP concentration changes will lead to changes in the concentration of Mg^{2+} in the reaction, when using high dNTP concentrations Mg^{2+} concentration should also be raised. When the excess dNTP concentration, dNTP binds to $Mg^{2+,}$ thereby reducing the concentration of Mg^{2+} in the reaction (Taylor, 1991). Reduced Mg^{2+} will result in reduced activity of taq polymerase enzyme, which in turn inhibit the annealing process so that amplification does not occur. When the presence of dNTPs is higher then the presence of Mg^{2+} in the appropriate concentration will lead to the amplification process, whereas if the Mg^{2+} concentrations were too low despite the presence of dNTPs pretty much the amplification will not occur (Blanchard et al., 1993, in Rahman et al , 2002).

Taq polymerase is also the other components contained in the PCR mix, use taq polymerase concentration is too high (> 4 unit/100 mL) to produce a product that is not specific

(Saiki, 1989 in Rahman et al., 2002). Additionally Haley et al. (1994) stated that the excess concentration of taq polymerase will cause DNA amplification in non-target areas.

PCR mix is a blend of the necessary components for amplification with compositions that have been set, so by using the PCR mix does not need to perform the optimization of these components one by one. From the results of the optimization of the volume of PCR mix, obtained the optimal volume for amplification is 12.5 mL.

DNA obtained from the isolation will serve as the template DNA in the amplification process. The DNA containing the target DNA which is part of the DNA to be amplified. The success of amplification is determined by the presence or absence of DNA binding sites. Primer will stick to the template DNA that have complementary nucleotide base composition of the base composition of the primer. In addition to the quality and quantity of DNA is very influential on the success of the PCR process. Low purity, for example due to the presence of secondary metabolites inhibit the attachment of the base composition of the DNA chain (Padmalatha and Prasad, 2006). The amount of DNA in the PCR reaction is also a component that needs to be optimized. DNA amount is too little or too much will affect the results of amplification (Sunandar and Imron, 2010). Weeden et al. (1992) stated that the concentration of the template DNA is too small often produce DNA bands Smir or unclear. low quantities of DNA template will affect the primary to stick to sites that are complementary to the DNA (karnodle, 1993).

In addition, the storage of DNA samples in TE (Tris EDTA) allows for residual EDTA participating in the amplification reaction. EDTA can bind Mg^{2+} so that no amplification and not generated DNA bands or DNA band were dim. In this study the DNA that has been stored for over 1 month showed no DNA bands, therefore re-isolation was performed in order to obtain fresh DNA template. The result of this optimization is known volume of template DNA that gives the best results is 2.5 mL, the volume of forward and reverse primer each 2 mL of total volume of 22 mL.

Annealing temperature in the PCR reaction is an equally important component for reacting optimally. Appropriate annealing temperature will cause the amplification process occurs optimally. At this stage the DNA primer will stick to the template DNA. As for the primers used in this study is JESPR 240, JESPR 247, JESPR 280, JEPR 299, and JESPR 303. The annealing temperature required by each primer is different that is at 500 C for primary JESPR 247, and 480 C for four other primary JESPR 280, 292 JESPR, JESPR 299, and 303 JESPR. Annealing temperature is too high or too low causes the amplification process does not occur completely, so that the resulting DNA bands are very thin or no DNA bands were formed. Composition of PCR components, the primary volume, and the volume of template DNA right and appropriate annealing temperature, can produce DNA bands were clear. Figure 1, shows the results of PCR using JESPR 247 with annealing temperature at 50^{o}C, while Figure 2 shows the results of PCR using JESPR 292 with annealing temperature at 48^{o}C.

DNA PCR results were separated based on the size of the base by electrophoresis. At first electrophoresis performed in 1.5% agarose gel and staining with ethidium bromide, the results did not show any separation of DNA bands. Furthermore, electrophoresis performed using 10% PAGE stained with silver nitrate, and it appears the separation of DNA fragments. Resolution acrylamide gel is higher than on agarose gel, causing the gel is able to detect a large number of alleles per locus (Maculay et al., 2001).

Figure 1: Results of PCR using JESPR 247 with annealing temperature at 50^{o}C.
From left: DNA marker, Kanesia 4, 6, 7, 8, 10, 11, 12, 13, and 14

Figure 2: Results of PCR using JESPR 292 with annealing temperature at 50^{o}C
From left: DNA marker, Kanesia 4, 6, 7, 8, 10, 11, 12, 13, and 14

CONCLUSION AND SUGGESTION

From these results it can be concluded that for the process of amplification by PCR reactions necessary optimal conditions for each PCR parameters. These parameters include the quantity and quality of their template DNA, PCR mix, primers, and annealing temperature. Optimal volume of PCR mix in this study was 12.5 mL, template DNA 2.5 mL, forward and reverse primer each 2 mL of total volume of 22 mL.

ACKNOWLEDGEMENT

The author would like to thank Mr. Prof.Dr.Moh.Amin, M.Sc., Dr.. Ir. Aris Winaya, MP, Dr. Ir Maftuchah, MP, who has given a lot of input and guidance in this study. Gratitude is also extended to the head of the lab. Biotechnology UMM Malang

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