The Use of RAPD Marker on Gambier's Breeding Program

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Abstract

Low productivity is one of the problems faced by farmers in gambier (**Uncaria gambir*)*** plants cultivation. This problem is mainly caused by the use of low yield gambir cultivars. Although breeding effort has been initiated since several years ago, no significant result has been achieved until now. This is due to inadequacy of molecular investigations in this species. Molecular aspects are commonly related with protocol for DNA preparation and molecular markers which are important so that it will speed up the breeding program. The objectives of the research were to optimize the DNA isolation procedures using different protocol and type of materials, to optimize DNA amplification procedures, and (3) to find out RAPD primers indicating high level of polymorphisms. The research was done from May to November 2009 in the Laboratory of Biotechnology and Plant Breeding, Faculty of Agriculture, Andalas University. DNA isolation procedure and type of materials were assessed. DNA isolation was performed using four different protocols. Type of materials tested consisted of seven treatments. Optimizing PCR condition and RAPD primer selection delivered basic information of optimum PCR condition. A number of 50 RAPD primers were randomly selected. The results recommended the application of CTAB-based method protocol, using young meristem leaves for DNA isolation.

Keywords: gambier, breeding, RAPD

Introduction

Low productivity is a major problem in gambier (*Uncaria gambir)* plant development. Crop productivity ranges between 400 and 600 kg per ha (Dinas Perkebunan Sumatera Barat, 1998; Roswita, 1990). Meanwhile the potential of this plant can reach 2100 kg per ha of dry resin (Sastrahidayat & Soemarsono, 1991). The low productivity is due to the use of low yield varieties. Meanwhile, the use of improved varieties is one determining factor in increasing crop productivity. In the cultivation of gambier, there is no information of using high yielding varieties.

Compared with other commodities, research and development of gambier plant breeding is still far behind. With the use of biotechnology is expected the lag, especially in the field of plant breeding can be pursued. Studies of molecular aspects of the basic information that will be useful as an initial step in gambier plant breeding programs

During the previous decade, strategy for the evaluation of genetic variability was carried out through the approach of anatomy, morphology, embryology, and physiology. This approach now has been fasilitated with molecular techniques. The development of biotechnology in particular science called molecular markers based on polymorphisms found in proteins or DNA, has been widely facilitate research in disciplines such as taxonomy, ecology, genetics, and plant breeding (Weising, *et al*, 1995).

In the field of plant breeding, using fingerprinting techniques have been applied to various aspects and types of plants. Various examples can be mentioned, among others, is its application to rice. Wu and Tanksley (1993) reported the identification of allele-specific microsatellite found in

indica and japonica rice types. The results are then followed by the use of PCR techniques to identify microsatelite polymorphism possessed by the upland rice and other rice cultivars. Virk *et al* (1995) using the RAPD technique for the distinction between the types of rice cultivation and to identify possible duplication of cultivars. Besides the rice crop, the application of molecular markers has also been applied to other fields. Meanwhile, for gambier plants, Fauza, *et al.* (2007) in early research on diversity studies of gambier concluded that RAPD markers have a broad genetic variability. But in this study there are several obstacles in the method of DNA isolation and RAPD-PCR method technique is not stable yet, and limited use of polymorphic primers for gambier plants.

This study aimed to obtain the DNA isolation method for the gambier plant, to find information on material characteristics for DNA isolation, to find a method (program) appropriate in RAPD-PCR technique for gambier plant, and to obtain primers polymorphic in plants gambier characterization techniques based on RAPD-PCR.

Materials and Methods

The research was done from May to November 2009 in the Laboratory of Biotechnology and Plant Breeding, Faculty of Agriculture Andalas University. Two informations namely: DNA isolation procedure and type of materials were assessed. Experiment of DNA isolation procedure performed four protocols as treatments, i.e.: (1) Saghai-Maroof, *et al.* (1984), (2) Doyle and Doyle (1990), (3) Krizman, *et al.* (2006), and (4) An Michiels et al. (2003). Experiment for type of materials applied seven treatments, namely: (1) fresh young leaf, (2) fresh old lead, (3) fresh young leaf stored at -20ºC for one week, (4) fresh young leaf stored at 4ºC, (5) young leaf stored at room temperature (25ºC) for one week, (6) young leaf stored with silica gel for one week (7) etiolated fresh young leaf. Optimizing PCR condition and RAPD primer selection; these experiments delivered basic information of optimum PCR condition that could be used in variability and pedigree analysis based on RAPD molecular technique. Optimization had been done by applying some levels of annealing temperature i.e.: 35°C; 37°C; 40°C; 42°C and 45°C. These annealing temperatures will be combined with one or two cycle groups. Besides some level of DNA concentration (5; 10; 15; 20; 25; 50; and 100 ng/reaction) and concentration of primer (5; 10; 20; 50; and 100 pmol/reaction) applied. A number of 50 RAPD primers were randomly selected.

Results and Discussion

Optimization of DNA Isolation

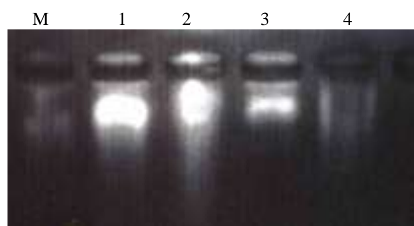
Of the four isolation procedures attempted, it seems that the protocol based on Doyle and Doyle (1990) produced the highest total DNA concentration. The ratio of the intensity of DNA fragments isolated by Doyle and Doyle (1990) showed 2-5 times higher than the standard λ DNA used. With the DNA isolation protocol based on Saghai-Maroof *et al.* (1984), there was no alleged DNA pellets obtained. While isolation by using a protocol based on Krizman *et.al* (2006) and based on An Michiels *et. al*, (2003) for the isolation of DNA-containing plant samples sap, the results obtained was also not optimum .

The success of protocols based on Doyle and Doyle was likely caused by the presence of phenol compounds was mixed with Chloroform-Isoamylalcohol in one stage of isolation. Phenol is known as a compound that is effective enough to separate the protein from cell organelles. It is known that plant cells are very rich in polyphenols. These substances are contaminants for the DNA isolation from gambier plant tissue. However for other species, the use of phenol during DNA isolation process is needed (Jamsari, 2003)

Of the several types and characteristics of materials used for DNA isolation was found that young leaves of gambier plants in fresh condition and young leaves stored in silica gel, were good

materials for the isolation of gambier plant DNA. It is characterized by the discovery of DNA pellet after the extraction process, which in turn is also evidenced by the results of electrophoresis analysis. While other materials did not show DNA pellet after the precipitation process.

At this stage the attempt to isolate DNA from various plant tissues by several methods was carried out. Isolation of DNA from fresh young leaves by the CTAB method (Doyle and Doyle, 1990) and DNA with DNA Kit produced better quality. While the isolation of DNA from young leaves that were stored with silica gel with CTAB method produced DNA with poorer quality than those from fresh leaves, but by using a DNA kit produced DNA with a fairly good quality. In principle, the resulting DNA can be used to the process of PCR-RAPD amplification. Optimization results of DNA with CTAB method are shown in Figure 1.



M = Lambda 50. 1,2,3 = fresh young leaves, 4 = young leaves stored in silica gel

Figure 1. The examples of optimization results of gambier plant DNA isolation.

Optimization of RAPD-PCR technique

Optimization of RAPD amplification was done by modifying the temperature and time of the stages of the amplification process of some methods and programs from various sources, including: method of Williams, *et al.* (1990) and Ready to Go PCR Kit (RTG-PCR Kit). Based on the results of the optimization program that will be applied in the amplification program was that recommended by Williams, *et al.* (1990), as showed in Table 1.

Table 1. RAPD PCR amplification program of gambier plant

Process	Temperature (°C)	Time	Total cycles
Initiation of denaturation	96	2 min	1
Denaturationi	94	30 sec	
Anneling	36	1 min	45
Extention	72	2 min	
Final extention	72	5 min	1
Pause	4	~	

Primer Selection

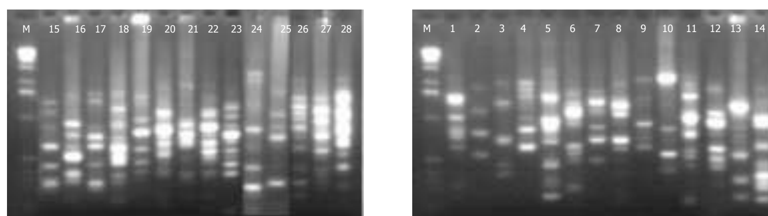
To obtain a high degree of polymorphism, primers were selected by using random operon dekamer primer technology (Almaeda, USA). As the DNA template used in the selection of this primer DNA pool consisting of five DNA genotype gambier based phenotypic appearance is thought to have a distance kinship. Selections of primer for subsequent experiments based on the number of bands (fragments) and the sharpness of the resulting ribbons. Selection was carried out on 50 types of primer RAPD method and program optimization. The primer selection was conducted at two-phase. In the first phase used 25 primers.Three of the 25 primers, were used were for the analysis of individual selection, the OPN-06, OPE-18 and OPY-08. Each band primer produced between 0-5 bands.

Table 2. List of RAPD primer and PCR product characteristics obtained

No	Primer Name	Secuences	Number of fragments	Fragment Characteristics
1.	OPA-11	CAA TCG CCG T	8	Fragment is quite clear, but it's rather difficult to be scoring
2.	OPA-12	TCG GCG ATA G	4	Fragments are clear and easy to be scoring
3.	OPA-15	TTC CGA ACC C	6	Fragments are clear and easy diskoring
4.	OPA-19	CAA ACG TCG G	8	Fragment is quite clear, but it's rather difficult to be scoring
5.	OPB-01	GTT TCG CTC C	14	Fragment is quite clear, but it's rather difficult to be scoring
6.	OPB-06	TGC TCT GCC C	10	Fragment is quite clear, but it's rather difficult to be scoring
7.	OPB-09	TGG GGG ACT C	7	Fragment is quite clear, but it's rather difficult to be scoring
8.	OPB-11	GTA GAC CCG T	9	Fragment is quite clear, but it's rather difficult to be scoring
9.	OPF-08	GGG ATA TCG G	5	Fragments are clear and easy to be scoring
10.	OPK-06	CAC CTT TCC C	8	Fragment is quite clear, but it's rather difficult to be scoring
11.	OPL-13	ACC GCC TGC T	11	Fragment is quite clear, but it's rather difficult to be scoring
12.	OPN-19	GTC CGT ACT G	7	Fragments are clear and easy to be scoring
13.	OPR-11	GTA GCC GTC T	8	Fragmen ts are clear and easy to be scoring
14.	OPT-16	GGT GAA CGC T	11	Fragments are clear and easy to be scoring
15.	OPW-11	CTG ATG CGT G	6	Fragments are clear and easy to be scoring
16.	OPX-01	CTG GGC ACG A	7	Fragments are clear and easy to be scoring
17.	OPX-04	CCG CTA CCG A	8	Fragments are clear and easy to be scoring
18.	OPX-07	GAG CGA GGC T	11	Fragment is quite clear, it's rather difficult to be scoring
19.	OPX-09	GGT CTG GTT G	7	Fragments a re clear and easy to be scoring
20.	OPX-15	CAG ACA AGC C	7	Fragments are clear and easy to be scoring
21.	OPX-19	TGG CAA GGC A	8	Fragments are clear and easy to be scoring
22.	OPY-04	GGC TGC AAT G	11	Fragments are clear and easy to be scoring
23.	OPY-20	AGC CGT GGA A	10	Fragments are clear and easy to be scoring
24.	OPB-17	AGG GAA CGA G	8	Fragments are clear and easy to be scoring
25.	OPF-05	CCG AAT TCC C	6	Fragments are clear and easy to be scoring
26.	OPE-18*	GGA CTG CAG A	12	Fragments are clear and easy to be scoring
27.	OPN-06*	GAG ACG CAC A	10	Fragments are clear and easy to be scoring
28.	OPY-08*	AGG CAG AGC A	13	Fragments are clear and easy to be scoring

* The best primer on the first stage selection.

The second stage was the repeatation of the selection of 25 primers and the best three in the first phase. DNA template used consisted of five different genotypes of first stage. Based on the results of 28 RAPD primer amplification with DNA pool gambier, all tested primer products were found in the DNA mixture. It means thar all the selected RAPD primers has the potential to be used in the selection of individual gambier plant DNA. Products produced ranged from 4-14 fragments of DNA. Fragments generated from the primer selection of this second stage were better than the first stage. This is due to the different DNA pool used in this experiment. Therefore RAPD primer that can be recommended for the analysis of kinship is the OPN-06, OPE-18 and OPY-08. More detail can be seen in Table 2 and the results of amplification of DNA pools with 28 primers RAPD gambier shown in Figure 2.



Description: sample mentioned in each of them according to the primary number in Table 2.

Figure 2. Amplification of gambier DNA pools with 28 primer RAPD.

Conclusions

The isolation of DNA for gambier generated good quality and quantity of DNA using the CTAB method by protocol of Doyle and Doyle (1990) using young leaf tissue. Meanwhile, a program that should be applied for RAPD-PCR amplification was a program recommended by Williams, *et al.* (1990). About 50 selected primers could be used in gambier DNA amplification, but three primers showed a high number of bands and fragments namely: OPE-18, OPN-06 and OPY-08.

References

An Michiels, W. Van den Ende, M. Tucker, L. Van Riet, and A. Van laere. 2003. Extraction of high quality Genomic DNA from latex-containing plants. *Analytical Biochemistry* (315): 85-89.

Dinas Perkebunan Sumatera Barat. 1998. Statistik Perkebunan. Dinas Perkebunan Sumatera Barat. Padang.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fress tissue. *Focus* 12: 13-15.

Fauza, H., I. Ferita, Murdaningsih,H.K., N. Rostini, dan R. Setiamihardja. 2007. Variabilitas genetik tanaman gambir berdasarkan marka RAPD. *Zuriat* 18 (1): 93-99.

Jamsari. 2003. Construction of High-Density Genetic and Physical Maps Around the Sex Gene M of *Asparagus officinalis* L. Published Dissertation-Schriftenreihe des Institut fur Plantzenbau und zuchtung. Cristian Alberchts Universitat zu Kiel (3) 1-171.

Krizman,M, J.Jakse, D.Baricevic, B.Javornik, and M.Prosek. 2006. Robust CTAB-activated charcoal protocol for plant DNA extraction. *Acta Agriculturae Slovenica*. 87:427-433

Roswita, D. 1990. Prospek tanaman gambir di Sumatera Barat. *Bul. BIP Padang* (01): 8-10.

Saghai-Maroof, M.A., Soliman, K.M., Sharm, P.D., Kubde, and S.A. Mulay. 1984. Ribosomal DNS spacer-length polymorphism in barley : Mendelian inheritance, chromosomal location and population dynamic. Proc. Natl. *Acad Sci USA* (81) : 8014-8018.

Sastrapradja, S., S. Dinimihardja, R. Soejono, N.W. Soetjipto, M.S. Prana. 1980. Tanaman Industri. PN Balai Pustaka. Jakarta. 132 hal.

Weising, K., H. Nybom, K. Wolf., and W. Meyer. 1995. DNA Fingerprinting in Plant and Fungi. CRC Press, Boca Raton. Florida. 322 p.

Williams, J.G.K., Kubelick, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V. 1980. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic acids Res*. 18:6531-6535.

Williams, J.K., A.R. Kubelik., K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrari primers are useful as genetic markers. *Nucleic Acid Research.* 18(22): 6531–6535.

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