

Genetic variation of somaclonal mutants from the 8th generation of Pekalongan accession rodent tuber (*typhonium flagelliforme lodd.*) based on RAPD-PCR analysis

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Abstract. Rodent tuber is a potential plant which can be developed as an anticancer drug. The rodent tuber plant has a low anticancer compound which does not have an economic value to be utilized on an industrial scale, particularly to explore as an anticancer drug. The material plant was used 30 clones of Rodent Tuber Pekalongan to increase its bioactive compounds using a combination of *in vitro* culture with gamma-ray irradiation. The selection process has been done until the 8th generation and obtained 14 somaclonal mutants that have higher bioactive compounds than control plant (non-mutant). This study aimed to analyze the genetic variation of 14 somaclonal mutants of rodent tuber using RAPD with 14 primers. The results were produced 513 total bands with the size of 200-5000 bp and 380 polymorphic bands between somaclonal mutants. OPB-18 primer produced the most polymorphic bands and five specific polymorphic bands of 1336 bp, 1070 bp, 901 bp, 861 bp and 728 bp in the somaclonal mutant M23 (20-4-2-1-1-1). OPD-20 primer produced seven specific polymorphic bands of 1574 bp, 1557 bp, 1501 bp, 1496 bp, 1234 bp, 1229 bp and 1086 bp in the M23 somaclonal mutant. The phylogenetic analysis showed that there were two main groups with the coefficient similarity between 0.77 and 0.83. The highest genetic variation obtained was found in the M23 somaclonal mutant with a genetic difference of 23% compared with control. There are five somaclonal mutants (M24, M22, M21, M16, M14) that have a genetic variation of up to 22% over the control. This study shows that OPB-18 and OPD-20 primers were efficient in detecting somaclonal mutant variants.

Keywords: *Typhonium flagellium* Lodd., somaclonal mutant, genetic variation, RAPD-PCR.



1. Background

Rodent tuber (*Typhonium flagelliforme* Lodd.) is a medicinal plant from Indonesia which belongs to Araceae family [5]. Rodent tuber is an herbal medicine plant that has anticancer activity as a raw material of anticancer drugs. Rodent tuber contains anticancer and antiviral components [26]. Rodent tuber has several great potentials for against cancers of the colon, prostate gland, cervix, liver, lung, breast, and leukemia [8, 10, 11, 14]. The hexane extract of rodent tuber is toxic to *Artemia salina* [23]. In addition, rodent tuber has antibacterial and antioxidant activity [15]. Choon et al. [3] said that rodent tuber has an activity as an anticancer and induces apoptosis.

The main problem in rodent tuber has the low of bioactive compounds. Propagation of vegetative seedlings that result in the genetic variation of these plants is low. One potential technology that can be used to increase the bioactive compounds of a plant is mutagenesis *in vitro* combined with somaclonal variation.

The genetic variation that occurs in chromosome structural changes in cell cycle progression [2]. According to van Harten [27] and Griffiths et al. [6], somaclonal can caused by the mitotic disorder used in the errors of chromosome instability and a small deletion within a gene. The genetic variation of plants produced through tissue culture can produce regenerated plants [12, 24].

The increase of genetic variation of rodent tuber of Pekalongan accession was conducted in 2011 using physical mutagen, i.e., gamma-ray irradiation at a dose of 20 Gy [17] and obtained 30 somaclonal mutants. The selection was performed at all generation based on morphology, and molecular detection was conducted until 14 somaclonal mutants were obtained. The genetic distance of the somaclonal mutants can be done by molecular analysis based on PCR with RAPD.

The genotypic analysis can be used by analyzing of plant DNA levels. DNA molecular analysis is one of the molecular biological forms to acquire genotype and genome mapping. The advantage is the DNA level indicating different factors between individuals with each other is because the same differences can be raised by different genotypes or vice versa, the identical genotypes can be represented by different phenotypes [21]. DNA analysis with RAPD can be used to identify genes or chromosomes and genome fingerprints and also to create genome maps [13].

The usage of the RAPD technique has been much to help the activities of plant breeding, among other genetic analysis, knowing the relationship to plant by seeing the distance of genetic similarity. The research conducted by Miklas et al. [13] using RAPD can distinguish between oil palm clones from tissue culture, and the differences in somaclonal mutants provide different polymorphic bands. The purpose of this study was to analyze genetic variation in somaclonal mutants in the 8th generation (MV8) with RAPD-PCR analysis.

2. Methodology

Plant material

Rodent tuber plant was obtained from Pekalongan, Central Java, Indonesia and 14 somaclonal mutants (8th generation) MV8 Pekalongan accessions was achieved through a combination of somaclonal variation with gamma-ray irradiation with a dose 20 Gy. MV8 somaclonal mutants were a collection of Purnamaningsih & Sianipar [17] (2018).

DNA isolation

DNA was isolated from the leaves of somaclonal mutants and control plant. The early process of DNA extraction method was followed by modifying Doyle & Doyle [4](1987). Each sample was measured at 2.5 g in total then inserted into a cooled mortar and added 0.1% PVP and crushed by adding liquid nitrogen to a fine powder. The powder was inserted into an Eppendorf tube containing 2 ml of extraction buffer [CTAB 10% w/v]; EDTA 0.5M pH 8.0; Tris HCl 1M pH 8.0; NaCl 5M] 10 µl mercaptoethanol 1% (w/v). The mixture was shaken using a vortex, heated 20 min at 60°C, then cooled in the room temperature and added 750 µl of chloroform solution: isoamyl alcohol (24:1) then mixed well by gentle inversion. The mixture was centrifuged for 10 min at 11,000 rpm. The upper portion was loaded to another Eppendorf tube and extracted with 1 ml of chloroform

solution:isoamethalcohol (24:1), centrifuged for 10 min at 11,000 rpm. The supernatant was supplied to another Eppendorf tube, then added 750 μ l cold isopropanol, gently mixed into homogeneous and stored at -20°C overnight, then centrifuged for 10 min at 11,000 rpm. The precipitate was dried vacuum for 1 hr. Dried DNA was dissolved in 200 μ l TE buffer (Tris-HCl 1M pH 8.0; EDTA 0.5M pH 8.0). The RNA was removed by adding 20 μ l RNase (10 mg/ml) in 200 ml of DNA solution. The mixture was incubated for 1 hr at 37°C, then placed in the refrigerator (4°C) overnight. The DNA sample solution was kept at -20°C until further use.

DNA assessment

The 1.5% agarose gel was prepared by dissolving 0.4 g of agarose in 40 ml of TAE 1x buffer (Tris base, glacial acetic acid, 0.5 M EDTA, pH 8.0). The gel was put into a tub of electrophoresis that has been filled TAE 1x solution until submerged. The DNA samples were placed into a gel well and electrically sprung for about 1 hr at 60 V. The thickness of the DNA bands shows the DNA quantity samples compared to the width of DNA bands of lambda. While the DNA quality was determined based on the integrity of white DNA band with no laddering or smearing band.

Data amplification and data analysis

The reagent composition in RAPD-PCR primers are presented in Table 1. 14 primers with 10-mer sizes were used in RAPD-PCR analysis. The amplification reaction was carried out using a Thermal Cycler Gene PCR (ABI 9700) with a thermal cycle of 45 times. The following steps for extracted DNA were first 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and 4 min at 72°C, after reaching 45 cycles there was an extension time step for 4 min at a fixed temperature of 72°C. The PCR product can be fractionated using 1.5% agarose gel (b/v) in TAE 1X 40 ml solution. Electrophoresis was run at 75 V for 1.5 hr. The electrophoresis gel results were documented by using KODAK Gel Logic 100 Imaging System hardware and KODAK Molecular Imaging software.

Table 1. Reagent composition in RAPD-PCR primers

Reagent	Concentration	Final volume (μ l) 1X
DNA Template (5 ng/ μ l)		5.0
dNTP (10 mM)	0.2 mM	0.2
PCR Buffer (10X) + MgCl ₂ (promega)	1x	2.5
Primer (10 pmol/ μ l)	1 μ l	1.0
Taq Polymerase (5U/ μ l)	1 U	0.2
ddH ₂ O		16.1
Total		25.0

Data analysis was carried out by using the scoring method of DNA band which presented on 1.5% agarose gel electrophoresis result. The molecular data of the DNA bands were converted to binary numbers (0 and 1) and formulated in the matrix. Scoring DNA bands were assisted by Gel Analyzer software to facilitate the position of DNA bands. The scoring results were then analyzed using the SARN-UPGMA (Unweighted Pair-Group Method with Arithmetic) program Sequential Agglomerative Hierarchical and Nested (NSW) version of NTSYS software version 2.1 [25]. The results were presented in the dendrogram that showed genetic similarity.

3. Results and Discussion

Analysis of genetic variation using RAPD marker

The results of genetic analysis of 14 somaclonal mutant rodent tuber including one accession of control plant using RAPD primers were presented in Table 2. A total number of bands produced by 14 RAPD primers over the 15 DNA samples was 513 and showed 380 polymorphic bands. Of these, the

lowest value of total bands was produced by the OPA-03 primer (20 bands with 14 polymorphic bands) while the highest ones were produced by OPB-18 (54 bands with 43 polymorphic).

Table 2. List of RAPD primers and the number of DNA bands produced by different primers in the 8th generation of rodent tuber somaclonal mutants and control plant.

Number	Primer	Sequence	Total bands	Polymorphic bands	Size (bp)
1	OPA-02	5'-TGCCGAGCTG-3'	27	14	300-1700
2	OPA-03	5'-AGTCAGCCAC-3'	20	14	300-2300
3	OPA-09	5'-GGGTAACGCC-3'	49	40	500-1650
4	OPA-14	5'-TCTGTGCTGG-3'	36	30	500-1550
5	OPB-18	5'-CCACAGCAGT-3'	54	43	200-1650
6	OPC-05	5'-GATGACCGCC-3'	27	21	300-2100
7	OPC-08	5'-TGGACCGGTG-3'	26	18	450-1550
8	OPC-11	5'-AAAGCTGCGG-3'	26	18	600-1200
9	OPC-14	5'-TGCGTGCTTG-3'	23	20	600-1100
10	OPD-08	5'-GTGTGCCCCA-3'	52	38	350-2000
11	OPD-10	5'-GGTCTACACC-3'	33	27	500-5000
12	OPD-20	5'-ACCCGGTCAC-3'	52	32	400-2000
13	OPE-03	5'-CCAGATGCAC-3'	35	28	250-1250
14	OPE-07	5'-AGATGCAGCC-3'	53	37	600-1500
Total			513	380	

The DNA bands in RAPD profiles generated by the primers OPB-18 and OPD-20 are shown in Figure 1. RAPD profiles for band similarity indices can clearly differentiate the somaclonal mutants and control plant. The size of amplified products ranged from 200 bp – 1650 bp in OPB-18 primer. Screening of 14 RAPD primers resulted in 2 primers (OPB-18 and OPD-20) which showed polymorphisms within the M23 somaclonal mutant. OPB-18 primer produced 5 specific polymorphic bands in size of 1336 bp, 1070 bp, 901 bp, 861 bp, and 728 bp. There were 7 specific polymorphic bands recorded by OPD-20 primer in size of 1574 bp, 1557 bp, 1501 bp, 1496 bp, 1234 bp, 1229 bp, and 1086 bp. The genetic variation has occurred in the somaclonal mutants and control plant as reported in the presence of polymorphic bands.

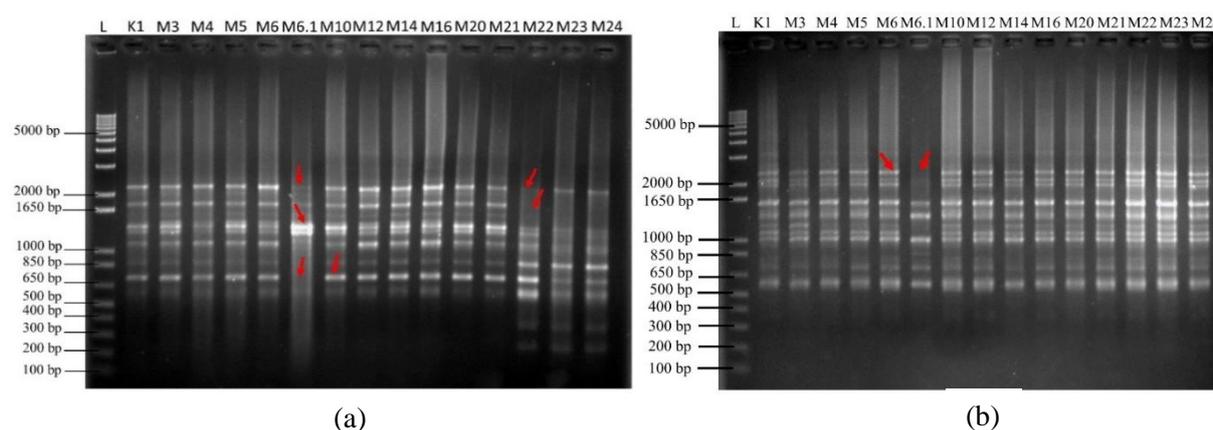


Figure 1 RAPD profiles generated from genomic DNA of 14 rodent tuber somaclonal mutants and control plant amplified by (a) OPB-18 primer and (b) OPD-20. (L) Marker; (K1) Control; (M3) 20-1-1-3-2-4; (M4) 20-1-1-2-2-3; (M5) 20-1-2-2-3; (M6) 20-1-2-1-2-6; (M6.1) 20-1-2-1-2-6; (M7) 20-1-2-2-4-3; (M8) 20-1-1-2-2-3; (M10) 20-1-3-4-4-8; (M12) 20-1-2-2-1-3; (M14) 20-1-2-2-4-3; (M16) 20-1-2-4-5; (M20) 20-1-1-1-3; (M21) 20-1-2-4-4; (M22) 20-1-3-4-4-8; (M23) 20-4-2-1-1-1; (M24) 20-1-3-4-4-9.

Phylogenetic analysis

The phylogenetic analysis showed that the 14 somaclonal mutants of rodent tuber used in this study were divided into two major groups at a coefficient similarity of 0.77 (Figure 2). The first group

consisted of 13 genotypes. The second group was represented only by 2 genotypes of somaclonal mutants M23 and M24. Furthermore, the first group was divided into two subgroups of IA subgroup and IB subgroup. Based on the genetic relationships, two somaclonal mutants have the closest relation of the IB subgroup, i.e., M16 and M20 with a genetic coefficient of 0.83, which means that both genotypes have 83% genetic similarity (Figures 2). Control plant (K1) shares 83% genetic similarity with the M3 somaclonal mutant. This result revealed that M3 somaclonal mutant and control plant has the closest genetic similarity. M4 and M5 formed a minor group with more than 80% similarity. All the species share more than 70% similarity among themselves.

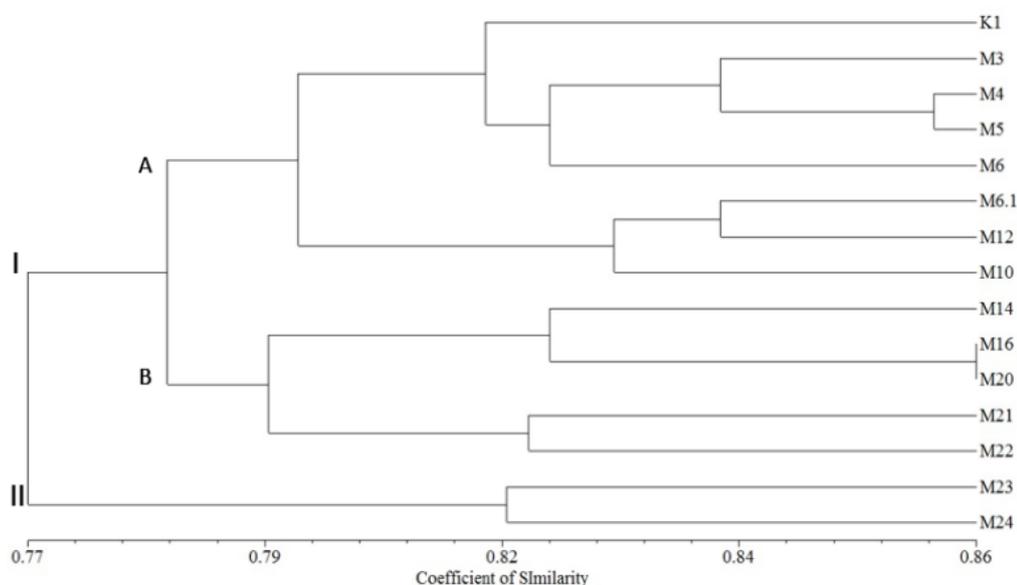


Figure 2. The dendrogram is showing the genetic relationships among 14 rodent tuber somaclonal mutants and control plant.

The highest similarity of 83% was indicated between control and M3 somaclonal mutant. This indicates that M3 is a mutant which has the smallest genetic differences with control to other somaclonal mutants, while the M23 somaclonal mutant is the most distinct mutant plant to control with a difference of 23%. There were five somaclonal mutant clones that have a genetic distance of 22% with control, i.e., M21, M22, M24, M14 and M16 (Figure 2).

The variation in the number of polymorphic bands and the genetic relationships indicates that the 14 primers used are effective in analyzing the genetic variability occurring in the 8th generation somaclonal mutant of rodent tuber. The primers of OPA-2, OPA-9, OPB-18, OPC-5, and OPC-8 proved to be effective for discovering genetic variation between species of *Typhonium flagelliforme*, *Typhonium trilobatum*, and *Typhonium roxburgii* [19]. The mechanism of genetic variation in plant tissue culture was derived from chromosome doubling, changes in DNA sequence and changes in the cytoplasmic [29, 9]. Genetic variation occurs in the *Etlingera elatior* (Jack) due to *in vitro* mutagenesis [30]. According to Yaycili & Alikamanoglu [28], the induction of gamma rays produces genetic diversity in mutant tolerance potato salt plants. Gamma rays can induce polymorphic DNA in *Emblca officinalis*, changes the DNA profile in *Solanum tuberosum*, and alters morphological characteristic in *Zingiber officinale* [20, 7, 18]. The RAPD marker is very effective for analyzing genetic variability induced through gamma rays. The RAPD technique is effective for detecting polymorphisms between different oil palm genotypes [16, 22].

Genetic analysis with RAPD-PCR showed genetic changes in somaclonal mutants. Atak et al. [1] used RAPD methods showed the genetic variability among *Rhododendron* mutants, which

successfully irradiated by gamma ray at doses 5 and 10 Gy. In contrast to phenotypic observations, although the morphology is the same, it may differ at DNA levels [21]. The results showed that utilization of somaclonal variation technology combined with gamma-ray irradiation is potential to increase the chances of obtaining acceptable somaclonal mutants that can be recommended for release as new improved varieties.

4. Conclusion

The RAPD-PCR profile results showed that there had been a genetic alteration of a somaclonal mutant of rodent tuber Pekalongan accession from mutation and somaclonal variation at the 8th generation. There were 513 total bands obtained by 14 primers. OPB-18 primer produced more bands and more amplified polymorphism bands. OPB 18 primer has recorded the most polymorphic bands and five specific polymorphic bands of 1336 bp, 1070 bp, 901 bp, 861 bp and 728 bp in the M23 somaclonal mutant. OPD 20 primer has produced the most specific polymorphic bands which are obtained seven specific polymorphic bands of 1574 bp, 1557 bp, 1501 bp, 1496 bp, 1234 bp, 1229 bp and 1086 bp in the M23 somaclonal mutant. There are five somaclonal mutants of M24, M22, M21, M16, and M14 which have genetic differences of up to 22% compared to the control. The M23 somaclonal mutant has the lowest genetic similarity about 77% compared with control plant. The present study clearly shows that genetic variability is present in the 8th generation of rodent tuber mutant plants via somaclonal variation and suggested that OPB-18 and OPD-20 primers are efficient in detecting somaclonal variation.

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6. References

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