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Development of *lectin* gene-based SNAP and ARMS markers as anticancer biomarkers in mutant rodent tuber (*Typhonium flagelliforme*) of Pekalongan accession, Indonesia

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Abstract. *Muflikhati Z, Sianipar NF, Reflinur, Anas. 2025. Development of* lectin *gene-based SNAP and ARMS markers as anticancer biomarkers in mutant rodent tuber (*Typhonium flagelliforme*) of Pekalongan accession, Indonesia. Biodiversitas 26: 424-433. Typhonium flagelliforme* is a medicinal plant with potential anticancer properties. This study aimed to develop Single Nucleotide Amplified Polymorphism (SNAP) and Amplification Refractory Mutation System (ARMS) markers from the *lectin* gene that contributed to anticancer activity found in gamma-irradiated mutant rodent tuber plants of the Pekalongan accession. The two markers were designed for the site of Single Nucleotide Polymorphisms (SNPs), which were identified at approximately 500 bp in the length of the *lectin* gene sequence. The SNAP Lec113 primer designed from a 113 bp silent mutation in the respective gene has successfully differentiated rodent tuber mutant lines across the wild-type, determined by the specific T allele at 351 bp. Meanwhile, the ARMS Lec241 primer, designed from a 241 bp missense mutation (arginine to threonine), distinguished mutants from their wild-type by the specific G allele at 193 bp. The two developed markers demonstrated their high specificity and sensitivity in detecting genetic variations spanning *lectin* gene involved in anticancer biosynthesis. The SNAP marker effectively distinguished among seven mutant samples, while the ARMS marker provided consistent results across experiments. These molecular markers offer a rapid and accurate method for identifying genetic variations in rodent tuber plants, providing a significant practical benefit for the field of genetic screening and mutation impact studies. The three-primer SNAP system presents a more cost-effective option for large-scale screening compared to the four-primer ARMS system. This study provides a foundation for targeted breeding programs and genetic studies in rodent tuber, potentially accelerating the development of varieties with enhanced medicinal properties. The markers enhance genetic screening efficiency and understanding of medicinal properties in rodent tuber, significantly advancing the field of natural product therapeutics and mutation impact studies.

Keywords: ARMS marker, *lectin* gene, mutant screening, SNAP marker, *Typhonium flagelliforme*

Abbreviations: ARMS: Amplification Refractory Mutation System, SNAP: Single Nucleotide Amplified Polymorphism

INTRODUCTION

Typhonium flagelliforme (G.Lodd.) Blume or rodent tuber plant, a native Indonesian herb rich in anticancer compounds, holds promise as a potential cancer drug ingredient. Genetic traits of the rodent tuber have been enhanced through in vitro gamma-ray radiation and tested for stigmasterol concentration, which is more effective at inhibiting breast cancer cells than the wild-type (Sianipar et al. 2016; Sianipar and Purnamaningsih 2018; Sianipar et al. 2024c)-similarly, fatty acid compounds (2-octanoic acid and 2-hexenoic acid) (Sianipar et al. 2023). The gamma-irradiation-treated mutant rodent tuber plants were cultivated in a controlled greenhouse environment with consistent and regulated conditions. Furthermore, these mutant plants are protected under plant variety protection (PVT) from the Ministry of Agriculture of the Republic of Indonesia (Binusantara 1, Nomor 00713/PPVT/S/2024), ensuring standardized and optimal cultivation practices to minimize variability. Mutant rodent tubers contain more anticancer compounds and can better inhibit cancer cells than wild-type plants (Purnamaningsih et al. 2018; Sianipar et al. 2023). This demonstrates the development of mutant rodent tuber plants in terms of anticancer bioactive components, such as *lectin*. *Lectin* can prevent breast cancer cells from proliferating without being toxic to normal cells (Alfarabi et al. 2015). It is a protein with antiproliferative abilities in certain cancer cells. Bioactive components like *lectin* can be regulated by a specific gene, namely the *lectin* gene (Alfarabi et al. 2015). This gene acts as a bioactive constituent, inhibiting cancer cell growth across various plant species. Partial sequences of the *lectin* gene from *T. flagelliforme* were successfully obtained at a size of 500 base pairs (bp) (Sianipar et al. 2024a). However, no known

specific molecular marker developed from the *lectin* gene can differentiate mutant rodent tubers from the wild-type Pekalongan accession.

Molecular markers help us to detect variations in DNA nucleotide sequences at specific locations in the genome because they are specific, allow for rapid identification, and are not influenced by environmental factors or plant age (Nadeem et al. 2018). Single Nucleotide Polymorphism (SNP) molecular markers are one-locus markers with two alleles at each locus (Dwiningsih et al. 2020). Based on the SNP sites of a genome, they can be developed into Single Nucleotide Amplified Polymorphism (SNAP) specific to one allele (Tarigan et al. 2021). In comparison to the other molecular markers, SNAP marker analysis has many advantages, such as a high probability of finding markers within target genes due to the high density of SNPs present in the genome (Xia et al. 2019). This marker only has 3 primers, 2 of which are designed based on allele differences. Additionally, SNAP markers are considered effective in detecting point mutations even though the amplification process is carried out separately for reference and alternate allele-specific primers. The software used to design SNAP markers is WebSnapper (Tarigan et al. 2021).

SNP marker development should involve more than one marker to increase the accuracy of SNP identification and provide a better understanding of genetic polymorphism, as each marker has different sensitivities to certain types of polymorphisms (Balladona et al. 2020). Another marker that can be developed is the Amplification Refractory Mutation System (ARMS) marker with specific allele properties (Zhu et al. 2021). ARMS serves as a marker for point mutation analysis, involving several primers, two of which are designed based on the SNP locus (Zhu et al. 2021). This approach introduces mismatched bases in the center of allele-specific primers. ARMS includes intentional polymorphisms at position -2bp to the third position of allele-specific primers to enhance amplification specificity. ARMS PCR is a rapid and reliable approach for detecting point mutations or minor deletions (Meng et al. 2017). Unlike other molecular mutation detection methods, it is simpler (requiring no sophisticated equipment), relatively inexpensive, and does not demand special skills (Meng et al. 2017). Several ARMS strategies exist, including single ARMS, multiplex ARMS, and double ARMS (Lo 1998). Sianipar et al. (2024a) reported variations in point mutations (SNPs) of the *lectin* gene in four rodent tuber mutants of Pekalongan accession through sequencing analysis. This study aimed to develop SNAP and ARMS markers from the *lectin* gene that contributed to the anticancer activity found in gamma-irradiated mutant rodent tuber plants of Pekalongan accession.

MATERIALS AND METHODS

Plant genetic and sequence materials

Plant genetic materials used in the present study comprised the rodent tuber mutant genotypes of Pekalongan accessions along with their wild-type. The mutant accession of Pekalongan was previously treated with gamma irradiation by Sianipar et al. (2020). The sample was stored at the laboratory of Food Biotechnology Research Center, Bina Nusantara University, Jakarta, Indonesia, and was taken in 2021. The tissues utilized for the template DNA consist of fresh leaves from wild-type and 8 mutant rodent tuber (Table 1).

The *lectin* gene sequence analyzed in this study was derived from wild-type rodent tuber and four mutant clones from previous research. These mutants were classified as having high and low anticancer properties, each with a sequence length of approximately 500 bp (Sianipar et al. 2024). The sequencing of the *lectin* gene revealed several point mutations. The identified sequences were analyzed to detect specific point mutations within the *lectin* gene. These mutations were then used as the basis for designing ARMS primers, enabling the differentiation between wild-type and mutant clones.

Genomic DNA extraction and quality test

The total genomic DNA of rodent tuber mutants and their wild-type plants obtained from fresh leaf tissues was extracted using a cetyltrimethylammonium bromide (CTAB)-based preparation method (Calderón-Cortés et al. 2010). After mixing with liquid nitrogen and mortar, the leaves were mixed with 1000 µL of extract buffer. The mixture was transferred into 2 mL microtubes, incubated in a water bath for 45 min, and homogenized every 15 min. Subsequently, 800 µL of chloroform-isoamyl alcohol solution was added to each tube, followed by adding sodium acetate at one-tenth of its volume to a separated 1.5 µL microtube and incubated for 10 min at room temperature. The micropipette was used to remove the supernatant, and the DNA pellet was added with 1X TE buffer solution (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]), and then treated with RNase (1 mg/mL).

The mixture was then gently mixed and maintained at 37°C for 30 min. Following this step, sodium acetate solution (pH 5.2) diluted to one-tenth of the DNA solution's volume was gently mixed in. Next, each sample underwent processing with 600 µL of ethanol (95%) to precipitate the DNA. The samples were combined gradually after a 45 min incubation at -20°C. The MiniSpin Plus (EU-IVD) (Eppendorf, UK) centrifuge removes the supernatant, spinning for 10 min at 13,500 rpm.

Table 1. Plant genetic materials used in the study

Identification numbers	Identification names	Collection
P1	KP Control (wild-type)	Binus
P2	PM 9	Binus
P4	PM 4	Binus
P7	KP 20-1-2-4-9	Binus
P11	KP 20-1-1-2-2-3	Binus
P12	PM 8-1	Binus
P13	KP 20-1-3-1-4-3	Binus
P15	KP 20-1-2-2-1	Binus
P16	PM 8-1-1	Binus

Once dried, TE buffer (200 µL, 1X) was used to resuspend the dried DNA, and the isolated DNA samples were tested using the Nanodrop Spectrophotometer 2000 (manufactured by ThermoScientificTM, USA) for concentration and purity measurement using the A260/A280 ratio. Finally, DNA samples were diluted with 1X TE solution (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]) to a final concentration of 10-18 ng/µL and kept at -20°C.

Sequence identification of Pekalongan rodent tuber *lectin* gene based on point mutations

The wild-type rodent tuber *lectin* gene sequence, approximately 500 bp in length (Sianipar et al. 2024), underwent verification analysis using the National Center for Biotechnology Information's (NCBI) Basic Local Alignment Search Tool (BLAST). Subsequently, Clustal alignment was performed using Geneious Prime 2023.1.1 (Biomatters, Ltd) to identify the exonic regions or segments encoding amino acids within the previously studied rodent tuber *lectin* gene sequence. The sequence was aligned against *lectin* gene sequences in other species, and those exhibiting high similarity to the wild-type rodent tuber *lectin* gene were selected for marker development purposes.

Geneious Prime 2023.1.1 aligned each gene sequence, which was then exported to MEGA software (version 11) to construct a tree of phylogenetics (Tamura et al. 2021). The tree of phylogenetic relationships was generated using the greatest likelihood approach and 1000 bootstrap replicates to identify similarities in *lectin* genes between rodent tuber and other plant species. The point mutation site was determined by aligning the sequence of the rodent tuber *lectin* gene from previous studies. The analysis showed several point mutations at base 113 bp, base 241 bp, base 269 bp, base 279 bp, and 323 bp (Sianipar et al. 2024). The software Geneious Prime (version 2023.1.1) was utilized for point mutation identification of the rodent tuber sequence, including amino acid translation based on these point mutations (Kearse et al. 2012). The location and type of point mutations, as well as amino acid changes resulting from them, were also analyzed. Point mutations that result in amino acid changes are classified as missense mutations, whereas those that do not affect amino acid sequences are classified as silent mutations.

Design of SNAP primer

SNAP primers were designed for *lectin* genes using WebSnapper software (http://ausubellab.mgh.harvard.edu) based on SNP sites of *lectin* gene sequences obtained from previous studies (Sianipar et al. 2024) with bi-allelic site allele types and those that cause amino acid changes. The specificity of primers to detect *lectin* genes was analyzed using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast). The parameters used for the design of the 113 bp position SNAP primers were a PCR product size range of 325-375 bp with a primer length of 20-30 bp and a primer melting temperature (Tm) of 55°C-65°C. Each primer combination was evaluated to ensure if at least three primers were required: two forward primers (wild-type allele and mutant allele) and a reverse primer (Table 2). PCR amplification was used for genotyping single SNP loci. Of which, a pair of wild-type allele primers (C) and reverse were used in the first reaction, while a pair of mutant allele primers (T) and reverse were used for the second reaction.

Design of ARMS primer

The specific *lectin* gene-based ARMS primers were designed based on previously identified point mutations (Sianipar et al. 2024). Point mutations with the potential to be developed for ARMS markers and having bi-allelic alternative alleles were then utilized for primer design. The primers were created using the web-based Primer1 (http://primer1.soton.ac.uk/primer1.html). The accuracy of the primer for detecting the *lectin* gene was verified using Primer BLAST available in NCBI. Next, to construct ARMS primers at position 241 bp, the specified criteria were considered: amplification product length between 100-300 bp, primer solution of 5 µM, primer length between 20 and 24 bp, and a Tm ranging from 62 to 70°C. The web-based OligoAnalyzerTM Tool was used to calculate the primer sequence's Tm.

SNAP marker amplification

The Polymerase Chain Reaction (PCR) was done in a total reaction volume of 12 µL, which included 5 µL of 1× MyTaqTM HS Red Mix (Bioline, UK) containing dNTPs, $MgCl_2$, MyTaq HS DNA polymerase, and reaction buffer, along with 2-5 µL of genomic DNA (2 to 5 ng template DNA), 0.5 µL of each 10 µM primer (forward primer for the wild-type allele, reverse primer, forward primer for the mutant allele, and reverse primer), and ddH_2O. The amplification process was performed using a CG1-96 Palm-Cycler Thermal Cycler (Corbett, USA) with the following PCR profile: an initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at the optimal temperature of 63°C, and extension for 30 sec at 72°C.

Table 2. The SNAP primers for *lectin* genes of mutant and wild-type rodent tuber plants

SNP position (bp)	Primer ID	Forward/reverse sequences (5'-3')	Melting temperature (°C)	Amplification size (bp)
113	Lec113_SNAP_F_C	GACTGCAACCTCGTCCTCTAGAAC	58.8	351
	Lec113_SNAP_R	CATCACCAGCATGTGGTTCCT	57.6	
	Lec113_SNAP_F_T	GACTGCAACCTCGTCCTCTAGAAT	58.1	

Notes: Lec113: A primer designed based on an SNP at position 113 in the *lectin* gene. F: Forward, R: Reverse

The reaction concluded with a final extension step for 5 min at 72°C. PCR products were electrophoresed on a 2.0% agarose gel (Vivantis.Sdn.Bhd.) in TBE buffer (Tris Borate EDTA) for 85 min at a steady of 75 volts. A DNA ladder of 100 bp (Geneaid.Ltd.) was added as a size marker to each agarose gel in order to determine the sizes of the PCR products. The gel was stained with a Florosafe DNA stain (1st BASE) and visualized using a Syngene UV Transilluminator (USA).

Scoring SNAP marker bands

The PCR outcomes were observed by displaying rodent tuber allelic DNA band. Clear and discrete DNA bands were assessed based on their presence or absence, and band intensity was compared between wild-type and mutant plants. The appearance of a band at a specific position indicates the presence of a particular allele, while its absence indicates the presence of allelic variation. For each sample, band patterns were compared to assess genetic differences, and scoring was done by assigning a value ("1" for presence and "0" for absence) to each band position. This scoring method allows quantitative comparison of allelic variation between wild-type and mutant plants.

ARMS marker amplification

PCR was done in a total reaction mixture of 12 µL including 1x MyTaqTM HS Red Mix (Bioline, UK) of 5 µL, 2 to 5 µL genomic DNA (3 to 5 ng template DNA), 10 µM primers (0.5 µL each), and adjusted with ddH_2O. PCR reactions were performed on a CG1-96 Palm-Cycler Thermal Cycler PCR machine (Corbett, USA) with a PCR profile: initial denaturation at 94°C for 4 min; then included 35 cycles of denaturation for 30 sec at 94°C; annealing for 30 sec at an optimum temperature of 65°C; extension for 30 sec at 72°C; and ended with a final extension cycle for 5 min at 72°C. Products of PCR were then separated using a agarose gel (2.0%) (Vivantis.Sdn.Bhd) in Tris Borate EDTA buffer (1st BASE) for 85 min at a steady 75 V. A DNA ladder of 100 bp (Geneaid.Ltd.) was inserted into each agarose gel as a size standard to determine the size of the PCR product. Gel was then stained using a Florosafe DNA stain (1st BASE) visualized under ultraviolet light with a Syngene UV Transilluminator (USA).

RESULTS AND DISCUSSION

Sequence identification of Pekalongan rodent tuber *lectin* gene based on point mutations

The result of the sequence comparison between the *lectin* gene of rodent tuber and the reference sequences database derived from other species (Table 3). Of these, the wild-type rodent tuber *lectin* gene sequence showed the highest significant sequence homology with the *T. flagelliforme lectin* gene, partial CDS (Accession number MK904840) accounting for 95.93% of identity percentage value, which covered 571 bp in length. This sequence also showed significant homology (90.63%) with that in the complete CDS sequence of *Pinellia ternata* mRNA *lectin* (Accession number LC764429) covering 810 bp in length. Furthermore, an 89.82% identity was observed with the complete CDS of *Pinellia pedatisecta* mannose-binding tuber *lectin PPA* gene (Accession number AY451853), which has a base length of 2739 bp. The e-values (e.g., 8e-176 for the species *P. pedatisecta*) are interpreted as 8 x 10^{-176}, which means the value is close to zero (Table 3).

This indicates a highly significant similarity between the two sequences, almost impossible to occur by chance, and most likely derived from an evolutionary or functional relationship. The choice of the *P. pedatisecta* mannose-binding tuber *lectin PPA* gene as a reference for Multiple Sequence Alignment (MSA) based on both start codon and amino acid translation was justified by its more extensive base sequence. Moreover, this gene represents a complete CDS, encoding the amino acids in a protein. Typically, CDS sequences of the *lectin* gene start with ATG (start codon) and end with a stop codon.

The sequence alignment between the *lectin* gene sequence of *P. pedatisecta* mannose-binding *lectin PPA* gene complete CDS and that of the rodent tuber from the previous study showed no gaps observed in either sequence (Figure 1). The approximately 500 bp segment of the *lectin* gene was obtained from the current study, which showed homology spanning from 1,212 bp to 1,703 bp out of the total 2,739 bp length with the *lectin* gene's complete CDS sequence. This result confirms with high confidence that the entire rodent tuber *lectin* gene sequence from the previous study constitutes a single exon.

Table 3. Sequence comparison analysis of *lectin* gene derived from Pekalongan rodent tuber and that from other species in reference sequence database

Species name	Gene name	E value	Percentage identity (%)	Accession length (bp)	Accession number
Typhonium flagelliforme	*Lectin* gene, partial cds	0.0	95.93	571	MK904840
Pinellia ternata	*Lectin* mrna, complete cds	0.0	90.63	810	LC764429
Pinellia pedatisecta	Mannose-binding tuber *lectin PPA* gene, complete cds	8e-176	89.82	2739	AY451853
Sauromatum venosum	*Lectin* mrna, complete cds	2e-167	88.93	1121	KX132811

Furthermore, the alignment result also underscores that the entire *lectin* gene sequence from the previous study indeed corresponds to an actual *lectin* gene within the exon. This finding is in line with Moraes Filho et al. (2017), who reported that 35 legume *lectin* gene sequences consisted of only one exon and lacked introns. Similarly, the *lectin* genes of *Salvia miltiorrhiza* were intronless and exhibited a sole exon type, as reported by Wenping et al. (2015).

The research has uncovered novel findings in the *lectin* gene sequence of rodent tuber and that in other plants. A phylogenetic structure of the *lectin* gene was successfully generated, revealing that the rodent tuber *lectin* gene shares its closest similarity with the *T. flagelliforme lectin* gene, partial CDS (Accession number MK904840) (Figure 2). In addition, a close relationship of the rodent tuber *lectin* gene with other *lectin* genes was observed, including those from *P. ternata* mRNA for *lectin*, complete CDS (Accession number LC764429), *Pinellia pedatisecta* mannose-binding *lectin PPA* gene, complete CDS (Accession number AY451853), *Typhonium divaricatum* mannose-binding *lectin ALA* gene, complete CDS (Accession number EF194099), *Sauromatum venosum lectin* mRNA, complete CDS (Accession number KX132811), *Arisaema lobatum* mannose-binding tuber *lectin* mRNA, complete CDS (Accession number AY557617), and *Remusatia vivipara lectin* (L1) gene, complete CDS (Accession number AU924066). This genetic relatedness, particularly the close similarity with the *T. flagelliforme lectin* gene, is a novel finding that adds to the understanding of the genetic relationships among various plant species (Family Araceae).

A comparison of amino acid translation sequences of rodent tuber *lectin* gene sequences in the wild-type and mutant lines on the basis of point mutation sites (Table 4). By using the *P. pedatisecta* mannose-binding *lectin PPA* gene as a start codon reference, three-point mutations resulting in amino acid changes were identified (Table 4). The variations in amino acids can be termed silent mutations if there is no variation in amino acid translation and missense mutations if there is a variation in amino acid translation (Iqbal et al. 2020). Silent mutations occurred at base 113 bp (replacing C in the AAC codon with a T in the AAT codon), while other mutations caused amino acid variations. For example, at base 241 bp, the AGG codon (coded for arginine) changed to the ACG codon (coded for threonine). Similarly, at base 279 bp, the CTG codon (coded for leucine) became the GTG codon (coded for valine). These mutations indicate missense changes. This mutation represents a significant finding, with potential implications for further studies in genetics and molecular biology.

Figure 1. Multiple sequence alignment of mutant and wild-type rodent tuber *lectin* gene sequences (KP-control, KP_20_1_2_1_2_6-low, PM4 low, PM6 high, PM9 high) with *P. pedatisecta lectin* gene sequences (AY451853.1)

Table 4. Amino acid translation of point mutation sites in the *lectin* gene sequence of wild-type rodent tuber and mutant clones based on the start codon

SNP position (bp)	Variation of point mutations	Type of base substitution	Type of point mutations	Changes in amino acid residues
113	C to T	Transition	Silent	Asparagine
241	G to C	Transversion	Missense	Arginine-Threonine
269	G to A	Transition	Silent	Proline
279	C to G	Transversion	Missense	Leucine-Valine
323	TT to TG	Transversion	Silent	Valine
	TT to AT		Missense	Valine-Aspartate

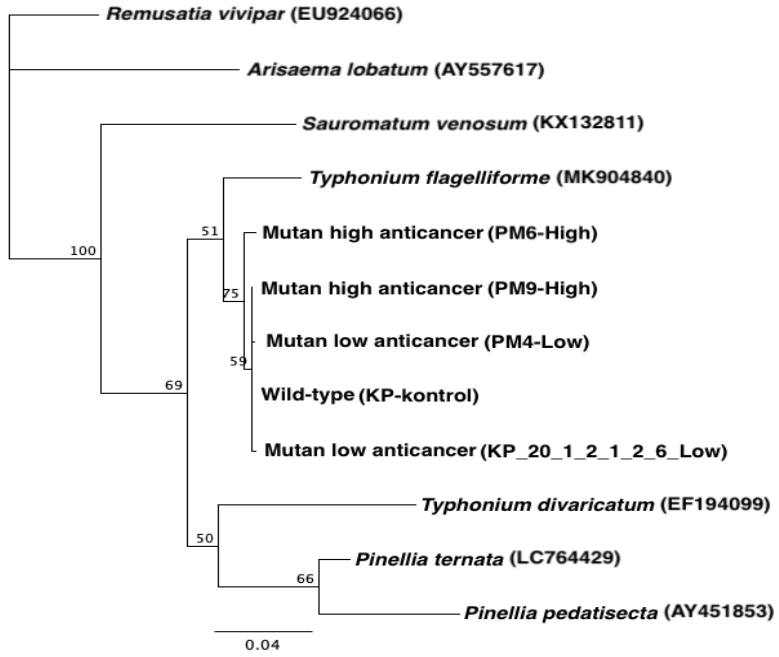

Figure 2. The phylogenetic tree of the *lectin* gene among species is based on the neighbor-joining consensus tree method

Substitutions of nitrogen bases in the genome lead to changes in amino acids (Chan et al. 2020), making it crucial because the structure of a protein can be altered (Kosova et al. 2021). This study revealed three out of five detected sites with point mutations-such as at 241 bp, 279 bp, and 323 bp-underwent missense mutations resulting in amino acid alterations. Of these, the mutation site of 241 bp was particularly noteworthy because it features a missense mutation without being close to other point mutation sites. In contrast, the point mutation at site 279 bp was neighbor to another mutation at site 269 bp, which could complicate the design of specific markers. The mutation site of 323 bp presents a challenge due to its presence as a triallelic nature (more than two-point mutations). This complexity could cause uncertainty, especially when it is used for marker development purposes. As research on anticancer bioactive components (*lectin*s) continues to evolve (Hafez and Shati 2016; Yassin et al. 2019; Sianipar et al. 2024b), leveraging information from identification sequences can facilitate marker development related to the *lectin* gene in rodent tuber plants.

SNAP marker amplification

The SNAP primer for the *lectin* gene successfully amplified the DNA of both wild-type and mutant rodent tuber plants (Figure 3). The SNAP primers pairs for the *lectin* gene, Lec113 (Lec113_SNAP_F_C: GACTGCAAC CTCGTCCTCTAGAAC; Lec113_SNAP_R: CATCACCAGC ATGTGGTTCCT; Lec113_SNAP_F_T: GACTGCAACCTCG TCCTCTAGAAT) amplified at the base position of 351 reflecting a consistency with the expected product size during primer design. In detail, the amplification of *lectin* gene using the Lec113 SNAP primer pairs specific to the C allele resulted in producing a specific amplicon across all rodent tuber genotypes (Figure 3, C-allele). Meanwhile, that specific to the T allele was successfully amplified in seven mutant samples but failed to amplify the wild-type clones (Figure 3, red arrow), revealing that the wild-type clones only possess the C allele at the 113 bp position. This result showed that the Lec113 SNAP primer pairs can discriminate the rodent tuber wild-type plants from the seven other mutants at the T allele, a finding of significant importance in the field of genetics and plant biology.

This finding also revealed the heterozygosity allele type of the *lectin* gene in the mutant clones, which is indicated by the presence of the specific amplification of the C allele in all samples and the T allele in seven mutant samples (Table 5). This finding aligns with Pesik et al. (2017), who confirmed the existence of heterozygous alleles in SNAP markers for Kopyor coconuts. The differences in *lectin* gene between mutant rodent tuber clones determined by the T allele and their wild-type plants showing the absence of respective allele confirming the Lec113 SNAP primer pairs designed in the present study showed both specificity and sensitivity of the primer to amplify the mutated sequence at the 113 bp position.

Table 5. The Lec113 SNAP primer across rodent tuber clones amplified the banding pattern of the *lectin* gene

Accession number	Accession name	Total DNA bands	
		C-allele	T-allele
P1	KP Control (wild-type)	1	0
P4	PM 4	1	1
P7	KP 20-1-2-4-9	1	1
P11	KP 20-1-1-2-2-3	1	1
P12	PM 8-1	1	1
P13	KP 20-1-3-1-4-3	1	1
P15	KP 20-1-2-2-1	1	1
P16	PM 8-1-1	1	1

Notes: "1" indicates the presence of a DNA band, and "0" indicates the absence of a DNA band

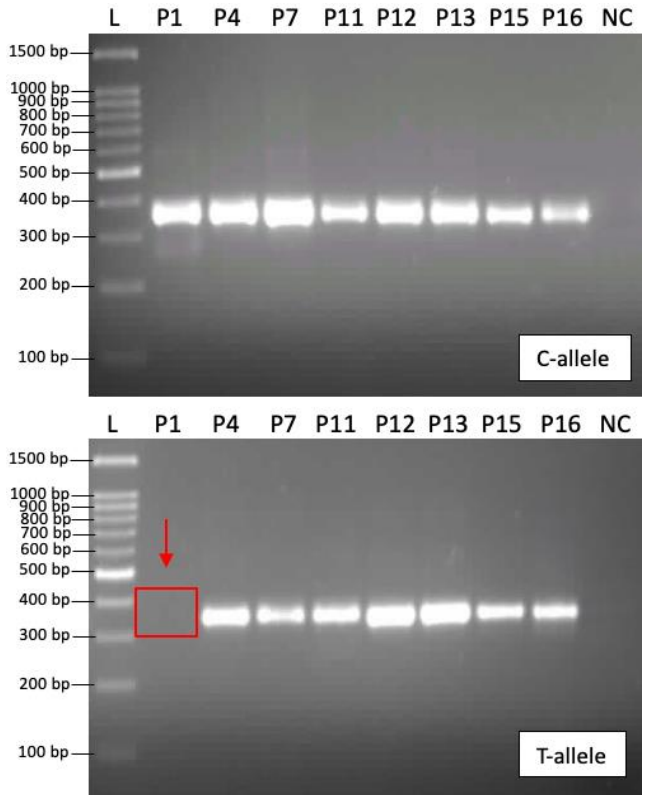

Figure 3. PCR amplification results with Lec113 SNAP primer. L: 100 bp DNA ladder. (P1) KP control; (P4) PM 4; (P7) KP20-1-2-4-9; (P11) KP 20-1-1-2-2-3; (P12) PM 8-1; (P13) KP 20-1-3-1-4-3; (P15) KP 20-1-2-2-1; (P16) PM 8-1-1; NC: Negative Control

The success story of molecular detection related to genetic variation in mutant rodent tuber clones still needs to be improved. For instance, Laurent et al. (2015) and Sianipar et al. (2017) applied RAPD markers to differentiate rodent tuber mutants and their wild-type plants. Still, it needs to provide more information on which site of observed RAPD loci are suggested for further guidance on elucidating the genes involved in bioactive compounds related to anticancer biosynthesis. Therefore, continuous efforts to understand genes involved in anticancer biosynthesis present in promising rodent tuber mutant clones have been made, one of which is DNA marker development derived from the *lectin* gene based on a specific SNAP marker. The reason behind the SNAP marker development is due to its specificity on the targeted alleles and co-dominant, which can easily be designed based on nucleotide sequence variations in the *lectin* gene.

This study aligns with the research of Tarigan et al. (2021), which successfully identified a SNAP marker related to the *CAT1* gene based on a C/T SNP at the 71st position in cacao plants. Additionally, Sukma et al. (2021) conducted molecular analysis related to disease resistance and disease-responsive genes (*Pto* and *Chi*). They created SNP-based molecular markers to identify resistance genotypes in *Phalaenopsis*. This study highlights the potential utility of the developed SNAP markers for other *T. flagelliforme* accessions. As demonstrated by Sianipar et al. (2024b), SNAP markers for *lectin* genes have been successfully applied to mutant plants of the Bogor rodent tuber accession. Specifically, the SNAP marker lec113 was effectively used in Bogor accessions, while lec241 successfully distinguished between mutant and wild-type Bogor accessions. This reinforces that SNAP lec113 is specific to Pekalongan accessions, aligning with the findings presented in this study. Besides SNAP markers, molecular marker development based on SNPs can also be achieved through other marker designs, such as ARMS markers (Han et al. 2017).

ARMS marker amplification

The ARMS primer for the *lectin* gene was successfully designed based on a missense mutation at point mutation position 241 bp, resulting in an allele change from guanine (G) to cytosine (C) (Table 6). The ARMS marker for the *lectin* gene was successfully amplified in rodent tuber DNA. The mutant and wild-type alleles of the rodent tuber were distinguished using ARMS primers (Lec241_ARMS_F_G: GAGCAGCGGCTCCCAGTCGGACAG and Lec241_ARMS_R: TGCCGTCGGCGTAGAGGACCTGG) (Figure 4).

The ARMS PCR product specific for the point mutation of the 241 bp *lectin* gene resulted in a 193 bp amplicon, which corresponded to the guanine allele (G). The G allele was amplified in the wild-type clone of the rodent tuber *lectin* gene and failed to produce the respective allele in the mutant clone (shown by the red arrow in Figure 4). These results indicate that the wild-type clone has a specific G allele in the *lectin* gene which differed from the mutant clone. In contrast, the ARMS primer pair for the C allele (Lec241_ARMS_R_C: ACGGCGGCGTACTTGCCGGG and Lec241_ARMS_F:

ACGGCGAACAGAGGACGGGACTGC) failed to amplify rodent tuber DNA fragments in both wild-type and mutant samples. The mutant rodent tuber plant lacks the G (guanine base) and C (cytosine base) alleles, while the wild-type has the G (guanine base) allele. This result suggests that using the designed ARMS primers has also enabled us to distinguish the mutant rodent tuber from the wild-type based on the specific guanine allele present in the specific site of the rodent tuber's *lectin* gene.

Ye et al. (2001) reported that ideally, designing ARMS primers should have a sequence length of at least 30 bp, whereas, the length of designed primers was 20-24 bp, making a discrepancy in the PCR profile. This discrepancy can affect the melting temperature (Tm) and annealing temperature (Ta) in the PCR profile, potentially leading to errors in primer binding; an annealing temperature of 65°C was utilized, which had been optimized for specificity. This mismatch in primer length may have reduced sensitivity to the main target, failing to amplify the C allele. According to Rachman (2022), to ensure the consistency of detection over a specific fragment, PCR amplification should be repeated at least twice. Following such a recommendation, PCR amplification using the ARMS Lec241 primer pair conducted in the present study was also repeated twice, yielding consistent results. Based on the ARMS Lec241 amplification results, it was found that mutant *T. flagelliforme* could be distinguished from wild-type plants based on the specific G allele at the 193 bp position.

Zhu et al. (2021) reported that ARMS is an allele-specific amplification technique combined with PCR (ARMS PCR), which was successfully used to identify sorghum species based on single nucleotide differences in genes. The study highlighted that ARMS primer pairs targeting forward and reverse alleles could differentiate between *non-wxc* sorghum and *wxc* sorghum. In this study, the primer pair of allele C (Lec241_ARMS_R_C and Lec241_ARMS_F) failed to amplify the DNA of both wild-type and mutant rodent tuber. This discrepancy occurs because the reverse primers contain a C sequence near the 3' end, allowing for non-specific binding to DNA despite the intentional mismatch (Medrano and de Oliveira 2014). Rejali et al. (2018) also noted that the absence of specific fragments (from allele-specific primers) could be due to the significant variability in the 3' end Untranslated Region (UTR). In contrast, the allele G ARMS primer pair (Lec241_F_G with Lec241_R) in this study successfully amplified and could distinguish wild-type rodent tuber from allele G-specific mutants at 193 bp.

ARMS PCR differs from other molecular mutation detection methods (such as sequencing and hybridization techniques), which require sophisticated equipment, high costs, and expertise. ARMS PCR is simpler, relying on the PCR principle with several primers, yet it maintains high sensitivity in mutation detection. This research aligns with Zhu et al. (2021), who successfully developed specific markers to distinguish waxy sorghum from wild-type sorghum based on point mutations. Similarly, Meng et al. (2017) stated that ARMS PCR is an effortless and sufficient approach for detecting allelic differences in point mutation loci and can potentially be used to distinguish sweet potato cultivars. In the previous study, the point mutation difference at the 241 bp *lectin* gene was caused by gamma-ray irradiation at six doses of Gy (Sianipar and Purnamaningsih 2018). The 241 bp point mutation of this *lectin* gene affects amino acid translation. Changes in amino acid due to point mutations can impact protein structure, potentially resulting in functional alterations (Iqbal et al. 2020). The increased presence of the G allele in mutant plants compared to wild-type suggests that this mutation might influence the synthesis of anticancer bioactive components, a key area for further investigation.

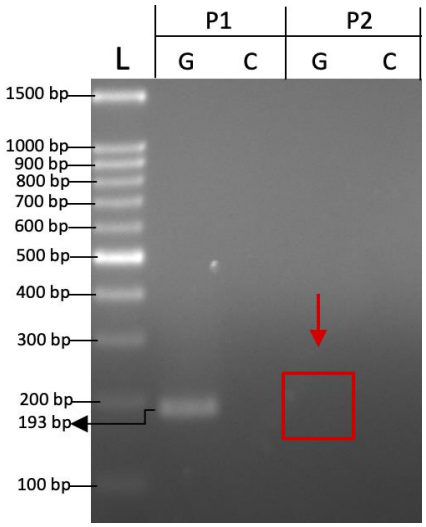

Figure 4. PCR amplification result with ARMS Lec241 primer. L: 100 bp DNA ladder, P1: KP Control; P2: PM 9, G: Guanine allele, C: Cytosine allele

Table 6. ARMS primers for the *lectin* gene were used in this study

SNP position (bp)	Primer ID	Forward/reverse sequences	Melting temperature (°C)	Amplification	Melting temperature (°C)
241	Lec241_ARMS_F_G	GAGCAGCGGCTCCCAGTCGGACAG	67.5	193	G-allele
	Lec241_ARMS_R	TGCCGTCGGCGTAGAGGACCTGG	67.0		
	Lec241_ARMS_F	ACGGCGAACAGAGGACGGGACTGC	67.0	129	C-allele
	Lec241_ARMS_R_C	ACGGCGGCGTACTTGCCGGG	67.9		

Note: Lec241: primer designed based on *lectin* gene with 241 bp point mutation

The ARMS *lectin* gene marker effectively distinguishes mutant Pekalongan rodent tuber from the wild-type. The mutant rodent tuber showed a significantly increased allele G level compared to wild-type plants. This suggests that the 241 bp point mutation may have distinct functional effects on synthesizing anticancer bioactive components. The SNAP and ARMS markers of the *lectin* gene that have been successfully designed can differentiate based on point mutations in wild-type and mutant Pekalongan rodent tuber plants. SNAP markers have a higher level of effectiveness compared to ARMS markers. This is because SNAP markers can differentiate more specifically in seven mutant samples. Moreover, SNAP markers have the advantage of being cheaper as they only use three primers compared to ARMS markers, which use four primers. Nevertheless, the amplification results from ARMS marker have a high level of consistency.

SNAP marker Lec113 bp and ARMS marker Lec241 can be used as rapid and efficient selection markers to identify the Pekalongan accession of rodent tuber mutants based on the *lectin* gene. Additionally, these markers can be used to evaluate point mutations of the *lectin* gene in all rodent tuber accessions. The long-term value of SNAP and ARMS markers lies in their ability to streamline genetic screening and reduce reliance on trial-and-error approaches, making them a cost-effective solution for accelerating plant improvement. This study has successfully developed molecular markers that provide a foundation for more targeted breeding programs (including implementing Marker-Assisted Selection) and genetic studies in rodent tuber. The ability to quickly and accurately identify genetic variations will accelerate the development of improved varieties with enhanced medicinal properties, contributing to the advancement of natural product-based therapeutics.

In conclusion, this study successfully developed SNAP and ARMS markers from the *lectin* gene, effectively distinguishing between wild-type and mutant rodent tuber clones. The SNAP Lec113 primer, based on a silent mutation, differentiated mutants from wild-type using a specific T allele at 351 bp. The ARMS Lec241 primer, targeting a missense mutation, distinguished mutants by a specific G allele at 193 bp. The SNAP marker demonstrated higher effectiveness in identifying seven mutant samples, while the ARMS marker showed consistent results. SNAP's cost-effectiveness (three primers vs. four) makes it preferable for large-scale screening. These molecular markers provide a foundation for targeted breeding programs and genetic studies in rodent tuber. The ability to rapidly and accurately identify genetic variations will accelerate the development of improved varieties with enhanced medicinal properties, advancing natural product-based therapeutics.

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