NUSANTARA BIOSCIENCE
Vol. 16, No. 2, pp. 201-209
November 2024

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n160206

Optimization design of tetra-primer ARMS-PCR using SNP lectin gene and in silico characterization of lectin protein in rodent tuber (*Typhonium flagelliforme*) mutant of Bogor accessions

NESTI F. SIANIPAR[1,2,♥], ZIDNI MUFLIKHATI[3], KHOIRUNNISA ASSIDQI[1,2]

[1]Department of Biotechnology, Faculty of Engineering, Universitas Bina Nusantara. Jl. K. H. Syahdan No. 9, Kemanggisan, Palmerah, Jakarta 11480, Indonesia. Tel.: +62-21-534-5830, ♥email: nsianipar@binus.edu

[2]Food Biotechnology Research Center, Universitas Bina Nusantara. Jl. K. H. Syahdan No. 9, Kemanggisan, Palmerah, Jakarta 11480, Indonesia

[3] Program of Biotechnology, Graduate School, Universitas Padjadjaran. Jl. Dipati Ukur No.35, Bandung 40132, Indonesia

Manuscript received: 26 February 2024. Revision accepted: 5 June 2024.

Abstract. *Sianipar NF, Muflikhati Z, Assidqi K. 2024. Optimization design of tetra-primer ARMS-PCR using SNP lectin gene and in silico characterization of lectin protein in rodent tuber (*Typhonium flagelliforme*) mutant* of Bogor accessions. *Nusantara Bioscience 16: 201-209.* Rodent tuber plant (*Typhonium flagelliforme* (G.Lodd.) Blume) contains several anticancer compounds. Mutant plants have a higher cytotoxic effect than wild-type plants. This study aimed to devise a tetra-primer ARMS PCR using lectin gene SNPs to differentiate *T. flagelliforme* mutants and wild-type of Bogor accessions. A protein modeling study was also conducted to investigate the impact of point mutations on the structure of protein. The tetra-primer ARMS design and in silico protein modeling analysis were based on mutation points from previously sequenced lectin genes. A pair of primers was successfully designed using the missense mutation type specific to the SNP site that causes amino acid variation. The ARMS lec183 tetra-primer focuses on a 183 bp mutation in the lectin gene that converts threonine to arginine to provide a successful lec183 primer. The ARMS lec183 primer pair did not differentiate *T. flagelliforme* mutant plants from wild-type of Bogor accessions. The tetra-primer ARMS lec183 could be amplified successfully in all *T. flagelliforme* samples at a size of 278 bp outer primer and 193 bp inner primer, as determined by primer size. In the mutant protein structure, the 183 bp mutation results in amino acid changes that closely match those in wild-type proteins.

Keywords: In silico lectin protein, lectin gene, specific primer design, tetra-primer ARMS PCR, *Typhonium flagelliforme*

Abbreviation: ARMS: Amplification Refractory Mutation System, SNPs: Single Nucleotide Polymorphisms

INTRODUCTION

The rodent tuber plant (*Typhonium flagelliforme* (G.Lodd.) Blume) contains anticancer compounds among the Indonesian herbs. The gamma irradiation of plants has been biotechnology studied to produce mutant plants (Sianipar et al. 2017). A *T. flagelliforme* mutant plant contains higher levels of anticancer compounds, such as palmitic acid, sitosterol, and stigmasterol than the wild-type plant (Sianipar et al. 2021, 2022). Sianipar et al. (2021) reported that stigmasterol from *T. flagelliforme* mutant plants has an IC_{50} value of 0.1623 M, which means it inhibits the proliferation of MCF-7 cancer cells. The Indonesian Ministry of Agriculture granted Plant Variety Protection to two stable mutants of these plants in 2020 (Sianipar et al. 2020b). Specific molecular markers are still being identified to distinguish *T. flagelliforme* mutant plants from wild-type ones (Sianipar et al. 2023).

The plant cell wall contains carbohydrates recognized by lectins (Petrova et al. 2021) and several proteins have a LecRLK architecture, forming LecRLK-type receptor-like kinases with kinases and transmembrane domains (Bellande et al. 2017). Proteins without kinase domains are LecRLPs, while soluble proteins without kinase and transmembrane domains are LecPs. Sianipar et al. (2015, 2016, 2020a) and Sianipar and Purnamaningsih (2018) demonstrate that gamma irradiation can produce superior *T. flagelliforme* mutant plants with higher cytotoxicity than the wild-type (control). There is an increase in stigmasterol content in *T. flagelliforme* mutant plants with the development of lectin genes linked to increased anticancer compounds. At a dosage of 20 g/mL, the compound inhibits the proliferation of uterine cancer cells by 50% (Alfarabi et al. 2015). These findings can be a basic reference to explain lectin gene function in superior *T. flagelliforme* mutant of Bogor accession.

Single Nucleotide Polymorphisms (SNPs) are highly polymorphic and can identify diversity with just one base variation (Islam et al. 2015). A unique SNP marker developed was the tetra-primer Amplification Refractory Mutation System (ARMS). The tetra-primer ARMS-PCR method is a high-efficiency, fast, and economical technique to find mutations or SNPs in a genome (Ye et al. 2001). ARMS-PCR uses tetra-primer primers containing a 3' end at the polymorphism and an additional variance at -2 bp to the 3' end of the primer. This design promotes greater amplification and reduces false positive results due to mismatch the primers (Garcés-Claver et al. 2007).

The ARMS-PCR method has been successfully used to *Schisandra chinensis* (Turcz.) Baill. (Kim et al. 2012) and

sweet potato (Meng et al. 2017). It is possible to generate mutant and wild-type alleles simultaneously with ARMS-PCR by using two sets of primers in single PCR tube. several studies have been conducted using internal DNA controls to investigate various mutations, such as Hou et al. (2013), Wang et al. (2014), Cai et al. (2019), and Park et al. 2020). Ye et al. (1992) developed tetra-primer PCR using four primers (two outer and two inner primers) for allele-specific amplification. Tetra-primer ARMS-PCR was created by merging this technique with ARMS in 2001. Regardless of gene genotype, outer primers significantly amplify target genes in one reaction step.

Sianipar et al. (2022) reported a sequence for lectin gene with a base length of about 500 bp in mutant and wild-type *T. flagelliforme* plants show numerous mutation points. The structure of proteins can be altered by point mutations that change amino acids (Kimball and Jefferson 2006). The tetra-primer ARMS analyzes sequence diversity through the gene point mutations, and successful ARMS-PCR requires well-designed primers. This study aimed to devise tetra-primer ARMS-PCR based on SNPs in lectin gene regions, and characterize lectin proteins in silico to identify specific allele differences and amino acid arrangement structures of *T. flagelliforme* mutants and wild-type plants.

MATERIALS AND METHODS

Plant materials

DNA samples were collected at Bina Nusantara (Binus) University, Jakarta, Indonesia in 2021. In the analysis of *T. flagelliforme* sequences, four mutant clones with sequence lengths of approximately 500 bp were compared to the wild-type ones (KB-control_61F). It was found that two mutant clones were classified as being high anticancer (KB-6-2-5-3_61F and BM-8-2_61) and low anticancer (KB-6-1-1-2_61F and KB-6-2-6-3_61F) (Sianipar et al. 2022). Therefore, a tetra-primer ARMS was built on top of these sequences, and a lectin protein model was developed. The study use DNA template samples of fresh leaf tissue from wild-type and 12 mutants of *T. flagelliforme* (Table 1).

Procedures

Genomic DNA extraction and quality test

Genomic DNA was extracted using Cetyltrimethylammonium Bromide (CTAB) method described by Calderón-Cortés et al. (2010). Samples were collected from *T. flagelliforme* mutants and wild-type of Bogor accessions. After mixing liquid nitrogen and mortar, a 1,000 mL extract buffer was combined with fresh leaf tissue. The mixture was incubated in 2 mL microtubes for 45 minutes and homogenized every 15 minutes. An 800 mL of isoamyl alcohol chloroform was added to 1.5 mL of the tube 0.01% sodium acetate supernatant. During the DNA strand examination, the tubes were kept closed. The supernatant was removed from the sample with a micropipette after 10 minutes of incubation at room temperature.

TE buffer was combined with the leftover DNA pellet at room temperature. Then, RNase was added and the solution was gently mixed and incubated at 37°C for 30 minutes. The sodium acetate solution was diluted to one-tenth by adding the DNA solution and stirring carefully, and the DNA was precipitated by processing samples in 600 mL of 95% ethanol. These samples were gently combined after incubation for 45 minutes at 20°C. The supernatant was removed from the solution by centrifugation for 10 minutes at 13,500 rpm with a MiniSpin plus (EU-IVD) (Eppendorf, UK). DNA was dried before being rehydrated with 200 μL of TE buffer. The instrument was calibrated at room temperature using TE buffer solution. A260/A280 ratios were determined using a Nanodrop Spectrophotometer 2000 manufactured by ThermoScientific™USA. Moreover, to test the quality including purity and concentration of the isolated DNA, Thermo Scientific™ USA's NanoDrop 2000 was utilized. DNA samples were stored at -20°C after diluting to a total concentration of 10-15 ng/μL for further use.

Table 1. Rodent tuber (*Typhonium flagelliforme*) mutant plants used in the study

No.	Accession numbers	Accession name	Types	Source
1.	B1	KB Control (wild-type)	Bogor accession	Binus collection
2.	B2	BM 8-2	Bogor accession	Binus collection
3.	B3	KB 6-2-6-3	Bogor accession	Binus collection
4.	B4	KB 6-2-5-3	Bogor accession	Binus collection
5.	B5	KB 6-1-1-2	Bogor accession	Binus collection
6.	B6	KB 6-1-3-4	Bogor accession	Binus collection
7.	B7	BM 8-8	Bogor accession	Binus collection
8.	B8	BM 8-4	Bogor accession	Binus collection
9.	B9	BM 8-9	Bogor accession	Binus collection
10.	B10	KB 6-2-8-2	Bogor accession	Binus collection
11.	B11	KB 6-9-3	Bogor accession	Binus collection
12.	B12	KB 6-9-5	Bogor accession	Binus collection
13.	B13	KB 6-3-3-6	Bogor accession	Binus collection

Design of tetra-primer ARMS

Gene-specific tetra-primer ARMS were developed based on SNP sites previously identified by Sianipar et al. (2022). SNP sites that caused amino acid changes were recorded as missense mutations. SNP sites that could be developed into tetra-primer ARMS and identified as having bi-allelic alternative alleles were then analyzed for primer design. Using the online tool Primer1 (http://primer1.soton.ac.uk/primer1.html) (Ye et al. 2001), a set of outer and inner PCR primers was created. The specificity of these outer primers, intended for detecting the lectin gene, was verified using NCBI BLAST primers. Next, a 183 bp position tetra-primer ARMS design was carried out with the following parameters: a PCR product range of 100-300 bp, a primer concentration of 5µM, a primer size range of 20-24 bp, and a primer melting temperature range of 62-70°C.

Tetra-primer ARMS PCR

Tetra-primer ARMS were designed and *T. flagelliforme* template DNA was used in the PCR machine for amplification. Next, 12 µL was used for the reaction mixture in the PCR reaction. This PCR mixture included 1x MyTaqTMHS Red Mix (Bioline, UK) (containing dNTPs, $MgCl_2$, MyTaq HS DNA polymerase, and reaction buffer) of 5 µL, 2-5 µL genomic DNA (3-5 ng template DNA), primers with a concentration of 10 µM each 0.5 µL, and added ddH_2O. Palm-Cycler Thermal Cycler PCR machine (Corbett, USA) with CG1-96 plates was applied to conduct the PCR procedures. The cycle parameters were set up: 4 minutes of initial denaturation at 94°C, followed by 30 seconds of denaturation at the same temperature, 30 seconds of annealing at 65°C, and 30 seconds of extension at 72°C. A last extension cycle lasting 5 minutes at 72°C made the reaction close. The PCR products were subsequently run on a 2% agarose gel (Vivantis.Sdn.Bhd.) in Tris Borate EDTA buffer and stained with a Florosafe DNA stain (1st BASE). Electrophoresis was run at 75 V for 85 minutes. DNA ladders (Geneaid Biotech Ltd.) were used as standards for estimating the size of the PCR product. An Agarose gel was illuminated with UV light using a UV transilluminator (Syngene, USA).

The amplification results were visualized by showing the DNA bands of internal control and alleles in mutant and wild-type *T. flagelliforme*. Tetra-primer ARMS are designed based on lectin gene mutation points that cause amino acid changes. Point mutations that cause amino acid changes can affect protein structure (Kimball and Jefferson 2006). Therefore, protein characterization and protein modeling can be done to determine the difference in protein structure due to missense mutation points.

Data analysis

Lectin genes with approximately 500 bp were used to characterize the proteins (Sianipar et al. 2022). Using the NCBI database, the CDS lectin gene sequence was adjusted based on the start codon by Multiple Sequence Alignment (MSA). Expasy translate (https://web.expasy.org/translate/) was used for translating gene sequences into proteins. This stage revealed differences in amino acids. For the identification of differences in protein types caused by point mutations, we used the Uniprot website (https://www.uniprot.org/blast). A manual comparison of *T. flagelliforme* mutant plants with wild-type revealed differences and similarities in the lectin proteins.

The amino acid arrangement was used for protein structure modeling through SWISS MODEL (https://swissmodel.expasy.org/). Protein data bank (PDB) files were downloaded based on the best-selected protein templates and further visualized with PyMol 2.0 software. Molecular weights and isoelectric points (pl) of mature proteins were estimated using the Compute pI/Mw tool (https://web.expansy.org/compute_pi/). An alteration in the isoelectric point can affect a protein's functional state (Kimball and Jefferson 2006).

RESULTS AND DISCUSSION

DNA quality

The test results indicated that the DNA concentration varied between 10 and 106.8 ng/µL, and the purity of A260/A280 was 1.4 to 2.0. Piskata et al. (2019) suggested that the optimal range for a pure DNA sample is from 1.7 to 2.0. Residual protein may produce higher values, whereas lower values represent extremely low DNA contents (Ke-xin et al. 2023). The DNA bands obtained in this study were reproducible and very clearly performed. The results of the DNA quality test showed that all samples of *T. flagelliforme* DNA were suitable for additional PCR analysis (Servusova and Piskata 2021).

Design of tetra-primer ARMS

The tetra-primer ARMS was successfully designed following the point mutation of the lectin gene sequence by Sianipar et al. (2022) (Table 2). The 183 bp mutation point has a G/C sequence mutation. The tetra-primer ARMS pair was constructed using the Primer1 software. ARMS PCR pairs with mismatches were designed to improve the specificity of alleles (Ye et al. 2001). Several mismatch bases were selected based on Little's (1997) guidelines. It was most effective to couple mismatches scored as "strong" at the 3' end (such as G/A or T/C mismatches) with a second mismatch rated as "weak" (such as A/C or T/G). A/A, T/T, C/C, or G/G are examples of "medium or intermediate" strength pairings that could be employed.

The ARMS method of detecting small deletions or point mutations has quick and accurate results. ARMS-PCR allows genotype-specific identification in a single PCR step, eliminating expensive post-PCR manipulations (Ye et al. 2001). ARMS method success largely depends on the primer's design and several criteria found to design ARMS primers. These criteria include primer base length, complementary primers, and the number of percent of GC or Tm (Bates 1995). This study calculated primer Tm with the web-based program OligoAnalyzer™Tool (https://sg.idtdna.com/calc/analyzer). Generally, the Tm values of all primer sequences designed in this study are fairly significant.

Table 2. A tetra-primer ARMS of lectin genes was used for this study

SNP position (bp)	SNP variation	Type of mutation in amino acid residues	Primer Id	Primer sequences (5'-3')	Tm (ºC)	Product Size (bp)
183	C/G	Missense (Threonine-Arginine)	Lec183_F_In_G	GAGCAGCGGCTCCCAGTCGGACAG	67.5	193
			Lec183_R_In_C	ACGGCGGCGTACTTGCCGGG	67.9	129
			Lec183_F_Out	ACGGCGAACAGAGGACGGGACTGC	67.0	278
			Lec183_R_Out	TGCCGTCGGCGTAGAGGACCTGG	67.0	

Note: Lec183: Primer designed based on the lectin gene with the 183 bp SNP, F: Forward, R: Reverse, In: Inner, Out: Outer

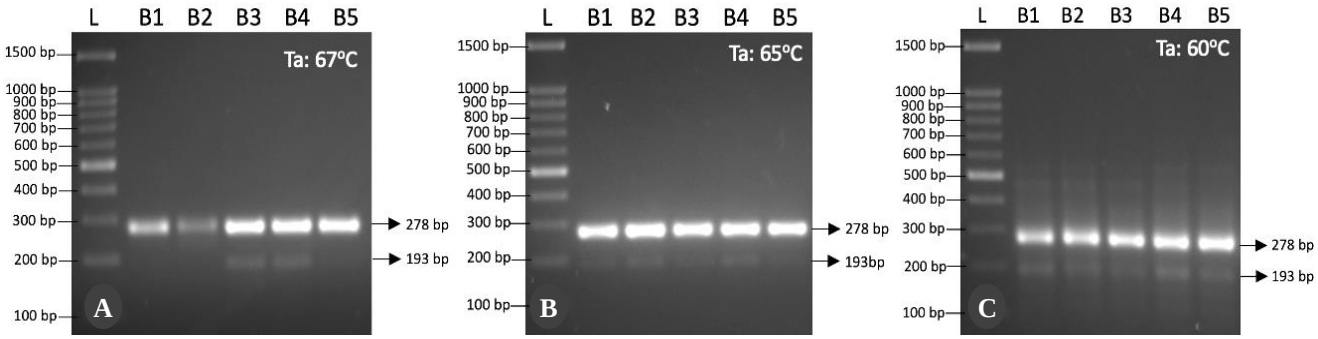

Figure 1. Electrophoresis outcomes of tetra-primer ARMS-PCR with different annealing temperatures in 5 *T. flagelliforme* mutant clones: A. 67°C, B. 65°C, C. 60°C. L: 100 bp DNA ladder, B1: KB Control, B2: BM 8-2, B3: KB 6-2-6-3, B4: KB 6-2-5-3, B5: KB 6-1-1-2, Ta: Annealing temperature

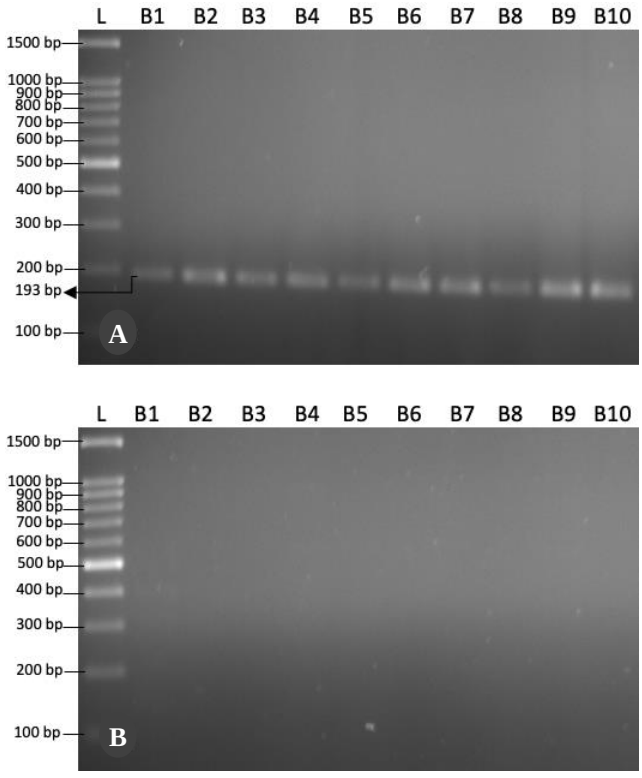

Figure 2. Electrophoresis results of inner primer ARMS separation in *T. flagelliforme* clones: A. G allele-specific reaction, B. C allele-specific reaction. L: 100 bp DNA ladder, B1: KB Control, B2: BM 8-2, B3: KB 6-2-6-3, B4: KB 6-2-5-3, B5: KB 6-1-1-2, B6: KB 6- 1-3-4, B7: BM 8-8, B8: BM 8-4, B9: BM 8-9, B10: KB 6-2-8-2

Tetra-primer ARMS-PCR

The ARMS tetra-primer was successfully designed in this study. ARMS primer lec183 was used to amplify *T. flagelliforme* mutants and wild-type based on the fragment size of the tetra-primer ARMS-PCR product (Lec183_F_In_G: GAGCAGCGGCTCCCAGTCGGACAG; Lec183_R_In_C: ACGGCGGCGTACTTGCCGGG; Lec183_F_Out: ACGGCGAACAGAGGACGGGACTGC; Lec183_R_Out: TGCCGTCGGCGTAGAGGACCTGG). The tetra-primer ARMS-PCR product was specific for the 183 bp lectin gene SNP containing 278 bp as internal control and 193 bp as the G/Guanin allele.

Three annealing temperatures were used to obtain optimum conditions and detect specific inner alleles (Figure 1). An internal control band at 278 bp was produced in five samples by the forward outer and reverse outer primer pairs. There were only a few samples that amplified the inner allele at 67°C. Only five samples successfully amplified the inner allele at 65°C and 60°C; all samples annealed at 60°C produced smears. The results indicate that too high annealing temperatures can cause the *T. flagelliforme* DNA to not adhere to the inner primer, while too low annealing temperatures can cause smears (Suhda et al. 2016). Different annealing temperatures affect the appearance of allele-specific bands in some samples (Zabala et al. 2017). Tanha et al. (2015) notes the ARMS-PCR method's strength is its ability to find the optimal PCR program (annealing temperature). According to this study, the ideal annealing temperature was 65°C.

Separating inner forward and inner reverse primers was performed to confirm the bands that appeared clearly (Figure 2). The inner forward/allelic G primer (Lec183_F_In_G:

GAGCAGCGGCTCCCAGTCGGACAG; Lec183_R_Out: TGCCGTCGGCGTAGAGGACCTGG) was successfully amplified in all samples at 193 bp according to the product size during primer construction (Figure 2.A). In contrast, the inner reverse/allele C primer (Lec183_R_In_C: ACGGCGGCGTACTTGCCGGG; Lec183_F_Out: ACGGCGAACAGAGGACGGGACTGC) was not amplified in all samples (Figure 2.B). This is because primers designed specifically for the G allele can attach to *T. flagelliforme* DNA, while primers specifically for the C allele cannot attach to *T. flagelliforme* DNA. Medrano and de Oliveira (2014) reported that inner reverse primers are used to recognize that they have a C sequence near the 3' end, which allows non-specific binding to DNA despite intentional mismatches.

DNA fragments of wild-type *T. flagelliforme* with three mutants were produced in the same reaction [all outer and inner primers (G/C)] and two specific alleles [inner primer of allele G (G) and inner primer of allele C (C)] were amplified separately to obtain the specific allele (Figure 3). The three mutant samples represent the results of amplifying all samples on the outer and inner primers of allele G. The outer primer pair (Lec183_F_Out: ACGGCGAACAGAGGACGGGACTGC; Lec183_R_Out: TGCCGTCGGCGTAGAGGACCTGG) was successfully produced in all samples at 278 bp according to the primer preparation. All samples with the inner forward primer or allele G (G) amplified separately showed bands at 193 bp.

Both *T. flagelliforme* wild-type (control) and mutant were successfully amplified and showed clear DNA bands in all samples. Three standalone PCR reactions were performed to examine reproducibility: one with an outer/internal control primer, one with an inner primer specific for the G allele, and one with an inner primer for the C allele. When amplified separately, the C allele (C) showed no bands (Figure 3). It has been suggested by Garcés-Claver et al. (2007) that the high variability in the 3' end UTR region may result in the absence of specific fragments from the inner allele primer. G allele data showed a 193-bp band when the inner forward primer is amplified separately. This G allele band is clearer than the mixing reaction (G/C). Zabala et al. (2017) reported that DNA fragments between two specific alleles can be separated if the amplicons have a minimum size of 50 bp.

Two outer primers can produce DNA fragments of different sizes, serving as a non-specific internal control band for allele detection. Combining the fragments with two allele-specific inner primers allows simultaneous amplification. The inner primers are asymmetrically located around the common primer, designed to anneal in opposite orientations, enabling the separation of wild-type and mutant amplicons by standard gel electrophoresis (Zabala et al. 2017). In this study, the designed tetra-primer ARMS lec183 was unable to differentiate the wild-type from the mutant. However, the ARMS lec183 tetra-primer was successfully amplified in all *T. flagelliforme* samples. Tóth et al. (2023) suggested that not all SNPs may be compatible with allele-specific methods due to limitations in primer design or challenging optimization.

Tetra-primer ARMS-PCR is used for genotyping SNPs. This method is considered more convenient than other techniques like PCR-RFLP (Restriction Fragment Length Polymorphism) because it does not require restriction enzyme incubation (Alvarez et al. 2016). In addition, tetra-primer ARMS only requires a small DNA concentration of 10-15 ng/μL. Tetra-primer ARMS-PCR comes with its own set of benefits and drawbacks. It is not suggested to use this method for SNP present in DNA sections that are abundant in guanine and cytosine. Also, it is not recommended to use it for unpurified DNA samples. However, this method has been successfully utilized in several applications, such as diagnosing human spinal muscular atrophy when SMN1 and SMN2 deletions were simultaneously identified (Baris et al. 2010). A similar approach has also been used to evaluate the diversity of *Panax* species (Yang et al. 2023), assess pork quality (Chai et al. 2010), and design cultivar markers for *Capsicum* species (Rubio et al. 2008). Tetra-primer ARMS are designed based on point mutations that cause amino acid changes (missense mutations). Additionally, Kimball and Jefferson (2006) associate missense mutation points in the genome with the protein structure associated with the change.

Lectin characterization and protein modeling

Lectins were characterized by translating DNA bases into protein amino acids and amino acid alignment by translating DNA bases into protein amino acids and aligning amino acids based on sequences of the lectin genes presented by Sianipar et al. (2022). The results of the alignment of lectin amino acids showed changes in amino acids due to point mutations (Figure 4). The amino acid change occurred from threonine (T) to arginine (R) (red box). Threonine is an amino acid with an uncharged closed R group, while arginine is a basic amino acid. BLAST results of the *T. flagelliforme* protein show that the *T. flagelliforme* mutant and wild-type lectins are highly similar to the bulb-type lectin domain-containing protein in *Colocasia esculenta* (red box) (Figure 5). This indicates that although there are differences in amino acids due to the mutation point, there is no change in the type of lectin protein in *T. flagelliforme* mutant and wild-type ones.

Protein modeling was performed to calculate the effect of mutations in the *T. flagelliforme* lectin sequence on the tertiary structure of the mature protein. Protein modeling showed no change in the tertiary structure of the wild-type lectin protein with the mutant despite the amino acid changes (Figure 6). The residue of amino acid in the wild-type lectin is threonine (T) and in the mutant lectin protein is arginine (R) at the 61st amino acid (red box). This indicates that lectin point mutations can change amino acids even though protein structure remains unchanged. The wild-type and mutant lectin proteins have the same estimated isoelectric value of 5.16 and molecular weight of 31.5 kDa. This study's results are similar to those of Alfarabi et al. (2015) which states that the molecular weight of lectins in *T. flagelliforme* is around 10-30 kDa. A similar claim was made by Luo et al. (2007), stating that lectins in *T. divaricatum* have a molecular weight of 12-48

kDa. This study's lectins of *T. flagelliforme* mutants are similar to those of *T. flagelliforme* (Alfarabi et al. 2015).

Proteins known as lectins have at least a single non-catalyzed domain that reversibly binds to particular carbohydrate molecules, and structurally, lectins consist of 4 types, namely merolectin, hololectin, chimerolectin, and superlectin (Peumans and van Damme 1995). Each type of lectin is distinguished by the number of domains that bind to carbohydrates. Mutant and wild-type *T. flagelliforme* have bulb-type lectin domain-containing protein. This type of lectin can bind specifically to mannose, a simple sugar C-2 epimer of glucose (Peumans and van Damme 1995). Through protein structure modeling, it is possible to determine an individual's protein type (Holle et al. 2017). Modeling of lectin proteins has also been carried out on *Acacia farnesiana* (L.) Willd. (AFAL) (Abrantes et al. 2013) and potatoes (van Damme et al. 2004).

Point mutation of the 183 bp lectin gene reported by Sianipar et al. (2022) affects amino acid translation. The in silico protein modeling analysis is based on the arrangement of amino acids that have changed due to point mutations. From this analysis, we can model the protein structure, molecular weight, and isoelectric point prediction due to point mutation changes that cause amino acid changes. Point mutations that cause changes in amino acids can affect protein structure (Kimball and Jefferson 2006). Protein structures that are naturally from wild-type sequences can be altered due to mutations in the protein sequence (Taverna and Goldstein 2002; Kimball and Jefferson 2006). the location of the mutation differs from the location of function, however, Yang et al. (2016) and Mitternacht and Berezovsky (2011) also found an impairment of function or changes at the level of functionality. These studies suggest that while the structure of the mutant protein closely resembles the structure of the wild-type protein, the topology of the mutant protein may be altered at locations far from the mutation site (Rajasekaran et al. 2017).

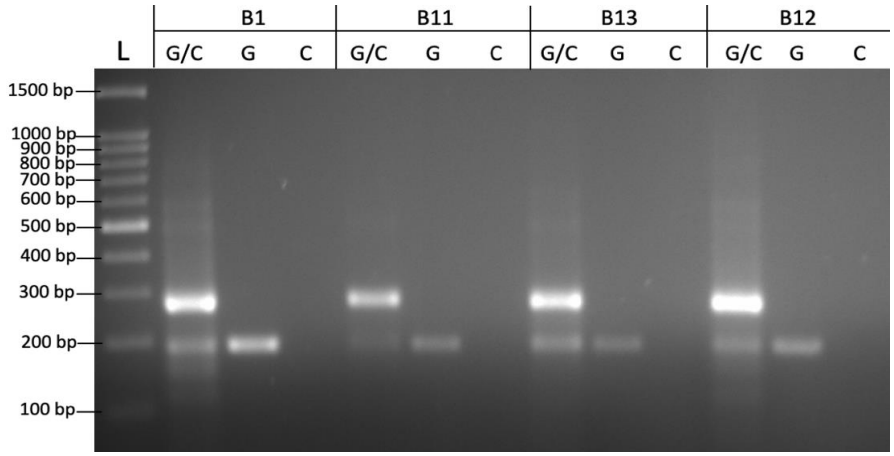

Figure 3. Electrophoresis results with mixing and separation ratio of outer primer and inner primer reactions in *T. flagelliforme* clones: G/C: All four primers in the same reaction, G: Guanine allele reaction, C: Cytosine allele reaction, L: 100 bp DNA ladder, B1: KB Control, B11: 6-9-3 KB, B12: 6-9-5 KB, B13: 6-3-3-6 KB

```
CLUSTAL O(1.2.4) multiple sequence alignment

KB-6-2-5-3_61F      NGDFDLVMQDDCNLVLYNGNWQSNTANRGRDCKLTLTDRGELIIQDGDGSNVWSSGSQSE       60
KB-Kontrol_61F      NGDFDLVMQDDCNLVLYNGNWQSNTANRGRDCKLTLTDRGELIIQDGDGSNVWSSGSQSE       60
KB-6-1-1-2_61F      NGDFDLVMQDDCNLVLYNGGWQSNTANRGRDCKLTLTDRGELIIQDGDGSNVWSSGSQSE       60
KB-6-2-6-3_61F      NGDFDLVMQDDCNLVLYNGGWQSNTANRGRDCKLTLTDRGELIIQDGDGSNVWSSGSQSE       60
BM-8-2_61F          NGDFDLVMQDDCNLVLYNGGWQSNTANRGRDCKLTLTDRGELIIQDGDGSNVWSSGSQSE       60
                    ******************.*****************************************

KB-6-2-5-3_61F      RGNYAAVVHPEGKLVIYGPSVFKINPWVPGLNSLRLGNIPSTSNMLFSGQVLYADGKLTA       120
KB-Kontrol_61F      TGNYAAVVHPEGKLVIYGPSVFKINPWVPGLNSLRLGNIPSTSNMLFSGQVLYADGKLTA       120
KB-6-1-1-2_61F      TGNYAAVVHPEGKLVIYGPSVFKINPWVPGLNSLRLGNIPSTSNMLFSGQVLYADGKLTA       120
KB-6-2-6-3_61F      TGNYAAVVHPEGKLVIYGPSVFKINPWVPGLNSLRLGNIPSTSNMLFSGQVLYADGKLTA       120
BM-8-2_61F          TGNYAAVVHPEGKLVIYGPSVFKINPWVPGLNSLRLGNIPSTSNMLFSGQVLYADGKLTA       120
                     ************************************************************

KB-6-2-5-3_61F      RNHML       125
KB-Kontrol_61F      RNHML       125
KB-6-1-1-2_61F      RNHML       125
KB-6-2-6-3_61F      RNHML       125
BM-8-2_61F          RNHML       125
                    *****
```

Figure 4. Alignment of lectin amino acids in wild-type ones and *T. flagelliforme* mutant. Amino acid residue substitutions in *T. flagelliforme* mutant (red box); the "*" mark indicates the position of a highly conserved residue; the "." mark indicates one of the conserved weak groups

Entry	Entry Name	Protein Names	Gene Names	Organism	Length
A0A843WNH9	A0A843WNH9_COLES	Bulb-type lectin domain-containing protein	Taro_044356	Colocasia esculenta (Wild taro) (Arum esculentum)	264 AA
R9RL27	CEA_COLES	Mannose-specific lectin CEA[...]	CEA	Colocasia esculenta (Wild taro) (Arum esculentum)	264 AA
B5LYJ9	LEC1_REMVI	Mannose-specific lectin 1[...]	L1, RVL	Remusatia vivipara (Hitchhiker elephant ear) (Arum viviparum)	256 AA

A

Entry	Entry Name	Protein Names	Gene Names	Organism	Length
A0A843WNH9	A0A843WNH9_COLES	Bulb-type lectin domain-containing protein	Taro_044356	Colocasia esculenta (Wild taro) (Arum esculentum)	264 AA
R9RL27	CEA_COLES	Mannose-specific lectin CEA[...]	CEA	Colocasia esculenta (Wild taro) (Arum esculentum)	264 AA
B5LYJ9	LEC1_REMVI	Mannose-specific lectin 1[...]	L1, RVL	Remusatia vivipara (Hitchhiker elephant ear) (Arum viviparum)	256 AA

B

Figure 5. BLAST results of *T. flagelliforme* lectin protein. A. Lectin protein of *T. flagelliforme* wild-type, B. Lectin protein of *T. flagelliforme* mutant

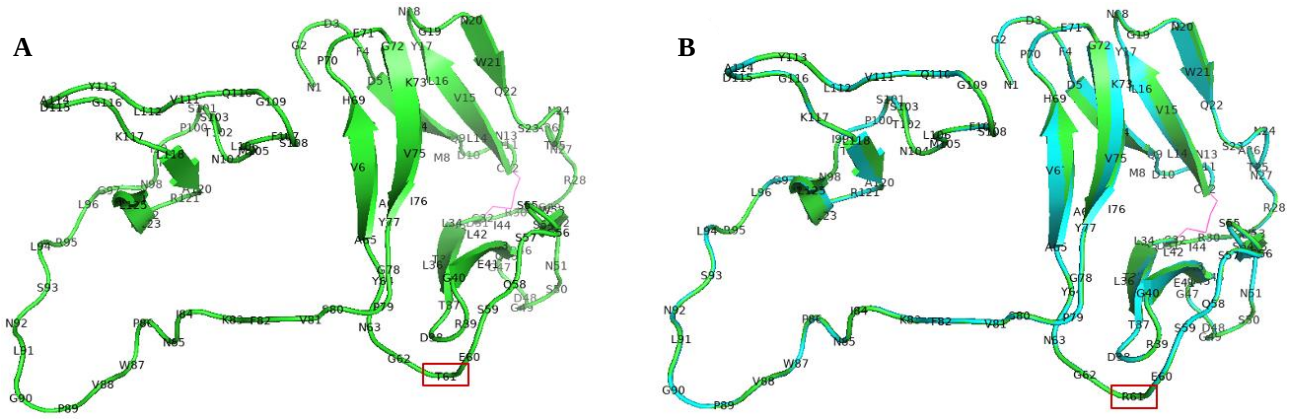

Figure 6. Predicted stereo structure models of lectin proteins. A. Superimposition of wild-type lectin protein (green) on the model protein of Mannose-specific lectin 2 (A5HMM7.1.A), B. Superimposition of mutant lectin protein (green) against the Mannose-specific lectin 2 protein model (A5HMM7.1.A; blue). Amino acid residue differences in mutant and wild-type lectins and (red squares) disulfide bonds (magenta)

Overall, the mutation point-based design of the tetra-primer ARMS lectin gene led to its effective amplification in both mutant and wild-type *T. flagelliforme* plants. The difference in mutation points in the 183 bp lectin gene is due to gamma irradiation treatment at six Gy doses (Sianipar et al. 2016, 2017; Sianipar and Purnamaningsih 2018). The lectin gene can impact environmental factors, such as temperature, light, and soil composition. These factors can influence the expression of the lectin gene, potentially affecting the plant's growth, development, and production of bioactive compounds (Mishra et al. 2019). The expression of lectin genes in *T. flagelliforme* mutants has not been directly studied. Protein structure modeling studies with similarities with the wild-type show the possibility of the same gene expression as the wild-type. Changes in point mutations that cause changes in protein structure can affect gene expression (Kim et al. 2004). This study's mutant protein structure is very similar

to the wild-type. The significance of lectin genes in producing *T. flagelliforme* plants has been reported by Alfarabi et al. (2015). The study stated that lectin isolates were successfully obtained and could inhibit breast cancer cells. The difference between *T. flagelliforme* mutants and wild-types in this study could not be obtained through the lectin gene approach. This allows the expression of the wild-type lectin gene to be very similar to the mutant, but the approach to genes controlling other anticancer compounds may differ.

In conclusion, tetra-primer ARMS lec183 (Lec183_F_In_G: GAGCAGCGGCTCCCAGTCGGACAG; Lec183_R_In_C: ACGGCGGCGTACTTGCCGGG; Lec183_F_Out: ACGGCGAACAGAGGACGGGACTGC; Lec183_R_Out: TGCCGTCGGCGTAGAGGACCTGG) was successfully designed based on the 183 bp point mutation of the lectin gene. Although the tetra-primer ARMS lec183 could not distinguish *T. flagelliforme* mutants from wild-type, the primer pair was successfully amplified in all *T. flagelliforme* samples at 278 bp outer primer and 193 bp inner primer G allele according to the product size during primer preparation. Amino acid variations based on the 183 bp mutation point of the lectin gene show that the mutant protein structure is very similar to the structure of the wild-type protein, which is a bulb-type lectin domain-containing protein. Therefore, research on primer design through genes encoding other anticancer compounds can be carried out to obtain molecular markers that differentiate *T. flagelliforme* mutants from wild-type plants.

ACKNOWLEDGMENTS

The Ministry of Education, Culture, Research, and Technology, Republic of Indonesia funded this study under project number 095/VRRTT/V/2023. The authors declare no conflicts of interest.

REFERENCES

Abrantes VE, Matias da Rocha BA, Batista Da Nóbrega R, Silva-Filho JC, Teixeira CS, Cavada BS, Gadelha CA, Ferreira SH, Figueiredo JG, Santi-Gadelha T, Delatorre P. 2013. Molecular modeling of lectin-like protein from *Acacia farnesiana* reveals a possible anti-inflammatory mechanism in carrageenan-induced inflammation. Biomed Res Intl 2013: 253483. DOI: 10.1155/2013/253483.

Alfarabi M, Rosmalawati S, Bintang M, Miftahudin, Rofa'ani E, Chaidir. 2015. Antip roliferation activity of tuber protein from *Typhonium flagelliforme* (Lodd.) Blume on MCF-7 cell line. Intl J Biosci 6 (12): 52-60. DOI: 10.12692/ijb/6.12.52-60.

Alvarez MF, Gomez MEV, Siewert S. 2016. Designing, optimization, and validation of tetra primer ARMS-PCR protocol for genotyping Single Nucleotide Polymorphism rs4731702 (C/T) of KLF14 gene associated with type 2 diabetes mellitus: A study in San Luis, Argentina. Open Access Libr J 3: e3270. DOI: 10.4236/oalib.1103270.

Baris I, Etlik O, Koksal V, Arican-Baris ST. 2010. Rapid diagnosis of spinal muscular atrophy using tetra-primer ARMS PCR assay: Simultaneous detection of SMN1 and SMN2 deletion. Mol Cell Probes 24 (3): 138-141. DOI: 10.1016/j.mcp.2009.12.001.

Bates G. 1995. Book Review: Current Protocols in Human Genetics. Edited by Dracopoli NC et al. Current Protocols, 1994. $295.00 loose leaf (750 pages) ISBN 0 471 03420 7. Trends Genet 11 (11): 458-459. DOI: 10.1016/s0168-9525(00)89147-7.

Bellande K, Bono JJ, Savelli B, Jamet E, Canut H. 2017. Plant lectins and lectin receptor-like kinases: how do they sense the outside? Intl J Mol Sci 18 (6): 1164. DOI: 10.3390/ijms18061164.

Cai L, Wang W, Wang F, Zhang R, Zhang L, Qi H, Gu W, Zhou N. 2019. A comparison of MASS-PCR and ARMS-PCR for the detection of lung cancer gene mutation. Transl Cancer Res 8 (7): 2564-2569. DOI: 10.21037/tcr.2019.10.37.

Calderón-Cortés N, Quesada M, Cano-Camacho H, Zavala-Páramo G. 2010. A simple and rapid method for DNA isolation from xylophagous insects. Intl J Mol Sci 11 (12): 5056–5064. DOI: 10.3390/ijms11125056.

Chai J, Xiong Q, Zhang PP, Shang YY, Zheng R, Peng J, Jiang SW. 2010. Evidence for a new allele at the SERCA1 locus affecting pork meat quality in part through the imbalance of Ca^{2+} homeostasis. Mol Biol Rep 37 (1): 613-619. DOI: 10.1007/s11033-009-9872-0.

Garcés-Claver A, Fellman SM, Gil-Ortega R, Jahn M, Arnedo-Andrés MS. 2007. Identification, validation, and survey of A Single Nucleotide Polymorphism (SNP) associated with pungency in *Capsicum* spp. Theor Appl Genet 115 (7): 907-916. DOI: 10.1007/s00122- 007-0617-y.

Holle S, de Schutter K, Eggermont L, Tsaneva M, Dang L, van Damme EJM. 2017. Comparative study of lectin domains in model species: New insights into evolutionary dynamics. Intl J Mol Sci 18 (6): 1136. DOI: 10.3390/ijms18061136.

Hou Y, Luo Q, Chen C, Zhou M. 2013. Application of tetra primer ARMS-PCR approach for detection of *Fusarium graminearum* genotypes with resistance to carbendazim. Australas Plant Pathol 42 (1): 73-78. DOI: 10.1007/s13313-012-0162-2.

Islam MS, Thyssen GN, Jenkins JN, Fang DD. 2015. Detection, validation, and application of genotyping-by-Sequencing Based Single Nucleotide Polymorphisms in upland cotton. Plant Genome 8 (1): plantgenome2014.07.0034. DOI: 10.3835/plantgenome2014.07.0034.

Ke-xin Y, Xiang C, Qing-qing H, Yi-an Y, Xiao-ming W, Ai-chun X, Jian G, Feng G. 2023. Development of a tetra-primer ARMS-PCR for identification of sika and red deer and their hybrids. Anal Sci 39: 1947-1956. DOI: 10.1007/s44211-023-00405-6.

Kim JS, Jang HW, Kim JS, Kim HJ, Kim JH. 2012. Molecular identification of *Schisandra chinensis* and its allied species using multiplex PCR based on SNPs. Genes Genom 34 (3): 283-290. DOI: 10.1007/s13258-011-0201-3.

Kim S, Binzel ML, Yoo KS, Park S, Pike LM. 2004. Pink (P), a new locus responsible for a pink trait in onions (*Allium cepa*) resulting from natural mutations of anthocyanidin synthase. Mol Genet Genom 272 (1): 18-27. DOI: 10.1007/s00438-004-1041-5.

Kimball SR, Jefferson LS. 2006. New functions for amino acids: Effects on gene transcription and translation. Am J Clin Nutr 83 (2): 500S-507S. DOI: 10.1093/ajcn/83.2.500s.

Little S. 1997. ARMS analysis of point mutations. In: Taylor GR (Ed.) Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA. CRC Press, Boca Raton.

Luo Y, Xu X, Liu J, Li J, Sun Y, Liu Z, Liu J, van Damme E, Balzarini J, Bao J. 2007. A novel mannose-binding tuber lectin from *Typhonium divaricatum* (L.) Decne (family Araceae) with antiviral activity against HSV-II and anti-proliferative effect on human cancer cell lines. J Biochem Mol Biol 40 (3): 358-367. DOI: 10.5483/bmbrep.2007.40.3.358.

Medrano RFV, de Oliveira CA. 2014. Guidelines for the tetra-primer ARMS-PCR technique development. Mol Biotechnol 56 (7): 599-608. DOI: 10.1007/s12033-014-9734-4.

Meng K, Xu JL, Li Q, Liu YJ, Wang X, Tang W, Yan H, Zhang YG, Fu MD. 2017. Development of SNP markers using RNA-seq technology and tetra-primer ARMS-PCR in sweet potato. J Integr Agric 16 (2): 464-470. DOI: 10.1016/S2095-3119(16)61405-3.

Mishra A, Behura A, Mawatwal S, Kumar A, Naik L, Mohanty SS, Manna D, Dokania P, Mishra A, Patra SK, Dhiman R. 2019. Structure-function and application of plant lectins in disease biology and immunity. Food Chem Toxicol 134: 110827. DOI: 10.1016/j.fct.2019.110827.

Mitternacht S, Berezovsky IN. 2011. Binding leverage as a molecular basis for allosteric regulation. PloS Comput Biol 7 (9): e1002148. DOI: 10.1371/journal.pcbi.1002148.

Park H, Kim S, Nie H, Kim J, Lee J, Kim S. 2020. Molecular identification of sweet potato accessions using ARMS-PCR based on

SNPs. J Plant Biotechnol 47 (2): 124-130. DOI: 10.5010/JPB.2020.47.2.124.

Petrova N, Nazipova A, Gorshkov O, Mokshina N, Patova O, Gorshkova T. 2021. Gene expression patterns for proteins with lectin domains in flax stem tissues are related to deposition of distinct cell wall types. Front Plant Sci 12: 634594. DOI: 10.3389/fpls.2021.634594.

Peumans WJ, van Damme EJM. 1995. Lectins as plant defense proteins. Plant Physiol 109 (2): 347-352. DOI: 10.1104/pp.109.2.347.

Piskata Z, Servusova E, Babak V, Nesvadbova M, Borilova G. 2019. The quality of DNA isolated from processed food and feed via different extraction procedures. Molecules 24 (6): 1186. DOI: 10.3390/molecules24061188.

Rajasekaran N, Suresh S, Gopi S, Raman K, Naganathan AN. 2017. A general mechanism for the propagation of mutational effects in proteins. Biochemistry 5 6(1): 294-305. DOI: 10.1021/acs.biochem.6b00798.

Rubio M, Caranta C, Palloix A. 2008. Functional markers for selection of potyvirus resistance alleles at the pvr2-eIF4E locus in pepper using tetra-primer ARMS-PCR. Genome 51 (9): 767-771. DOI: 10.1139/G08-056.

Servusova E, Piskata Z. 2021. Identification of selected tuna species in commercial products. Molecules 26 (4): 1137. DOI: 10.3390/molecules26041137.

Sianipar NF, Assidqi K, Abbas BS. 2020a. The effects of subculture on the mutant plant regeneration of rodent tuber (*Typhonium flagelliforme*) in vitro mutagenesis using gamma-ray irradiation. IOP Conf Ser: Earth Environ Sci 426: 012180. DOI: 10.1088/1755-1315/426/1/012180.

Sianipar NF, Assidqi K, Hadisaputri YE, Salam S. 2023. Mutant plant tipobio variety of rodent tuber (*Typhonium flagelliforme*): Fatty acids compounds and in vitro anticancer activity. E3S Web Conf 01032: 4-8. DOI: 10.1051/e3sconf/202338801032.

Sianipar NF, Hadisaputri YE, Assidqi K, Salam S, Yusuf M, Destiarani W, Purnamaningsih R, Gautama So I. 2021. Characterization and investigation of stigmasterol isolated from rodent tuber mutant plant (*Typhonium flagelliforme*), its molecular docking as anticancer on MF-7. Preprints 2021: 2021070278. DOI: 10.20944/preprints202107.0278.v1.

Sianipar NF, Purnamaningsih R, Chelen C. 2016. Effect of gamma irradiation on protein profile of rodent tuber (*Typhonium flagelliforme* Lodd.) in vitro mutant based on 1D and 2D-page analyses. Jurnal Teknologi 78 (10-4): 35-40. DOI: 10.11113/jt.v78.9888.

Sianipar NF, Purnamaningsih R, Darwati I, Laurent DC. 2015. The effects of gamma irradiation and somaclonal variation on morphology variation of rodent tuber mutant (*Typhonium flagelliforme* Lodd.) lines. KnE Life Sci 2 (1): 637. DOI: 10.18502/kls.v2i1.236.

Sianipar NF, Purnamaningsih R, Darwati I. 2020b. Binus University. Plant Variety Protection (PVP) Rights. Tipobio. No. HKI: 00490/PPVT/S/2020. Center for Plant Variety Protection and Agricultural Licensing, Ministry of Agriculture of the Republic of Indonesia.

Sianipar NF, Purnamaningsih R, Gumanti DL, Rosaria, Vidianty M. 2017. Analysis of gamma irradiated-third generation mutants of rodent tuber (*Typhonium flagelliforme* Lodd.) based on morphology, RAPD, and GC-MS Markers. Pertanika J Trop Agric Sci 40 (1): 185-202.

Sianipar NF, Purnamaningsih R. 2018. Enhancement of the contents of anticancer bioactive compounds in mutant clones of rodent tuber (*Typhonium flagelliforme* Lodd.) based on GC-MS analysis. Pertanika J Trop Agric Sci 41 (1): 305-320.

Sianipar NF, Reflinur R, Ashan MD, Assidqi K, Widyaningrum D, Purnamaningsih R. 2022. Determination of lectin genes in superior mutant of rodent tuber bogor accession (*Typhonium flagelliforme*) based on PCR amplification. Proc 7th Intl Conf Biol Sci (ICBS 2021) 2022: 278-283. DOI: 10.2991/absr.k.220406.039.

Suhda S, Paramita DK, Fachiroh J. 2016. Tetra primer ARMS PCR optimization to detect single nucleotide polymorphisms of the CYP2E1 gene. Asian Pac J Cancer Prev 17 (7): 3065-3069. DOI: 10.4236/oalib.1104145.

Tanha HM, Naeini MM, Rahgozar S, Rasa SMM, Vallian S. 2015. Modified tetra-primer ARMS PCR as a single-nucleotide polymorphism genotyping tool. Genet Test Mol Biomarkers 19 (3): 156-161. DOI: 10.1089/gtmb.2014.0289.

Taverna DM, Goldstein RA. 2002. Why are proteins so robust to site mutations? J Mol Biol 315 (3): 479-484. DOI: 10.1006/jmbi.2001.5226.

Tóth T, Csaba Á, Bokor A, Ács N. 2023. Variable Fragment Length Allele-Specific Polymerase Chain Reaction (VFLASP), a method for simple and reliable genotyping. Mol Cell Probes 69: 101910. DOI: 10.1016/j.mcp.2023.101910.

van Damme EJM, Barre A, Rougé P, Peumans WJ. 2004. Potato lectin: An updated model of a unique chimeric plant protein. Plant J 37 (1): 34-45. DOI: 10.1046/j.1365-313X.2003.01929.x.

Wang YZ, Zhu Z, Zhang HY, Zhu MZ, Xu X, Chen CH, Liu LG. 2014. Detection of hepatitis B virus A1762T/G1764A mutant by amplification refractory mutation system. Braz J Infect Dis 18 (3): 261-265. DOI: 10.1016/j.bjid.2013.09.005.

Yang G, Hong N, Baier F, Jackson CJ, Tokuriki N. 2016. Conformational tinkering drives evolution of a promiscuous activity through indirect mutational effects. Biochemistry 55 (32): 4583-4593. DOI: 10.1021/acs.biochem.6b00561.

Yang Z, Lo YT, Quan Z, He J, Chen Y, Faller A, Chua T, Wu HY, Zhang Y, Zou Q, et al. 2023. Application of a modified tetra-primer ARMS-PCR assay for rapid *Panax* species identity authentication in ginseng products. Sci Rep 13: 14396. DOI: 10.1038/s41598-023- 39940-7.

Ye S, Dhillon S, Ke X, Collins AR, Day INM. 2001. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 29 (17): 88. DOI: 10.1093/nar/29.17.e88.

Ye S, Humphries S, Green F. 1992. Allele specific amplification by tetra-primer PCR. Nucleic Acids Res 20 (5): 1152. DOI: 10.1093/nar/20.5.1152.

Zabala AS, Gomez MEV, Álvarez MF, Siewert S. 2017. Tetra primer ARMS PCR optimization to detect Single Nucleotide Polymorphism of the KLF14 gene. Open Access Libr J 4: e4145. DOI: 10.4236/oalib.1104145.