AL-KAUNIYAH: Jurnal Biologi, 17(2), 2024, 377-386
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

PRIMER DESIGN AND OPTIMIZATIONOF ANNEALING TEMPERATURE FOR GENE AMPLIFICATION GSTL2 ON RICE

DESAIN PRIMER DAN OPTIMASI SUHU ANNEALING UNTUK AMPLIFIKASI GEN GSTL2 PADA TANAMAN PADI

Violita*, Afifatul Achyar, Zulyusri, Yusni Atifah, Dwi Hilda Putri, Rezi Nabilah
Universitas Negeri Padang, Indonesia,
Jl. Prof. Hamka kampus Universitas Negeri Padang air tawar, 25131 Padang Utara
**Corresponding author:* violita@fmipa.unp.ac.id

Submitted: 14 June 2023; Revised: 19 July 2023; Accepted: 17 August 2023

Abstract

Glutathione S-Transferase is a superfamily enzyme that has many roles for living things, especially in the detoxification of Reactive Oxygen Species (ROS), one of which is rice plants. One gene of this family that has a role for plants is GSTL2 This gene is known to have a contribution to *Arabidopsis* and *Oryza sativa* L. against abiotic stress. To find out how this gene expression is requires a primer to specifically amplify this gene, and an optimal annealing temperature to support the success of the primer. This study aims to design primers and determine the optimal annealing temperature for gene amplification GSTL2. Primers were designed using the Primer3, which were then analyzed with Genious Prime based on good primer criteria and optimizing the annealing temperature which was carried out using gradient PCR. The results of this study obtained a primer pair is forward 5'-TTCGAAGGGCCAGCATTACT-3' primer reverse 5'-CAATGTCCACCAAGCTGAA-3'. The primer pair has a length of 20 nt, with melting temperature (Tm) 59–59,4 °C, and GC content 50%. The primer forward there is a secondary structure in the form of a self-dimer with (Tm) 6.2 °C. The primer pair can amplify gene sequences GSTL2 by producing a PCR product 224 bp. The annealing temperature of 60 °C resulting in a single thick, bright DNA strand.

Keywords: Annealing; Glutathione S-Transferases; GSTL; *Oryza sativa*; Primer design

Abstrak

Glutathione S-Transferase merupakan enzim superfamily yang memiliki banyak peranan bagi makhluk hidup terutama dalam detoksifikasi Reactive Oxygen Species (ROS), salah satunya tumbuhan. Salah satu gen dari famili ini yang memiliki peranan bagi tanaman adalah GSTL2. Gen ini diketahui memiliki kontribusi bagi tanaman Arabidopsis dan Oryza sativa L. terhadap stres abiotik. Untuk mengetahui bagaimana ekspresi gen GSTL2 ini dibutuhkan primer untuk mengamplifikasi gen ini secara spesifik, di samping itu suhu annealing yang optimal untuk menunjang keberhasilan primer. Penelitian ini bertujuan untuk mendesain primer dan menentukan suhu annealing yang optimal untuk mengamplifikasi gen GSTL2. Primer didesain menggunakan tools Pick Primer dan Geneious Prime, yang kemudian dianalisis secara in silico berdasarkan kriteria primer yang baik. Serta optimasi suhu annealing yang dilakukan menggunakan gradient PCR. Hasil penelitian ini diperoleh sepasang primer dengan panjang masing-masing primer 20 nt, primer forward 5'-TTCGAAGGGCCAGCATTACT-3' primer reverse 5'-CAATGTCCACCAAGCTGAA-3'. Pasangan primer dapat mengamplifikasi sekuen gen GSTL2 dengan menghasilkan produk PCR sebesar 224 bp pada suhu annealing 60 ℃.

***Kata kunci**: Annealing; Desain primer; Glutathion S-Transferase; GSTL; Oryza sativa*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v17i2.32859

© 2024 The Author(s). This is an open article under CC-BY-SA license (http://creativecommons.org/licenses/by-sa/4.0/)

INTRODUCTION

Glutathione S-Transferase (GSTs, E.C.2.5.1.18) or glutathione transferases refers to the most multipurporse natural enzyme superfamily, by catalyzing a wide range of reactions involving glutathione conjugation(GSH; γ-Glu–Cys–Gly) (Estévez & Hernández, 2020; Labrou et al., 2015). In addition, GST is an enzyme that is well-known for its role in detoxification reactions phase II (Kumar & Trivedi, 2018). This enzyme has been found in eukaryotic and prokaryotic, cytosol (Frova, 2003), chloroplasts, mitochondria, plastids, peroxisomes, nucleus, and plasma membranes (Lallement et al., 2014).

Glutathione s-transferase is categorized into three families including cytosolic, microsomal, and mitochondrial GSTs (Kumar & Trivedi, 2018). It consists of 14 classes including Dehydroascorbate reductase (DHAR), Elongation factor 1Bc (EF1Bc), Phi (GSTF), Hemerythrin (GSTH), Iota (GSTI), Lamda (GSTL), Theta (GSTT), Tau (GSTU), Zeta (GSTZ), Tetra-chloro-hydroquinone dehalogenase (TCHQD) (Liu et al., 2013), Glutathionyl hydroquinone reductase (GHR), Microsomal prostaglandin E synthase type 2 (mPGES-2), Ure2p (Lallement et al., 2014).

In plant, Glutathione s-transferase has an important role for its growth and development, (Hossain & Fujita, 2013), mainly involved in defense mechanisms against biotic and abiotic stress (Estévez & Hernández, 2020; Vaish et al., 2020). One of the most damaging abiotic stress is drought, because the response to drought stress is a very complex process involving various mechanisms at different levels (Basal et al., 2020). Drought can decrease the rate of photosynthesis, by inducing closure and decrease in total stomata size (Zagoto & Violita, 2019) which has an effect on the rate of photosynthesis due to differences in the production and use of electrons and photosynthetic apparatus which causes plants to experience oxidative stress due to the formation of Reactive Oxygen Species (ROS) (Verma et al., 2019).

Glutathione S-Transferase has peroxidation activity in detoxification ROS by catalyzing hydrogen peroxide (H_2O_2) and fatty hydroperoxides (ROOH) (Hossain & Fujita, 2013). GST works in detoxifying ROS by releasing Glutathione (GSH), and producing glutathione disulfide (GSSG) as its oxidized form which can be secreted from cells, sequestered in vacuoles or catabolized by Glutathione Reductase with the help of NADPH and hydrogen ions into GSH and $NADP^+$ (Hossain & Fujita, 2013; Nianiou-Obeidat et al., 2017; Zagorchev et al., 2013).

One of the plants that is very vulnerable to drought stress is rice. According to Maisura et al. (2014), rice is a semi-aquatic plant that grows normally in waterlogged conditions. Drought can affect the growth of rice plants at both the morphological, anatomical, and even physiological levels (Violita & Azhari, 2021).

There have been many previous studies reporting the role of glutathione s-transferase against drought stress; CaGST on *Capsium annum* (Islam et al., 2019), ThGSTZ1 in transgenic plants *Arabidopsis thaliana* (Yang et al., 2014), and OsGSTL2 on *Arabidopsis* (Kumar et al., 2013). OsGSTL2 or GSTL2 has many synonym in the previous studies like GSTZ5 and Protein IN2 homolog (Hu et al., 2011).

The method that can be used to investigate gene expression is Quantitative Polymerase Chain Reaction (q-PCR) (Maddocks & Jenkins, 2017). The important thing that determine success in gene expression analysis is primer design (Biedendieck et al., 2011). The designed primer must fulfill the criteria of a good primer (Chuang et al., 2013; Rodríguez et al., 2019). In addition, an important aspect that determines the success of q-PCR is determining the annealing temperature (Ta). Annealing temperature (Ta) is an important variable for primer performance (Bustin & Huggett, 2017), especially for obtaining specific genes.

In previous studies, there were primers for GSTL2 gene in rice plant (Hu et al., 2011) but after conducting an analysis on Primer-BLAST it showed that the primer pair has 20 nt with GC content primer forward 45% and primer reverse 50%, temperature melting (Tm) forward 55,73 °C and reverse 58,89 °C, and produce PCR amplicon 300 bp. In this analysis showed this primer has high amount of GC content especially on primer reverse. This high amount of GC content in primer can impact the primer temperature melting (Tm) (Rodríguez-Lázaro et al., 2013). According to Thornton and Basu (2011) the Tm difference between primer pair should not be more than 1–2 °C,

and this primer pair has Tm difference up to 3 °C. In addition, based on specificity analysis it showed this primer pair could amplify genomic DNA (gDNA) such as *Oryza sativa* Japonica Changxianggeng chromosome 11 (CP101151.1), *Oryza sativa* Indica chromosome 3 (CP056054.1), *Oryza sativa* Japonica chromosome 3 (AP014959.1), and Oryza sativa chromosome 3 BAC OSJNBa0044H10 (AC084405.6) which has the same amplicon of 300 bp, so it can affect the result of gene expression.

In the study of gene expression, a primer design specifically amplified on RNA is needed. One of the solutions to avoid contamination from genomic DNA is DNase treatment (Dotti & Bonin, 2011). However, the application of DNase treatment requires greater costs, so that can be done to avoid gDNA is design primer specific. Furthermore, a primer design is needed to obtain a specific primer design. The purpose of this study was to design a specific primer and optimize the annealing temperature for GSTL2 gene in rice plant.

MATERIALS AND METHODS

This research was conducted in October 2022-January 2023 at the Laboratory of Biotechnology and Genetics, Padang State University. This research was carried out based on molecular biology and computational testing (bioinformatics) and optimization of annealing temperatures through laboratory testing.

The tools used in this study were the Geneious Prime software and the Pick Primer tools on the NCBI website https://www.ncbi.nlm.nih.gov/tools/primer-blast/, autoclave, centrifuge, vortex, thermal cycle, electrophoresis, spin down, micropipette, blue tip, yellow tip, white tip, micropestle, Nanodrop, and UV-TEC. The material used in this study was a rice root sample GENEzol^{TM}, ddH2O, SensiFAST^{TM} cDNA Synthesis Kit, , liquid nitrogen, agarose, TAE 1x, Nuclease Free Water (NFW), GoTaq® Green master mix 2x (PROMEGA, cat. M7128).

Primer Design

Primer design was carried out using Primer3 NCBI. Primers are designed on GSTL2 sequence of *Oryza sativa* L. (DQ323738) with an amplicon target of 150–250 bp. The primer candidate that was obtained analyzed using Geneious Prime software to fulfill the ideal primer criteria including; primer length and difference, GC content, melting temperature (Tm), and the presence of secondary structure check like dimers and hairpins (Rodríguez et al., 2019).

Primer Specificity Analysis

The selected primer pairs were analyzed in silico to test the specificity of the primers and the similarity of PCR products produced with the database at GenBank NCBI, using Primer BLAST tools on the NCBI sites (Pranata & Ahda, 2022). Primer specificity was tested by alignment the primer sequences using PrimerBLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi (Syamsurizal et al., 2021). Primer specificity was tested with primer pair specificity cheking parameters; specificity check was activated, search mode automatic, database nr, no exclusion, and organism was blank. Primer with the best criteria and specificity will be selected and sent to PT. Genetics Science Indonesia for primer ordering.

RNA Extraction

RNA extraction was carried out by follow the official protocol from GENEzol^{TM}. The 3 days old rice roots were taken and weighed as much as 40 mg, then, a liquid nitrogen added and crushed using a micropestle until smooth. The crushed sample then used in RNA isolation followed the official protocol from GENEzolTM with a few modifications. The isolated RNA then checked for the concentration and purity using the NanoDrop spectrophotometer. The results of RNA isolation are stored at -80 °C or can be continued to the cDNA synthesis stage.

cDNA Synthesis

The RNA samples will be synthesized using the SensiFAST^{TM} cDNA Synthesis Kit. cDNA synthesis was carried out by preparing a mixture of cDNA synthesis reactions, then homogenized

with a vortex. Samples were run for 30 minutes on a thermal cycler, with annealing settings of 25 °C for 10 minutes, the reverse transcription step at 42 °C for 15 minutes, and inactivation at 85 °C for 5 minutes, then the cDNA samples could be stored temporarily at 4 °C. The volume of extracted RNA added to a mixture of cDNA synthesis reaction was calculated using the following formula, RNA volume (µl) = $\frac{1000\ ng}{\text{RNA concentration } (\frac{ng}{\mu l})}$.

Optimization Annealing Temperature

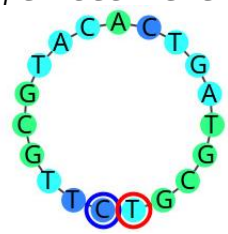
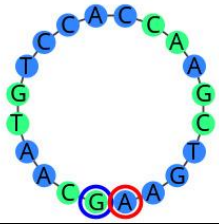
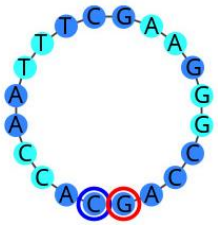
Optimization of the primer annealing temperature was carried out by gradient PCR. The cDNA then amplified using GoTaq® (PROMEGA, cat. M7128) by PCR machine. It was set with a pre-denaturation temperature at 95 °C for 3 minutes, followed by 35 cycles with a denaturation 95 °C for 30 seconds, annealing was carried out by gradient PCR with temperature variation start with temperature around 5 °C under Tm both primers, elongation at 72 °C for 30 seconds. And the last cycle at 72 °C for 5 minutes. The amplification results from the PCR gradient were electrophoresis with 1.5% agarose gel. The electrophoresis results were then observed with UV-TEC.

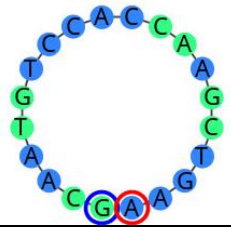
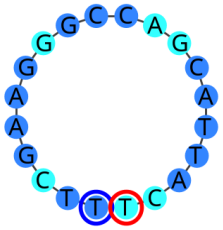
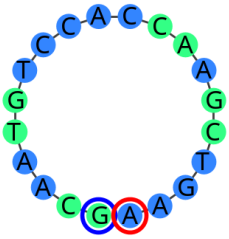
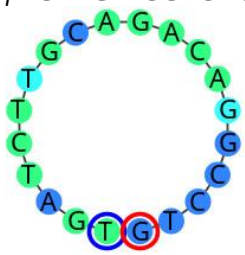
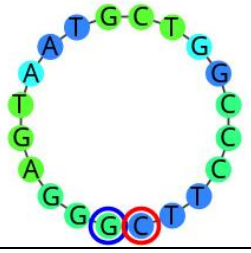
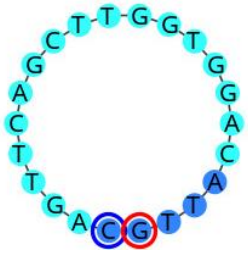
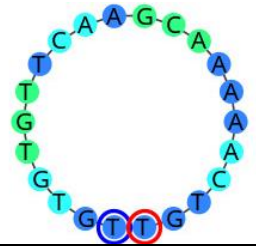
RESULTS

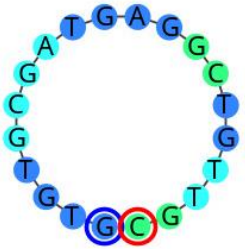
Primer Design

Primer design performed on GSTL2 sequence of *Oryza sativa* L. (DQ323738) resulted in six primer candidates (Table 1). Based on the results of the primer analysis based on ideal primer criteria, primer pair number three was selected. Each primer has 20 nt, with temperature melting (Tm) 59–59.4 °C, with %GC 50%. In the primer forward there is a secondary structure in the form of self-dimer with Tm 6.2 °C (Table 2).

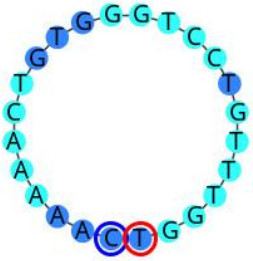
Table 1. Candidate primers

No.	Sequence (5'--> 3')	Characteristics					
		Length (nt)	Tm (°C)	%GC	Secondary structure check: Tm hairpin	Secondary structure check: Tm self-dimer	PCR product (bp)
1.	*GST-f* CTTGCGTACACTGATGCGT	20	60.0	50	-	-	165
							
	GST-r GCAATGTCCACCAAGCTGAA	20	59.0	50	33.8	-	
							
2.	*GST-f* CACCAATTTCGAAGGGCCAG	20	59.4	55	-	6.2	231
							
	GST-r GCAATGTCCACCAAGCTGAA	20	59.0	50	-	-	

							
3.	GST-f TTCGAAGGGCCAGCATTACT	20	59.0	50	-	6.2	224
							
	GST-r GCAATGTCCACCAAGCTGAA	20	59.4	50	-	-	
							
4.	*GST-f* TGATCTTGCAGACAGGCCTG	20	60.0	55	37.2	31.6	150
							
	GST-r GGGAGTAATGCTGGCCCTTC	20	60.5	50	35.9	-	
							
5.	*GST-f* CAGTTCAGCTTGGTGGACATTG	22	60.0	50	-	12.9	186
							
	GST-r TGTGTGTTCAAGCAAAAACTGT	22	57.8	36	-	-	
							
6.	*GST-f* GTGTGCGATGAGGCTGTTGC	20	62.5	60	38.8	-	255



GST-r CAAAAACTGTGGGTCCTGTTTGGT 24 62.1 45 36.8 -



The results of aligning the primer pair sequences using Primer-BLAST showed that primer pair number three successfully amplified the GSTL2/GSTZ5 gene specifically in rice (*Oryza sativa* L.) in both Japonica and Indica varieties in the NCBI database (Table 2).

Table 2. Results of Primer-BLAST using NCBI database

Primer sequence	Number of recognized targets				
	Oryza sativa GSTZ5	*Oryza sativa* Japonica cDNA	*Oryza sativa* Indica cDNA	Protein IN2-homolog	Total
Forward: TTCGAAGGGCCAGCATTACT Reverse: GCAATGTCCACCAAGCTGAA	(DQ323738.1) 1	(AK289120.1) (AK065887.1) (AK061849.1) 3	(CT828851.1) 1	(NM_001417548.1) 1	6
	PCR Product: 224 bp				

Optimization of Annealing Temperature (Ta)

The results of annealing temperature optimization with PCR gradient visualized using agarose obtained bright and thick DNA bands for each given temperature variation. The band formed corresponds to the length of the target gene PCR product which is 224 bp (Figure 1). Based on the results of the optimization, the Ta used is the highest temperature which is 60 °C.

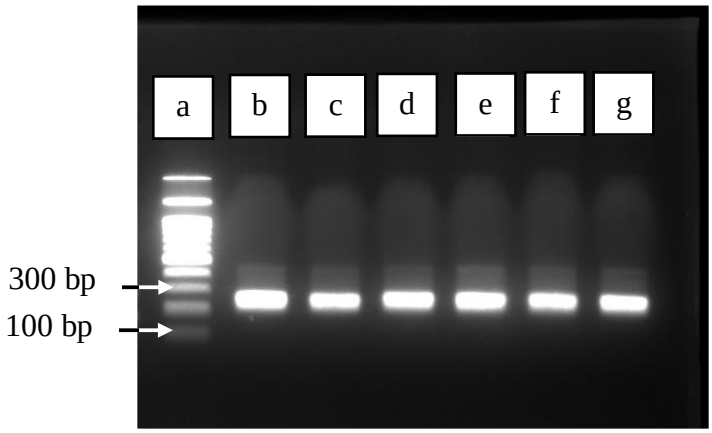


Figure 1. Electropherogram of Gradient PCR. Ladder 100 bp (a), annealing temperature 50 °C (b), 52.2 °C (c), 54.4 °C (d), 56.7 °C (e), 58.7 °C (f), and 60 °C (g)

DISCUSSION

Design Primer

Primer designs are performed on gene sequences of GSTL2 *Oryza sativa* L. with accession no. DQ323738 using the Primer3 on NCBI produced 6 primer candidates (Table 1). Based on the

analysis using Genious Prime, primer pair number 3 primer forward GST-f (5'-TTCGAAGGGCCAGCATTACT-3' and reverse GST-r 5'-GCAATGTCCACCAAGCTGAA-3'). The information regarding the characteristics of the design primer results selected from Geneious Prime can be seen in (Table 2).

A good primer must fulfill the ideal primer characteristics such as; primer length, %GC, melting temperature (Tm) and Tm difference between primer pair, the presence of dimers and hairpins (Rodríguez et al., 2019). Primer length should be between 18–24 mer, because shorter primers have a higher efficiency of attachment to their targets than longer primers (Hung & Weng, 2016).

Percentage of GC base is a percentage value that indicates the ratio of G and C base nucleotides. The suitable percentage of GC base is between 40–60%. It is important to know The percentage of GC base in the primer because it will affect the primer annealing process indirectly (Chuang et al., 2013), according to Bustin and Huggett (2017) the % GC should be as close to 50% as possible. Due to the high percentage of GC can make primers "sticky" so as to increase the possibility of mispriming on unwanted parts (Hung & Weng, 2016). Besides that, the oligonucleotides with high %GC will complicate the denaturation process during PCR which can lead to decreased amplification efficiency (Lim et al., 2011), especially repeating Guanine (G) bases should be avoided (Bustin & Huggett, 2017).

The amount of base content of GC and TA can affect the value melting temperature (Tm). Primers that are rich in GC content had higher Tm values than those rich in TA (Rodríguez-Lázaro et al., 2013). According to Thornton and Basu (2011) to optimize the primer work, preferably the selected primer has a Tm in the range of 59–68 °C with optimal Tm 63–64 °C. In addition, the difference in Tm between the forward and reverse primers should not be more than 1–2 °C. Based on the results of the primer candidates obtained that fulfilled the Tm requirements, only primer pair number 5 had a Tm difference of 3 °C.

Based on these criteria, primer pair number 3 was chosen. Primer pair no. 3 each has a base length of 20 nucleotides with %GC on 50% and Tm ~59 °C. On forward primer found secondary structure form self dimers at a temperature of 6.2, while the reverse primer free from secondary structure hairpin nor self dimers. Although there are self dimers in the forward primer this is acceptable because the dimer formed has a relatively low Tm (Putri et al., 2021) and is far from the Tm of the two primer (Thornton & Basu, 2015) which is equal to 6.2 °C. This shows that self dimers formed can separate at this temperature.

This primer pair produces an amplicon of 224 bp. The size of the PCR amplicon taken has a range of 150–250. According to Wang et al. (2012) , this range was chosen because the relatively short size of the amplicon is very important for the efficiency of PCR amplification, especially if the quality of the RNA obtained is low. In addition, if the amplicon size is below 100 bp, it will be difficult to distinguish specific target PCR products from primer dimers during gel electrophoresis. Where all the primer candidates that were successfully designed produced amplicon according to the standard, namely in the range of 150–250 bp.

The results of aligning the primer pair sequences using Primer-BLAST can be seen in (Table 2). Primer-BLAST is a tool that can be used to check primer specificity with pre-existing primers (Ye et al., 2012). Based on the results of checking through Primer-BLAST, the selected primer succeeded in amplifying the gene GSTL2/GSTZ5 specifically for rice (*Oryza sativa* L.) for both Japonica and Indica varieties in the NCBI database. In addition, it can be seen that the primer pairs are designed not to amplify genomic DNA, so as to prevent genomic DNA amplification from occurring which can affect the resulting PCR product. In addition to B-like IN2-homologous Protein is a synonym of gene GSTL2/GSTZ5 on the NCBI database with accession number NM_001417548 (https://www.ncbi.nlm.nih.gov/nuccore/NM_001417548). It can also be known where this gene can be found on chromosome 3 where the gene GSTL2 to be (Hu et al., 2011).

Optimization of Annealing Temperature (Ta)

Optimization of annealing temperature is carried out to obtain optimal annealing temperature for the primer. Optimization of annealing temperature can be done with gradient PCR by applying several different annealing temperatures. According to Rodríguez et al. (2019) optimizing the annealing temperature by testing several annealing temperatures, it is recommended to start with a temperature of around 5 °C under Tm both primers to obtain optimal experimental annealing conditions. The gradient PCR result are visualized in the Figure 1.

Based on the results of the PCR gradient visualized using 1.5% agarose, it shows a single DNA band that is thick and bright for each variation of annealing temperature tested on the primer. This single band indicates its absence as an unspecific band which indicates that the selected primer does not contain genomic DNA contamination, the primer specifically produces a PCR product according to the target with a length of 224 bp.

Although it can be seen at any temperature it produces thick, bright DNA bands. However, the annealing temperature chosen is the highest temperature, which is 60 °C. This is because using a higher annealing temperature will reduce the risk of forming DNA bands from non-specific amplicons. According to Thornton and Basu (2015) the annealing temperature (Ta) is usually not far from the primer Tm, normally it is between 55–60 °C. However, to increase the specificity of the primer it is recommended to use high temperatures (Erjavec, 2019; Thornton & Basu, 2015). In Patel et al. (2015) the use of a higher annealing temperature can increase the bonding of a greater specific primer.

CONCLUSION

The results of this study obtained a pair of primers designed using Primer3 (NCBI) and analysis with Geneious Prime. The designed primer has succeeded in amplifying GSTL2 gene of *Oryza sativa* L. specifically by producing a PCR product of 224 bp. The primer has an annealing temperature of 60 °C resulting a single thick, bright DNA strand. Therefore, this primer pair can be used in future studies such as to determine the expression GSTL2 gene in rice plant.

ACKNOWLEDMENTS

The researcher expresses his high appreciation and thanks to the Institute for Research and Community Service, Padang State University for providing financial supports to this research with research contract number: No. 947/UN35.15/LT/2022.

REFERENCES

Basal, O., Szabó, A., & Veres, S. (2020). Physiology of soybean as affected by PEG-induced drought stress. *Current Plant Biology*, *22*.

Biedendieck, R., Borgmeier, C., Bunk, B., Stammen, S., Scherling, C., Meinhardt, F., … Jahn, D. (2011). Systems biology of recombinant protein production using bacillus megaterium. In *Methods in Enzymology*, *500*. doi: 10.1016/B978-0-12-385118-5.00010-4.

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular Detection and Quantification*, *14*(November), 19-28. doi: 10.1016/j.bdq.2017.11.001.

Chuang, L. Y., Cheng, Y. H., & Yang, C. H. (2013). Specific primer design for the polymerase chain reaction. *Biotechnology Letters*, *35*(10), 1541-1549.

Dotti, I., & Bonin, S. (2011). DNase treatment of RNA. *In Guidelines for Molecular Analysis in Archive Tissues*, *3*(1), 87-90. doi: 10.1002/zamm.19630430112%250Ahttp://www.sciencedirect.com/.

Erjavec, M. S. (2019). Annealing temperature of 55 °C and specificity of primer binding in pcr reactions. *In Synthetic Biology-New Interdisciplinary Science.*, *IntechOpen*, 1-16.

Estévez, I. H., & Hernández, M. R. (2020). "Plant glutathione s-transferases: An overview." *Plant Gene*, *23*, 100233. doi: 10.1016/j.plgene.2020.100233.

Frova, C. (2003). Minireview the plant glutathione transferase gene family: Genomic structure, functions, expression and evolution. *Physiologia Plantarum*, *4*(119), 469-479.

Hossain, M. A., & Fujita, M. (2013). Hydrogen peroxide priming stimulates drought tolerance in

mustard (*Brassica juncea* L.) seedlings. *Plant Gene and Trait*, *4*(20), 109-123.

Hu, T. Z., He, S., Huang, X. Y., Deng, L., & Wang, G. X. (2011). Cloning, molecular characterization and heterologous expression of a glutathione s-transferase gene in rice. *Russian Journal of Bioorganic Chemistry*, *37*(3), 344-350.

Hung, J. H., & Weng, Z. (2016). Designing polymerase chain reaction primers using Primer3Plus. *Cold Spring Harbor Protocols*, *2016*(9), 821-826.

Islam, S., Sajib, S. Das, J. Z. S., Arabia, S., & Islam, T. (2019). Genome-wide identification of glutathione s-transferase gene family in pepper, its classification, and expression profiling under different anatomical and environmental conditions. *Scientific Reports*, *July 2018*, 1-15.

Kumar, S., Asif, M. H., Chakrabarty, D., Tripathi, R. D., Dubey, R. S., & Trivedi, P. K. (2013). Differential expression of rice lambda class gst gene family members during plant growth, development, and in response to stress conditions. *Plant Molecular Biology Reporter*, *31*(3), 569-580.

Kumar, S., & Trivedi, P. K. (2018). Glutathione s-transferases: Role in combating abiotic stresses including arsenic detoxification in plants. *Frontiers in Plant Science*, *9*.

Labrou, N. E., Papageorgiou, A. C., Pavli, O., & Flemetakis, E. (2015). Plant gstome: Structure and functional role in xenome network and plant stress response. *Current Opinion in Biotechnology*, *32*, 186-194. doi: 10.1016/j.copbio.2014.12.024.

Lallement, P.-A., Brouwer, B., Keech, O., Hecker, A., & Rouhier, N. (2014). The still mysterious roles of cysteine-containing glutathione transferases in plants. *Frontiers in Pharmacology*, *5*(August), 1-22.

Lim, J., Shin, S. G., Lee, S., & Hwang, S. (2011). Design and use of group-specific primers and probes for real-time quantitative PCR. *Frontiers of Environmental Science and Engineering in China*, *5*(1), 28-39.

Liu, Y-J., Han, X-M., Ren, L-L., Yang, H-L., Zeng, Q-Y. (2013. Functional divergence of the glutathione s-transferase supergene family in *Physcomitrella* patens reveals complex patterns of large gene family evolution in land plants. *Plant Physiology*, *161*, 773-786.

Maddocks, S., & Jenkins, R. (2017). Chapter 4 - quantitative pcr: Things to Consider. In S. Maddocks & R. Jenkins (Eds.), *Understanding PCR* (pp. 45–52). Amsterdam: Academic Press.

Maisura, M. A., Lubis, I., Junaedinand, A., & Ehara, H. (2014). Some physiological character responses of rice under drought conditions in a paddy system. *Journal of the International Society for Southeast Asian Agricultural Sciences*, *20*(1), 104–114.

Nianiou-Obeidat, I., Madesis, P., Kissoudis, C., Voulgari, G., Chronopoulou, E., Tsaftaris, A., & Labrou, N. E. (2017). Plant glutathione transferase-mediated stress tolerance: functions and biotechnological applications. *Plant Cell Reports*, *36*(6), 791-805.

Patel, S. V, Bosamia, T. C., Bhalani, H. N., Singh, P., & Kumar, A. (2015). Polymerase chain reaction (pcr). *Agrobios Newsletter: A Monthly Magazine of Agricultural and Biological Sciences Publishing*, *XIII*, *148*.

Pranata, A., & Ahda, Y. (2022). Desain primer untuk identifikasi gen luciferase pada kunang-kunang genus *Lamprigera* (*Lampyridae: Coeloptera*). *Prosiding Seminar Nasional Biologi*, *1*(2), 1739-1747.

Putri, A. I., Achyar, A., Putri, D. H., & Ahda, Y. (2021). Primer design, in silico pcr and optimum annealing temperature for *Escherichia coli* detection in refillable drinking water samples. *Tropical Genetics*, *1*(2), 52-60.

Rodríguez-Lázaro, D., Cook, N., & Hernández, M. (2013). Real-time pcr in food science: PCR diagnostics. *Current Issues in Molecular Biology*, *15*(2), 39-44.

Rodríguez, A., Rodríguez, M., Córdoba, J. J., & Andrade, M. J. (2019). Design of primers and probes for quantitative real-time pcr methods. *PCR Primer Design*, *1275*(3), 31-56.

Syamsurizal, A. M. D., Fevria, R., Atifah, Y., Badriyya, E., Achyar, A., & Robiansyah, I. (2021). Design of primer *Ipomoea batatas* chloroplast gene matK. *Tropical Genetics*, *2*(1), 12-16.

Thornton, B., & Basu, C. (2011). Real-time pcr (qpcr) primer design using free online software.

Biochemistry And Molecular Biology Education, *39*(2), 145-154.

Thornton, B., & Basu, C. (2015). Rapid and simple method of qpcr primer design. *Methods in Molecular Biology*, *1275*, 173-179.

Vaish, S., Gupta, D., Mehrotra, R., Mehrotra, S., & Kumar, M. (2020). Glutathione s-transferase : A versatile protein family. *3 Biotech*, *10*(7), 1-19. doi: 10.1007/s13205-020-02312-3.

Verma, G., Srivastava, D., Tiwari, P., & Chakrabarty, D. (2019). ROS modulation in crop plants under drought stress ros generation: An overview. In M. Hasanuzzaman, V. Fotopoulos, K. Nahar, & M. Fujita (Eds.), Reactive oxygen, nitrogen, and sulfur species in plants: Production, Metabolism, Signaling and defense mechanisms: Chapter 13 (pp. 311-336). Online Library Wiley.

Violita, V., & Azhari, S. (2021). Effect of peg-8000 imposed drought stress on rice varieties germination. *Journal of Physics: Conference Series*, *1940*(1). doi: 10.1088/1742-6596/1940/1/012071.

Wang, X., Spandidos, A., Wang, H., & Seed, B. (2012). PrimerBank: A pcr primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Research*, *40*(D1), 1144-1149.

Yang, G., Wang, Y., Xia, D., Gao, C., Wang, C., & Yang, C. (2014). Overexpression of a gst gene (thgstz1) from *Tamarix hispida* improves drought and salinity tolerance by enhancing the ability to scavenge reactive oxygen species. *Plant Cell, Tissue and Organ Culture*, *117*(1), 99-112. doi: 10.1007/s11240-014-0424-5.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-blast: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, *13*(134).

Zagorchev, L., Seal, C. E., Kranner, I., & Odjakova, M. (2013). A central role for thiols in plant tolerance to abiotic stress. *International Journal of Molecular Sciences*, *14*(4), 7405-7432.

Zagoto, A. D. P., & Violita, V. (2019). Leaf anatomical modification in drought of rice varieties (*Oryza sativa* L.). *Eksakta : Berkala Ilmiah Bidang MIPA*, *20*(2), 42-52. doi: 10.24036/eksakta/vol20-iss2/201.