

Primer Design and Optimization of Annealing Temperature for Analysis of Glutathione Reductase Gene Expression in Rice (*Oryza sativa* L.)

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Abstract

Glutathione Reductase (GR) belongs to the NADPH-dependent flavoprotein oxidoreductase family and is found in both prokaryotes and eukaryotes. The GR gene is considered to play a key role in the elimination of oxidative reaction products by looking at the level of gene expression of GR rice in dealing with drought stress using qPCR. One of the important steps to develop a specific, effective and efficient qPCR is the primer design. Several studies analyzing GR gene expression in rice have also designed primers. However, the primer still lacks an ideal characteristic of primer, as it still has a secondary structure. This studies aims to design rice GR specific primers and optimize the annealing temperature for GR gene expression analysis on rice. Primers were designed using the Primer3 and Geneious Prime and checked for specificity using the Primer-BLAST tool. The selected primer pairs were then optimized for annealing temperature using gradient PCR. The best primer design results were GR-Forward 5'-ACGATTGCAGCCAGTGAAGA-3' and GR-Reverse 5'-TGCGGCAATACTATCAACATCC-3', with an amplicon length of 204 bp, primer base lengths of 20 and 22 nucleotides, T_m values of 60°C and 58.9°C, %GC of 50% and 45.5%, respectively. This primer pair had no secondary structure, both hairpin and self dimer. Gradient PCR showed the optimum annealing temperature for this primer pair was 52.2°C so that the primer can be used as a specific primer to analyze the GR gene expression in rice using qPCR.

Keywords: annealing temperature, glutathione reductase, primer design, rice

1. Introduction

Glutathione reductase, also known as GSR or GR belongs to the NADPH-dependent flavoprotein oxidoreductase family and is found in both prokaryotes and eukaryotes. GR contains different domains such as NADPH binding domain, FAD binding domain and interface domain for joining two GR subunits [1]. Although GR is localized to chloroplasts, cytosol, and mitochondria, more than 80% of its activity in photosynthetic tissues is in chloroplast isoforms [2]. GR is known to be involved in protecting photosynthesis against oxidative stress [1]. One of which occurs due to drought stress [3].

Glutathione reductase plays an important role in cellular defense against reactive oxygen metabolites efficiently maintaining the cellular pool of reduced glutathione (GSH) by catalyzing the reduction of oxidized glutathione (GSSG)

to reduced glutathione (GSH) with a concomitant increase in NADPH oxidation. GR converts GSSG to GSH, thereby assisting in maintaining a high GSH/GSSG ratio under various abiotic stresses [2].

Glutathione reductase is one of the antioxidant enzymes produced by plants as a defense mechanism against drought stress. Based on the results of Violita (2007), GR enzyme activity increased with the length time of drought stress treatment in soybean plants. The highest increase in GR activity occurred on the 10th day of the drought stress treatment. This increase in GR activity is related to the role of GR in protecting plants from oxidative stress due to a decrease in the relative moisture content of the leaves. GR enzymes protect cells from oxidative damage through the regulation of cyclic ascorbate glutathione (ASH-GSH) [2]. GR enzyme activity in the ASH-GSH cycle is controlled by genes GR differ depending

on the type of group. This is in line with the results of Refli & Purwestri, (2016) research, that transcription patterns of GR gene change in different ways in response to drought stress and salinity in rice plants. GR gene transcription regulates glutathione reductase activity in plants. Therefore, GR enzymes may have less activity in drought-treated rice seedlings compared to those treated with salinity. This may be due to more GR genes which involves setting strategies for adapting rice seedlings to salinity compared to drought strategies [4].

To find out how the mechanism of the GR enzyme in dealing with drought stress, further research on the expression of the GR gene in dealing with drought stress is required. This can be accomplished by examining differences in the activity levels and transcription of genes encoding the GR enzyme, which is expected to play a vital role in reduced glutathione defense (GSH) and the removal of oxidative reaction products [4].

Gene expression is comprised of two stage such as transcription and translation. To express its genetic information, cells carry out various processes, including copying genetic information from DNA to mRNA (messenger RNA), this process is called transcription, and then followed by the translation of the genetic information contained in the mRNA molecule into protein, this process is called translation [5].

The technique most frequently used to analyze differential mRNA expression is quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR is an effective method for observing changes in gene expression during processes such as cellular differentiation because it is highly sensitive and specific [6]. The qRT-PCR was used to see the quality of gene expression through formation of complementary DNA (cDNA) formed from RNA and calculate the quantity of cDNA amplification produced [7]. In the cDNA amplification stage with PCR, a pair of primers is needed (forward and reverse), to limit the area to be amplified [8].

PCR success depends on the primer used. Primers are single-stranded oligonucleotide molecules consisting of approximately 18-30 bases. A good primer is determined by several primer criteria. These criteria include: primer length between 18-22 mer, %GC ranging from 40% - 60%, T_m (melting temperature) 58-60°C and not more than 65°C, no primer interactions (dimers and hairpins), primer stability, repeats, runs and false priming [9].

The key to PCR success is inseparable from bioinformatics studies when designing specific primers for target genes [10]. The success of gene amplification by PCR using specially designed primers is largely determined by the accuracy of the primer attachment temperature (annealing) with a DNA template. If the temperature is too low it can cause the primer to stick to the genomic DNA or attach to a non-specific place, while the temperature is too high it will prevent the amplification process from occurring [11]. For this reason, it is necessary to optimize the PCR process so that the PCR

results obtained are optimal [12]. Several studies analyzing GR gene expression in rice have also designed primers [13], [14]. However, the primer still lacks an ideal characteristic of primer, as it still has a secondary structure, both hairpin and dimer. This secondary structure can reduce PCR efficiency. Based on this, the purpose of this study were to design rice GR specific primers and optimize the annealing temperature for GR gene expression analysis on rice.

2. Methodology

This study was research based on molecular biology and computational testing (bioinformatics). Gene nucleotide sequence of GR (NCBI accession number: XM_015757647.2) which will be modified referring to research [13], [14]. Primers were designed using the program primer3 on the site Primer3web (<https://primer3.ut.ee/>). Generated primer candidates were analyzed further using Geneious Prime software [15], then target gene specificity was analyzed using primer-BLAST on the NCBI website [10]. The primers obtained were tested for their annealing temperature using gradient PCR, electrophoresis using 1.5% agarose gel and visualization using Gel Doc.

2.1 Primer Design

GR accession number of (XM_015757647.2) was entered on column search on the NCBI site. The symbol for this gene is LOC4348623 with the locus tag OSNPB_100415300 found in chloroplasts [16]. Then the primer generated using 'pick primer' tools. The length of the PCR product was adjusted for qPCR analysis in the "PCR product size" column, which was a minimum of 150 and a maximum of 250 bp.

2.2 Primer Analysis Using Geneious Prime and Primer-BLAST

The FASTA file of GR sequence (XM_015757647.2) was downloaded from the NCBI website, then imported to the Geneious Prime software. All candidate primer sequences that have been designed using 'pick primer' on the NCBI website were copied and saved as primer sequences in Geneious Prime. The primer criteria were shown on primer annotation and also the DNA fold displayed for each primer. Then a primer pair that fits the criteria for a good primer was chosen. After obtaining the best primer, then the specificity of the primer was checked using the primer-BLAST tool on the NCBI website. Selected primer pair was synthesized in IDT, Singapore.

2.3 Rice Root RNA Extraction

TRNA extraction from rice root was using GENEzol™ Reagent (Cat. GZR100). All stages of extraction followed the GENEzol procedure™. Samples were weighed 50–100 mg and then crushed using liquid nitrogen with the help of a micropestle. To the samples 500 µl GENEzol was added and

incubated for 10 minutes at room temperature. After that, 200 µl of chloroform was added to the sample tube as much as 500 µl by shaking gently for 10 seconds. Sample mixture was centrifuged at 16,000 rpm for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to a new tube. Isopropanol alcohol was added according to the volume of RNA into the tube and then incubated at room temperature for 10 minutes. Sample was centrifuged at 16,000 rpm for 10 minutes at 4°C then the supernatant was discarded without disturbing the pellet. RNA pellets were washed using 70% ethanol and centrifuged at 16,000rpm, 5 minutes at 4°C. The supernatant was discarded again without disturbing the pellet then air dried the RNA pellet for 10 minutes at room temperature. To resuspend the RNA pellet 50 µl of nuclease-free water was added then incubated at 55°C to dissolve the pellets.

2.4 cDNA Synthesis

cDNA synthesis was performed using reverse transcriptase (RT) enzymes in combination with qPCR with the Sensifast cDNA Synthesis Kit. qRT-PCR was carried out by preparing a cDNA synthesis reaction mixture. The sample was briefly homogenized with a vortex, then put into the thermocycler and run for 30 minutes, with annealing settings at 25°C for 10 minutes, reverse transcription stage at 42°C for 15 minutes, and inactivated at 85°C for 5 minutes and the cDNA can be directly used as a template for qPCR and stored at -20°C.

2.5 Gradient PCR

Optimization of annealing temperature was conducted using gradient PCR. PCR reaction in 10 µl consisting of 0.5 µl rice root cDNA, 0.4 µl of 10 µM forward and reverse primers, 5 µl of 2x GoTaq PCR master mix (Promega) and 3.7 µl nuclease-free water. PCR temperature cycle, i.e. initial step 95°C for 3 minutes, followed by 35 cycles of denaturation 95°C for 30 seconds, annealing temperature was set gradient 50-60°C for 30 seconds, elongation 72°C for 30 seconds, and final elongation 72°C for 5 minutes. The PCR products were separated using electrophoresis using 1.5% agarose gel with 1x TAE buffer. Electrophoresis was carried out at 100 V for 35 minutes. Furthermore, the electrophoresis result was visualized using Gel Doc.

3. Results and Discussion

3.1 Primer Design

Based on the design results on the program 'pick primer', 10 forward primers and 10 reverse primers (10 primer sets) were obtained Table 1. Forward primer is a primer located at the front end of the DNA target and serves to mark the front end of the DNA strand to be duplicated; in other words, forward primer will go from the 5' end to the 3' end. Meanwhile, the reverse primer is located at the back end of the DNA target [9].

Of the 10 sets of primer candidates generated, each has a

different amplicon length and nucleotide arrangement. Amplicons are target DNA strands that have been successfully duplicated during the PCR process [17]. The length of the PCR amplification amplicons depends on the purpose of the research to be carried out. Standard PCR amplicon lengths are 100-500 bp. Products with a length of 1000 bp require 1 minute for the extension process at the PCR stage [18]. Based on the data of the primer candidates in Table 1, each primer has a length of <2000 bp so that the required extension time for PCR is also less than 1 minute.

3.2 Primer Analysis Using Geneious Prime and Primer-BLAST

Analysis of primer candidates was conducted based on good primer criteria using Geneious Prime. In general, the optimal base length for qPCR primer was 18-22 nucleotides [19]. Based on the primer candidate data (Table 1), all primer candidate sets 1 to 10 are good primers forward and reverse which have primer length of about 20-22 nucleotides, this meets the criteria for optimal base length.

Melting temperature (Tm) is the temperature at which 50% of the DNA double strands separate. The optimal primer Tm for qPCR is 58°C – 60°C [20]. In this study, 9 sets of primer pair candidates (forward and reverse) have a Tm between 58-60°C. The already meets the optimal primer Tm for qPCR. While primer set 6 has reverse primer Tm of 54.2°C so that it does not meet the criteria for a good Tm (Table 1). If the Tm primer is too low, the primer tends to anneal elsewhere and produce non-specific products. While if Tm is too high (> 65°C), it will reduce the effectiveness of annealing which can lead to failure of the DNA amplification process. Primer pair must have a difference in the Tm value of not more than 5 because it can cause a decrease in the amplification process or even no amplification process occurs. Tm can be calculated manually using the formula $Tm = 2(A+T) + 4(G+C)$. The Tm of a primer must be chosen carefully because it has a significant impact on temperature annealing used in the PCR process [19].

Percentage (%) GC is the number of percentages of guanine and cytosine in a primer. %GC should be in the range of 40-60% [9]. In this study, all sets of primers forward and reverse already met the criteria for a good %GC (41.7% - 55%), except primer set 8 because the reverse primer only has 39.1% (Table 1). Primers with %GC below 50% require a base length of more than 18bp to keep Tm above the recommended minimum. The 9 sets of primer pair candidates have fulfilled the primer criteria of %GC because they are in the range of 40-60% [17].

The ideal PCR product size or primer amplicon length for qPCR is between 100-250 bp to increase amplification efficiency [20]. In this study, only 2 primer sets (forward and reverse) which met the ideal amplicon length criteria, namely set 2 with an amplicon length of 196 bp and set 10 with an am-

plicon length of 204 bp. Meanwhile, the other primer candidates do not meet the ideal amplicon length criteria (Table 1).

Table 1. Specification of GR primer candidates

Set	Primer	Sequence (5' > 3')	Tm	% GC	Amplicon Size
1	Forward	TGTTGCAGTCTGACCAGTCG	60.2	55.0	289 bp
	Reverse	TGCGGCAATACTATCAACATCC	58.9	45.5	
2	Forward	CAGCCAGTGAAGAACGGAGA	59.7	55.0	196 bp
	Reverse	GCGGCAATACTATCAACATCCTG	59.8	47.8	
3	Forward	AACCGCGGAAACAACAGACA	60.7	50.0	262 bp
	Reverse	GCGGCAATACTATCAACATCCTG	59.8	47.8	
4	Forward	CCGCGGAAACAACAGACAGA	60.9	55.0	260 bp
	Reverse	GCGGCAATACTATCAACATCCT	58.6	45.5	
5	Forward	TTGCAGTCTGACCAGTCGAT	59.0	50.0	288 bp
	Reverse	ATGCGGCAATACTATCAACATCC	59.2	43.5	
6	Forward	TTGTGTTGCAGTCTGACCAGT	60.1	47.6	291 bp
	Reverse	GCGGCAATACTATCAACATCC	54.2	47.6	
7	Forward	TCTAACCGCGGAAACAACAGA	59.9	47.6	268 bp
	Reverse	CATGCGGCAATACTATCAACATCC	60.4	45.8	
8	Forward	CGCGGAAACAACAGACAGAT	58.9	50.0	263 bp
	Reverse	TCATGCGGCAATACTATCAACAT	58.6	39.1	
9	Forward	ACCGCGGAAACAACAGACA	60.2	52.6	265 bp
	Reverse	TCATGCGGCAATACTATCAACATC	59.5	41.7	
10	Forward	ACGATTGCAGCCAGTGAAGA	60.0	50.0	204 bp
	Reverse	TGCGGCAATACTATCAACATCC	58.9	45.0	

A good primer can't have a secondary structure like a hairpin and dimer. Stability secondary structure determined by the free energy (ΔG) and the melting temperature. This causes the primer to not anneal to the DNA template. A hairpin is a structure formed by polynucleic acid base pairing between complementary single-stranded sequences of either DNA or RNA. The formation of hairpin structures in primers should be avoided, but it is very difficult to obtain primers without hairpin structures. Primer also may not bond with their partner primer which is called pair dimer nor self-dimer which is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Of the primer candidates that have been designed, only 2 primer sets meet these criteria, namely primer set 2 and primer set 10 (Table 1).

Based on the analysis of primer above, the selected primers are primer sets of 10 that have met the criteria for good primers, namely with base lengths of 20 and 22 nucleotides, Tm 58.9 and 60°C, %GC 50% and 45.5 %, amplicon length 204 bp and has no secondary structure (hairpin and self-dimer). The specifications for the selected primer candidates can be seen in Table 2. This primer set 10 is then named by GR primer.

The specificity of the designed primers was checked using the Primer BLAST tool on the NCBI website (Achyar et al., 2021). NCBI Primer BLAST results (Figure 1) indicate that primer pairs can amplify GR mRNA sequence with amplicon length GR of 204 bp (Figure 2).

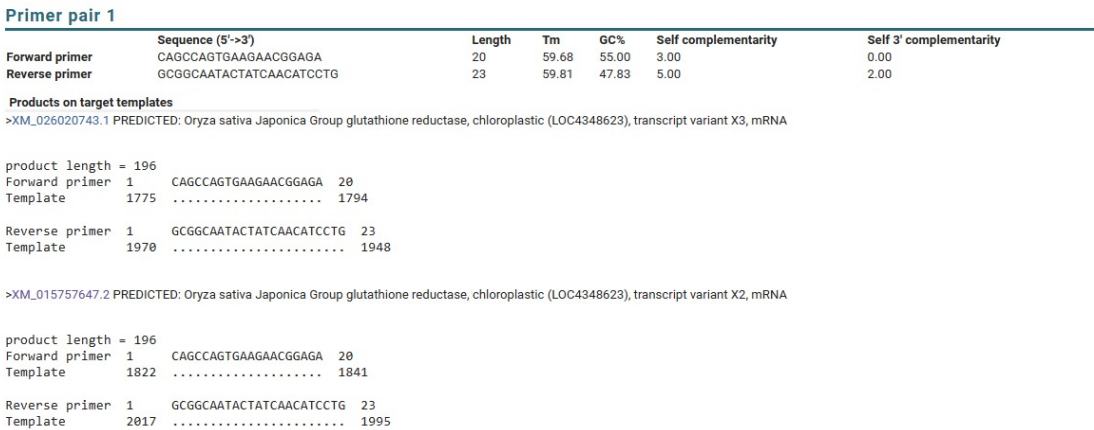


Figure 1. NCBI primer BLAST results from primer set 10

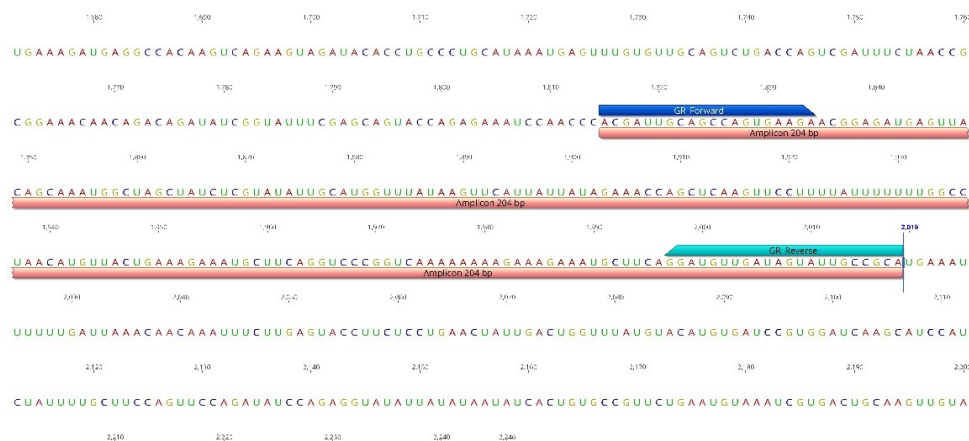


Figure 2. Amplicon length of primer set 10

Specificity checks were carried out to determine the number and type of genes GR that can be detected using the primer as well as other organisms that can also be detected by the primer [21].

Table 2. Selected primers (primer set 10) characteristics analyzed using Geneious Prime

No	Primary characteristics	DNA Fold
1	Name: GR_Forward Type: Primer Bind (primer_bind) (Created by primer3) Length: 20 Interval: 1,815 -> 1,834 %GC: 50.0 Hairpin Tm: None Self Dimer Tm: None Tm: 60.0 Sequence: ACGATTGCAGCCAGTGAAGA Product size: 204 bp Mismatches: 0 # Local Off-target Sites: 0 Local Off-target: XM_015757647	
2	Name: GR_Reverse Type: Primer Bind (primer_bind) (Created by primer3) Length: 22 Interval: 2,018 -> 1,997 %GC: 45.5 Hairpin Tm: None Self Dimer Tm: None Tm: 58.9 Sequence: TGC GCAATACTATCAACATCC Product size: 204 bp Mismatches: 0 # Local Off-target Sites: 0 Local Off-target: XM_015757647	

3.2 Primer annealing temperature (Ta) optimization

Optimization of annealing temperature (Ta) using gradient PCR aims to test primer pairs in order to obtain the optimum Ta in amplifying target genes [22]. The optimal Ta is typically 5°C lower than the Tm of the primer-ssDNA template. The Ta is determined by the number and order of nucleotides in the primer [23].

Based on the results of agarose gel electrophoresis of primer set 10 (Figure 3), the optimum annealing temperature is at 52.2°C because the resulting DNA bands are brighter and thicker than the other DNA bands. According to Iqbal et al., (2016), bands that are clear, brilliant, unbroken or not smeared are considered to meet the requirements for good DNA bands.

In this study all samples had bands that were clearly visible, brilliant, thick and no unspecific band nor dimer. The size of the amplicon is also in accordance with the results of the design. Mubarak et al. (2020) stated that an annealing temperature that is too high prevents optimal primer-template binding, whereas an annealing temperature that is too low can result in non-specific binding and, consequently, non-specific PCR products. After the primers hybridize to the templates, extension occurs [23].

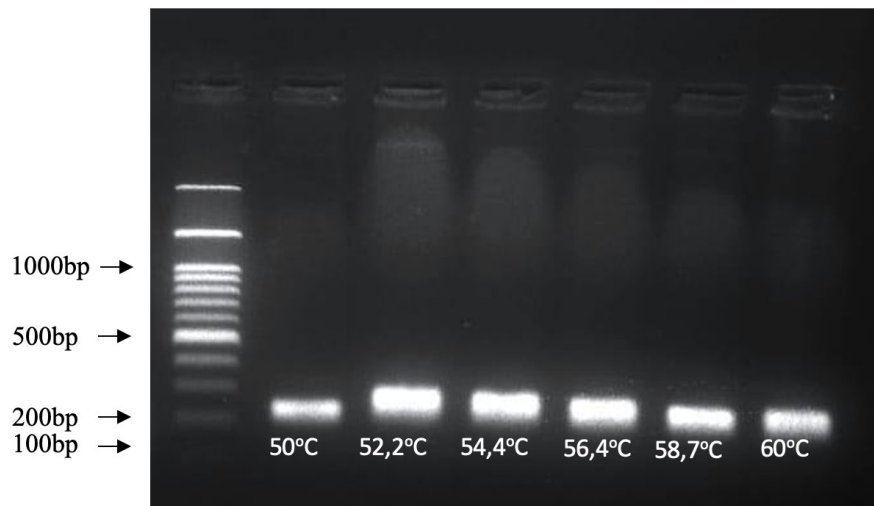


Figure 6. Visualization of electrophoresis results of PCR products with primer set 10 on cDNA of rice roots stressed by drought at a temperature range of 50-60°C using a 100 bp marker

4. Conclusion

The best primer design results were primer set 10 which is named GR primer. The characteristic of GR primer are GR-forward 5'-ACGATTGCAGCCAGTGAAGA-3' and GR-reverse 5'-TGCGGCAATACTATCAACATCC-3', amplicon length 204 bp, base lengths 20 and 22 nucleotides, T_m values 60 °C and 58.9 °C, %GC 50% and 45.5%, respectively. Both primers had no secondary structure (hairpin and self dimer) with 52,2°C as optimum annealing temperature so that these primers can be used as a specific primer to amplify the GR gene in rice using qPCR.

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