JPPIPA 12(5) (2026)

Jurnal Penelitian Pendidikan IPA

http://jppipa.unram.ac.id/index.php/jppipa/index

Design and Experimental Validation of qPCR Primer for Drought-Responsive CaHsfA2 Gene in *Capsicum annuum* for Sustainable Crop Production

Suwirmen[1*], Zozy Aneloi Noli[1], Anthonie Agustien[1], Djong Hon Tjong[1], Amanda Nurhafitri[1]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, Indonesia

Received: February 02, 2026
Revised: April 30, 2026
Accepted: May 25, 2026
Published: May 31, 2026

*Corresponding Author:
Suwirmen
suwirmen.bio1963@gmail.com

DOI: 10.29303/jppipa.v12i5.14897

Open Access

© 2026 The Authors. This article is distributed under a (CC-BY License)

Abstract: Drought stress severely reduces plant productivity by disrupting photosynthesis and cellular homeostasis. Plants respond through regulatory networks involving stress-responsive genes, including heat shock proteins (HSPs), regulated by heat shock transcription factors (HSFs). Among these, HsfA2 plays an important role in abiotic stress tolerance, including drought. Although CaHsfA2 expression has been reported in *Capsicum annuum*, validated primers for quantitative PCR (qPCR) analysis remain limited. This study aimed to design and validate specific primers for CaHsfA2 expression analysis. Primer candidates were designed from sequence NM_001324561.1 using Primer-BLAST and evaluated based on length, GC content, melting temperature (Tm), and self-complementarity. Total RNA was extracted from chili roots subjected to drought stress (50% field capacity) and nanopriming with *Padina minor*, followed by cDNA synthesis, gradient PCR and agarose gel electrophoresis.. Secondary structures were analyzed using OligoAnalyzer. Of ten primer pairs, four met the criteria, and primer set 1 (1_CaHsfA2) showed optimal characteristics (%GC 55%, Tm 60°C, product length 190 bp) and minimal secondary structure potential ($\Delta G > -9$ kcal/mol). Gradient PCR (55.3–59.2°C) produced a single band (~193 bp), consistent with the predicted size (190 bp), with an optimal annealing temperature of 57.3°C, confirming its specificity and suitability for qPCR-based gene expression analysis under drought conditions.

Keywords: CaHsfA2; C. annuum; Design Primer; Drought stress; qPCR

Introduction

Abiotic environmental factors are key determinants of crop productivity, with drought being one of the most detrimental stresses affecting plant growth. Drought induces stomatal closure, reduces CO_2 entry, impairs photosynthetic rate, and ultimately inhibits plant growth and productivity (Hasanuzzaman et al., 2020; Zandalinas et al., 2021). Drought stress occurs as a consequence of insufficient rainfall or deficient soil moisture (Zhou et al., 2017). To survive under such conditions, plants activate complex physiological and molecular defense mechanisms to maintain cellular function stability (Ohama et al., 2016). Therefore, understanding the molecular basis of drought tolerance is crucial for enhancing plant resilience and supporting sustainable agriculture.

At the cellular level, plants respond to drought stress through complex regulatory networks that modulate the expression of stress-responsive genes, including heat shock proteins (HSPs) (Y. Li et al., 2025; Tian et al., 2021). HSPs function as molecular chaperones to maintain protein stability and prevent protein misfolding under stress conditions. Their expression is regulated by heat shock transcription factors (HSFs), which play important roles not only in heat stress but also in other abiotic stresses, including drought (Gomez-Pastor et al., 2017; Wang & Xu, 2025). One of the common

---

How to Cite:
Suwirmen, Zozy Aneloi Noli, Agustien, A., Tjong, D. H., & Nurhafitri, A. (2026). Design and Experimental Validation of qPCR Primer for Drought-Responsive CaHsfA2 Gene in Capsicum annuum for Sustainable Crop Production. *Jurnal Penelitian Pendidikan IPA*, *12*(5), 128–135. https://doi.org/10.29303/jppipa.v12i5.14897

HSFs in plants is HsFA2. This gene contributes to drought resilience by maintaining protein homeostasis and redox balance (Y. Li et al., 2025).

Red chili peppers (*Capsicum annum* L.) are a horticultural commodity that plays a significant role in Indonesian agriculture. However, chili pepper production in Indonesia often faces serious challenges due to drought, which can reduce plant growth and yield. Drought is one of the primary environmental stresses with significant impacts. Drought stress causes negative physiological effects in chili peppers, such as reduced seed viability, decreased chlorophyll content, and disrupted photosynthesis, which ultimately suppresses plant productivity (Roziqoh et al., 2023; Suwirmen et al., 2025). Therefore, chili pepper sensitivity to drought is a primary concern in chili pepper production (Li, 2009).

Gene expression analysis is generally performed using quantitative PCR (qPCR), a method used to measure transcript abundance. However, the accuracy of qPCR is highly dependent on the quality of the primers used. Proper primer design is the initial step in the PCR or qPCR process and determines the overall amplification performance (Praja, 2021). Poorly designed primers result in nonspecific amplification, secondary structure formation, and inaccurate quantification in qPCR. According to Mubarak et al. (2020), good primer criteria include a length of 18–25 bp, a melting temperature (Tm) of 52–58°C, a GC content of 45–60%, and a secondary structure with the lowest free energy. Therefore, this study designed specific primers for the HsFA2 gene in chili peppers, designated as CaHsfA2.

Several studies have successfully examined the expression of the HsFA2 gene in *Solanum lycopersicum* (Fragkostefanakis et al., 2016), *Nicotiana tabacum* (Kotak et al., 2004), *Solanum melongena* (Gong et al., 2021), and *Solanum tuberosum* (Tang et al., 2016). Gene expression analysis in *Capsicum annuum* has also been conducted (Guo et al., 2015). However, none of these studies have focused specifically on designing CaHsfA2 primers for use in qPCR analysis. Therefore, this study aims to design CaHsfA2 primers for qPCR through the design and validation of specific primers on chili pepper cDNA.

Method

Design Primer

The primers were designed based on the sequence from the study by Guo et al. (2014) with NCBI accession number NM_001324561.1. Based on this nucleotide sequence, a forward and reverse primer pair was designed using the NCBI Primer Designing Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) to generate primer recommendations. Subsequently, the primer pair was selected based on recommended criteria, including a melting temperature (Tm) 50-65°C) (Chuang et al., 2013; Wang, 2016), GC content of 45–60%, primer length of 18–25 bp, and minimal self-complementarity as well as 3′ self-complementarity (Mubarak et al., 2020). Furthermore, the specificity of the primer candidates toward other species was analyzed using NCBI Primer-BLAST.

Secondary Structure Analysis of Primers

Primer candidates were reselected based on their secondary structure using OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer. Hairpins and dimers formed by the forward and reverse primers must have a ΔG value greater than -9 kcal/mol (Prediger, 2024).

Chili Pepper Root RNA Extraction

Total RNA was extracted from roots of 60-day-old chili pepper plants subjected to drought stress (50% field capacity) and nanopriming treatment with *Padina minor*, using the Total RNA Mini Kit (Plant) from Geneaid. Total RNA was extracted from approximately 50 mg of chili pepper root tissue according to the kit protocol. The sample was frozen with liquid nitrogen and ground into a fine powder, then lysed using 500 µL RB buffer supplemented with 5 µL β-mercaptoethanol, incubated at 60°C for 5 minutes, and filtered using a filter column. The clear filtrate was mixed with absolute ethanol to precipitate the RNA, then applied to an RB column and centrifuged. To reduce genomic DNA contamination, in-column treatment with DNase I + DNase I RB was performed. This was followed by washing steps using W1 buffer and wash buffer, followed by drying of the column matrix. The purified RNA was eluted using 50 µL RNase-Free Water and its quantity was measured using a NanoSpectrophotometer. Subsequently, a sample with a concentration of 100 ng/µL was obtained after dilution and stored in a -80°C freezer.

cDNA Synthesis

cDNA synthesis was performed using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover according to the manufacturer's instructions. Briefly, total RNA (100 ng/ µL) was used as a template and denatured at 65°C for 5 minutes, followed by immediate cooling on ice. Genomic DNA contamination was removed by incubating the RNA sample with 4× DN Master Mix at 37°C for 5 minutes. Subsequently, reverse transcription was carried out by adding 5× RT Master Mix II and incubating at 37°C for 15 minutes, followed by an optional step at 50°C for 5 minutes. The reaction was terminated by heating at 98°C for 5 minutes. The synthesized cDNA was then used PCR analysis and stored at −20°C until further use.

PCR Gradient

Optimization of primer annealing temperature using a PCR Gradient. The PCR reaction consists of 12.5 µL Bioline Redmix PCR, 10.5 µL nuclease-free water, 1 µL cDNA isolate, and 1 µL (10 µM) each of forward and reverse primers. The PCR cycle consists of an initial step at 95°C for 1 minute, denaturation at 95°C for 15 seconds repeated for 35 cycles, annealing at 55–60°C, extension at 72°C for 30 seconds, and a final extension for 5 minutes. The PCR products were separated by 2% agarose gel electrophoresis (100V for 50 minutes) using 1x TAE buffer. They were then visualized using Uvitec gel documentation.

Result and Discussion

Design Primer

The design of specific primers for the CaHsfA2 gene, based on a 1436 bp sequence from GenBank with accession number NM_001324561.1, was performed using the NCBI primer design tool as an initial step. Based on this, 10 primer recommendations were obtained from the CaHsfA2 gene sequence (Table 1). Primer sets 1 through 10, consisting of forward and reverse primers, ranged in size from 19–21 bp, with product lengths of 142–846 bp, GC content of 47–60%, self-complementary regions of 2–6 bp, self-complementary regions of 0–3 bp, and and a relatively uniform melting temperature (Tm) around 60°C**.**

When designing primers, primer length is a critical parameter that determines the success of amplifying specific fragments of the target gene. Based on the primer set recommendations in Table 1, primer lengths range from 19–21 bp and fall within the ideal range of 18–30 bp (Borah, 2011). Shorter primers (<18 bp) can reduce binding specificity to the template, increasing the risk of mispriming and non-specific binding (Hung & Weng, 2016). However, if the primer is too long, it may induce the formation of secondary structures (Syamsidi et al., 2021). Therefore, the primer lengths most commonly used for PCR or qPCR are 20–22 bp (Chen et al., 2023; Hidayah et al., 2025; Syamsidi et al., 2021).

Tabel 1. Primer Design of CaHsfA2 from NCBI Primer-BLAST

Set		Sequence (5′-3′)	Size (bp)	GC (%)	Tm (°C)	Self 3′ comp	PL (bp)
1*	F	TGGAGCAGAGGAAGTACCGA	20	55	59.96	0	190
	R	GTGCTAAGAACGCCAGAGGA	20	55	59.75	0	
2	F	CCTCTGGCGTTCTTAGCACA	20	55	60.04	3	142
	R	TGCACCCCACGACTCTATCA	20	55	60.61	1	
3	F	GGAGACTGACAATGACCCCC	20	60	59.75	0	451
	R	AGGAACAACAAGAGGTCCCC	20	55	59.23	3	
4	F	AAAGCTTCAGTTTGCGGGGA	20	50	60.47	0	180
	R	GCTTTATGCACCCCACGACT	20	55	60.68	1	
5*	F	TCTTGTTGTTCCTCTGGCGT	20	50	59.53	0	153
	R	ATGCACCCCACGACTCTATC	20	55	59.25	0	
6	F	TGACAGCAAGTGGAACCGAT	20	50	59.60	2	245
	R	GAGGTCCCCGCAAACTGAAG	20	60	60.96	2	
7	F	GGTGGATCCTGACAGATGGG	20	60.	59.53	0	846
	R	AAGAGGTCCCCGCAAACTG	19	58	59.93	1	
8	F	GACTGACAATGACCCCCAGT	20	55	59.31	1	459
	R	AAGAACGCCAGAGGAACAACA	21	47	60.13	0	
9*	F	TTTGCGGGGACCTCTTGTTG	20	55	60.82	0	169
	R	CTTTATGCACCCCACGACTCT	21	52	60.07	1	
10*	F	GTTTGCGGGGACCTCTTGTT	20	55	60.82	0	164
	R	GCACCCCACGACTCTATCATT	21	52	59.86	1	

PL: Product Length

*indicate primers set that showed criteria of a good primer

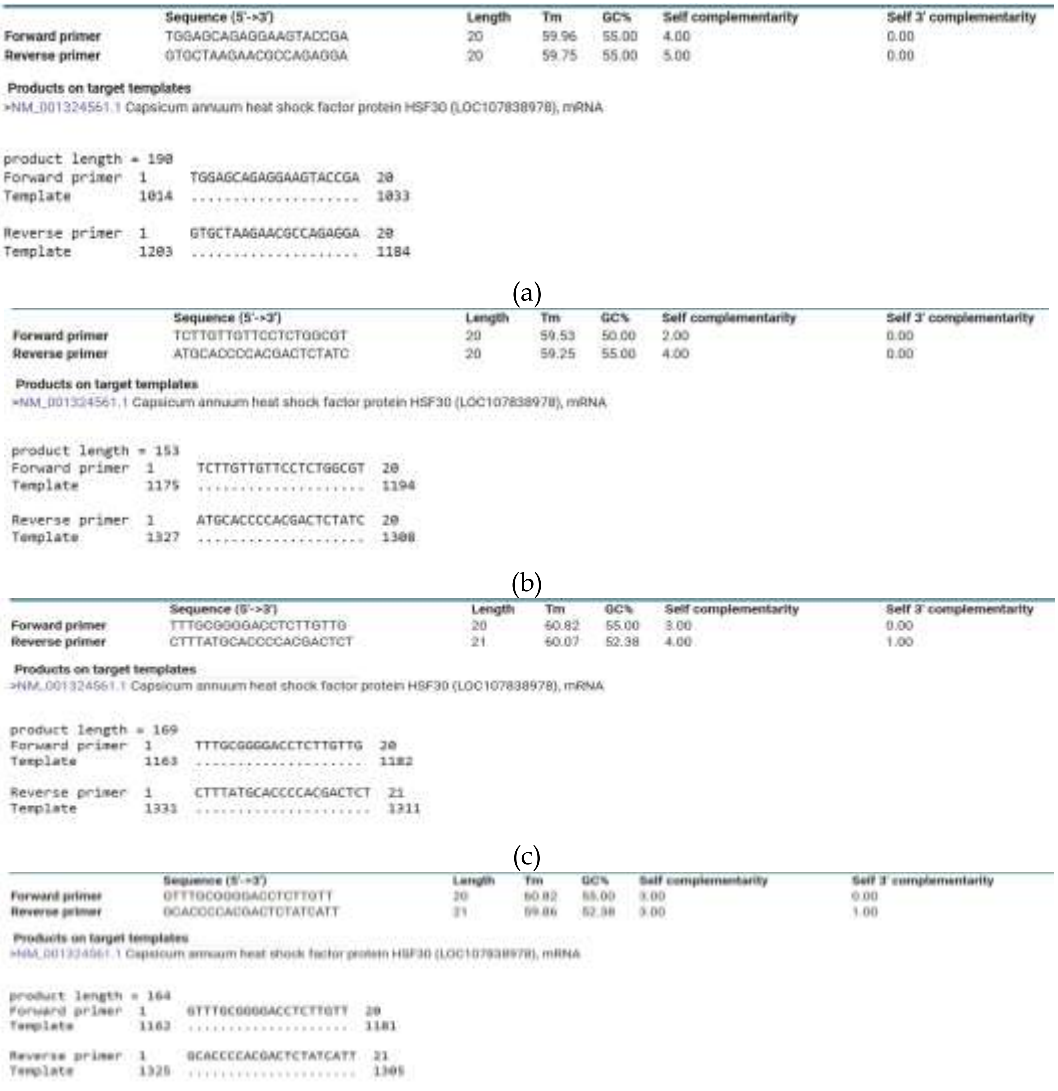
The composition of Guanine (G) and Cytosine (C) bases affects the strength of the primer's binding to the template during the PCR amplification process. This is because G-C base pairs form three hydrogen bonds, whereas A-T base pairs form only two hydrogen bonds (Alberts et al., 2017). Therefore, the GC content of primers is generally recommended to be within the range of 40–60%, with an ideal percentage of 50% (Prediger, 2024). The higher the G and C content in a primer, the higher the Tm required to dissociate these bonds (Atifah & Achyar, 2023). Ideally, Tm should be within the range of 50–65°C (Chuang et al., 2013; Wang, 2016). Based on these parameters, the primer sets in

Table 1 have shown GC% and Tm values that meet the criteria.

Another important parameter to consider when selecting primers is 3′ self-complementarity. This value arises when there is base pairing at the 3′ end of the primer that can trigger the formation of primer dimers, which can be extended by DNA polymerase (Thornton & Basu, 2011). This, in turn, affects the efficiency of primer annealing to the target sequence. The maximum value for self-3′ complementarity is 3, and the lower this value, the better the primer is for use (Shen et al., 2010). In Table 1, all recommended primer sets have self-3′ complementarity values below 3 for both the forward and reverse primers. However, the primer sets with the lowest self-3′ complementarity (a value of 0) are set 1 and set 5.

The PCR product length is the number of base pairs (bp) of the DNA fragment amplified by a pair of primers. This parameter plays a crucial role, particularly in qPCR, as it affects amplification efficiency and detection sensitivity. Shorter amplicons generally provide better amplification efficiency (Bustin et al., 2009), with a recommended range of approximately 70–150 bp (Prediger, 2024). However, some studies still use sizes up to ±150–250 bp as long as reaction efficiency remains optimal (Khaira et al., 2023).

Based on these primary criteria, this study selected primer sets with the lowest self-3′ complementarity (sets 1 and 5) and product lengths of 150–200 bp (sets 1, 5, 9, and 10). This range was chosen as a compromise between qPCR amplification efficiency and visualization requirements during the initial validation stage, as fragments that are too short are difficult to observe clearly on agarose gel electrophoresis.



	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGGAGCAGAGGAAGTACCGA	20	59.96	55.00	4.00	0.00
Reverse primer	GTGCTAAGAACGCCAGAGGA	20	59.75	55.00	5.00	0.00

Products on target templates

>NM_001324561.1 Capsicum annuum heat shock factor protein HSF30 (LOC107838978), mRNA

```
product length = 190
Forward primer  1     TGGAGCAGAGGAAGTACCGA  20
Template        1014  ....................  1033

Reverse primer  1     GTGCTAAGAACGCCAGAGGA  20
Template        1203  ....................  1184
```

(a)

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TCTTGTTGTTCCTCTGGCGT	20	59.53	50.00	2.00	0.00
Reverse primer	ATGCACCCCACGACTCTATC	20	59.25	55.00	4.00	0.00

Products on target templates

>NM_001324561.1 Capsicum annuum heat shock factor protein HSF30 (LOC107838978), mRNA

```
product length = 153
Forward primer  1     TCTTGTTGTTCCTCTGGCGT  20
Template        1175  ....................  1194

Reverse primer  1     ATGCACCCCACGACTCTATC  20
Template        1327  ....................  1308
```

(b)

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTTGCGGGGACCTCTTGTTG	20	60.82	55.00	3.00	0.00
Reverse primer	CTTTATGCACCCCACGACTCT	21	60.07	52.38	4.00	1.00

Products on target templates

>NM_001324561.1 Capsicum annuum heat shock factor protein HSF30 (LOC107838978), mRNA

```
product length = 169
Forward primer  1     TTTGCGGGGACCTCTTGTTG  20
Template        1163  ....................  1182

Reverse primer  1     CTTTATGCACCCCACGACTCT  21
Template        1331  .....................  1311
```

(c)

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTTTGCGGGGACCTCTTGTT	20	60.82	55.00	3.00	0.00
Reverse primer	GCACCCCACGACTCTATCATT	21	59.86	52.38	3.00	1.00

Products on target templates

>NM_001324561.1 Capsicum annuum heat shock factor protein HSF30 (LOC107838978), mRNA

```
product length = 164
Forward primer  1     GTTTGCGGGGACCTCTTGTT  20
Template        1162  ....................  1181

Reverse primer  1     GCACCCCACGACTCTATCATT  21
Template        1325  .....................  1305
```

(d)

Figure 1. NCBI Primer-BLAST result from primer set 1 (a), set 5 (b), set 9 (c), set 10 (d)

Based on BLAST analysis using NCBI Primer-BLAST, primer sets 1, 5, 9, and 10 are specific to the species *C. annuum*. According to Gheno et al. (2026), Primer-BLAST is a tool that can be used to verify primer specificity against genomic databases. This step is crucial to avoid false positives in diagnostic testing. Next, the secondary structure characteristics of primer sets 1, 5, 9, and 10 were analyzed to assess the potential for hairpin and dimer formation, which could affect amplification performance.

Secondary Structure Analysis

Forward and reverse primer candidates from sets 1, 5, 9, and 10 analyzed using Oligo Analyzer showed relatively small ΔG hairpin values (–1.57 to 1.47 kcal/mol), indicating a weak tendency for hairpin formation. Self-dimer values ranged from -7.05 to -0.96 kcal/mol; while most primers remained within a safe range, those from sets 5 and 9 exhibited a stronger tendency toward dimerization. Base complementarity at the 3′ end ranged from 2 to 4 bp, which is still tolerable. Meanwhile, heterodimers had ΔG values ranging from -9.21 to -0.96 kcal/mol, with the strongest interactions found in sets 9 and 10.

The formation of secondary structures, namely dimers and hairpins, must be taken into account when designing primers (VanGuilder et al., 2008). This is because secondary structures are artifacts that can form during PCR and qPCR, affecting the efficiency and specificity of amplification. A hairpin forms when a portion of the nucleotides (two or three) in a primer hybridizes with itself, creating a loop structure that can generate nonspecific products or even inhibit amplification (Singh & Singh, 2000). Dimerization occurs when two identical primers (self-dimers) or a forward-reverse primer pair (heterodimers) hybridize due to base complementarity. The formation of these dimer structures can lead to amplification of the primer itself rather than the target amplicon (Gheno et al., 2026).

Oligoanalyzer tools are used to predict the formation of hairpins and dimers based on Gibbs free energy (ΔG) values (Caro et al., 2022; Prediger, 2024). A larger (more positive) ΔG value indicates that the secondary structures (hairpins and dimers) gave weak bonds and are less likely to form. The ΔG value must be more positive than -9 kcal/mol, and complementarity must not exceed 4 bp (Handoyo & Rudiretna, 2001; Meagher et al., 2018). Therefore, based on these criteria, primer set 1 exhibited the lowest tendency for secondary structure formation compared to the other sets; consequently, it was selected as the best primer candidate and named 1_CaHsfA2 to proceed to primer synthesis.

Table 2. Secondary structure analysis based on ΔG value from OligoAnalyzer

Set		Hairpin (ΔG : kcal/mol)	Self-dimer (ΔG : kcal/mol)	Complementarity (bp)	Heterodimer(ΔG : kcal/mol)
1	F	-0.54 – 0.27	-3.61 – -0.96	2 - 4	-6.69 – -0.96
	R	-0.25 – 0.58	-3.61 – -0.96	2	
5	F	0.67 – 1.47	-3.61 – -3.07	2 - 3	-5.02 – -1.34
	R	0.56 – 0.7	-7.05 – -0.96	2 - 4	
9	F	-1.57	-3.61 – -1.34	2 - 4	-9.21 – -1.34
	R	0.56	-7.05 – -0.96	2 - 4	
10	F	-1.57	-3.61 – -1.34	2 - 4	-9.21 – -1.34
	R	-0.23	-3.61 – -0.96	2	

Note: Negative ΔG values indicate stable secondary structure formation, while positive values indicate non-spontaneous structure formation.

Annealing Temperature Optimization via PCR Gradient

Annealing temperature optimization using a PCR gradient was performed to determine the optimal temperature at which the primer can specifically bind to the DNA template (Roux, 1995). Figure 2 shows the 2% agarose gel electrophoresis profile of the PCR gradient results for primer 1_CaHsFA2 against the *C. annuum* cDNA genome. A clear single band of approximately 193 bp was observed across the temperature range of 55.3–59.2°C. This band size is consistent with the expected amplicon length of 190 bp predicted in silico, with the slight difference likely due to the limited resolution of agarose gel electrophoresis This indicates that the primer specifically amplifies the target on the DNA template and aligns with the predicted product length of 190 bp (Table 1). This temperature range was determined based on the five temperatures below the Tm value in Table 1. The optimal annealing temperature was set at 57.3°C because it produced the brightest band compared to the other temperatures. The strong band intensity without smearing indicates that amplification occurred efficiently and that the PCR product quality was good (Iqbal et al., 2016). According to Gheno et al. (2026), an annealing temperature that is too low can cause nonspecific amplification, while a temperature that is too high inhibits primer binding to the target DNA.

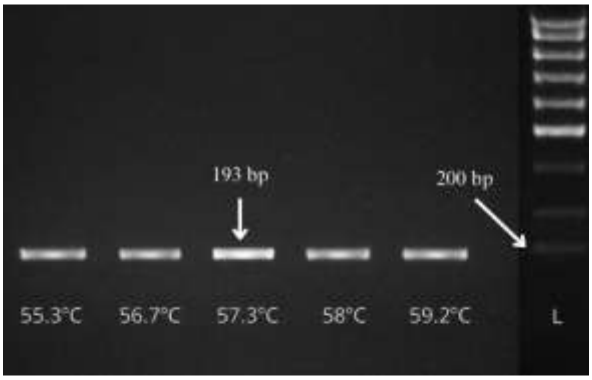


Figure 2. PCR electrophoresis profile of *C. annuum* root cDNA samples at five temperature gradients: 100 bp DNA ladder

The annealing temperature is the stage at which the primer hybridizes complementarily with the template DNA (Mubarak et al., 2020). This temperature must be determined experimentally because primer design software only predicts Tm as an initial estimate (Bustin et al., 2009). A higher GC content increases the Tm value and the required annealing temperature. Generally, the optimal annealing temperature is approximately 5°C below Tm (Mubarak et al., 2020), however this serves only as a guideline and may vary depending on primer characteristics and reaction conditions. In this study, although the predictred Tm was around 60oC, gradient PCR analysis (55–59°C) showed that 57.3^{o}C produced the most specific amplification with a clear single band. The use of a slightly higher annealing temperature likely enhanced primer specificity by reducing non-specific binding. Therefore, 57.3°C was selected as the optimal annealing temperature for primer 1_CaHsfA2 in qPCR analysis.

Conclusion

This study successfully designed and experimentally validated a specific primer targeting the CaHsFA2 gene in *Capsicum annuum*. Among ten candidates, primer set 1 termed as 1_CaHsfA2 (Forward: TGGAGCAGAGGAAGTACCGA; Reverse: GTGCTAAGAACGCCAGAGGA) showed the most optimal criteria (%GC 55%, Tm 60^{o}C, PL 190 bp) and minimal secondary structure potential, and produced a clear single band of approximately 193 bp, consistent with the predicted size of 190 bp while gradient PCR analysis identified an optimal annealing temperature of 57.3°C. These results confirm that the selected primer is specific and reliable for downstream gene expression analysis, providing a robust molecular tool for qPCR-based studies of heat-stress-related gene regulation in chili.

Acknowledgments

The authors would like to express their sincere gratitude to all individuals and institutions who contributed to the completion of this study, both directly and indirectly.

Author Contributions

Conceptualization, S., Z.A.N., A.A.; methodology, S., D.H.T.; formal analysis, S., A.N.; investigation, S., A.N.; data curation, S.; writing—original draft preparation, S., A.N.; writing—review and editing, S., Z.A.N., A.A., D.H.T., and A.N.; supervision, Z.A.N., A.A., and D.H.T.; funding acquisition, S. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the Directorate of Research and Community Service (DPPM), Ministry of Higher Education, Science, and Technology (Kemdiktisaintek), Fiscal Year 2025, under Contract Number 103/UN16.19/PT.01.03/PL/2025 through Universitas Andalas.

Conflicts of Interest

The authors declare no conflict of interest.

References

Alberts, B., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K., & Walter, P. (2017). *Molecular biology of the cell*. Norton & Company eBooks. https://doi.org/10.1201/9781315735368

Atifah, Y., & Achyar, A. (2023). Design of Specific Primer for Methallothionein Gene of Tor Fish (Tor tambra. *Natural Science Journal of Science and Technology*, *11*(02). https://doi.org/10.22487/25411969.2022.v11.i02.16216

Borah, P. (2011). Primer designing for PCR. *Science Vision*, *11*(3), 134–136. Retrieved from https://sciencevision.org/storage/journal-articles/February2019/aPFH6nHcXUQRMoYOsaBI.pdf

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum information for publication of Quantitative Real-Time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Caro, R. E. S., Cagayan, J., Gardoce, R. R., Manohar, A. N. C., Canama-Salinas, A. O., Rivera, R. L., Lantican, D. V, Galvez, H. F., & Reaño, C. E. (2022). Mining and validation of novel simple sequence repeat (SSR) markers derived from coconut (Cocos nucifera L.) genome assembly. *Journal of Genetic Engineering and Biotechnology*, *20*(1), 71. https://doi.org/10.1186/s43141-022-00354-z

Chen, Z., Halford, N. G., & Liu, C. (2023). Real-Time Quantitative PCR: Primer Design, Reference Gene Selection, Calculations and Statistics. *Metabolites*, *13*(7), 806. https://doi.org/10.3390/metabo13070806

Chuang, L., Cheng, Y., & Yang, C. (2013). Specific primer design for the polymerase chain reaction. *Biotechnology Letters*, *35*(10), 1541–1549. https://doi.org/10.1007/s10529-013-1249-8

Fragkostefanakis, S., Mesihovic, A., Simm, S., Paupière, M. J., Hu, Y., Paul, P., Mishra, S. K., Tschiersch, B., Theres, K., Bovy, A., Schleiff, E., & Scharf, K. (2016). HSFA2 controls the activity of developmentally and Stress-Regulated heat stress protection mechanisms in tomato male reproductive tissues. *Plant Physiology*, *170*(4), 2461–2477. https://doi.org/10.1104/pp.15.01913

Gheno, B. P., Fröder, H., Lopes, T. S., Rissi, V. B., Silva, A. E., & S. (2026). Steps for primer development using open-access platforms. *Biologia*, *81*(1). https://doi.org/10.1007/s11756-025-02088-y

Gomez-Pastor, R., Burchfiel, E. T., & Thiele, D. J. (2017). Regulation of heat shock transcription factors and their roles in physiology and disease. *Nature Reviews Molecular Cell Biology*, *19*(1), 4–19. https://doi.org/10.1038/nrm.2017.73

Gong, C., Pang, Q., Li, Z., Li, Z., Chen, R., Sun, G., & Sun, B. (2021). Genome-Wide Identification and Characterization of Hsf and Hsp Gene Families and Gene Expression Analysis under Heat Stress in Eggplant (Solanum melongema L. *Horticulturae*, *7*(6), 149. https://doi.org/10.3390/horticulturae7060149

Guo, M., Lu, J., Zhai, Y., Chai, W., Gong, Z., & Lu, M. (2015). Genome-wide analysis, expression profile of heat shock factor gene family (CaHsfs) and characterisation of CaHsfA2 in pepper (Capsicum annuum L. *BMC Plant Biology*, *15*(1), 151. https://doi.org/10.1186/s12870-015-0512-7

Guo, M., Yin, Y., Ji, J., Ma, B., Lu, M., & Gong, Z. (2014). Cloning and expression analysis of heat-shock transcription factor gene CaHsfA2 from pepper (Capsicum annuum L. *Genetics and Molecular Research*, *13*(1), 1865–1875. https://doi.org/10.4238/2014.march.17.14

Handoyo, D., & Rudiretna, A. (2001). Prinsip umum dan pelaksanaan Polymerase Chain Reaction (PCR. *Unitas*, *9*(1), 17–29. Retrieved from https://repository.ubaya.ac.id/35/

Hasanuzzaman, M., Bhuyan, M., Zulfiqar, F., Raza, A., Mohsin, S., Mahmud, J., Fujita, M., & Fotopoulos, V. (2020). Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants*, *9*(8), 681. https://doi.org/10.3390/antiox9080681

Hidayah, N., Ahda, Y., Achyar, A., & Atifah, Y. (2025). Desain Primer Gen Ednrb Ekson 4 dan Optimasi PCR untuk Analisis Mutasi Gen pada Hirschsprung Disease. *Jurnal Biogenerasi*, *10*(2), 1468–1476. https://doi.org/10.30605/biogenerasi.v10i2.5954

Hung, J., & Weng, Z. (2016). Sequence Alignment and Homology Search with BLAST and ClustalW. *Cold Spring Harbor Protocols*, *2016*(11), 93088. https://doi.org/10.1101/pdb.prot093088

Iqbal, M., Buwono, I. D., & Kurniawati, N. (2016). Analisis Perbandingan Metode Isolasi DNA Untuk Deteksi White Spot Syndrome Virus (WSSV) Pada Udang Vaname (Litopenaeus vannamei. *Jurnal Perikanan Kelautan*, *7*(1). Retrieved from http://journal.unpad.ac.id/jpk/article/view/13941

Khaira, A., Achyar, A., Zulyusri, Z., Atifah, Y., Putri, D. H., & Violita, V. (2023). Primer Design and Optimization of Annealing Temperature for Analysis of Glutathione Reductase Gene Expression in Rice (Oryza sativa L. *3BIO Journal of Biological Science Technology and Management*, *5*(1), 142–148. https://doi.org/10.5614/3bio.2023.5.1.3

Kotak, S., Port, M., Ganguli, A., Bicker, F., & Koskull-Döring, P. (2004). Characterization of C-terminal domains of Arabidopsis heat stress transcription factors (Hsfs) and identification of a new signature combination of plant class A Hsfs with AHA and NES motifs essential for activator function and intracellular localization. *The Plant Journal*, *39*(1), 98–112. https://doi.org/10.1111/j.1365-313x.2004.02111.x

Li, D. (2009). Research Advance of heat stress and heat tolerance in pepper. *Northern Horticulture*. Retrieved from https://en.cnki.com.cn/Article_en/CJFDTotal-BFYY200909038.htm

Li, Y., Gong, K., Wang, X., Sun, Z., & Ding, F. (2025). Heat shock transcription factors as central integrators of plant stress responses: from thermotolerance to Multi-Stress resilience. *Biology*, *14*(12), 1800. https://doi.org/10.3390/biology14121800

Meagher, R. J., Priye, A., Light, Y. K., Huang, C., & Wang, E. (2018). Impact of primer dimers and self-amplifying hairpins on reverse transcription loop-mediated isothermal amplification detection of viral RNA. *The Analyst*, *143*(8), 1924–1933. https://doi.org/10.1039/c7an01897e

Mubarak, S. M., Al-Koofee, D. A., Radhi, O. A., Ismael, J. M., & Al-Zubaidi, Z. F. (2020). An optimization and common troubleshooting solving in polymerase chain reaction technique. *Systematic Reviews in Pharmacy*, *11*(2), 427–436. https://doi.org/10.5530/srp.2020.2.63

Ohama, N., Sato, H., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2016). Transcriptional Regulatory Network of Plant Heat Stress Response. *Trends in Plant Science*, *22*(1), 53–65. https://doi.org/10.1016/j.tplants.2016.08.015

Praja, R. K. (2021). In Silico Oligonucleotide Primer Design For Campylobacter Jejuni Cytolethal Distending Toxin B Gene Amplification. *Oceana Biomedicina Journal*, *4*(1), 53. https://doi.org/10.30649/obj.v4i1.88

Prediger, E. (2024). *A practical guide for PCR and qPCR primer design*. Retrieved from https://sg.idtdna.com/page/support-and-education/decoded-plus/how-to-design-primers-and-probes-for-pcr-and-qpcr/

Roux, K. H. (1995). Optimization and troubleshooting in PCR. *Genome Research*, *4*(5), 185– 194. https://doi.org/10.1101/gr.4.5.s185

Roziqoh, W., Perdani, A. Y., Wahyuni, Y., & Su'udi, M. (2023). Upaya Peningkatan Ketahanan Cabai Merah (Capsicum annuum L.) Terhadap Cekaman Kekeringan dengan Iradiasi Gamma. *Jurnal Agrotek Tropika*, *11*(4), 547. https://doi.org/10.23960/jat.v11i4.6676

Shen, L., Chepelev, I., Liu, J., & Wang, W. (2010). Prediction of quantitative phenotypes based on genetic networks: a case study in yeast sporulation. *BMC Systems Biology*, *4*(1), 128. https://doi.org/10.1186/1752-0509-4-128

Singh, S., & Singh, M. (2000). Genotypic basis of response to saliniity stress in some crosses of spring wheat Triticum aestivum L. *Euphytica*, *115*(3), 209–214. https://doi.org/10.1023/a:1004014400061

Suwirmen, N., A., Z., Marta, F. D., & Chairul. (2025). Application of fern Davallia denticuluta L. extract using different solvents as biostimulants for Kopay chili (Capsicum annuum L.) production. *Jurnal Agronomi Indonesia (Indonesian Journal of Agronomy)*, *53*(2), 278–286. https://doi.org/10.24831/jai.v53i2.62870

Syamsidi, A., Aanisah, N., Fiqram, R., & Jultri, I. A. (2021). Primer Design and Analysis for Detection of mecA gene. *Journal of Tropical Pharmacy and Chemistry*, *5*(3), 245–253. https://doi.org/10.25026/jtpc.v5i3.297

Tang, R., Zhu, W., Song, X., Lin, X., Cai, J., Wang, M., & Yang, Q. (2016). Genome-Wide identification and function analyses of heat shock transcription factors in potato. *Frontiers in Plant Science*, *7*, 490. https://doi.org/10.3389/fpls.2016.00490

Thornton, B., & Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, *39*(2), 145–154. https://doi.org/10.1002/bmb.20461

Tian, F., Hu, X., Yao, T., Yang, X., Chen, J., Lu, M., & Zhang, J. (2021). Recent advances in the roles of HSFs and HSPs in heat stress response in woody plants. *Frontiers in Plant Science*, *12*, 704905. https://doi.org/10.3389/fpls.2021.704905

VanGuilder, H. D., Vrana, K. E., & Freeman, W. M. (2008). Twenty-Five years of quantitative PCR for gene expression analysis. *BioTechniques*, *44*(5), 619–626. https://doi.org/10.2144/000112776

Wang, C. (2016). *Primer Design*. Centre for Medical Parasitology University of Copenhagen. Health Science, Denmark.

Wang, Y., & Xu, J. (2025). The heat shock transcription factors regulate response mechanisms to abiotic stresses in plants. *Crop Design*, *4*(3), 100109. https://doi.org/10.1016/j.cropd.2025.100109

Zandalinas, S. I., Fritschi, F. B., & Mittler, R. (2021). Global warming, climate change, and environmental pollution: recipe for a multifactorial stress combination disaster. *Trends in Plant Science*, *26*(6), 588–599. https://doi.org/10.1016/j.tplants.2021.02.011

Zhou, R., Yu, X., Ottosen, C., Rosenqvist, E., Zhao, L., Wang, Y., Yu, W., Zhao, T., & Wu, Z. (2017). Drought stress had a predominant effect over heat stress on three tomato cultivars subjected to combined stress. *BMC Plant Biology*, *17*(1), 24. https://doi.org/10.1186/s12870-017-0974-x