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Design and validation of PIP gene primer for quantitative PCR in *Capsicum annuum* using in silico and experimental approaches

Amanda Nurhafitri*, Suwirmen
Biology Study Program, Faculty of Mathematic and Natural Sciences, Universitas Andalas, Jl. Dr. Mohammad Hatta, Limau Manis, Pauh, Padang, 25175, Indonesia

*Corresponding e-mail: amanda28nurhafitri04@gmail.com

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Abstract

Capsicum annuum L. is an economically important horticultural crop whose productivity is strongly affected by drought stress. Plasma Membrane Intrinsic Protein (PIP), an aquaporin gene involved in water transport and osmotic regulation, requires specific and efficient primers for accurate gene expression analysis using quantitative PCR (qPCR). However, the design and validation of qPCR primers targeting the PIP gene in *C. annuum* have not been well explored. This work aims to design and validation of PIP gene–specific primers for qPCR analysis in *C. annuum* through combined in silico and experimental methods. Primers were designed using the NCBI Primer-BLAST tool with reference to the CaPIP sequence (XM_016711608.2), followed by in silico evaluation of primer specificity and secondary structure using Primer-BLAST and OligoAnalyzer. Nine primer pairs were initially generated and evaluated based on primer length, %GC, Tm, self3' complementarity and amplicon size. Secondary structure analysis revealed strong self-dimer formation in pair 8, whereas pair 2 showed weak secondary structure within acceptable ΔG threshold (-9 kcal/mol). Experimental validation was conducted using gradient PCR to optimize the annealing temperature, followed by visualization on 2% agarose gel. Primer 2_CaPIP produced specific and clear amplification, with an optimal annealing temperature of 57.3°C. This study provides a validated CaPIP primer set suitable for qPCR-based gene expression analysis in *C. annuum*, supporting future molecular studies on drought stress tolerance.

Introduction

Capsicum annuum L. is an economically important horticultural crop whose productivity is highly sensitive to abiotic stresses, particularly drought. The productivity and adaptability of chili peppers are influenced by genetic factors and environmental conditions, with drought and salinity being major limiting factors affecting plant growth and yield (AlHarbi et al., 2014; Ntanasi et al., 2025). Climate change exacerbates water deficit conditions, which can disrupt plant physiological processes such as water balance, photosynthesis, and nutrient uptake, thereby increasing the risk of crop failure (Oktavianti et al., 2019). In Indonesia, the Central Statistics Agency (BPS) reported a decline in red chili productivity from 25.42 tons/ha in 2021 to 20.63 tons/ha in 2022. This decline may be associated with the increasing impact of abiotic stress, highlighting the importance of understanding the physiological and molecular mechanisms underlying drought tolerance in *C. annuum*.

As part of the aquaporin gene family, Plasma Membrane Intrinsic Protein (PIP) plays an essential role in facilitating water transport and maintaining osmotic balance in plant cells (Deshmukh et al., 2016; Li et al., 2025). PIP gene expression is strongly linked to plant responses to abiotic stress and to the regulation of water use efficiency (Afzal et al., 2016; Zhang et al., 2019). In *C. annuum*, the CaPIP gene is an

important candidate gene for study due to its potential in regulating adaptive responses to environmental conditions, particularly drought.

To understand the mechanism of PIP in responding to drought, further research requires analysis of PIP gene expression in *C. annuum* during drought. A technique commonly used to analyze gene expression is qPCR (Khaira et al., 2023). This analysis can be performed because qPCR can detect gene expression through the formation of complementary DNA (cDNA) from RNA, which is quantified through an amplification process (Bustin & Muller, 2005). Amplification of cDNA by PCR relies on a specific pair of forward and reverse primers to restrict the region being amplified (Pradnyaniti et al., 2013).

Primers are crucial for the success of PCR. Primers are oligonucleotides used to mark DNA polymerization from specific genes (Mubarak et al., 2020). Good primers are determined by several criteria. These criteria are a primer length of 18–22 bp, a GC percentage between 40–60%, minimal 3' self-complementarity, and amplification product length (Prediger et al., 2024; Sasmito et al., 2014). These parameters can be analyzed in silico based on bioinformatics studies available in several software programs such as NCBI Primer-BLAST and Oligoanalyzer. Additionally, experimental evaluation of primers is essential to identify the optimal annealing temperature (Ta) for effective primer binding to the target sequence in PCR (Syamsurizal et al., 2019). Reliable primers are essential for accurate gene expression analysis using qPCR, enabling precise quantification of target gene transcripts in various physiological and stress-related studies (Chen et al., 2023; Zhang et al., 2023).

Several studies have reported primer design for analyzing PIP gene expression in *Capsicum annuum*. Sahitya et al. (2018) designed primer pairs CaAQP-6 (F/R) and CaAQP-7 (F/R), while Yin et al. (2015) designed the CaPIP1-1 (F/R) primer pair for gene expression analysis. However, to date, there has been no research specifically focused on the design of PIP gene primers along with their optimization and validation for qPCR application in the *C. annuum* genome. Therefore, this study aims to design and validate PIP gene primers suitable for qPCR through an in silico and experimental approach in *C. annuum*. The designed primers showed appropriate characteristics based on in silico analysis and successfully amplified the target fragmen during experimental validation. These findings provide a reliable primer set for future studies on PIP gene expression in response to abiotic stress, particularly drought, in chili pepper.

Materials and methods

This research was conducted from August to November 2025 at the Genetics and Biomolecular Laboratory and Plant Physiology Laboratory, Department of Biology, Faculty of Mathematics and Science, Andalas University, Padang.

1. Method and Research Design

This research was conducted through bioinformatics and experimental design. Bioinformatics design was used to design primers from the PIP gene sequence from NCBI and analyze the secondary structure from OligoAnalyzer. Experimental design was used to optimize the annealing temperature of the PIP gene primer with the *C. annuum* genome through gradient PCR and visualized with electrophoresis.

2. Procedures

a. Design Primer

The reference sequence measuring 1223 bp with accession number XM_016711608.2 was entered into the search field on NCBI (Yin et al., 2014). Then, primer selection will appear by pressing the pick primer tools. The primer candidates recommended by NCBI primer BLAST will be selected based on primer length of 18–22 bp, GC percentage between 40–60%, lowest 3' self-complementarity, and product length (Prediger et al., 2024; Sasmito et al., 2014). Primer specificity was evaluated using Primer-BLAST by aligning the primer sequences against the NCBI database to ensure that each primer pair produced a single target amplicon in *Capsicum annuum* and showed no significant amplification with non-target species.

b. Secondary Structure Analysis of Primers

Secondary structure analysis includes dimers and hairpins using OligoAnalyzer software by IDT (https://sg.idtdna.com/pages/tools/oligoanalyzer). The sequences of the forward and reverse primers selected from the NCBI primer picker tool were entered into the analysis column on

OligoAnalyzer. The dimer structures to be analyzed included self-dimers and heterodimers, as well as the hairpin structures of each primer. Primer secondary structures were considered acceptable when the predicted ΔG values were greater than −9 kcal/mol, indicating weak or unstable secondary structures (Prediger, 2024).

c. RNA Extraction

Total RNA was extracted using Geneaid Total RNA Mini Kit (Plant) from four-day-old germinated chili seeds of *Capsicum annuum* that were germinated in the Plant Physiology Laboratory, Universitas Andalas, Indonesia. Approximately 50 mg of chili seeds tissue was processed following the manufacturer's protocol. The samples were frozen in liquid nitrogen and ground into a fine powder, followed by lysis with 500 µL RB buffer supplemented with 5 µL β-mercaptoethanol, incubation at 60 °C for 5 minutes, and filtration using a filter column. The clear filtrate was mixed with absolute ethanol to bind the RNA, then applied to the RB column and centrifuged. To reduce genomic DNA contamination, DNase I + DNAse I RB treatment was performed in-column. This was followed by a washing step using W1 buffer and wash buffer, followed by a column matrix drying step. Pure RNA was eluted using 50 µL and its quantity was checked with a nanospectrophotometer. RNA purity was assessed based on the absorbance ratios, with acceptable ranges of 1.8–2.2 for A260/280 and >1.7 for A260/230 (Wieczorek et al., 2012). Then, a sample with a concentration of 50 ng/µL was obtained after dilution and stored in a freezer at −80 °C.

d. cDNA synthesis

cDNA synthesis was performed using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover according to the kit protocol. Total reaction volume of 8 µL consisting of 4x DN Master mix 2 µL, RNA template 50 ng/µL 2 µL, and nuclease-free water 4 µL. The reaction mixture was gently mixed and placed in a thermocycler. The reverse transcription reaction was carried out at 37 °C for 15 min, followed by 50 °C for 5 min, and enzyme inactivation at 98 °C for 5 min. The synthesized cDNA was then stored at −20 °C until further use.

e. PCR Gradien

Optimization of the primer annealing temperature was performed using PCR gradient analysis. The total volume of the PCR reaction was 25 µL, consisting of 12.5 µL Bioline RedMix PCR, 10.5 µL nuclease-free water, 1 µL cDNA template, and 1 µL each of forward and reverse primers. Each PCR reaction was performed once**.** The PCR cycling conditions were performed according to Yin et al. (2015), consisting of an initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 55–59°C, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The amplified products were separated by electrophoresis on a 2% agarose gel prepared in 1× TAE buffer. Fragment sizes were estimated using a 100 bp DNA ladder (Bioline) and visualized using a Uvitec gel documentation system.

Results and discussion

1. RNA Extraction

RNA extraction from *Capsicum annuum* seeds was successfully carried out and its quality was analyzed using a Nano spectrophotometer. The RNA concentration obtained was 321.4 ng/µL with an absorbance ratio of A260/280 of 2.1 and A260/230 of 2.2. These values are within the good range for further analysis, namely 1.8–2.2 for the A260/280 ratio and >1.7 for the A260/230 ratio (Wieczorek et al., 2012). According to Gudenschwager et al. (2012), an A260/230 ratio above 1.8 indicates pure total RNA without polyphenol and polysaccharide contamination, while an A260/280 ratio in the range of 1.8–2.2 indicates low protein contamination. Therefore, the results obtained indicate that RNA isolated from *C. annuum* seeds has good purity and is suitable for further molecular analysis.

2. Design Primer

Designing primers is highly recommended using the BLAST primer software available on the NCBI website (Ye et al., 2012). Based on NCBI Primer-BLAST, nine primer pairs were recommended for amplifying the PIP gene in *C. annuum*. Based on Table 1, the forward and reverse primers from pair 1 to pair 9 have a similar size of 20 bp. The GC percentage ranges from 50% to 55%, the melting temperature

is 60°C, and the 3' self-complementarity is between 0 and 3 bp. The product length of pairs 1 to 9 varies from 80 to 286 bp.

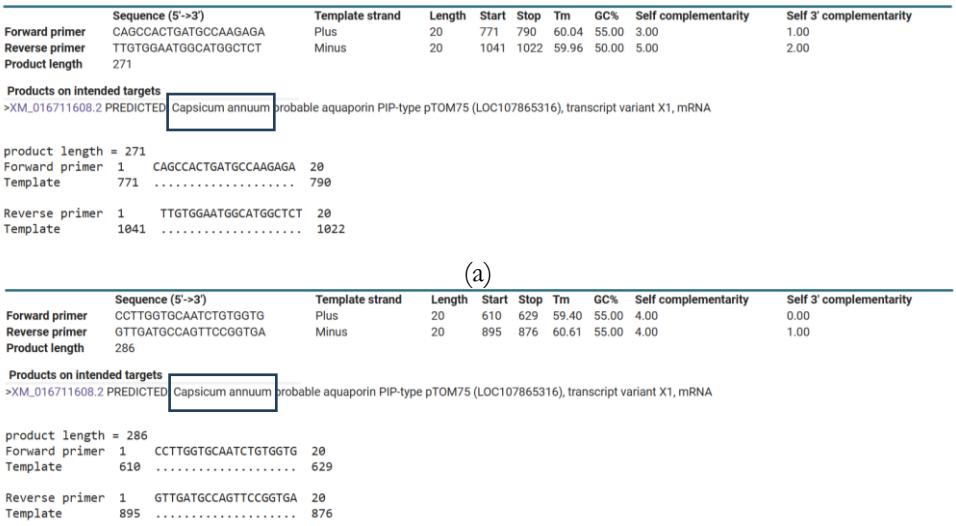
Table 1. Characteristics of PIP primer candidates from NCBI Primer Tools

Pair	Forward/ Reverse	Sequence (5'-3')	Size (bp)	%GC	Tm (°C)	Self 3'Comp	Amplicon (bp)
1	F	CATCAACCCAGCTGTGACCT	20	55	60	1	265
	R	TCTCTTGGCATCAGTGGCTG	20	55	60	3	
2	**F**	**CAGCCACTGATGCCAAGAGA**	**20**	**55**	**60**	**1**	**271**
	R	**TTGTGGAATGGCATGGCTCT**	**20**	**50**	**60**	**2**	
3	F	TGCTTGGGCTTTTGGTGGTA	20	50	60	2	80
	R	CAGCTGGGTTGATGTGTCCT	20	55	60	0	
4	F	TGTTGCTTGGGCTTTTGGTG	20	50	60	0	129
	R	GCCCTGGTCAAGGACAACTT	20	55	60	3	
5	F	TCAAGGTGTTGCTTGGGCTT	20	50	60	0	90
	R	ACAGCTGGGTTGATGTGTCC	20	55	60	0	
6	F	CAAAGACTTGGTGGTGGTGC	20	55	60	2	229
	R	TGATGCCAGTTCCGGTGATG	20	55	60	2	
7	F	CAAGGTGTTGCTTGGGCTTT	20	50	60	0	87
	R	AGCTGGGTTGATGTGTCCTC	20	55	60	1	
8	**F**	**CCTTGGTGCAATCTGTGGTG**	**20**	**55**	**60**	**0**	**286**
	R	**GTTGATGCCAGTTCCGGTGA**	**20**	**55**	**60**	**1**	
9	F	ATTCAAGGTGTTGCTTGGGC	20	50	60	2	99
	R	AAAGGTCACAGCTGGGTTGA	20	50	60	2	

Guanine (G) and Cytosine (C) are nucleotide bases that have three hydrogen bonds. Therefore, GC bonds are stronger than Adenine (A) and Thymine (T) bases, which only have two hydrogen bonds (Marliana et al., 2015). This is an important consideration when selecting primers. In primer design, the amount of guanine and cytosine is referred to as the GC percentage (%GC). The GC value should be in the range of 40-60% (Wang, 2016; Masnaini et al., 2023) with an ideal percentage of 50% (Prediger et al., 2024). If the primer has a low %GC, the efficiency of the primer to bind to the template sequence will decrease (Masnaini et al., 2023). This is related to the Tm of the primer because the number of G and C bases affects the melting temperature of the primer (Sari et al., 2018). The data in Table 1 shows a %GC of 50–55%, which is within the ideal range for use.

Specific primers are composed of a pair of primers, namely forward and reverse primers (Li et al., 2012). The forward primer directs DNA synthesis in the 5'–3' orientation, whereas the reverse primer facilitates synthesis from the opposite strand in the 3'–5' direction (Baker et al., 2016; Aurora et al., 2025). Primer length is a critical factor influencing binding specificity to the target template sequence. In general, primers with lengths ranging from 18 to 22 base pairs are considered optimal (Sasmito et al., 2014). Primers shorter than this range tend to exhibit reduced specificity and may anneal to non-target sequences, while excessively long primers increase the likelihood of secondary structure formation (Syamsidi et al., 2021). Consequently, primer lengths of 20–22 bp are most commonly applied in PCR and qPCR analyses (Hidayah et al., 2025; Chen et al., 2023; Syamsidi et al., 2021; Li et al., 2020). As shown in Table 1, all primer pairs recommended by NCBI Primer-BLAST possess a uniform length of 20 bp.

Another parameter to consider when selecting primers from the pairs listed in Table 1 is the self3' complementarity value. This value appears when there is a base match at the 3' end of the primer that can trigger the formation of primer dimers that can be extended by DNA polymeraseThis factor influences the efficiency of primer binding to the target sequence. Therefore, the self 3' complementarity value has a tolerance limit of no more than 3 bases (Handoyo & Rudiretna., 2001). In Table 1, the self 3' complementarity values that meet the requirements are in pairs 2, 3, 4, 5, 7, and 8. However, pairs 3, 4, 5, and 7 have short amplification product lengths and are not suitable for amplification with qPCR. An amplification product that is too short can cause mispriming, allowing other fragments to be amplified by that fragment (Astari et al., 2021). Holm et al (2021) stated that an amplification product length of 200–400 bp can increase the quantification cycle difference between DNA from living and dead cells. This is important in qPCR to better reflect the actual biological conditions of an activity being observed via qPCR.



	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGCCACTGATGCCAAGAGA	Plus	20	771	790	60.04	55.00	3.00	1.00
Reverse primer	TTGTGGAATGGCATGGCTCT	Minus	20	1041	1022	59.96	50.00	5.00	2.00
Product length	271								

Products on intended targets

>XM_016711608.2 PREDICTED: Capsicum annuum probable aquaporin PIP-type pTOM75 (LOC107865316), transcript variant X1, mRNA

```
product length = 271
Forward primer  1     CAGCCACTGATGCCAAGAGA  20
Template        771   ....................  790

Reverse primer  1     TTGTGGAATGGCATGGCTCT  20
Template        1041  ....................  1022
```

(a)

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCTTGGTGCAATCTGTGGTG	Plus	20	610	629	59.40	55.00	4.00	0.00
Reverse primer	GTTGATGCCAGTTCCGGTGA	Minus	20	895	876	60.61	55.00	4.00	1.00
Product length	286								

Products on intended targets

>XM_016711608.2 PREDICTED: Capsicum annuum probable aquaporin PIP-type pTOM75 (LOC107865316), transcript variant X1, mRNA

```
product length = 286
Forward primer  1     CCTTGGTGCAATCTGTGGTG  20
Template        610   ....................  629

Reverse primer  1     GTTGATGCCAGTTCCGGTGA  20
Template        895   ....................  876
```

(b)

Figure 1. NCBI Primer BLAST results from primer (a) pair 2 and (b) pair 8

The specificity of the selected primer pairs 2 and 8 was examined using Primer-BLAST from NCBI. Based on Figure 1a and 1b, each primer candidate from pairs 2 and 8 is specific to the species *C. annuum*. There were no significant matches to the genomes of other species. This indicates that the possibility of primers binding and amplifying non-target sequences is very low. Ye et al. (2012) stated that Primer-BLAST is an effective tool for evaluating and designing primers specific to genomes. Primer specificity is a key factor in the success of PCR amplification, as non-specific primers can produce non-specific products and reduce reaction efficiency (Hafzari et al., 2024). Therefore, pair 2 and pair 8 were selected for in silico secondary analysis by OligoAnalyzer.

3. Secondary Structure Analysis

The secondary structures in the form of hairpins, self-dimers, and heterodimers of pair 2 and pair 8 were analyzed using OligoAnalyzer software. The results of the secondary structure analysis showed that both primer pairs had relatively low hairpin ΔG values (Table 2). This indicates a tendency toward the formation of weak hairpin structures. However, a significant difference is seen in the potential for self-dimer formation. Pair 8 shows a very negative self-dimer ΔG value of -9.75 kcal/mol, while pair 2 has a higher and more positive self-dimer ΔG value, indicating a less stable secondary structure.

Table 2. Analysis of the primary secondary structure of CaPIP from Oligoanalyzer

Pair_Gen	Sequence (5' – 3')	Hairpin (ΔG : kcal/mol)	Selfdimer (ΔG : kcal/mol)	Heterodimer (ΔG : kcal/mol)
2_CaPIPF	CAGCCACTGATGCCAAGAGA	-0.47 – -0.4	-3.55 – -1.47	-5.38 – -1.57
2_CaPIPR	TTGTGGAATGGCATGGCTCT	-0.24 –0.73	-5.38 – -1.57	
8_ CaPIPF	CCTTGGTGCAATCTGTGGTG	-0.26 – 0.73	-7.05 – -1.47	-5.09 – -1.94
8_ CaPIPR	GTTGATGCCAGTTCCGGTGA	-0.15 – 0.66	-9.75 – -1.47	

Secondary structure analysis was conducted using OligoAnalyzer to support the development of high-quality primer designs (Caro et al., 2022). This tool enables the assessment of potential secondary structures, including hairpins and primer dimers, by analyzing Gibbs free energy (ΔG) values. The probability of primer annealing to the target sequence and subsequent extension by DNA polymerase is influenced by changes in Gibbs free energy (ΔG), as this parameter reflects the thermodynamic stability of primer interactions and the tendency to form non-specific structures (Meagher et al., 2018). Hairpin structures occur when a primer folds back and binds to itself (Chuang et al., 2013), whereas primer dimers arise from interactions between primers, either as self-dimers or as heterodimers formed between forward and reverse primers (Fakih et al., 2021).

According to Prediger (2024), the ΔG value in hairpins, self-dimers, and heterodimers should be in the weak range, with a threshold of around -9.0 kcal/mol, because a very negative ΔG value reflects a stable secondary structure. Yang et al. (2020) stated that the ΔG value in the primer must be greater than -6 kcal/mol. Based on these criteria, pair 8 has the potential to form a stable self-dimer, thereby inhibiting amplification efficiency. Conversely, the ΔG value of the self-dimer in pair 2 indicates that the secondary structure formed is unstable or may not form at all. Therefore, pair 2 was selected and named 2_CaPIP, then proceeded to the primer synthesis stage and further experimental testing.

4. Annealing temperature optimization by PCR Gradient

Annealing temperature (Ta) is regarded as a critical factor influencing primer performance and the success of amplification reactions (Fraige et al., 2013). Ta refers to the temperature range at which primers can stably anneal to DNA or RNA templates (Syamsidi et al., 2021). In comparison with melting temperature (Tm), Ta more accurately reflects the actual conditions required for optimal primer–template binding (Bustin & Huggett, 2017). Consequently, experimental optimization of the annealing temperature is essential, since primer design software typically provides only theoretical Tm values (SantaLucia, 2007).

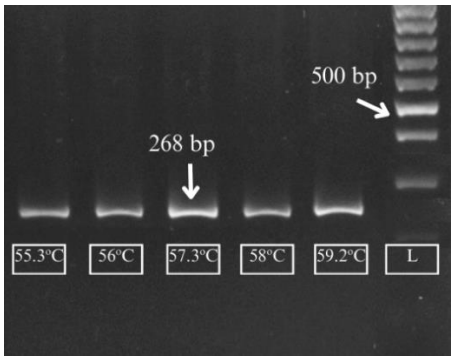


Figure 2. Electrophoresis profile of the 2_CaPIP pair 1 primer with *C. annuum* cDNA. Ta: 55-59°C. L: 100 bp ladder.

Optimization of the PCR annealing temperature was performed using five temperatures below the melting temperature of primer pair 2 (Table 1), ranging from 55–59 °C, producing an expected amplicon of 268 bp. According to Mubarak et al. (2020), the optimal annealing temperature is typically 5°C below the Tm. Based on the PCR gradient results shown in the electrophoresis profile in Figure 2, all samples exhibited clear, thick, specific bands without dimers. The presence of a single band at all temperatures indicates that the specificity of the primers has been confirmed (Chen et al., 2023). The results of optimizing several temperatures show that the band at 57.3°C is the brightest and thickest compared to other bands. Clear, thick bands without smearing reflect good DNA bands due to maximal PCR amplification (Irmawati et al., 2003). Therefore, the annealing temperature of 57.3°C is the most optimal for the 2_CaPIP primer to amplify *C. annuum* cDNA.

Conclusion

The best primer design result is primer pair 2, named 2_CaPIP. The characteristics of the primers are 2_CaPIPF CAGCCACTGATGCCAAGAGA and 2_CaPIPR TTGTGGAATGGCATGGCTCT, with a base length of 20 bp, amplicon 271 bp, Tm 60oC, and %GC of 50% and 55%, respectively. Both primers have very small ΔG hairpin and dimer values, far below -9 kcal/mol, with an optimum annealing temperature (Ta) of 57.3°C. The validated 2_CaPIP primer pair provides a reliable tool for qPCR-based expression analysis of the CaPIP gene in *Capsicum annuum*, supporting future studies on aquaporin-mediated water regulation and drought stress tolerance.

Author Statements

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Competing of interest: The authors declare that there is no conflict of interest regarding the publication of this paper

Author's contributions: AN conducted the experiments, analyzed the data, and drafted the manuscript. S designed the research proposal, supervised the study, and served as the corresponding author.

Generative AI: Generative AI tools were used only for language editing and improving the clarity of the manuscript. The authors take full responsibility for the content of the manuscript**.**

Data availability: The data supporting the findings of this study are available within the article.

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