BIODIVERSITAS
Volume 25, Number 6, June 2024
Pages: 2645-2651

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250634

Identification and validation of reference genes for gene expression study using Quantitative Real-Time PCR in shallot (*Allium cepa* L. Aggregatum group) based on bulb development phase

ENDANG SULISTYANINGSIH♥, WIDHI DYAH SAWITRI, VALENTINA DWI SUCI HANDAYANI, ADDIN WAHYU WICAKSONO, CLARENCIA MARGARETH SILALAHI, RUDI HARI MURTI

Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
Tel./fax.: +62-274- 563062, ♥email: endangsih@ugm.ac.id

Manuscript received: 15 February 2024. Revision accepted: 22 June 2024.

Abstract. *Sulistyaningsih E, Sawitri WD, Handayani VDS, Wicaksono AW, Silalahi CM, Murti RH. 2024. Identification and validation of reference genes for gene expression study using Quantitative Real-Time PCR in shallot (*Allium cepa *L. Aggregatum group) based on bulb development phase. Biodiversitas 25: 2645-2651.* Shallot (*Allium cepa* L. Aggregatum group) is cultivated vegetatively using bulbs (produce many bulbs) and generatively using TSS (True Seed of Shallot) which tends to produce single bulbs. Bulb aggregation is influenced by activity of genes and the gene expression could be analyzed using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The qRT-PCR analysis requires reference gene data (housekeeping gene) as a comparison gene against our target gene. This study aimed to select and validate three housekeeping genes in shallot as an initial study for qRT-PCR analysis. The research flow began with the preparation of shallot 'TukTuk' leaves and bulb samples in bulb formation from bulb initial phase to mature phase. Furthermore, a specific primer design based on actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences were obtained from gene bank data. The results showed that the actin, tubulin, and GAPDH genes were successfully amplified and the products were 142 bp, 116 bp, and 136 bp in size, respectively and the genes were expressed almost constantly in leaves and bulbs in all phases of bulb formation. The tubulin gene had the potential as a reference gene for qRT-PCR analysis in shallot with the most consistently expressed.

Keywords: *Allium cepa* L. Aggregatum group, bulb, seed, q-PCR, reference gene, shallot

INTRODUCTION

The pungency character of shallot (*Allium cepa* L. Aggregatum group) bulbs is an important spice and seasoning as an essential ingredient for dish preparation in tropical countries, including Indonesia. Moreover, shallot is a medicinal remedy for common ailments (Moldovan et al. 2022). Sutardi et al. (2022) reported that shallot is commonly cultivated vegetatively using bulbs with an advantage in a short period of cultivation (about 2 months). However, the problem of low available quantity of shallot bulbs appears in off-season cultivation because of high prices and low supply. Therefore, True Seed Shallot (TSS) becomes an alternative material planting for shallot (Rosliani et al. 2016; Saidah et al. 2019).

According to Sulistyaningsih et al. (2020), two ('Sanren' and 'Lokananta') out of five cultivars of TSS showed a high ability of 10-61% in bulb aggregation in the first plant generation planted from seeds (G0). Three other cultivars, namely 'TukTuk', 'Biru Lancor', and 'Pancasona' showed low ability in bulb aggregation of less than 9.52%. Aggregation is an important character of shallot for bulb productivity improvement. Periode of bulb development and aggregation differ between shallot planted from bulb and seed. Shallot planted from seed needs 4 months from seed germination to bulb harvest (Pangestuti et al. 2022). However, shallot planted from bulbs needs 2 months from bulb cultivation to bulb harvest (Sutardi et al. 2022). Aggregation ability in shallot is related to several gene expressions. Designing superior shallot cultivars requires various basic analyses to measure gene expression levels. Eisenberg and Levanon (2013) and Tong et al. (2021) reported that analysis of quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) provides a more sensitive, reproducible, and precise approach for detecting gene expressions using a range of fluorescent report dyes that correlate the yield of PCR product with fluorescence intensity.

Understanding the genes involved in aggregation ability in shallot requires investigations on their expression profiles across different tissues and organs through qRT-PCR method. The emergence of qRT-PCR addresses the evident requirement for quantitative analysis in gene expressions. To elucidate the regulation of the genes encoding aggregation ability in shallot, it begins with investigating the expression of the genes of interest. However, the appropriate reference genes for normalizing the gene expression are still unavailable in shallot, although gene references have been reported in Chinese chives (*Allium tuberosum* Rottler ex Spreng.) (Tong et al. 2021) and Chinese onion (*Allium waliichii* Kunth) (Lin et al. 2023). The limited report concerning genome sequence database in shallot is facing more important challenges. It might be caused by shallot cultivated in limited areas compared to onion and Chinese chives (Jones 2017).

A prerequisite is selecting optimal internal control genes for the normalizations under given experimental conditions. The ideal reference genes in the relative quantifications should be expressed constantly and stably in all examined samples regardless of experimental conditions, such as different developmental stages, biological processes, treatments, and even different organs or tissues (Valente et al. 2014; Pabuayon et al. 2016). Several reference genes that are commonly used for analysis of gene expression in plants consist of actin (ACT), β-tubulin (TUB), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Radchuk 2008; de Lima Rebouças et al. 2013), but recent studies have shown that their expressions are also affected by specific treatments, tissue difference, or other experimental conditions (Valente et al. 2014; Pabuayon et al. 2016). This study aimed to select and validate three housekeeping genes (ACT, TUB, and GAPDH) in shallot as an initial study for qRT-PCR analysis. Our knowledge in this study was the first analysis of reference genes and will facilitate gene expression studies in shallot.

MATERIALS AND METHODS

Plant materials

The variety of shallot 'TukTuk' produced by East West Seed Company was planted in the Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia experimental station during the wet season (November to February). This study used two types of planted materials, namely TSS (G0) and bulb (G1, bulb from G0) for the gene expression analysis. Samples from leaves and bulbs were collected for RNA extraction and cDNA library construction. Since the bulb development from TSS and bulb differed in the time course, the samples were collected 6 Weeks After Planting (WAP) (bulb initial phase), 8 WAP (bulb enlarge phase), and 11 WAP (bulb mature phase) for G0 (plants from seeds), while 4 Weeks After Planting (WAP) (bulb initial phase), 5 WAP (bulb enlarge phase), and 7 WAP (bulb aggregation and mature phase) in the G1 generation (plants from bulbs) with three plants as replications each observation time. Fresh samples of leaves and bulbs were washed in running water to remove dirt and blotting paper was used to remove excess water. For qRT-PCR, materials from leaves and bulbs were harvested at three different time points, frozen immediately before use in liquid nitrogen and stored at −80°C (Zhang et al. 2016).

Procedures

Shallot cultivation

The planting medium for shallot cultivation was a 45 × 43 cm polybag containing a mixture of manure and soil at a ratio of 1:1. Bulbs were planted in polybags with 2 bulbs per polybag. Fertilizers were applied twice at a dose of 2.8 g/polybag, respectively. The first fertilizer was applied 15 Days After Planting (DAP) using NPK compound 15-15-15 fertilizer (N: Nitrogen, P: Phosphate, and K: Potassium) and the second fertilizer was applied 30 DAP.

Design of set primers and selection of candidate reference genes

Three candidate reference genes, namely ACT, TUB, and GAPDH have been identified based on commonly used reference genes in the National Center for Biotechnology Information (NCBI). Since the database of shallot genome sequence is not available in NCBI, thus, we select those three candidate genes from the same genus of shallot, such as onion (*Allium cepa*) and garlic (*Allium sativum*). All sequences from NCBI were analyzed by multiple sequence alignment using ClustalW (https://www.genome.jp/tools-bin/clustalw) to determine the conserved regions in the nucleotide sequence. Based on the information of multiple sequence alignment, the nucleotide conserved region is selected for qRT-PCR primer design. The primers were obtained by inserting the conserved sequence using Primer-BLAST Tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the following criteria: primer size of 20-21 bp, GC content of 45-65%, melting temperature of 50-60°C, and product size of 70-200 bp (Table 1) (Lorenz 2012). The specificity of the primer pairs was assessed via qRT-PCR and a melting curve analysis. Finally, the amplification products were optimized and verified via 1% agarose gel electrophoresis.

RNA extraction, cDNA synthesis, and qRT-PCR

RNA from shallot leaves and bulbs was extracted using the GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The RNA concentration was measured using a BioDrop (Biochrom, UK) and RNA samples with an A260/A230. A ratio of >1.8 was used for cDNA synthesis. For qRT-PCR, 500 ng of RNA was used for reverse transcription in a 10 µL reaction volume using the Thermo Scientific Revert Aid First Strand cDNA Synthesis (Thermo Fisher Scientific).

Table 1. Genes and primer sets of the candidate reference genes used for qRT-PCR

Gen	Primer sequence (Forward/Reverse)	Length (bp)	GC%	Tm (°C)
Actin	5'-GAGAATATGATGAGTCGGGG-3'/	142	50	52.1
	5'-TGGACCTTAAGCAAATCCAA-3'		40	51.9
Tubulin	5'-AGCGAAATTCTGGGAAGTAG-3' /	116	45	52.2
	5'-AGCTCGCTTCATTGTAGTAG-3'		45	51.9
GAPDH	5'-TCCTAATTCAAACACACCCT-3' /	136	40	51.3
	5'-ACGACAAACATAGGAGCATTT-3'		38	52.4

Note: bp: Base pair; Tm: Melting temperature

The qRT-PCR amplification was performed in 96-well plates using ExcelTaq 2X Fast q-PCR Master Mix SYBR Kit (Smobio, China) on a CFX96 Touch Real-Time PCR Detection System machine (Biorad, USA). Reactions were performed in a total volume of 10 µL containing 5 µL of 2x qPCR mixture, 1 µL of 10-fold diluted cDNA, 0.8 µL each of forward and reverse primer (10 µM), and 2.4 µL of nuclease-free water. The qRT-PCR conditions were as follows: 95°C for 2 min (predenaturation) followed by 40 cycles of 95°C for 15 sec (denaturation), 55°C for 30 sec (annealing), and 65°C for 5 sec (extension). After completion of amplification, a melting curve analysis was performed. Therefore, the technical and biological replications were performed thrice. The validation of reference genes was also analyzed to test the stability of the candidate reference genes. In this study, we compare the gene expression of targeted genes from different organs in shallot. The same band intensity will be visualized when the resulting amplification is stable. The primer for qRT-PCR (Table 1) and relative gene expression were analyzed using the Livak or $2^{-\Delta\Delta CT}$ method.

Relative quantification of sucrose phosphate synthase

The ACT and TUB were used as reference genes for the relative quantification of Sucrose Phosphate Synthase (SPS) in shallot. The previous study showed that SPS in shallot 'TukTuk' was up-regulated by UV-B induction (Widiastuti et al. 2024). The sequence of SPS set primers is 5'-TCAGGTGCCTTGAATGTTCC-3' for forward primer and 5'-AAACCCCACTGCTCATCGAT-3' for reverse primer. The qRT-PCR conditions were as follows: 95°C for 2 min (predenaturation) followed by 40 cycles of 95°C for 15 sec (denaturation), 55°C for 30 sec (annealing), and 65°C for 5 sec (extension). The experiments were replicated thrice with at least three plants each time.

Data analysis

The primer candidates for qRT-PCR were designed by selecting only the conserved region and the sequence was inserted into Primer-BLAST Tools software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers of gene reference were optimized using PCR, and only the single band was selected for further qRT-PCR analysis. The stability reference gene was evaluated using standard deviation values. The reference gene obtained with the lowest Standard Deviation (SD) showed the smallest variation and was considered the most stable (Antiabong et al. 2016; Dong et al. 2022; Song et al. 2022).

RESULTS AND DISCUSSION

Characters of bulb development from True Seed Shallot (TSS) and seed bulb

True Seed Shallot (TSS) and seed shallot cultivar 'TukTuk' bulbs were monitored until they formed a bulbous shape. The shallot cultivar 'TukTuk' bulb development was evaluated during bulb development concerning the phenological growth stage (Pangestuti et al. 2022). The bulb's initial phase from TSS was 6 WAP, the second phase in which the bulb was enlarged was 8 WAP, and the third phase approached maturity at 11 WAP. Conversely, the bulb developmental stage from the seed bulb was faster than TSS. The bulb's initial phase of TSS was 4 WAP, the second phase was 5 WAP when the bulb was aggregated, and the third stage was 7 WAP when bulbs matured. Shallot plants from TSS required a longer period of 4 months to finish the vegetative phase (bulb formation) than shallot plants from seed bulbs. Interestingly, 'TukTuk' tends to produce single bulb when cultivated using TSS and produce single bulb or multiple bulbs when cultivated seed bulbs.

Multiple bulbs could be generated when they were cultivated using seed bulbs. However, a dominantly single bulb could be obtained when 'TukTuk' was cultivated using TSS. Shallot cultivars from TSS showed different abilities in multiple bulbs (Sulistyaningsih et al. 2020). The phenomenon of yielding single bulb in 'TukTuk' was similar phenomenon of single and large bulbs in onion. Single and large bulbs commonly exist in onion cultivation in which the material planting used is seed as reported by Lee et al. (2020) in South Korea and Tsuchiya et al. (2021) in Japan as subtropical countries that use onions in their culinary.

Selection of candidate reference genes and primer specificity

Three candidate reference genes, namely Actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-tubulin (TUB), were selected based on the nucleotide sequence data of the genus *Allium* in NCBI (Figure 1). To identify the primer specificity, the amplified products were first evaluated using the regular PCR method and visualized using agarose gel electrophoresis. Only one clear band of the expected size appeared in each lane, and no primer dimers or non-specific amplification could be detected (Figure 2).

Actin drives a dynamic equilibrium of monomers and polymers within living cells' nuclei. It plays a fundamental role in plant morphogenesis, determining the cell division plane, directed cell expansion, and the wall material cell deposition. During polarized pollen tube elongation, microtubules act as deposition of proteins/lipids at the tip membrane and track for vesicular transport (Gavazzi et al. 2017). Moreover, Gilliland et al. (2003) explained that actin (ACT) is the most strongly expressed in young plant tissues and has the greatest response to physiological cues, such as plant cell morphogenesis, including cell wall component, growth of hairy root, trichome, pollen tube, cell elongation and meristem apical. Blessing et al. (2004) reported that gene regulation, including mRNA processing, chromatin remodeling, and global gene expression were activities in the cells that required actin. Polymeric actin is now specifically linked to transcription by RNA polymerase I, II, and III. An active process requiring both actin polymers and myosin drives RNA polymerase I transcription. Moreover, it is implicated in long-range chromatin movement. This mechanism brings activated genes from separate chromosomal territories together and then participates in their compartmentalization near nuclear speckles. Nuclear speckle formation requires polymeric actin and factors promoting polymerization, such as profilin and PIP2, which

are concentrated there. This mechanism will relate to cell division. de Lima Rebouças et al. (2013) stated that the β-actin gene encodes a structural protein of the cytoskeleton and is perhaps the most widely used gene for normalization in gene expression experiments.

Microtubules are polymers from α- and β-tubulin monomers. Tubulins are often expressed at high levels in most tissues because the cytoskeleton requires many microtubules. However, the expression of almost all tubulin genes is spatially and temporally controlled during growth, development, and in response to specific signals. The different patterns of tubulin expression suggest that tubulin isoforms may play specific roles during plant development. Therefore, tubulin genes are expressed highly redundantly in vegetative tissues and most abundant in the meristematic leaf tissue during leaf growth (Radchuk 2008).

GAPDH is widely known as a glycolytic enzyme catalyzing. Nevertheless, various GAPDH functions have been found unrelated to glycolysis. Some of these functions presume the interaction of GAPDH with DNA, but its translocation to the nucleus mechanism is not fully understood. When in the nucleus, GAPDH participates in the transcription and initiation of apoptosis of genes involved in cell proliferation and anti-apoptotic pathways and plays a role in telomere length regulation. GAPDH also participates in the transcription of genes involved in anti-apoptotic pathways and cell proliferation through its direct interaction with DNA and/or proteins (Kosova et al. 2017).

GAPDH gene sequences used in this study were obtained from NCBI database, namely *Allium ampeloprasum* glyceraldehyde-3-phosphate dehydrogenase (GAPC2) gene, partial cds (MF158631.1); *A. cepa* var. *aggregatum* GAPC2 gene, partial cds (KX373357.1); *A. schoenoprasum* GAPC2 gene, partial cds (GU206352.1); and unverified: *A. sativum* GAPC2-like mRNA, complete sequence (KP116306.1). Alignment results showed that the selected gene sequences are mostly similar and. nearly exhibited a significant degree of homology with conserved regions (Figure 1.A). Based on this alignment result, we selected the conserved region sequences and designed the primer for gene expression analysis. The size of PCR product that has been designed was not longer than 200 bp. This pair of primers was further validated using PCR amplification and observed for the primer specificity whether only single band can be produced.

Actin gene sequences used in this study were obtained from NCBI database, namely *A. cepa* β-actin mRNA, complete cds (MF919598.1); *A. sativum* actin mRNA, partial cds (AY821677.1); *A. cepa* actin mRNA, partial cds (GU570135.2); and *A. cepa* actin-like mRNA, partial sequence (JN797640.1); The certain sequences have similar among *Allium* species (Figure 1.B), although the alignment result of ACT was more lowly conserved than GAPDH. The conserved region that has been decided to design the primer was more challenging since there are not many conserved regions among ACT from *Allium* species.

Tubulin gene sequences used in this study were obtained from NCBI database, namely *A. sativum* tubulin mRNA, partial cds (AY148156.1); *A. cepa* tubulin mRNA, complete cds (MF919599.1); and unverified: *A. cepa* tubulin-like mRNA, partial sequence (KY072875.1). Similar to ACT, the conserved region among TUB from *Allium* species was not higher compared to GAPDH. Therefore, only certain conserved region that exhibited a significant degree of homology was selected for further primer design (Figure 1.C). By using the information of conserved region from each region, primer candidates of ACT, TUB, and GAPDH could be designed by selecting only the conserved regions and the sequence inserted into Primer-BLAST Tools software. This strategy has been applied for primer design method since the target sequence cannot be found in the Genebank.

Figure 1. Multiple sequence alignment of: A. GAPDH; B. ACT; C. TUB genes

Although three candidate reference genes were specifically amplified, the ACT, TUB, and GAPDH transcript levels differed among phenological bulb growth stages in leaves (6,8,11 WAP) and bulbs (4,5,7 WAP) (Figure 2). Bulb growth stages were observed as follows: 6 WAP (bulb initial phase), 8 WAP (bulb enlarge phase), and 11 WAP (bulb mature phase) for plants from seeds, while 4 WAP (bulb initial phase), 5 WAP (bulb enlarge phase), and 7 WAP (bulb aggregation and mature phase) in plants from bulbs. Expression level of GAPDH was not stable between leaves and bulb organs, which was the low intensity of amplified bands in all bulb growth stages of plants from TSS (Figure 2). The visual appearance of PCR products showed that the PCR results of actin and GAPDH genes had different DNA band thicknesses in leaves and bulbs. This result indicates that the level of gene expression in each organ (leaves and bulbs) in shallot was different. However, tubulin genes produced the thickness of the DNA band of PCR results with almost the same in leaves and bulbs. The most stable tubulin gene in leaves was obtained in the bulb's initial phase (6 WAP from TSS and 4 WAP from seed bulb) and the most stable tubulin gene in bulbs was obtained in all bulb development phases (6,8,11 WAP from TSS and 4,5,7 WAP from seed bulb). Therefore, to prove that the amplified genes are actin, GAPDH, and tubulin genes, PCR products should be sequenced using the DNA Sanger method in further studies. Amplified DNA bands of actin, GAPDH, and tubulin genes are shown in Figure 2. It has been reported that the expression of plastidial GAPDH plays an important role in plant metabolism and is involved in abiotic stress response (Ling et al. 2014; Bao et al. 2016; Li et al. 2019; Li et al. 2020; Chen et al. 2023). Moreover, Wang et al. (2019) and Wang et al. (2023) reported that GAPDH was the most unstable gene during cold and drought stress conditions and in exclusive tissues, which was consistent with the screening results of reference genes in garlic under salt stress. Therefore, GAPDH was not selected for further analysis using qRT-PCR to evaluate the amplification specificity through melting curves for pairs of ACT and TUB genes.

Validation the candidate reference genes using qRT-PCR

The amplification primer specificity through melting curves for pairs of ACT and TUB genes was assessed using RT-qPCR. A single peak in the melting curve was obtained after amplifying the two genes (Figures 3.A and 3.B). Two candidate reference genes, actin (ACT) and β-tubulin (TUB) were validated by qRT-PCR. The reference gene with the lowest Standard Deviation (SD) was considered the most stable (Figure 3.C). Dong et al. (2022) also reported a similar result in their study. Our result showed that TUB has a lower SD than ACT. This means the tubulin gene is a stable reference gene in the bulb development of shallot. Lim et al. (2014) stated that the accuracy of qPCR results strongly relied on selecting one or more reference genes stably expressed across different tissues or organ samples.

Different reference genes' influences on the target mRNA's expression were also observed using a gene-encoding Sucrose Phosphate Synthase (SPS). SPS was selected as a model gene, up-regulated by UV-B exposure for 6 hours (Widiastuti et al. 2024) for additional RT-qPCR analysis. It is in good agreement that the most stable gene used TUB as a reference for normalization (Figure 3.C). The relative expression level of SPS showed significant up-regulation using TUB compared to ACT. Moreover, the relative expression level of SPS, which TUB normalized, showed a lower SD value than ACT as a gene reference (Figure 4). Based on the three housekeeping genes tested, the tubulin gene (TUB) had the potential as a reference gene for qRT-PCR analysis in shallot, because it was consistently expressed in leaves and bulbs in the bulb development stage.

These three reference genes (ACT, TUB, and GAPDH) are selected because their expression levels are assumed to remain stable across different conditions, tissues, or treatments (Radchuk 2008; de Lima Rebouças et al. 2013). Stable expression ensures that any changes in gene expression observed in experimental conditions can be attributed to the treatment or condition under study rather than to variations in the reference gene itself. However, it is important to note that no gene can be guaranteed to have completely stable expression across all conditions and organs in a plant system. Different tissues or treatments may still affect the expression of some reference genes. Therefore, the selection of stable gene expression in different organs is essential since there is limited information on shallot genome sequence in the gene bank database. Overall, this is the first study to evaluate the candidate reference genes from two different organs in shallot, such as leaves and bulbs. In order to explore the physiological research in shallot including aggregation in bulb development phase, the stable expression of reference genes were required. Therefore, the three candidate reference genes (ACT, TUB, and GAPDH) were selected for further analysis. The obtained candidate primers were validated using PCR analysis to reveal the expression level in leaf and bulb organs.

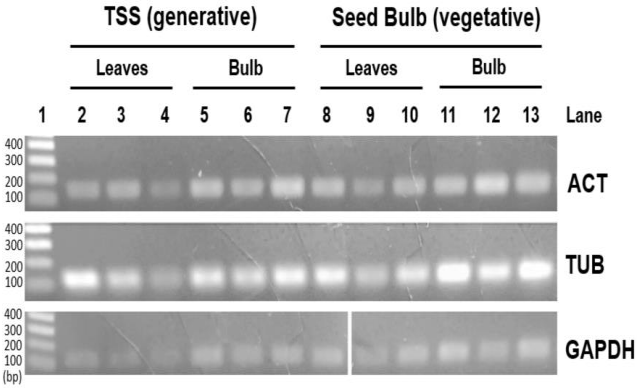

Figure 2. Amplification products of 3 candidate reference genes (ACT, TUB, GAPDH). Lane 1 (M): 1 kb DNA ladder marker; lanes 2-3-4: 6-8-11 WAP from leaves of TSS; lanes 5-6-7: 6-8-11 WAP from the bulb of TSS; lanes 8-9-10: 4-5-7 WAP from leaves of seed bulb; lanes 11-12-13: 4-5-7 WAP from bulb of seed bulb

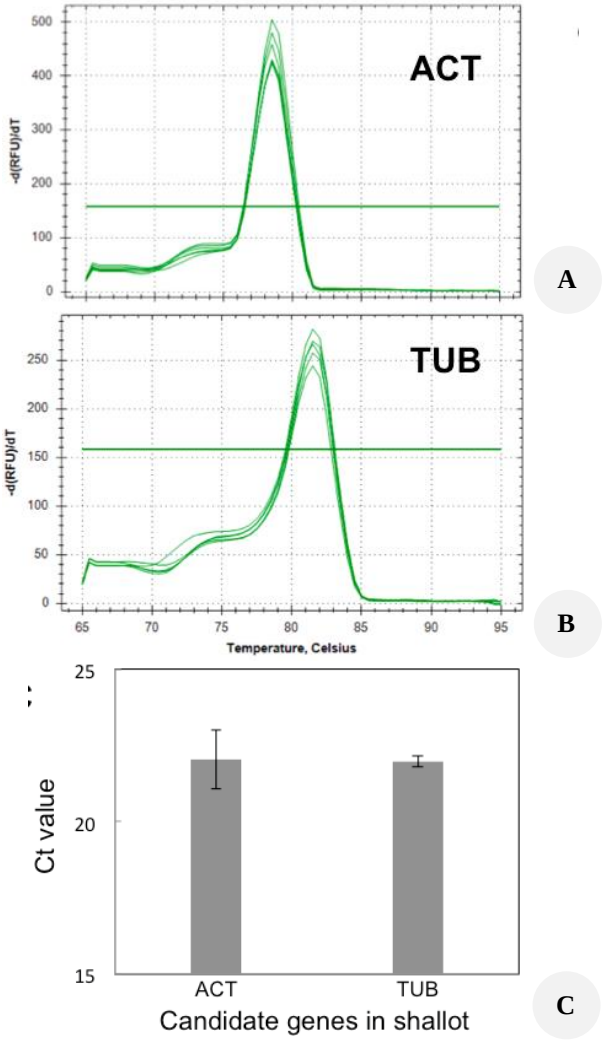

Figure 3. Melt curve analysis for qRT-PCR analysis of reference genes: A. Actin (ACT); B. Tubulin (TUB); C. Expression level of the two candidate reference genes evaluated in shallot. Values are measured as the cycle threshold (Ct, mean of triplicate samples) and are inversely proportional to the number of templates

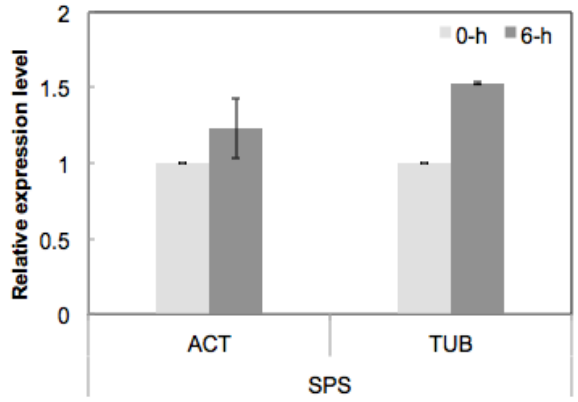

Figure 4. The relative expression level of SPS with different reference genes, ACT and TUB, after UV-B exposure for 6 hours as a case study

Our result showed that the expression level of ACT and TUB in bulb and leaves were the same band intensity compared to GAPDH (Figure 2). On the other hand, there are some considerations regarding the stability of reference genes during different growth periods. During vegetative growth, plants are primarily focused on leaf, stem, root development, and bulb development especially in shallot. Reference genes that are commonly stable across various tissues in vegetative growth stages include those involved in basic cellular functions such as metabolism, protein synthesis, and cellular structure maintenance. Therefore, it is important to carefully select and validate reference genes for each specific experimental condition to ensure accurate normalization of gene expression data across different growth stages of the plant. It showed the GAPDH band patterns were not homogeneously amplified. Thus, it indicated that expression of GAPDH was not stable compared to ACT and TUB (Figure 2). It suggested that its expression level remains relatively constant and can therefore be used to accurately normalize the expression levels of other genes of interest in the experimental samples. This normalization step is crucial for accurate interpretation of gene expression data, ensuring that observed differences in gene expression are due to biological factors rather than technical variations.

Finally, the model gene with a case study of SPS was selected to validate the most stable and suitable reference genes (Widiastuti et al. 2024). In practice, validation of target gene expression typically involves performing qRT-PCR to measure the expression levels of target genes in experimental samples. These measurements are then normalized using the validated reference genes, providing a reliable basis for comparing gene expression levels across samples or conditions. In Figure 4, the relative expression of SPS was compared by using ACT and TUB as reference gene. Based on this result, it exhibited that a higher standard error was shown in ACT compared to TUB. Therefore, TUB has a stable expression and potential for further gene expression analysis in shallot.

In conclusion, selecting appropriate reference genes is a critical first step in gene expression studies, validating the expression of target genes ensures the accuracy, reliability, and interpretability of results. Reference genes should ideally have stable expression across all experimental conditions, tissues, developmental stages, and treatments being studied. This stability expression in TUB ensures that variations in the expression of target genes are not due to differences in reference gene expression. The expression levels of TUB remain constant under the experimental conditions being investigated in leaves and bulb organs in bulb development phase (initial and bulb mature stages) both from TSS and seed bulb. We recommend the tubulin gene for housekeeping gene in qRT-PCR analysis of shallot, especially in bulb development phase. Our result provides a basis for the selection of housekeeping gene as reference genes to normalize qRT-PCR data in shallot and lay molecular analysis for studying the mechanism of aggregation bulb in shallot.

ACKNOWLEDGEMENTS

We expressed gratefully Academic Excellent Grant 2023 No. 7725/UNI 1.P.II/Dit-Lit/PT.01.03/2023 from Universitas Gadjah Mada, Yogyakarta, Indonesia to support funding for this research.

REFERENCES

Antiabong JF, Ngoepe MG, Abechi AS. 2016. Semi-quantitative digital analysis of polymerase chain reaction-electrophoresis gel: Potential applications in low-income veterinary laboratories. Vet World 9 (9): 935-939. DOI: 10.14202/vetworld.2016.935-939.

Bao W, Qu Y, Shan X, Wan Y. 2016. Screening and validation of housekeeping genes of the root and cotyledon of *Cunninghamia lanceolata* under abiotic stresses by using quantitative real-time PCR. Intl J Mol Sci 17 (8): 1198. DOI: 10.3390/ijms17081198.

Blessing CA, Ugrinova GT, Goodson HV. 2004. Actin and ARPs: Action in the nucleus. Trends Cell Biol 14 (8): 435-442. DOI: 10.1016/j.tcb.2004.07.009.

Chen Y, He Q, Li X, Zhang Y, Li J, Zhang L, Yao X, Zhang X, Liu C, Wang H. 2023. Identification of accurate reference genes for qRT-PCR analysis of gene expression in *Eremochloa ophiuroides* under multiple stresses of phosphorus deficiency and/or aluminum toxicity. Plants 12 (21): 3751. DOI: 10.3390/plants12213751.

de Lima Rebouças E, do Nascimento Costa JJ, Passos MJ, de Sousa Passos JR, van den Hurk R, Silva JRV. 2013. Real time PCR and importance of housekeepings genes for normalization and quantification of mRNA expression in different tissues. Braz Arch Biol Technol 56 (1): 143-154. DOI: 10.1590/S1516-89132013000100019.

Dong Z, Zhan B, Li S. 2022. Selection and validation of reference genes for gene expression studies using quantitative real-time PCR in prunus necrotic ringspot virus-infected *Cucumis sativus*. Viruses 14 (6): 1269. DOI: 10.3390/v14061269.

Eisenberg E, Levanon EY. 2013. Human housekeeping genes, revisited. Trends Genet 29 (10): 569-574. DOI: 10.1016/j.tig.2013.05.010.

Gavazzi F, Pigna G, Braglia L, Giani S, Breviario D, Morello L. 2017. Evolutionary characterization and transcript profiling of β-tubulin genes in flax (*Linum usitatissimum* L.) during plant development. BMC Plant Biol 17 (1): 237. DOI: 10.1186/s12870-017-1186-0.

Gilliland LU, Pawloski LC, Kandasamy MK, Meagher RB. 2003. Arabidopsis actin gene ACT7 plays an essential role in germination and root growth. Plant J 33 (2): 319-328. DOI: 10.1046/j.1365-313X.2003.01626.x.

Jones RN. 2017. Cytogenetics. In: Rabinowitch HD (eds). Onions and Allied Crops. Volume I: Botany, Physiology, and Genetics. CRC Press, Boca Raton. DOI: 10.1201/9781351075169.

Kosova AA, Khodyreva SN, Lavrik OI. 2017. Role of glyceraldehyde-3 phosphate dehydrogenase (GAPDH) in DNA repair. Biochemistry 82 (6): 643-654. DOI: 10.1134/S0006297917060013.

Lee J, Moon J-S, Kim J, Park G-O, Kwon J-H, Ha I-J, Kwon Y-S, Chang Y-H. 2020. Evaluation of onion cultivars as affected by bulb maturity and bulb characteristics of intermediateday yellow onions in South Korea. J Hortic Sci Biotechnol 95 (5): 645-660. DOI: 10.1080/14620316.2020.1742586.

Li L, Li N, Fang H, Qi X, Zhou Y. 2020. Selection and validation of reference genes for normalisation of gene expression in *Glehnia littoralis*. Sci Rep 10 (1): 7374. DOI: 10.1038/s41598-020-63917-5.

Li X, Wei W, Li F, Zhang L, Deng X, Liu Y, Yang S. 2019. The plastidial glyceraldehyde-3-phosphate dehydrogenase is critical for abiotic stress response in wheat. Intl J Mol Sci 20 (5): 1104. DOI: 10.3390/ijms20051104.

Lim F-H, Fakhrana IN, Rasid OA, Idris AS, Parveez GKA, Ho C-L, Shaharuddin NA. 2014. Isolation and selection of reference genes for *Ganoderma boninense* gene expression study using quantitative real-time PCR (qPCR). J Oil Palm Res 26 (2): 170-181.

Lin Y, Liu G, Rao Y, Wang B, Tian R, Tan Y, Peng T. 2023. Identification and validation of reference genes for qRT-PCR analyses under different experimental conditions in *Allium wallichii*. J Plant Physiol 281: 153925. DOI: 10.1016/j.jplph.2023.153925.

Ling H, Wu Q, Guo J, Xu L, Que Y. 2014. Comprehensive selection of reference genes for gene expression normalization in sugarcane by real time quantitative RT-PCR. PLoS One 9 (5): e97469. DOI: 10.1371/journal.pone.0097469.

Lorenz TC. 2012. Polymerase chain reaction: Basic protocol plus troubleshooting and optimization strategies. J Vis Exp 63: e3998. DOI: 10.3791/3998.

Moldovan C, Frumuzachi O, Babotă M, Barros L, Mocan A, Carradori S, Crişan G. 2022. Therapeutic uses and pharmacological properties of shallot (*Allium ascalonicum*): A systematic review. Front Nutr 9: 903686. DOI: 10.3389/fnut.2022.903686.

Pabuayon IM, Yamamoto N, Trinidad JL, Longkumer T, Raorane ML, Kohli A. 2016. Reference genes for accurate gene expression analyses across different tissues, developmental stages and genotypes in rice for drought tolerance. Rice 9 (1): 32. DOI: 10.1186/s12284-016-0104-7.

Pangestuti R, Sulistyaningsih E, Kurniasih B, Murti RH. 2022. Effect of seed soaking with plant growth regulators combination on the aggregation ability of shallot from seeds. Ilmu Pertanian 8 (3): 186-195. DOI: 10.22146/ipas.90993.

Radchuk VV. 2008. The transcriptome of the tubulin gene family in plants. In: Blume YB, Baird WV, Yemets AI, Breviario D (eds). The Plant Cytoskeleton: A Key Tool for Agro-Biotechnology. NATO Science for Peace and Security Series C: Environmental Security. Springer, Dordrecht. DOI: 10.1007/978-1-4020-8843-8_11.

Rosliani R, Hidayat IM, Sulastrini I, Hilman Y. 2016. Dissemination of technology for shallot (*Allium ascalonicum* L.) seed production using True Shallot Seed (TSS) in Indonesia. Acta Hortic 1143: 345-352. DOI: 10.17660/ActaHortic.2016.1143.49.

Saidah, Muchtar, Syafruddin, Pangestuti R. 2019. The effect of plant spacing at the growth and yield of shallot from true shallot seed in Sigi District, Central Sulawesi. Pros Sem Nas Masy Biodiv Indon 5: 209-212. DOI: 10.13057/psnmbi/m050211.

Song J, Cho J, Park J, Hwang JH. 2022. Identification and validation of stable reference genes for quantitative real time PCR in different minipig tissues at developmental stages. BMC Genomics 23 (1): 585. DOI: 10.1186/s12864-022-08830-z.

Sulistyaningsih E, Pangestuti R, Rosliani R. 2020. Growth and yield of five prospective shallot selected accessions from true seed of shallot in lowland areas. Ilmu Pertanian 5 (2): 92-97. DOI: 10.22146/ipas.52457.

Sutardi, Kristamtimi, Purwaningsih H, Widyayanti S, Arianti FD, Pertiwi MD, Triastono J, Praptana RH, Malik A, Cempaka IG, Yusuf, Yufdy MP, Anda M, Wihardjaka A. 2022. Nutrient management of shallot farming in sandy loam soil in Tegalrejo, Gunungkidul, Indonesia. Sustainability 14 (19): 11862. DOI: 10.3390/su141911862.

Tong J, Hu M, Han B, Ji Y, Wang B, Liang H, Liu M, Wu Z, Liu N. 2021. Determination of reliable reference for gene expression studies in Chinese chive (*Allium tuberosum*) based on the transcriptome profiling. Sci Rep 11 (1): 16558. DOI: 10.1038/s41598-021-95849-z.

Tsuchiya Y, Nakamura Y, Mikami T. 2021. Japanese bulb onion: Production, consumption, and cultivars. J Hortic Res 29 (1): 75-82. DOI: 10.2478/johr-2021-0002.

Valente V, Teixeira SA, Neder L, Okamoto OK, Oba-Shinjo SM, Marie SK, Scrideli CA, Paçó-Larson ML, Carlotti Jr CG. 2014. Selection of suitable housekeeping genes for expression analysis in glioblastoma using quantitative RT-PCR. Ann Neurosci 21 (2): 62-63. DOI: 10.5214/ans.0972.7531.210207.

Wang G, Tian C, Wang Y, Wan F, Hu L, Xiong A, Tian J. 2019. Selection of reliable reference genes for quantitative RT-PCR in garlic under salt stress. PeerJ 7: e7319. DOI: 10.7717/peerj.7319.

Wang Q, Guo C, Yang S, Zhong Q, Tian J. 2023. Screening and verification of reference genes for analysis of gene expression in garlic (*Allium sativum* L.) under cold and drought stress. Plants 12 (4): 763. DOI: 10.3390/plants12040763.

Widiastuti A, Sawitri WD, Idris M, Handayani VDS, Winona B, Silalahi CM, Matra DD, Doni F, Setiyadi AH. 2024. Unraveling the potential UV-B induced gene expression of the primary and secondary metabolisms in shallot. Rev Agric Sci 12: 111-127. DOI: 10.7831/ras.12.0_111.

Zhang C, Zhang H, Zhan Z, Liu B, Chen Z, Liang Y. 2016. Transcriptome analysis of sucrose metabolism during bulb swelling and development in onion (*Allium cepa* L.). Front Plant Sci 7: 1425. DOI: 10.3389/fpls.2016.01425.