



Article

Reference Gene Selection and Gene Expression Analysis during Gall Development of *Zizania latifolia*

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Abstract: The stem tips of *Zizania latifolia* at different development stages were used as research materials. The expression stability of nine candidate reference genes (*ACT1*, *H2B*, *UBI*, *EF-1 α* , *GAPDH*, β -*actin*, *60S*, *SKIP* and *AQP*) were evaluated using qRT-PCR. The data were analyzed with GeNorm and NormFinder software. Present results indicated that the expression of *ACT1* was stable and that it could be used as the optimal reference gene for studying the development stage of gall formation. *ACT1* was selected as the reference gene to verify the expression level of the correlative genes in the gall formation stage of *Z. latifolia*. Our results were consistent with the previous transcriptome sequencing results. This study revealed that *ACT1* was the classic reference gene for the analysis of correlative genes in all of the gall development stages of *Z. latifolia*.

Keywords: *Zizania latifolia*; stage of gall formation; real-time quantitative PCR; reference gene



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1. Introduction

Real-time quantitative PCR (qRT-PCR) has become an important tool in modern molecular biology for the study of gene expression variation due to its high sensitivity, high specificity, good reproducibility, and wide range of dynamic quantitative monitoring [1]. This technique provides support for the analysis of complex pathogenesis, genetic traits, resistance and basic defense, and biometabolic pathways in plants and animals. However, as reported by Ginzinger [2], Derveaux [3], and the researchers mentioned further, in the practical application of qRT-PCR technology, there may be some bias and instability in qRT-PCR data results due to uncertainties in RNA extraction quality, reverse transcription efficiency, and template quality [4]. There were two methods for mRNA quantification using qRT-PCR: “absolute quantification” and “relative quantification”. The objective of “relative quantification” is to determine the relative proportion of target genes in a sample—which usually requires one or more housekeeping genes with stable expression in various tissues, stages of growth and development as reference genes to correct and standardize the data [5]—and to obtain relative accurate and reliable data results compared with non-reference genes. Therefore, the selection of stable reference genes for “relative quantification” analysis is an important prerequisite for the study of gene expression using the qRT-PCR technique. The commonly used reference genes are ribosomal rRNA (*18S rRNA* and *25S rRNA*), microtubulin gene (*Tubulin*), actin gene (*Actin*), transcriptional elongation factor gene (*EF-1 α*), cyclophilin gene (*CYP*), and glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) for plants due to the expression levels of these genes being relatively stable [6–9]. However, an increasing number of studies have shown that although these reference genes are constitutively expressed in different plants with high conserved and relatively consistent expression levels, their expression varies somewhat across cells, tissues, organs, and stages of growth and development. Many genes have proven to be

somewhat variable in in-depth studies, and no single reference gene can be applied to all experimental studies under all conditions for plants [10,11]. Therefore, with the increasing requirements for quantitative analysis, simultaneous validation screening of new reference genes or using multiple reference genes has become an effective strategy to solve the drawbacks [12]. Before using the qRT-PCR technique for quantitative analysis, a stable and suitable candidate reference gene should be validated and screened based on test materials and set conditions as the best and stable quantitative reference applicable for further study.

Zizania latifolia is an aquatic herb of the genus *Zizania*. The stem of *Z. latifolia* infected by *Ustilago esculenta* expands to form a part that can be used as a vegetable, which is called Jiaobai. Jiaobai is one of the most important aquatic vegetables in China [13]. It has been reported that *Z. latifolia* is rich in protein, amino acids, polysaccharides, dietary fiber, and other nutrients, and it has a crunchy and fresh taste, which is why it is consumed by many Chinese people [14]. In recent years, due the wide range of products, diverse patterns, excellent quality, and outstanding benefits of *Z. latifolia*, it has been flourishing in Chinese bazaars, exhibiting a good scaling developmental pattern, industrialization, and branding. The product organ of *Z. latifolia* is the enlarged flesh stem infected and formed by *Ustilago esculenta*. *U. esculenta* is an endophytic fungus, which proliferates in the stem of *Z. latifolia*. *U. esculenta* prevents the formation of flowering organs and stimulates the plant to secrete growth hormones to form edible product organs, which is quite different from the effects caused by other pathogenic bacteria infesting the plant [15]. Therefore, it is important to study the formation mechanism of the fleshy stem expansion in *Z. latifolia* and extract relevant functional genes from it to reveal the adaptive evolution mechanism and trait improvement of *Z. latifolia* after infestation by *U. esculenta*. The transcriptome sequencing work of *Z. latifolia* has already been completed, based on which several differentially expressed genes related to stress responses and hormone syntheses have been mined [16].

In the present study, *ACT1*, *H2B*, *UBI*, *EF-1 α* , *GAPDH*, *β -actin*, *60S*, *SKIP*, and *AQP* were selected as candidate reference genes for qRT-PCR using fleshy stem tips at different developmental stages during the gestation period of *Z. latifolia*. The expression stability of the relevant genes was analyzed using reference gene evaluation software GeNorm v 3.5 and Normfinder v 0.953 [17]. The *YUCCA* gene is a rate-limiting enzyme-coding gene for IAA biosynthesis, which plays an important role in IAA biosynthesis and regulation [18]. *ZINPR1* is a key regulatory gene in the salicylate-mediated SAR pathway, which initiates the expression of the downstream disease-resistant gene *ZIPR1* [19]. *ZILEA3* is an important gene for plant resistance to osmotic stresses, which is mainly induced by water stress and ABA [20]. The expression patterns of the key genes *ZIYUCCA*, *ZINPR1*, *ZIPR1*, and *ZILEA3* during the occurrence of a fleshy stem expansion in *Z. latifolia* were analyzed by using the best reference genes that were validated and screened, and their reliability was also verified to ensure that the mechanism of *Zizania* formation could be studied and to lay a theoretical foundation for the future directed breeding of new *Z. latifolia* varieties.

2. Materials and Methods

2.1. Test Materials

Z. latifolia cultivator ‘Zhejiao 10’, a double season Jiaobai, selected by Jinhua Agricultural Science Research Institute, was used as the experimental material. The material was planted in the field at the National Aquatic Vegetable Breeding Innovation Base (Jinhua). Sample collection was conducted in May 2021 during the critical period of the occurrence of Jiaobai seedling (3 days before and 1, 5, 9, and 13 days after pregnancy) (Figure 1). After cleaning the fleshy stems with 75% anhydrous ethanol, a blade was used to remove the tip of the stem. Each experiment was completed with three replicates. All collected samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.



Figure 1. Different stages of gall formation in *Z. latifolia*.

2.2. RNA Extraction, Quality Testing, and cDNA Reverse Transcription

Total RNA was extracted from frozen samples using the Trizol kit (TaKaRa, Shiga, Japan). The integrity and purity of the extracted RNA were evaluated by 2% agarose gel electrophoresis and Nanodrop ND2000 ultraviolet spectrophotometer (Thermo, Waltham, MA, USA). Only the A260/280 value of the total RNA of each sample ranging between 1.8 and 2.1 met the requirements of subsequent experiments. The above RNA samples (1 µg) were used as a template for cDNA synthesis using a full gold reverse transcription kit (AE311-03, TransGen Biotech Company, Beijing, China). The cDNAs were stored at −20 °C until analyzed.

2.3. Primer Design of Candidate Reference Genes and Pregnancy-Related Genes in Jiaobai

Referring to the research results of reference genes in rice [6], wheat [7], Chinese cabbage [4], dandelion [8], and eggplant [9], nine commonly used candidate reference genes were selected from the transcriptomes of *Z. latifolia* (Table 1): actin1 (*ACT1*), histone (*H2B*), polyubiquitin (*UBI*), protein translation elongation factor (*EF-1α*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actin, 60S rRNA 60S, ask-interacting protein (*SKIP*), and aquaporin (*AQP*). At the same time, key genes—*ZIYUCCA*, *ZINPR1*, *ZIPR1*, and *ZILEA3*—which play key roles for fleshy Jiaobai stem development, were selected as the target genes for identifying and screening the reliability of reference genes (Table 2). The target gene sequences were obtained by blast analysis with the previous transcriptome data of the research group. All primers were designed with Premier 5.0 online tool and were synthesized by Sheng-gong Biotechnology Co., Ltd. (Shanghai, China).

Table 1. Primers used for qRT-PCR.

Reference Gene	Primer Sequence	Product Length (bp)	Tm (°C)
<i>ACT1</i>	F: GTCAAGGCAGGTTTTGCTGG R: CACCCACGTAGGCATCCTTT	118	60
<i>H2B</i>	F: AAGAAGGCGAAGAAGAGCGT R: GGCGAGCTTCTCGAAGATGT	141	60
<i>UBI</i>	F: GCACAGATCCTCCCTCTTCG R: CGTCCATCCCAAGCTCAAGT	143	60
<i>EF-1α</i>	F: TTCCGATACCGCCGATCTTG R: GTCTCCGGTAAGACCCTCCT	115	60
<i>GAPDH</i>	F: TGCTGCCTTCTTCTCCCTG R: GGACATGAAGTCGTCGGAGG	161	60

Table 1. *Cont.*

Reference Gene	Primer Sequence	Product Length (bp)	Tm (°C)
<i>β-actin</i>	F: GGATTGGGCCTCATCACCAA R: TGGAACCGGAATGGTCAAGG	145	60
<i>60S</i>	F: AGCTTTTCCCTGGCCTTTGT R: TCTGTTCTGTGCCTGACCAC	170	60
<i>SKIP</i>	F: CAGGTCATATTCCTCCCCGC R: TGAGACACAGTCATGGGCAC	145	60
<i>AQP</i>	F: TGGACCTGGGACTCATTTGC R: CGAGCAGGGTAGGCATGATT	141	60

Table 2. The key genes primer information of gall development in *Z. latifolia*.

Pregnancy-Related Gene	Primer Sequence	Product Length (bp)	Tm (°C)
<i>ZIYUCCA</i>	F: CCTAGAAGGTAGCAAGCAGA R: TAAACCCAGTGAAAAAACG	179	60
<i>ZINPR1</i>	1F: GCTTTGGCAAGGATAATGTTTC 1R: GTTCCCGAGTTCCACTGTTT	214	56
<i>ZIPR1</i>	1F: CACTACACGCAGATCGTGTGG 1R: GTAGTAGTTGCAGGTCATGAAG	96	56
<i>ZILEA3</i>	1F: GCCGGCGAGRCCAAGGSMC 1R: GSCGGWYTTGTCCTTGCCGG	300	58

2.4. Stability Analysis of Candidate Reference Genes and Identification of Optimal Reference Genes

The cycle threshold (Ct) value was recorded for each qRT-PCR experiment using geNorm and NormFinder analyses to analyze the stability of the reference genes [17]. Using the $2^{-\Delta\Delta C_t}$ method, we converted the Ct value to the relative expression value for their respective analysis. The data analysis was conducted using SPSS 26.0 software.

3. Results

3.1. Total RNA Quality Determination and Primer Specificity Analysis

All the RNAs samples from the five periods were analyzed with 2% agarose gel electrophoresis. The results showed that the 18S and 28S RNA bands were bright and clear without obvious trailing, indicating that all the extracted RNA had suitable integrity. The picture of the RNA agarose gel electrophoresis can be found in Supplementary Figure S1. RNA concentration and quality were determined (Table 3). Moreover, the A260/280 and A260/230 of RNA were between 1.85 and 1.96 and were lower than 2.0, indicating that the extracted RNA was of high purity, free from protein or inorganic salt contamination, and could be used for cDNA synthesis. At the same time, the melting curve of the nine reference genes displayed a single peak, with no primer dimers and secondary bands being present, indicating that the selected genes could be used in subsequent experiments (Figure 2).

Table 3. Spectrophotometer test results of total RNA.

Sampling Time (Day)	A260/A280	A260/A230
−3	2.13	2.02
1	2.08	1.83
5	2.14	1.96
9	2.13	1.93
13	2.11	1.88

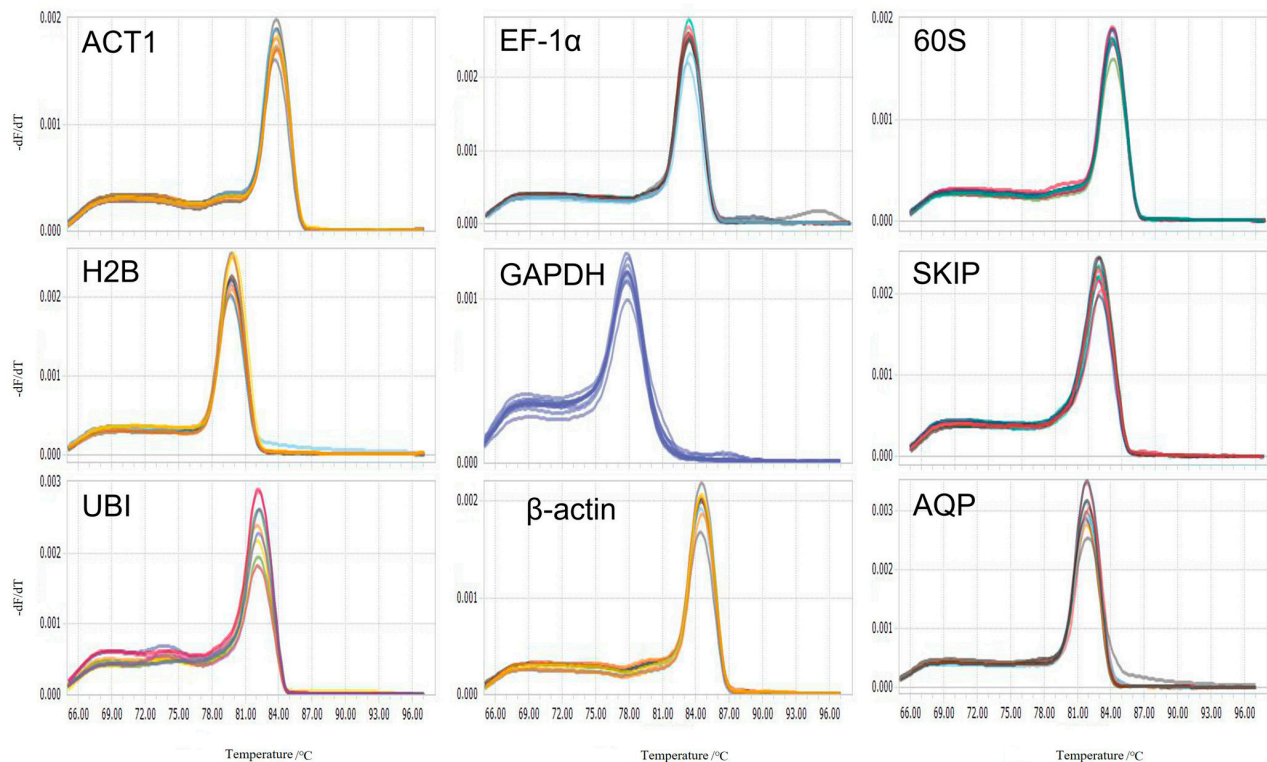


Figure 2. Melting curves of candidate reference genes.

3.2. Abundance Analysis of Candidate Reference Genes

The expression levels of candidate reference genes could be reflected by measuring the cycle threshold (C_t) values based on qRT-PCR, and the lower C_t value corresponded to higher gene expression. The C_t values of the nine candidate reference genes in the shoot tips of fleshy stems in different development stages of Jiaobai were between 17.61 and 32.07 (Figure 3). The C_t value of *H2B* were between 17.61 and 21.78, while the C_t values of *GAPDH* were between 30.13 and 30.40. The C_t value of *H2B* was low, indicating that it had the highest expression. Moreover, the C_t value of *GAPDH* was high, indicating that the expression of *GAPDH* was low.

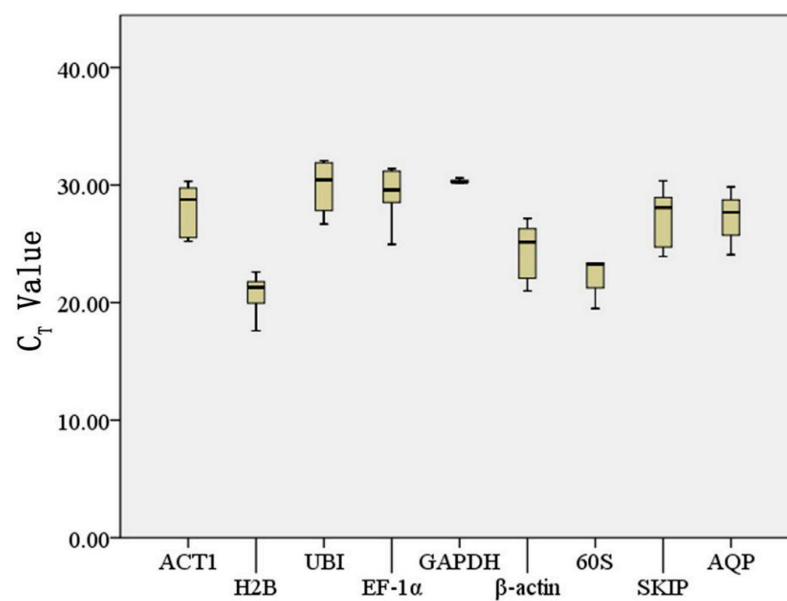


Figure 3. C_T value distribution of nine candidate reference genes.

3.3. Expression Stability of Candidate Reference Genes during Pregnancy of *Z. latifolia*

3.3.1. geNorm Analysis

The geNorm software determines the expression stability of reference genes by calculating the M value (expression stability), and the lower M value corresponds to higher stability. The strong expression stability of the nine candidate reference genes was $ACT1 = UBI > SKIP > \beta\text{-actin} > H2B > EF\text{-}1\alpha > GAPDH > 60S > AQP$ during the pregnancy of *Z. latifolia*. The results indicated that the expression levels of *ACT1* and *UBI* were the most stable, while the stability of *AQP*'s expression level was generally the worst in the shoots during pregnancy.

geNorm determined the optimal number of candidate reference genes by calculating the $Vn/n + 1 = 0.15$ value to obtain more accurate and reliable results. The program applied $Vn/n + 1 = 0.15$ as a threshold value. When $Vn/n + 1 < 0.15$, this means that the reference genes have reached the expression requirement of the target gene for correction; otherwise, an $n + 1$ target gene is required for correction. B, $V2/3 > 0.15$. The results indicated that three genes were the most suitable reference genes for the gall development of Jiaobai (Figure 4). Therefore, *ACT1* and *UBI* were selected as the reference genes in the gall stages of Jiaobai and verified with *SKIP*.

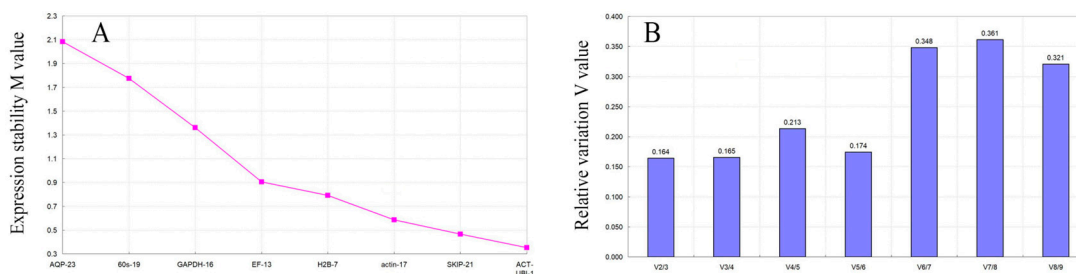


Figure 4. Expression stabilities of nine candidate reference genes (A) and optimal number of reference genes (B) analyzed by GeNorm.

3.3.2. NormFinder Analysis

NormFinder determines the stability of a reference gene by the value of gene expression's stability, with lower stability representing a more suitable reference gene. The expression level's stability of the nine candidate reference genes was as follows: $ACT1 > UBI > H2B > SKIP > \beta\text{-actin} > EF\text{-}1\alpha > GAPDH > AQP > 60S$ (Table 4). The reference gene with the highest stable expression among the nine candidate genes was *ACT1*, the worst expression stability was seen in *60S* and *AQP*, and the expression stability of *UBI* and *H2B* was moderate.

Table 4. Expression stabilities of nine candidate reference genes analyzed by NormFinder.

Reference Gene	Stability Value	Ranking
<i>ACT1</i>	0.255	1
<i>UBI</i>	0.333	2
<i>H2B</i>	0.414	3
<i>SKIP</i>	0.442	4
$\beta\text{-actin}$	0.623	5
<i>EF-1α</i>	0.813	6
<i>GAPDH</i>	1.621	7
<i>AQP</i>	1.970	8
<i>60S</i>	1.973	9

3.4. Analysis of Key Genes during Gall Development of Jiaobai

ACT1 was selected as the reference gene to analyze the expression of four target genes during the fleshy stem enlargement of *Z. latifolia*. The results showed that the expression of auxin synthesis rate-limiting enzyme gene *ZIYUCCA* was downregulated at the early

stage and significantly upregulated at the middle stage. During the late stage, *ZIYUCCA*'s expression level decreased and gradually stabilized (Figure 5). The expression level of *ZILEA3* significantly decreased in the early stage and then increased gradually. *ZIPR1* and *ZINPR1* showed similar expression patterns during the fleshy stem enlargement of *Z. latifolia*, both of which were significantly upregulated to more than ten times the initial expression level at the middle stage of growth (5 d) and then downregulated to the initial expression level. The results were consistent with the transcriptome analysis in the early stage of Jiaobai development.

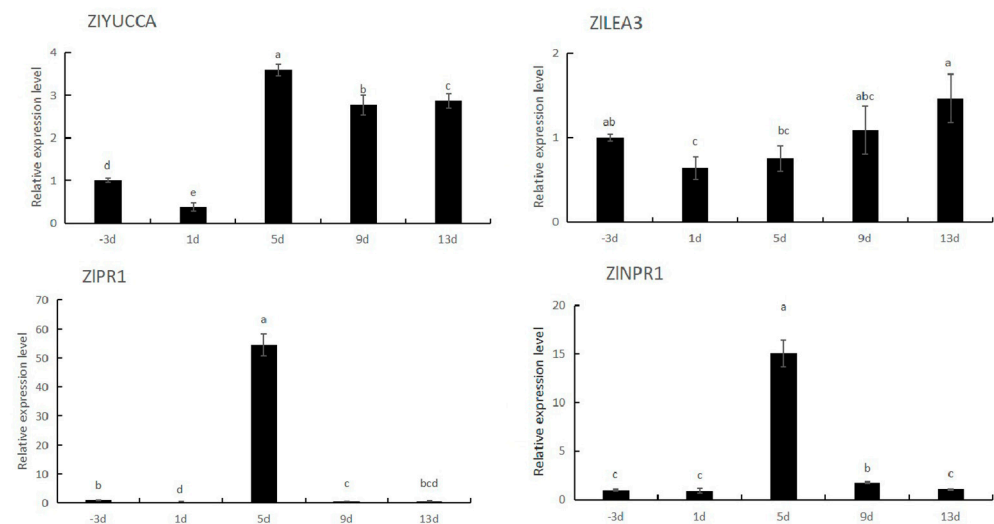


Figure 5. Gene expression analysis of the stage of gall formation in *Z. latifolia* with *ACT1* as reference gene (Different lowercase letters represent significant differences).

4. Discussion

Jiaobai is the product of the symbiosis of the *Z. latifolia* plant and *U. esculenta*. The expansion of the plant's base to form a fleshy stem during the pregnancy stage is a typical feature of *Z. latifolia*. With continued research in the field of molecular biology, the mechanism of *Z. latifolia* will be further revealed through the study of the difference in its functional genes' expression, which will become a hot spot in the future. At present, qRT-PCR is one of the common technical methods used to detect gene expression in molecular biology research, and screening suitable reference genes is the key to detecting the expression level of target genes at different developmental stages or different tissue sites. *Actin* is an important skeleton protein of cells, as a traditional reference gene for many developments of plants. *Actin* is widely distributed in the cytoplasm and has a high copy number. It is highly conserved between different species and has a relatively constant expression in various tissues and cells. So, *Actin* has often been used as a reference gene for gene expression analysis, including being applied to the period related to the synthesis of blunt anemone anthocyanin [21], walnut adventitious root stage [22], as well as the germination stages of various organs and seeds of rice [10]. In addition, in the study of *Solanum aculeatissimum*, a wild relative of eggplant, it was also found that the expression level and stability of *Actin* were less affected by the treatment of different plant growth regulators [23]. By monitoring the expression of *Actin* in different parts of *Abelmoschus esculentus*, different developmental stages, and under different treatment conditions, Li Yongping also confirmed that *Actin* is expressed in a stable manner and can be used as a suitable reference gene for *A. esculentus* [24]. *ACT1* was expressed stably under the conditions of this study and was the most suitable reference gene for the occurrence stage of gall formation in *Z. latifolia*.

The *YUCCA* gene, which encodes flavin monooxygenase, was originally identified clonally from *Arabidopsis* mutants with reduced IAA levels [18]. As a rate-limiting enzyme-coding gene for IAA biosynthesis, *YUCCA* plays an important role in IAA biosynthesis

and regulation. Overexpression of *YUCCA* can significantly increase the content of IAA in *Arabidopsis*, rice, potato, and other plants, and produce the phenotype of IAA oversynthesis. Rice expressing the antisense *OsYUCCA1* exhibited a defective phenotype similar to that of rice IAA-insensitive mutants [25]. Endogenous hormones played crucial roles in the development stage of *Z. latifolia* gall, in which IAA was thought to play the major role. In the early stage of succulent stem expansion, the IAA content of the stem tip increases rapidly and remains high during the expansion process. The results of the present study demonstrated that the expression level of *ZIYUCCA* was decreased on the first day of the white fleshy stem's expansion and peaked on the fifth day. However, it decreased slightly in the following days but remained at a high level, which was consistent with the trend of how IAA content varied [26]. The expression level of *ZIYUCCA* continuously increased. This signifies that it may participate in the expansion process of white fleshy stems by regulating the biosynthesis of IAA. As an important gene for plant resistance to osmotic stress, the expression level of *ZILEA3* was mainly induced by water stress and ABA, which was closely related to plant stress resistance [20]. During the expansion of fleshy stems, the expression of *ZILEA3* decreased and then increased, but ABA content remained almost unchanged [26]. It was speculated that it may maintain the water content in Jiaobai by adjusting the osmotic pressure during the expansion of the fleshy stems. *ZINPR1* is a key regulatory gene in the salicylate-mediated SAR pathway, and it initiates the expression of the downstream disease-resistant gene *ZIPR1*. In the present study, *ZIPR1* and *ZINPR1* were significantly upregulated to more than ten times the initial expression level in the middle stage of succulent stem expansion (5 d), which reflected the dynamics of the rapid expansion of *U. esculenta* in the middle stage of positive stem expansion well, being consistent with the results of previous research [19].

In the present study, qRT-PCR was used to verify and screen the candidate reference genes during gall formation in *Z. latifolia*. The most suitable reference gene, *ACT1*, was determined, which laid a theoretical foundation for the subsequent gene expression analysis in *Z. latifolia*.

5. Conclusions

In this study, we evaluated the expression stability of nine candidate reference genes (*ACT1*, *H2B*, *UBI*, *EF-1 α* , *GAPDH*, β -*actin*, *60S*, *SKIP*, and *AQP*) by qRT-PCR, and we analyzed the data using GeNorm and NormFinder software. The results showed that *ACT1* was the classic reference gene for the analysis of correlative genes in all of the gall development stages of *Z. latifolia*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10070759/s1>, Figure S1: The picture of the RNA agarose gel electrophoresis of the five periods.

Author Contributions: M.Y. and S.Z. designed this research study; Y.L., H.Y. and Q.G. performed the research experiments; Y.L. and M.Z. analyzed the data and wrote this manuscript; M.Y., S.Z., Z.Z. and X.Z. revised this manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within this article.

Conflicts of Interest: The authors declare no conflicts of interest.

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