Evaluation and Selection of Suitable qRT-PCR Reference Genes for Light Responses in Tea Plant (Camellia Sinensis)

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Research

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Abstract

Background: Tea plant (Camellia sinensis) is an important woody economic crop used for processing leaf-type beverages. Tea has been proved to be beneficial to human health because it is rich in tea polyphenols and other active ingredients. Numerous studies have shown that light is a necessary environmental condition to control the growth and metabolism of C. sinensis. Gene expression experiments are always performed to explore the transcriptional regulation mechanism of plants widely based on the technique of quantitative real time polymerase chain reaction (qRT-PCR). The screening and application of reference genes are necessary for the normalization of gene expression under specific conditions. However, the reference genes for systematic analysis of light-induced transcription mechanisms are still not available in C. sinensis.

Results: In this research, we identified actin family genes that are always used as reference genes with high frequency and without distinction for various expression experiments in C. sinensis. Six pairs of distinctive primers (corresponding to CsACT1, CsACT2, CsACT(3-4), CsACT(5-6), CsACT(7-8), and CsACT(9-10) genes) were designed to evaluate their expression stability in response to light quality (LQ), light intensity (LI), and photoperiod (PD). Simultaneously, six other family members (CsUBC1, CsCLATHRIN1, CsGAPDH, CsTBP, CsTIP41, and CseIF-4a) of C. sinensis commonly used as reference genes were also investigated. The stability rankings of gene expression were calculated by the statistical algorithms of geNorm, BestKeeper, NormFinder, and RefFinder softwares.

Conclusions: CsACT(5-6), CsTIP41, and CsACT(3-4) were the most stable genes for light quality (LQ), light intensity (LI), and photoperiod (PD) treatments, respectively. This study provides a basis for the selection of reference genes for future research on the transcription mechanism of light response in C. sinensis. Moreover, the analysis of actin family members of C. sinensis will help to understand the individual transcription mechanism of housekeeping family.

Background

Quantitative real time polymerase chain reaction (qRT-PCR) is a universal technique to analyze gene expression because of its high sensitivity, good repeatability, and fast speed [1, 2]. In the process of using qRT-PCR to analyze gene expression, the purity and concentration of the sample cannot be absolutely guaranteed that may cause experimental errors [2, 3]. In order to make the results more accurate, it is necessary to use the reference gene as a reference to standardize the expression level of the target gene and correct the discrepancy of transcription efficiency and cDNA usage [2, 4]. Stable expression is the basis for selection of reference genes because of the selection of unstable reference genes will lead to test deviation. Some genes necessary for maintaining basic cell functions have been found in plants, called housekeeping genes, whose expression levels are less affected by environmental conditions and developmental stages [5, 6]. The housekeeping genes or other genes used as reference genes cannot be stably expressed in all transcriptional conditions, and the expression stability of genes is usually biased for specific conditions [7–9]. Therefore, it is necessary to select the reference genes for specific conditions to ensure the accuracy in qRT-PCR experiments.

Tea plant (Camellia sinensis) is an important woody cash crop, and its leaves are popular all over the world as a beverage. Tea leaves are rich in tea polyphenols, theeamine, and polysaccharides, which contribute to human health, such as anti-cancer, anti-aging, anti-inflammatory, and prevention of cardiovascular and cerebrovascular diseases [10, 11]. Light is the energy basis for the survival of green plants, and it is also an important signal factor regulating various biological functions of plants [12]. Before picking the fresh tea leaves, shading method for limiting partial direct light can effectively reduce the bitter and astringent substances of tea [13–16]. Moreover, the discrepancies of light quality and photoperiod also affected the accumulation of flavor substances and the formation of biological properties of tea leaves, such as the biosynthesis of flavonoid and aroma volatiles [17, 18], the conversion of leaf color [18], and the development of leaf and flower [18, 19]. The evidences from the transcriptomes and metabolomes of C. sinensis have shown that light signal transduction is regulated by photoreceptors, such as cryptochromes, phototropins, phytochromes, and UVR8 [18, 20]. To further investigate the light-induced molecular mechanism of C. sinensis, it is inevitable to accurately detect and analyze the expression profiles of genes involving in light signal regulation. However, systematic screening of reference genes for light response research of C. sinensis is still lacking.

Housekeeping genes from evaluated or not evaluated have been frequently used as reference genes in extensive researches on gene expression [3, 21–24]. Some of housekeeping genes, such as the actin (ACT) gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, eukaryotic translation initiation factor (eIF) gene, TATA-box binding protein (TBP) gene, and ubiquitin-conjugating enzyme (UBC) gene, have been identified as reliable reference genes in plants [22, 23, 25]. In the expression experiments of C. sinensis, housekeeping genes, especially ACT gene, were the most frequently used reference genes [16, 17, 20, 26]. Suitable reference genes in C. sinensis have been screened and evaluated under biotic and abiotic stress, biotic and abiotic stresses, tissue development, and hormone treatment [27–31]. In general, the stable expression of reference genes under certain experimental conditions may not be stable under other conditions. Therefore, it is necessary to reevaluate the stable reference genes under new experimental conditions. In addition, it is worth noting that the evaluation of expression stability among family members of housekeeping genes is often ignored in previous studies [27–31].

In order to detect whether there is any difference in the expression stability of actin family members when C. sinensis is exposed to various light conditions, six pairs of distinctive primers (corresponding to CsACT1, CsACT2, CsACT(3-4), CsACT(5-6), CsACT(7-8), and CsACT(9-10) genes from C. sinensis genome) were investigated in this study. In addition, six common reference genes of C. sinensis from other families, including CsUBC1, CsGAPDH, CsTBP, CseIF-4a, clathrin adaptor complex subunit (CsCLATHRIN1), and tap42-interacting protein of 41 kDa (CsTIP41) were evaluated together [28, 30]. The expression stability of these candidate genes was calculated and evaluated to obtain the most suitable reference genes in response to light quality (LQ) (red light and blue light), light intensity (LI) (shading and darkness), and photoperiod (PD) (0–24 h) based on four different
statistical algorithms (geNorm [32], NormFinder [33], BestKeeper [34], and RefFinder [35]). This study aims at providing a basis for selecting suitable reference genes for future light response research in C. sinensis.

Results

Identification and analysis of actin family genes

Thirteen homologous actin genes were obtained from the C. sinensis genome using Arabidopsis thalianaAtACT1 gene (AT2G37620.1) as the retrieval sequence (Additional file 1: Table S1). Ten CsACT genes encoding complete sequences were eventually confirmed and renumbered from CsACT1 to CsACT10 (Additional file 1: Table S1). Multiple alignments showed that the amino acid sequences of actin family members of A. thaliana and C. sinensis are highly conserved (Additional file 2: Fig. S1). Nevertheless, the actin family members of C. sinensis still have many variant sites (Additional file 2: Fig. S1). To further analyze the phylogenetic relationship and subfamily classification of actin family members, a neighbor-joining phylogenetic tree was constructed based on the amino acid sequence of actin proteins of A. thaliana, Populus trichocarpa, Oryza sativa, and C. sinensis (Fig. 1 and Additional file 3: Table S2). All actin family members could be divided into three groups, namely, Group I, Group II, and Group III. CsACT1-4, CsACT5-8, and CsACT9-10 of C. sinensis were assigned to Group I, Group II, and Group III, respectively.

In addition, the cis-acting elements in the upstream region (1,500 bp) of actin family genes were analyzed to predict the possible transcriptional regulation in C. sinensis. A total of 48 types of cis-acting elements were obtained from the promoter region of actin family of C. sinensis (Additional file 4: Table S3). The ranking of total element numbers for each member of actin family as follows: CsACT2 (163) > CsACT6 (122) > CsACT5 (121) > CsACT7 (116) > CsACT8 (116) > CsACT1 (112) > CsACT3 (89) > CsACT9 (84) > CsACT4 (83) > CsACT10 (83). The most frequent elements for all members were 'AT-~TATA-box/TATA/TATA-box' (core promoter element around -30 of transcription start) and 'CAAT-box' (common cis-acting element in promoter and enhancer regions), which play roles in maintaining the basic transcription of genes [36,37]. The number ranking of these two basic elements for each member of actin family as follows: CsACT2 (110) > CsACT7 (69) > CsACT8 (69) > CsACT1 (65) > CsACT5 (61) > CsACT6 (60) > CsACT3 (46) > CsACT4 (44) > CsACT9 (34) > CsACT10 (33). Other cis-acting elements mainly included light-responsive, hormone-responsive, short function, anaerobic induction, low-temperature responsive, and uncommented elements.

Expression profiles of candidate reference genes

Based on the classification and nucleotide sequence characteristics of actin family genes of C. sinensis, six pairs of distinctive primers were designed to detect the individual expression of CsACT1, CsACT2, CsACT(3-4), CsACT(5-6), CsACT(7-8), and CsACT(9-10) genes, respectively (Table 1). Objective to more comprehensively evaluate and develop the most suitable reference genes in response to light quality (LQ), light intensity (LI), and photoperiod (PD), the actin family members, together with six other commonly used as reference genes (CsUBC1, CsGAPDH, CsTBP, CseIF-4α, CsCLATHRIN1, and CsTIP41), were investigated (Table 1). The relative expression levels of the candidate reference genes were determined by the threshold cycle (Cq) values, which were obtained from the number of amplification cycles when the fluorescence signal reaches above the baseline threshold in qRT-PCR experiment. In order to unify the parameters, the baseline threshold was set to the average level (2551.05).

The distribution of all Cq values was described by a box and whiskers plot (Fig. 2a). The lower Cq values correspond to higher expression levels, and the higher Cq values correspond to lower expression levels. CsACT1 gene with low Cq values (mean Cq = 28.40) had high expression level, while CsACT2 gene with high Cq values (mean Cq = 34.98) had low expression level. The Cq values of CsACT(7-8), CsTIP41, and CseIF-4α genes were more centralized, whereas the Cq values of most genes, such as CsACT1, CsACT(3-4), CsACT(5-6), CsACT(9-10), and CsUBC1, were relatively dispersed.

In addition, the Cq values of all genes from LQ, LI, and PD treatments were calculated, respectively. The relative positions of Cq values of all genes on the three box and whiskers plots almost did not change, but the distribution range of these Cq values changed greatly (Fig. 2b, Fig. 2c, and Fig. 2d). In LQ treatment, except for the Cq values of CsACT(5-6) and CseIF-4α, the data of other genes were relatively dispersed, especially CsACT2 and CsACT(9-10) (Fig. 2b). In LI treatment, the Cq values of CsTIP41, CsTBP and CseIF-4α were more centralized, while the data of CsACT2, CsACT(3-4), CsACT(9-10), and CsCLATHRIN1 were more dispersed (Fig. 2c). In PD treatment, the Cq values of CsACT1, CsACT(3-4), CsACT(9-10), and CsCLATHRIN1 were more centralized, the data of other genes were relatively dispersed, especially CsUBC1 and CsGAPDH (Fig. 2d).

Stability evaluation of gene expression in response to LQ treatment

For LQ treatment, the calculated results of NormFinder, RefFinder, and BestKeeper softwares were relatively consistent. These three softwares showed that CsACT (5-6) were the most stable genes and CsACT2 was the most unstable gene. CsACT2 with largest calculated value (M = 1.81) was the most unstable gene, while CsACT(3-4) and CsGAPDH with least calculated value (M = 1.172) were the most stable genes in geNorm analysis. As an integrated evaluation software, RefFinder ranked the expression stability of genes in response to LQ treatment as follows: CsACT(5-6) > CsACT1 > CsGAPDH > CsACT(3-4) > CseIF-4α > CsUBC1 > CsTBP > CsCLATHRIN1 > CsACT(7-8) > CsTIP41 > CsACT(9-10) > CsACT2 (Table 2).

Stability evaluation of gene expression in response to LI treatment

For LI treatment, CsTIP41 was identified as the most stable gene from the calculated results of geNorm, NormFinder, BestKeeper, and RefFinder softwares. CsACT(9-10) with largest calculated value were the most unstable gene by the analysis of geNorm, NormFinder, and RefFinder softwares, while the expression stability of CsCLATHRIN1 ranked last (CV ± SD = 5.59 ± 1.85) according to the calculated results of BestKeeper software. Based on the data integration of RefFinder software, the expression stability of genes in response to LI treatment was ranked as follows: CsTIP41 > CseIF-4α >
CsUBC1 > CsACT1 > CsTBP > CsGAPDH > CsACT(7-8) > CsACT(5-6) > CsACT2 > CsACT(3-4) > CsCLATHRIN1 > CsACT(9-10) (Table 2). Compared with the stability rankings of genes in LQ treatment, the rankings of CsTIP41 and CsACT(5-6) from LI treatment were obviously different, that is, CsTIP41 ranked first from ‘tenth’ and CsACT(5-6) ranked eighth from ‘first’ (Table 2).

Stability evaluation of gene expression in response to PD treatment

For PD treatment, the data calculations of the several softwares had yielded three results for the most stable gene. CsACT(3-4) with least calculated value were the most stable genes from the calculated results of geNorm and RefFinder softwares, while CsACT(5-6) and CsACT1 were considered to be the most stable genes in the calculated results of NormFinder and BestKeeper, respectively. The expression stability of CsGAPDH ranked last according to the calculated results from geNorm, NormFinder, and RefFinder softwares, while CsUBC1 with largest calculated value (CV ± SD = 5.39 ± 1.62) was the most unstable gene in BestKeeper analysis. In response to PD treatment, the expression stability of genes from the data integration of RefFinder software was ranked as follows: CsACT(3-4) > CsACT(9-10) > CsACT(5-6) > CsACT1 > CsCLATHRIN1 > CsTIP41 > CsACT2 > CsACT(7-8) > CsTBP > CseIF-4α > CsUBC1 > CsGAPDH (Table 2). Obviously different from the stability rankings of genes in LQ and LI treatment, the stability rankings from PD treatment ranked CsACT(9-10) and CsCLATHRIN1 in the top five, and ranked CseIF-4α, CsUBC1, and CsGAPDH in the bottom three (Table 2).

Stability evaluation of gene expression in response to total treatments

For total treatments, the stability ranking of CsTIP41 ranked first according to the calculated results of geNorm, NormFinder, and RefFinder softwares, while CseIF-4α with least calculated value (CV ± SD = 3.26 ± 1.05) ranked first in BestKeeper analysis. CsACT2 ranked in last was the most unstable gene in geNorm and NormFinder analysis, while the last ranked genes from the calculated results of BestKeeper and RefFinder softwares were CsACT(3-4) and CsACT(9-10), respectively. In response to total treatments, the stability ranking of gene expression from the data integration of RefFinder software was as follows: CsTIP41 > CseIF-4α > CsTBP > CsACT(5-6) > CsUBC1 > CsACT(7-8) > CsACT1 > CsGAPDH > CsACT(3-4) > CsCLATHRIN1 > CsACT2 > CsACT(9-10) (Table 2).

Reference gene validation

In *C. sinensis*, CsCHS1, CsMYB4, and CsDFR genes were confirmed to regulate flavonoid biosynthesis in light signal transduction [18,20]. To validate the reliability of candidate reference genes for the light-induced expression, the expression levels of CsCHS1, CsMYB4, and CsDFR genes in response to light were normalized by the most stable reference genes in *C. sinensis*. Based on the result of the stability evaluation, CsACT(5-6), CsTIP41, and CsACT(3-4) were used as reference genes of LQ, LI, and PD treatments, respectively (Fig. 3 and Additional file 5: Table S4). In LQ treatment, the expression levels of CsCHS1, CsMYB4, and CsDFR genes induced by red light reached the highest value at 4, 12, and 4 h, respectively. The expression levels of CsCHS1, CsMYB4, and CsDFR genes were also induced by blue light, and reached the highest value at 12, 4, and 4 h, respectively. Compared to the response to red light, the expression levels of CsCHS1 and CsDFR genes in response to blue light were first down-regulated at 1 h (Fig. 3a). In LI treatment, the expression levels of CsCHS1 and CsDFR genes were silenced during treatments, while the expression levels of CsMYB4 was up-regulated in certain periods of shading and darkness treatments (Fig. 3a). In PD treatment, the expression levels of CsCHS1, CsMYB4, and CsDFR genes fluctuated regularly, which was mainly manifested the trend that the gene expression was inhibited in the dark, but up-regulated in light (Fig. 3b).

In addition, the reliability of the most stable gene CsTIP41 for total treatments also was evaluated by normalizing the expression levels of CsCHS1, CsMYB4, and CsDFR genes in LQ and PD treatments (Fig. 4). In red light treatment, the expression levels of CsCHS1 and CsDFR genes was inhibited, while the expression levels of CsCHS1 gene were up-regulated at 4 h and 12 h. In blue light treatment, the expression levels of CsCHS1 and CsMYB4 were up-regulated after 4 h, while the expression trend of CsDFR genes was decreasing. Compared with the normalization results of CsACT(5-6) reference gene in LQ treatment, the relative expression levels of CsCHS1, CsMYB4, and CsDFR genes in response to light were obviously different in spite of the gene expression trends was similar. In PD treatment, the expression trends of CsCHS1, CsMYB4, and CsDFR genes were up-regulated and then down-regulated when exposed to darkness or light by using CsTIP41 as reference gene. Compared with the normalization results of CsACT(3-4) reference genes in PD treatment, the main differences were that the expression levels of CsCHS1, CsMYB4, and CsDFR normalized by CsACT(3-4) began to increase when exposed to darkness, and were abnormally down-regulated when exposed to light at 18 h.

Discussion

qRT-PCR for gene expression analysis is a widely adopted method, which is more sensitive than traditional semi-quantitative methods, and can truly reflect the transcription levels of genes [1, 3]. However, many factors, including the quality of total RNA, cDNA synthesis efficiency, and PCR amplification efficiency between different samples, can lead to deviations in the experiment results of gene expression [2, 3]. The selection and application of stable reference genes in the normalization of gene expression are the necessary ways to make the results more accurate of qRT-PCR analysis [2]. In this study, the expression stabilities of *actin* family genes (CsACT1-10) and six common reference genes (CsUBC1, CsCLATHRIN1, CsGAPDH, CsTBP, CsTIP41, and CseIF-4α) in response to light were evaluated in *C. sinensis*. The stability calculation of these twelve genes will improve the accuracy of qRT-PCR analysis for future light-responsive researches of *C. sinensis*.

When the reference genes in the expression experiment have not been evaluated and cannot be obtained, the normalization of gene expression generally adopts the alternative of selecting some common reference genes, such as *ACT* and *GAPDH* genes [38, 39]. However, the stability of *ACT* and *GAPDH* genes has been proved to be not absolutely reliable under different experimental conditions [27, 28, 30]. Reference genes are mainly derived from the selection of housekeeping genes [9]. The detection primers for housekeeping genes may simultaneously detect the expression of multiple genes in this
family, because the nucleotide sequences of housekeeping gene family members are usually highly similar [40, 41]. Therefore, the expression stability of reference genes actually refers to the expression stability of all genes specifically combined by the detection primers. In this study, the members of actin family were identified and classified to explore the individual differences in C. sinensis. Although highly conservative, the sequences of actin family genes of C. sinensis still have a large number of mutation sites. The cis-acting element analysis of CsACTs showed that the basic transcription elements were dominant in the promoter region. Interestingly, the expression levels of some members of CsACTs were correlated with the number of these transcription basic elements, such as CsACT1, CsACT5, CsACT6, CsACT9, and CsACT10. An exception, the relative expression levels of CsACT2 gene with most cis-acting elements were generally lower. It is suggested that the transcription of CsACT2 gene may be inhibited by other factors. In addition, some light-responsive and hormonal responsive elements were found in the promoter region of CsACTs, which indicates both of light and hormone conditions may affect the transcriptional expression of CsACTs.

Although the calculation results of geNorm, NormFinder, BestKeeper, and RefFinder were moderate different, the RefFinder method for stability ranking of this study was still used as the main reference based on its comprehensive evaluation advantages [35]. The stability rankings of CsACTs, CsUBC1, CsGAPDH, CsTBP, CselF-4a, CsCLATHRIN1, and CsTIP41 genes were not consistent in response to LQ, LI, and PD treatments, respectively. Several actin genes, including CsACT(5–6), CsACT1, CsACT(3–4), and CsGAPDH gene were relatively stable in LQ treatment, while the stability of CsACTs (except for CsACT1) and CsGAPDH genes were relatively poor in LI treatment. By contrast, the stability of ACT and GAPDH genes for responding to different light quality treatments were observed to be poor in the red callus of Vitis davidii (Rom. Caill.) Foëx, while the ACT and GAPDH genes for responding to different light intensity treatments were relatively stable in Solanum lycopersicum [42, 43]. It indicates that the stability of the common ACT and GAPDH genes for light treatments varies greatly among different species. For the stability in PD treatment, actin family members, including CsACT(3–4), CsACT(9–10), CsACT(5–6), and CsACT1, were ranked in the top four. This result is not consistent with previous observations, which may be related to the differences of detected number of actin family genes and their primer design in C. sinensis [27, 28]. The fifth ranked CsCLATHRIN1 gene for PD treatment used the same primers as before and got similar results [27, 28]. The poor stability of CsUBC1 for PD treatment of long day was similar to the previous observation [27]. The expression of CsUBC1 seems to be more stable in short day [27, 28]. In addition, the last ranked CsGAPDH gene for PD treatment was different from the previous observations, which may also be due to the differentiated primer design [27, 28].

CsCHS1, CsMYB4, and CsDFR genes were involved in light-dependent regulation of flavonoid biosynthesis in C. sinensis [18, 20]. A recent study found that blue light could induce the expression of CsCHS1 and CsDFR genes [18]. The induced expression of CsCHS1 and CsDFR genes was also observed when using the most stable CsACT(5–6) as reference genes in LQ treatment. Another study, except for CsMYB4, the expression levels of CsCHS1 and CsDFR genes were significantly inhibited in shading in previous study [20]. In this study, the similar results were observed based on the application of CsTIP41 reference gene. In addition, the expression trends of CsCHS1, CsMYB4, and CsDFR genes fluctuated regularly by using CsACT(3–4) reference genes in PD treatment, which may be regulated by the biological clock. After the above comparison, it is not difficult to see that CsTIP41 gene is not the best reference gene in LQ and PD treatments, although CsTIP41 is calculated as the most stable reference gene for total treatments. Therefore, it would be more ideal to use the best reference genes in the expression experiments of LQ, LI, and PD treatments, respectively.

Conclusions

In conclusion, this study identified actin family genes (CsACT1-10) and evaluated the reference genes (CsACTs, CsUBC1, CsCLATHRIN1, CsGAPDH, CsTBP, CsTIP41, and CselF-4a) for the normalization of light-responsive expression experiments in C. sinensis. CsACT(5–6), CsTIP41, and CsACT(3–4) genes are the most suitable reference genes in LQ, LI, and PD treatments, respectively. The characteristics of structure, classification, and transcription of actin family members of C. sinensis were investigated. The individual transcription of CsACTs may be related to their number and composition of cis-acting elements in the promoter region.

Materials And Methods

Plant material and light conditions

One-year-old cutting seedlings of tea plant (C. sinensis cv. ‘Fuding Dabaicha’) were planted in light incubators (programmed with 75% ± 5% relative humidity, 16 h white light (25 °C) with light intensity of 180 μmol·m⁻²·s⁻¹ in a day) one month before light treatments. The light-experimental seedlings were divided into two groups. The first group of seedlings was subdivided into four series, which were exposed to red light (180 μmol·m⁻²·s⁻¹), blue light (180 μmol·m⁻²·s⁻¹), shading (20 μmol·m⁻²·s⁻¹), and darkness, respectively. The first and second leaves (mix later as a sample) of first group were picked at 1h, 4h, and 12h. The second group of seedlings was exposed to white light consistent with the pre-treatment conditions. The samples were picked every two hours from 8 pm the first day to 8 pm the next day. The relative position of picked tissues in the second group was consistent with that of the first group. The picked sample at the beginning of the experiment (0 h) served as a control for all light treatments. Three biological repetitions were performed for each treatment. The leaf materials of tea plant were stored in the refrigerator at -80 °C after quick freezing in liquid nitrogen.

Total RNA isolation and cDNA reverse transcription

The total RNA of tea leaves was extracted using the Quick RNA Isolation Kit (Huayueyang Biotech Co., Ltd., Beijing, China), and then was reverse transcribed into cDNA using the Goldenstar™ RT6 cDNA Synthesis Kit (TsingKe Biotech Co., Ltd., Beijing, China) for expression analysis. The reaction volume for reverse transcription was as follows: 4 μL of 5× Goldenstar™ Buffer, 1 μL of dNTP Mix, 1 μL of Goldenstar™ Randomer, 1 μL of DTT, 1 μL of
Goldenstar™ RT6, 1 ng of total RNA template, plus RNase-free water to the total volume of 20 μL. The reaction condition was as follows: 25 °C for 10 min, 50 °C for 15 min, and 85 °C for 5 min.

**Bioinformatics analysis of actin family genes**

The homologous actin genes of *C. sinensis* were obtained by querying *C. sinensis* genome (TPID: Tea Plant Information Archive, http://tpia.teaplant.org/) [44] based on the amino acid sequence of *A. thaliana* AtACT1 gene (AT2G37620.1). The sequences of *A. thaliana* were obtained from TAIR (https://www.arabidopsis.org/). The sequences of *P. trichocarpa* and *O. sativa* were obtained from JGI (https://phytozome.jgi.doe.gov/pz/portal.html). The multiple alignments of CsACT proteins were performed using DNAMAN 6.0 software. MEGA 5.0 software was used to construct phylogenetic tree with neighbor-joining method. The analysis of the cis-acting elements of the promoters for CsACT genes was performed by online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**Primer design and qRT-PCR assay**

All detection primers of reference genes were designed using Primer Premier 5.0 software. qRT-PCR was performed on CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction volume for qRT-PCR amplification was 20 μL, including 10 μL of 2× T5 Fast qPCR Mix (SYBR Green I) (TsingKe Biotech Co., Ltd., Beijing, China), 2 μL of cDNA template (10× dilution), 1 μL of each primer, and 6 μL ddH2O. The amplification reaction condition was as follows: 95 °C for 1 min, 40 cycles at 95 °C for 10 s, and 55 °C for 12 s. All qRT-PCR assays included three technical repetitions.

**Data analysis**

Cq values of fluorescence curves were obtained by standardizing the baseline threshold to mean 2551.05. Four softwares, including geNorm [32], NormFinder [33], BestKeeper [34], and RefFinder [35], were employed for calculating the expression stability of candidate genes in response to light treatments, respectively. Cq values are converted into relative quantities according to the formula: $2^{-\Delta Ct} (\Delta Ct = \text{the corresponding Cq value} - \text{minimum Cq})$ [45]. The calculations of geNorm and NormFinder are based on these converted $2^{-\Delta Ct}$ and ΔCt quantities, respectively; BestKeeper and RefFinder are based on the raw Cq values.

**Declarations**

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**Authors' contributions**

ZW and JW initiated and designed the research. JW, QT, and ZW performed the experiments. JW, QT, KS, LZ, and ZW analyzed the data. ZW contributed reagents/materials/analysis tools. JW wrote the paper. ZW and JW revised the paper.

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**Availability of data and materials**

All data generated or analyzed during this study are available in this published article and its additional files.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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### Tables

**Table 1 The primer sequences for the candidate reference genes**

TPID: Tea Plant Information Archive (http://tpia.teaplant.org/).
| Gene          | TPID/GeneBank ID          | Primer sequence (5'-3') forward/reverse          | Amplicon length (bp) | Ref.   |
|--------------|---------------------------|-------------------------------------------------|----------------------|--------|
| *CsACT1*     | CSS0023400.1              | ATCTGGCATCACACCTTCTACAAT/AATAGCAACATACATAGCAGGAGTG | 156                  | Novel  |
| *CsACT2*     | CSS0026008.4              | TCTCCTCACTGAAGCACCTTCT/CAATGAGTGATGGCTGGAAGAGAA | 167                  |        |
| *CsACT(3-4)* | CSS0041919.1; CSS0010930.1 | CTTTGATTATGAGCAGGAGTTGGAA/CAATGAGTGATGGCTGGAAGAGAA | 143                  |        |
| *CsACT(5-6)* | CSS0050441.1; CSS0008920.1 | TGAGAGATCTCGTTGCCCTGAAGTC/TCCTGCTCATACGCTGCAGATC | 192                  |        |
| *CsACT(7-8)* | CSS0022984.1; CSS0013216.1 | TTGAGATCTCGGAGATGGTTAGCC/AGCAAATTCTCTTCACATCACGG | 195                  |        |
| *CsACT(9-10)* | CSS0010824.1; CSS0002724.1 | AATTGTTGGGATTTCTGGAAGAAG/GGCTAGGATCTTGGTAAGGTCT | 133                  |        |
| *CsUBC1*     | CSS0013210.1; KA281185.1  | TGCTGGTGAGGTTTTCTTCTTTG/AAAGCATATGCTCCCATTGCTGTTT | 124                  | Hao et al. [27] |
| *CsCLATHRIN1* | KA291473.1                | TAGAGGCGGTAGTGGAGACCTGCT/TTACAAACCGGGCTGTCGATGAGATTT | 129                  | Hao et al. [27] |
| *CsGAPDH*    | CSS0028943.1              | CCCTCTAATGCTCTCCTCCTCCTTG/TTGCTGAGCCACTACAT | 158                  | Wu et al. [30] |
| *CsTBP*      | CSS0013302.1              | GGCGGATCAAGTTGGAGAGGAG/ACGCTTGAGGTATTCGGATGATTTTA | 166                  | Wu et al. [30] |
| *CsTIP41*    | CSS0015264.1; CSS0002878.1 | TGGAGTTGAAGTGGAGCGACCGA/CTCGGAAAGTGGAGTTGGAGAAGC | 176                  | Wu et al. [30] |
| *Cself-4a*   | CSS0020804.1              | TGAGTTACTTTGCGTATGGGAGAA/CCTTTGCTGAAATTGAGGAGGCT | 145                  | Wu et al. [30] |

Table 2 Gene expression stability ranked by geNorm, NormFinder, BestKeeper, and RefFinder

SD [±Cq]: standard deviation of the Cq; CV [%Cq]: coefficient of variance expressed as a percentage of the Cq level.
| Group          | Rank | Gene      | Stability | NormFinder | Stability | BestKeeper | SD[±Cq] | CV[%Cq] | RefFinder |
|---------------|------|-----------|-----------|------------|-----------|------------|---------|---------|-----------|
| Light quality |      | CsACT(3-4) | 1.17      | CsACT(5-6) | 0.89      | CsACT(5-6) | 0.70    | 2.27    | CsACT(5-6) | 1.41     |
| Light intensity | 1    | CsGLAPDH  | 1.17      | CsACT1     | 0.89      | CsTBP      | 1.03    | 2.92    | CsTBP     | 7.24     |
| Potoperiod    | 2    | CsTBP     | 1.54      | CsCLATHRIN1| 1.38      | CsTBP      | 1.07    | 3.21    | CsCLATHRIN1| 7.52     |
| Total         |      | CsACT(7-8)| 1.59      | CsACT(7-8) | 1.43      | CsCLATHRIN1| 1.16    | 3.53    | CsCLATHRIN1| 9.46     |
### Additional Files

**Additional file 1:** Table S1. List of actin family genes in *C. sinensis*.

**Additional file 2:** Figure S1. Multiple alignment of the amino acid sequence of CsACT proteins in *C. sinensis*.

**Additional file 3:** Table S2. List of actin family genes in *A. thaliana*, *P. trichocarpa*, and *O. sativa*.

**Additional file 4:** Table S3. The cis-acting elements and their functions of predictive promoter region of CsACT family genes in *C. sinensis*.

**Additional file 5:** Table S4. Detection primers for the expression validation of CsCHS1, CsMYB4, and CsDFR in *C. sinensis*.

### Figures

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**Figure 1**

![Circular diagram showing the evolutionary relationships among actin family genes in different species, with three main groups: Group I, Group II, and Group III.](image)

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| 5 | CsACT(5-6) | 1.51 | CsACT1 | 1.28 | CsTBP | 1.27 | 3.67 | CsUBC1 | 5.63 |
|---|------------|------|--------|------|-------|------|------|--------|------|
| 6 | CsACT1     | 1.60 | CsUBC1 | 1.30 | CsACT2 | 1.32 | 3.77 | CsACT(7-8) | 5.86 |
| 7 | CsACT(7-8) | 1.66 | CsACT(7-8) | 1.32 | CsUBC1 | 1.33 | 4.36 | CsACT1 | 6.22 |
| 8 | CsACT(3-4) | 1.71 | CsACT(3-4) | 1.33 | CsCLATHRIN1 | 1.39 | 4.32 | CsGAPDH | 8.54 |
| 9 | CsCLATHRIN1 | 1.74 | CsCLATHRIN1 | 1.41 | CsACT(5-6) | 1.40 | 4.61 | CsACT(3-4) | 8.56 |
| 10| CsACT(9-10) | 1.77 | CsACT(9-10) | 1.51 | CsACT1 | 1.47 | 5.18 | CsCLATHRIN1 | 8.74 |
| 11| CsGAPDH    | 1.82 | CsGAPDH | 1.56 | CsACT(9-10) | 1.48 | 4.66 | CsACT2 | 10.09 |
| 12| CsACT2     | 1.86 | CsACT2 | 1.61 | CsACT(3-4) | 1.55 | 5.22 | CsACT(9-10) | 10.24 |
Phylogenetic tree analysis of ACT proteins from C. sinensis, Arabidopsis, poplar, and rice.

Figure 2

Cq values of twelve candidate reference genes of C. sinensis. Raw Cq values of all samples from total treatments (a), LQ treatment (b), LI treatment (c), and PD treatment (d) were described using a box and whiskers plot, respectively. The line across the box is the median. The outer box is determined from 25th to 75th percentiles, and the inner box represents the mean value. The whiskers represent percentiles from 5th to 95th, and outliers are depicted by asterisks.
Figure 3

Relative expression normalization of CsCHS1, CsMYB4, and CsDFR genes using best reference genes in C. sinensis. CsACT(5-6) (a), CsTIP41 (a), and CsACT(3-4) (b) as reference genes for LQ, LI, and PD treatments, respectively.

Figure 3

Relative expression normalization of CsCHS1, CsMYB4, and CsDFR genes using best reference genes in C. sinensis. CsACT(5-6) (a), CsTIP41 (a), and CsACT(3-4) (b) as reference genes for LQ, LI, and PD treatments, respectively.
Figure 4

Relative expression normalization of CsCHS1, CsMYB4, and CsDFR genes using CsTIP41 as reference gene in C. sinensis. CsTIP41 as reference gene for LQ (a) and PD (b) treatments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1.xlsx
- Additionalfile2FigureS1.pdf
- Additionalfile3TableS2.xlsx
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