Selection of reference genes for quantitative gene expression analyses in pedunculate oak (*Quercus robur* L.) using real-time quantitative PCR

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Abstract. Drought and desertification are global issues leading to numerous negative consequences, such as reducing the arable land area, decreasing soil fertility, and crop yield. An effective method to fight against degradation and desertification of agricultural lands is agroforestry, thus there is a need to obtain drought-resistant tree species for use in agroforestry on arid lands. One of the key species used for creating forest strips is pedunculate oak (*Quercus robur* L.). Understanding the molecular mechanisms of adaptation to drought conditions is necessary to obtain resistant tree species. Real-time quantitative PCR method allows studying gene expression changes under various stress factors, but accurate results require careful selection of reference genes. The aim of this study is to select reference genes for quantitative gene expression analysis of *Q. robur* using RT-qPCR. Modeling of soil drought in the laboratory conditions was conducted to evaluate the stability of housekeeping genes expression in *Q. robur*. The RT-qPCR data were analyzed using the RefFinder web tool. Results from various algorithms showed that ACT7 had the most stable expression, while EF1α had the least stable expression. ACT7 is recommended as the reference gene for gene expression studies of *Q. robur* under drought conditions.

1 Introduction

Droughts occur periodically in the Volgograd region and have a negative impact on agriculture and the economy of the region. At the same time, there is a decrease in precipitation, an increase in average air temperature, and the development of desertification processes in the region. The increase in average air temperature can lead to more intense evaporation of moisture from the soil, making it more prone to droughts. Furthermore, climate change may also affect the amount and distribution of precipitation, further complicating the situation with water supply and reducing crop yields [1].

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Forest strips play an important role in agrolandscapes as they effectively help to counteract negative climatic conditions [2]. They contribute to increasing crop yields, preserving soil fertility, protecting crops from drought and dry winds, as well as preventing water and wind soil erosion. Forest strips create a favorable microclimate that promotes increased humidity and reduced wind flows, which have a positive impact on plants and soil. Moreover, tree roots improve soil structure, preventing erosion and enriching it with mineral elements. Therefore, the implementation of forest strips in agrolandscapes is an important element of a set of measures to improve conditions for agriculture, protect soil, and increase crop yields [3]. However, negative climate changes affect all plants, leading to their death.

Due to this, the development of drought-resistant plants used in agroforestry is currently relevant. One important species used in these activities is pedunculate oak (*Quercus robur* L.). This species has a strong root system that helps retain soil and prevent erosion. Additionally, *Q. robur* is highly resistant to adverse weather conditions and is long-lived.

Real-time quantitative PCR is widely used in various fields of research due to its high sensitivity and accuracy [4]. Special attention needs to be paid to the selection of reference genes for evaluating differential gene expression, as they play a crucial role in PCR result normalization [5]. The most commonly used reference genes are GADPH, EF1α, ACT, and some others. Developing specific primers for reference genes of *Q. robur* can help characterize genes associated with drought resistance. Studying the expression patterns of these genes will provide a better understanding of the molecular mechanisms underlying *Q. robur* drought resistance.

The aim of this study is to select reference genes for quantitative gene expression analysis in *Q. robur* using real-time quantitative PCR.

2 Materials and methods

2.1 Drought modeling and plant material

To study the stability of housekeeping genes expression in *Q. robur* under drought stress, soil drought modeling was conducted in laboratory conditions. Two groups were formed: with regular watering (intact) and without watering, with 10 6-month-old oak seedlings in each group. The constant air temperature in laboratory was maintained at 30°C throughout the experiment. Due to the fact that *Q. robur* is a relatively drought-resistant species of woody plants, a long-term drought was simulated. Leaf samples were collected at the beginning of the experiment and after 336 hours in tubes with RNA stabilizer IntactRNA (Evrogen, Russia) and then stored at -80°C.

2.2 RNA extraction

Total RNA was extracted using the R-Plants kit (Biolabmix, Russia) from 50 mg samples of *Q. robur* leaves. Sample homogenization was carried out using a portable homogenizer with replaceable pestles. During the RNA extraction process, the samples were always kept in a cooling rack at +4°C, centrifugation was also carried out at this temperature. Then, the total RNA samples were treated with DNase I (Magen, China), followed by reprecipitation in sodium acetate and ethanol. The RNA concentration was measured using a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA).

2.3 Primer design

The online service Primer-BLAST was used to design primers for housekeeping genes actin-7 (ACT7), EF1α, and GAPDH in *Q. robur*. The primer sequences were selected for CDS of the corresponding genes according to the following criteria: primer length 18-22 bp, amplicon length up to 200 bp, optimal T_m of 57-63°C with a difference of no more than 3°C between the forward and reverse primers, GC-content 20-80%, self-complementarity score no more than 8, and self 3'-complementarity score no more than 3 (Table 1). In addition, the forward and reverse primers for each gene were separated by at least one intron in the corresponding genomic DNA.

Table 1. Primers, amplicon length and average melting temperatures of amplicons for candidate reference genes of *Q. robur* for RT-qPCR.

Symbol	ACT7	EF1α	GAPDH
GenBank accession number	XM_050436545.1	XM_050436719.1	XM_050420371.1
Forward primer	5′ CGGAGGTTGGCTTG CTCTT 3′	5′ AGTGCGGCGAGAGA GTCTA 3′	5′ GGTGAAGGACGAGAA GACCC 3′
Reverse primer	5′ ATCCAGCCTTCACCA TTCCAG 3′	5′ AGCAGCCTCCTTCTC AAACC 3′	5′ GCTGGGAGCAGAGAT CACAA 3′
Amplicon length (bp)	147	192	196
Amplicon T_m (°C)	82.8	82.7	84.5

2.4 RT-qPCR analysis

RT-qPCR was carried out using a Gentier 96E thermocycler (Tianlong, China) with the OneTube RT-PCR SYBR kit (Evrogen, Russia) containing ROX as a reference dye. Before PCR, all samples were diluted with nuclease-free water to a concentration of 20 ng/µl. The total volume of the reaction mixture was 25 µl, containing 5 µl of 5X OneTube PCRmix SYBR, 1 µl of each primer (10 mM), 0.5 µl of LowROX, 2 µl of RNA sample, 0.5 µl of reverse transcriptase and 15 µl of nuclease-free water. Three replicates were used for each sample. Two types of negative controls were used to check for contamination of RNA and reagents: without RNA and without revertase. The RT-qPCR protocol included: reverse transcription at 55°C for 15 min, polymerase activation and revertase inactivation at 95°C for 1 min, followed by denaturation at 95°C for 15 s, primer annealing at 62°C for 20 s, elongation at 72°C for 20, for a total of 50 cycles, and a melt curve analysis from 55 to 95°C with 0.5°C increments. Threshold cycle (C_t) values were calculated automatically.

2.5 Statistical analysis

The RefFinder web tool was used to identify suitable reference genes for *Q. robur* (http://blooge.cn/RefFinder/). This tool is designed to evaluate and rank reference genes in gene expression studies and combines several common analysis methods such as Genorm, NormFinder, BestKeeper, and others to provide a more accurate and reliable evaluation of reference genes [6]. RefFinder was used to process and analyze the threshold cycles of the genes for their evaluation by various algorithms. Also, quantitative data were analyzed using Statistica 12.0 (StatSoftInc., USA) with the calculation of indicators used to characterize

nonparametric samples: median, Q_1, Q_3 (Me [Q_1 ÷ Q_3]). To prove the validity of the differences, the Kruskal-Wallis test was applied for multiple groups and the Mann-Whitney test for two independent groups ($p < 0.05$).

3 Results and discussion

During RT-qPCR, the values of threshold cycle values for each candidate reference genes were obtained (Figure 1). Amplification with primers designed for ACT7, EF1α and GAPDH resulted in the formation of a single product with average melting temperature of 82.8°C, 82.7°C, and 84.5°C, respectively (Table 1). The symbol * indicates significant differences in C_t values ($p<0.05$) compared to GAPDH (Mann-Whitney test).

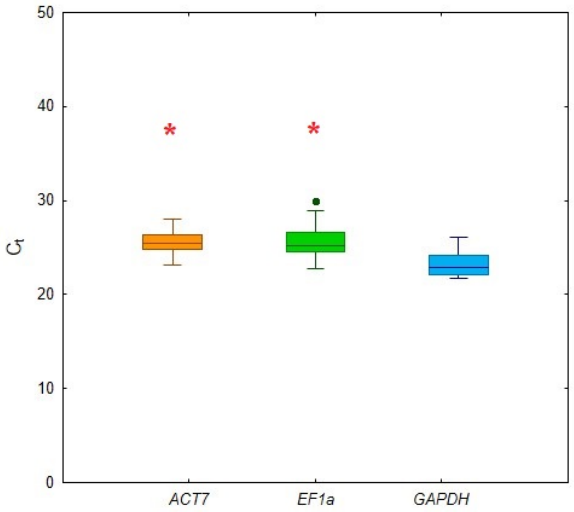

Fig. 1. The results of the evaluation of the expression of three candidate reference genes ACT7, EF1α and GAPDH by RT-qPCR.

The highest expression of *Q. robur* leaves was observed in GAPDH, the median value of the threshold cycles was 22.8, while ACT7 and EF1α had median C_t values of 25.4 and 25.2, respectively. The Kruskal-Wallis test revealed differences in the median values of threshold cycles among the three housekeeping genes under study ($p<0.05$). The C_t values of GAPDH significantly differed from the threshold cycles of ACT7 and EF1α under drought stress ($p<0.05$).

As a result of the evaluation of reference genes using the RefFinder web tool, the data presented in Table 2 and Figure 2 were obtained. Each method has its specific algorithm for calculating values to determine gene stability. Nonetheless, in all cases, the lower values indicate more stable gene expression.

Table 2. The results of the stability evaluation of reference genes based on various methods

Gene/Method	Delta CT	BestKeeper	Genorm	NormFinder
ACT7	**2.188**	**0.88**	**1.345**	2.15
GAPDH	2.295	1.26	1.677	2.15
EF1α	2.333	1.331	1.773	2.272

Apart from NormFinder in the considered methods the most stable reference gene is ACT7, while the least stable is EF1α. In the Genorm, the scores of ACT7 and EF1α are the

same – 2.15. When evaluated by the BestKeeper, EF1α had 1.5 times less score of expression stability under drought conditions, and 1.3 times score when evaluated by Genorm. Delta CT and NormFinder provided similar estimates of expression stability for all three reference genes under study.

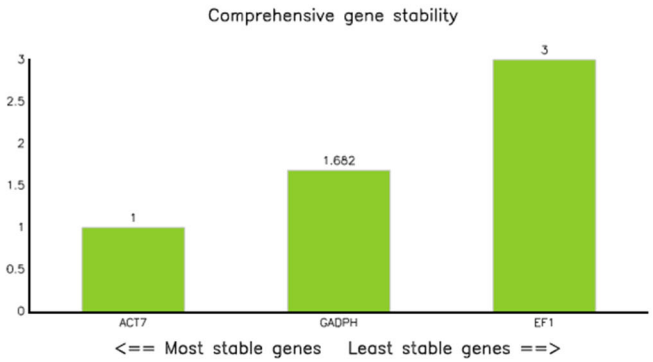

Fig. 2. Evaluation of reference gene expression stability based on RefFinder ranking.

Based on the final ranking in RefFinder and taking into consideration other analysis methods, it was revealed that among the three reference genes under study, ACT7 expression is the least susceptible to changes under simulated soil drought conditions. The second gene in terms of expression stability was GAPDH, and EF1α had the least stable expression among the three genes.

4 Conclusion

The evaluation of *Q. robur* reference genes using various algorithms showed that ACT7 has the most stable expression, while EF1α has the least stable expression. The final ranking in RefFinder also confirmed that ACT7 has the most stable expression under drought conditions. Therefore, for obtaining the most reliable results in gene expression studies of *Q. robur* under drought conditions, ACT7 may be recommended as a reference gene for RT-qPCR analysis.

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