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Selection of reference genes for gene expression analysis by RT-qPCR in *Robinia pseudoacacia* L. under drought conditions

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Abstract. *Robinia pseudoacacia* L., or black locust, is a valuable tree species known for its rapid growth in drought conditions. Due to its adaptation to diverse climates, *R. pseudoacacia* is widely used in agroforestry to combat soil erosion, restore land, strengthen sand, and increase pasture productivity. However, to effectively utilize *R. pseudoacacia* in drought conditions, it is essential to understand the molecular mechanisms underlying its adaptation to this stressor. One of the key tools for investigating these mechanisms is reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). To achieve accurate results, it is crucial to select appropriate reference genes to normalize the expression data of genes involved in stress response. The aim of this study is to identify the most suitable reference genes for quantitative gene expression analysis in *R. pseudoacacia* using RT-qPCR. An analysis was performed via RT-qPCR to evaluate the stability of expression of several housekeeping genes under simulated soil drought in laboratory conditions. Based on gene expression stability data analyzed with RefFinder, one gene was identified as the most stable under drought conditions. Results from various algorithms indicated that *ACT_v2* is the most stable reference gene for *R. pseudoacacia* under drought conditions, while *ACT_v1* showed the least stability.

Keywords: gene expression, reference gene, RT-qPCR, drought, desertification, *Robinia pseudoacacia* L.

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Introduction. Desertification and land degradation, driven by global climate change, present urgent challenges worldwide. Agroforestry practices offer a sustainable, long-term, and environmentally friendly solution to address these issues. Additionally, forest strips provide stable ecological conditions for agricultural landscapes [1, 3, 10]. *Robinia pseudoacacia* L., a fast-growing tree species, is frequently used in agroforestry for soil protection against erosion, microclimate improvement, and enhancement of crop yield [8, 9]. Moreover, *R. pseudoacacia* forms symbiotic relationships with nitrogen-fixing bacteria of the genus *Rhizobium*, enabling the conversion of atmospheric nitrogen into ammonia, which is readily absorbed by other plants. This advantage is especially beneficial in nutrient-poor and degraded areas, where it can significantly improve soil fertility [13].

However, to use *R. pseudoacacia* effectively in agroforestry under drought conditions, it is crucial to understand the molecular mechanisms of its adaptation moisture deficiency. Plant adaptation to stress, such as drought, involves complex changes in gene expression, necessitating a comprehensive approach to study these processes [2, 7, 15, 19].

A vital tool for investigating these mechanisms is RT-qPCR [6]. Accurate gene expression analysis with RT-qPCR requires precise data normalization, achieved through the application of reference genes with stable expression under various stress conditions [4]. Selecting such reference genes is a key task in research on the molecular mechanisms of plant adaptation [12].

The aim of this study was to select the most stable reference genes for quantitative gene expression analysis in *R. pseudoacacia* under drought conditions.

Materials and Methods. To study the stability of housekeeping gene expression in *Robinia pseudoacacia* under drought conditions, a laboratory modeling of soil drought was conducted. Seeds of *R. seudoacacia* were collected from the arboretum of the FSC of agroecology RAS and subjected to

thermal scarification. The seeds were then planted in individual plastic pots with a volume of 0.75 L and cultivated in the "Phytotron" greenhouse at the same research center. The soil substrate was prepared by mixing soil with sand at a ratio of 3:1. After reaching three months of age, the seedlings were divided into two groups: control and drought, with eight plants per group. Over a four-day period, soil moisture was maintained at 75-80 % field capacity in the control group and at 5-10 % in the drought group. Soil moisture was measured daily in the morning using the Landtek MC-7828 SOIL moisture meter (AZ Instrument, China). The average room temperature ranged between 29 and 32 degrees Celsius, with air humidity ranging from 27 to 29 percent. On the fourth day, leaf samples were collected for subsequent RNA extraction.

Total RNA was extracted from the leaves of *R. seudoacacia* using the R-Plants kit (Biolabmix, Russia). Leaf samples (50 mg each) were homogenized with a Precellys® 24 homogenizer (Bertin Technologies, France). During extraction, samples were kept on a cooling rack at +4 °C, and centrifugation was also performed at this temperature. Total RNA samples were then treated with DNase I (Magen, China), followed by rprecipitation in 3M sodium acetate and ethanol. RNA concentration was measured using a Qubit® 4 fluorometer (Thermo Fisher Scientific, USA). Aliquots of the extracted RNA were stored at –80 °C and subjected to only one freeze-thaw cycle prior to RT-qPCR.

For RT-qPCR, predesigned primers for the housekeeping genes *ACT* and *GAPDH*, as reported by Wang J. et al. [18], were used, along with an additional pair targeting another *ACT* sequence, as described by Liu S. et al. [11]. The nucleotide sequences of the primers and their annealing temperatures (T_m) are provided in Tabl. 1.

Table 1

Primer sequences and annealing temperatures

Name	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Annealing Temperature (Tm), °C
ACT_v1	TTGCCTTGGATTATGAACA	GATGGCTGGAACAGAACTT	54
ACT_v2	CAAAGTGATGCCCTTGTGAC	TCGTGGATTGGGTCGGTG	60
GAPDH	TCAACAATGCCAAACCTG	GTGTCAACGAGCACGAAT	54

RT-qPCR was performed using the OneTube RT-PCR SYBR dye kit (Evrogen, Russia) with a Gentier 96E thermal cycler (Tianlong, China). LowROX (Evrogen, Russia) was used as the reference dye. Each reaction mix had a total volume of 25 µL, including 5 µL of 5X OneTube PCRmix SYBR, 1 µL of each primer (10 µM), 0.5 µL of LowROX, 2 µL of RNA, 0.5 µL of reverse transcriptase, and 15 µL of nuclease-free water. Prior to reactions, all samples were adjusted to an RNA concentration of 20 ng/µL by dilution with nuclease-free water. The RT-qPCR protocol included reverse transcription at 55 °C for 15 min, polymerase activation and reverse transcriptase inactivation at 95 °C for 1 min, followed by 50 cycles of denaturation at 95 °C for 15 sec, primer annealing at the specified temperature (Tabl. 1) for 20 sec, and extension at 72 °C for 20 sec, with melt curve analysis from 54 to 95 °C in 0.5 °C increments. Two replicates were used for each sample. The values of the threshold amplification cycles (C_t) were calculated automatically.

Threshold cycle values were analyzed using Statistica 12.0 (StatSoft Inc., USA). Median, first quartile, and third quartile values were calculated. The Friedman test ($p < 0.05$) was applied to evaluate differences in expression stability among the three reference genes. For post hoc analysis, the Wilcoxon test with Bonferroni correction ($p < 0.017$) was used.

The evaluation and selection of reference genes for *R. pseudoacacia* were conducted using the freely available RefFinder web tool [20], which integrates four computational programs: geNorm [14], Normfinder [5], BestKeeper [14], and the comparative delta Ct method [16]. This tool calculated the geometric mean weight for each candidate reference gene across the four algorithms and ranked them from least to most stable based on this metric. The gene with the lowest geometric mean weight was deemed the most stable reference gene.

Results and discussion. As a result of conducting RT-qPCR on *R. pseudoacacia*, cycle threshold (C_t) values were obtained for each candidate reference gene under study (Fig. 1). Analysis of melting curves revealed a single melting peak for each primer pair, with average melting temperatures for the amplification products of 81.2 °C, 82.8 °C, and 81 °C for *ACT_v1*, *ACT_v2*, and *GAPDH*, respectively.

The highest expression level was observed for *ACT_v2*, with a median C_t value of 23.2. *ACT_v1* and *GAPDH* showed lower transcriptional activity, with median C_t values of 28.2 and 28.3, respectively. Comparison of the amplification results for the three genes using the Friedman test revealed significant differences in their expression levels ($p < 0.05$). Subsequent pairwise comparison of C_t values among candidate genes showed statistically significant differences between *ACT_v1* and *GAPDH* compared to *ACT_v2* ($p < 0.017$), but no significant difference was found between *ACT_v1* and *GAPDH* (Fig. 1).

An evaluation of the stability of candidate reference genes using the RefFinder ranked the genes in the following order from most to least stable: *ACT_v2*, *GAPDH*, *ACT_v1*. According to the comparative delta C_t method (Fig. 2A), *ACT_v2* and

GAPDH showed similar stability scores, at 1.471 and 1.494, respectively, while *ACT_v1* had a score of 1.61. The BestKeeper method (Fig. 2B), in contrast, indicated a greater difference between *ACT_v2* and *GAPDH*, with scores of 0.899 and 1.275, respectively, while *GAPDH* and *ACT_v1* showed similar stability levels. The NormFinder method (Fig. 2C), similar to comparative delta C_t, indicated close stability estimates for *ACT_v2* and *GAPDH*, at 0.915 and 0.995, respectively, while *ACT_v1* had a stability score of 1.294. With the geNorm method (Fig. 2D), *ACT_v2* and *GAPDH* both had identical stability scores of 1.354, whereas *ACT_v1* had a stability score of 1.525.

The final ranking in RefFinder (Fig. 3) showed that among the three candidate reference genes in

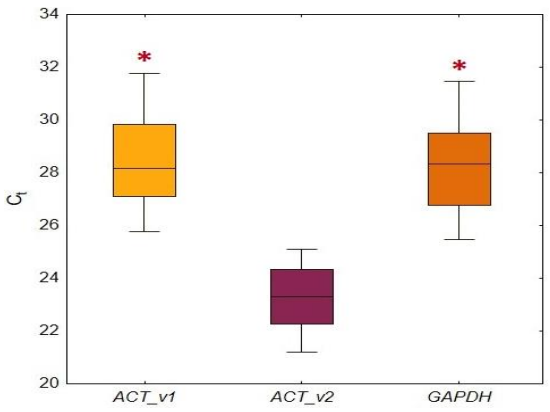

Fig. 1. Expression analysis of three candidate reference genes (*ACT_v1*, *ACT_v2*, and *GAPDH*) in *R. pseudoacacia* using RT-qPCR. * – statistically significant differences from *ACT_v2*, $p < 0.017$

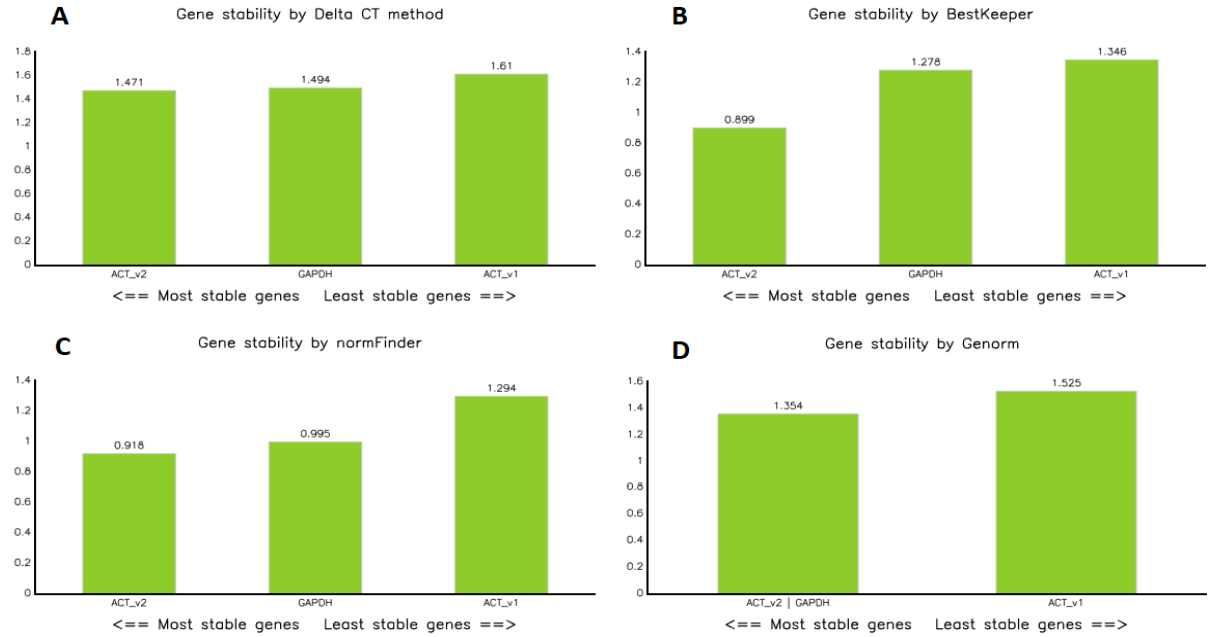

Fig. 2. Stability assessment results for candidate reference genes in RefFinder: A – gene stability ranking using the comparative delta C_t method; B – gene stability ranking using the BestKeeper method; C – gene stability ranking using the NormFinder method; D – gene stability ranking using the geNorm method

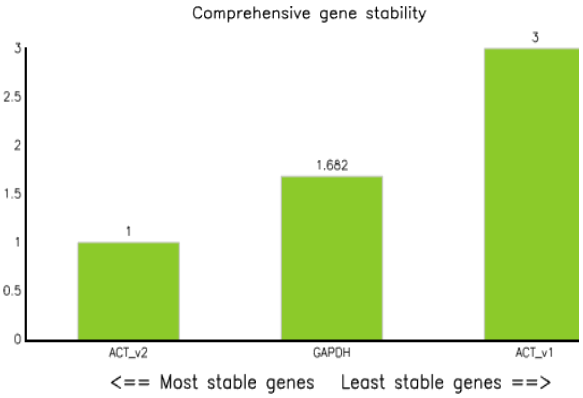

Figure 3. Final ranking of reference gene stability in RefFinder

R. pseudoacacia, *ACT_v2* had the most stable expression under drought conditions, with a stability score of 1. *GAPDH* had slightly lower stability with a score of 1.682, and *ACT_v1* had the lowest stability with a final score of 3.

Conclusion. The evaluation of reference genes in *Robinia pseudoacacia* using four different methods integrated within the RefFinder tool identified that *ACT_v2* has the most stable expression under drought conditions, while *GAPDH* displays moderate stability, and *ACT_v1* shows the least stability. Consequently, *ACT_v2* can be recommended as a reference gene for gene expression analysis via RT-qPCR in *R. pseudoacacia* under drought conditions. The experimental data obtained in this study can serve as a foundation for fundamental research on the mechanisms of drought adaptation in *R. pseudoacacia* and may also aid in the selection of individuals with valuable breeding traits.

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Отбор референсных генов для анализа экспрессии генов методом ОТ-ПЦР-РВ у *Robinia pseudoacacia* L. в условиях засухи

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Аннотация. Робиния псевдоакация (*Robinia pseudoacacia* L.) – ценная древесная порода, быстро растущая в засушливых условиях. Благодаря адаптации к различным климатическим условиям она широко используется в агролесомелиорации для борьбы с эрозией почв, восстановления земель, укрепления песков и повышения пастбищной продуктивности. Однако, для эффективного использования *R. pseudoacacia* в условиях засухи необходимо понимать молекулярные механизмы ее адаптации к данному стрессовому фактору. Одним из ключевых инструментов для исследования этих механизмов является метод полимеразной цепной реакции в реальном времени с обратной транскрипцией (ОТ-ПЦР-РВ). Для достижения высокоточных результатов важно подобрать референсные гены, которые необходимы для нормализации данных экспрессии генов, ответственных за стрессовые реакции. Цель данной работы – отобрать наиболее подходящие референсные гены для количественного анализа экспрессии генов *R. pseudoacacia* с помощью ПЦР в реальном времени. С помощью метода ОТ-ПЦР-РВ проведено исследование стабильности экспрессии нескольких генов домашнего хозяйства в условиях моделирования почвенной засухи в лабораторных условиях. На основе данных об экспрессии изучаемых генов с помощью инструмента RefFinder был выбран один наиболее стабильный в условиях засухи ген. Результаты, полученные различными алгоритмами, показали, что *ACT_v2* является наиболее стабильным референсным геном *R. pseudoacacia* в условиях засухи, в то время как *ACT_v1* продемонстрировал наименьшую стабильность.

Ключевые слова: экспрессия генов, референсный ген, засуха, опустынивание, *Robinia pseudoacacia* L.

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Конфликт интересов. Автор заявляет об отсутствии конфликта интересов.
Author's contribution. Author of this research paper have directly participated in the planning, execution and analysis of this study. Author of this paper have read and approved the final version submitted.
Conflict of interest. Author declare no conflict of interest.