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Sequence based prediction of 26S rRNA in medicinal plants for qRT-PCR purposes

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Abstract
Reverse transcription quantitative real-time PCR is worlwide most often used for the analysis of relative expression levels of genes under various experimental treatments or in various comparative studies. Methodological approach of qRT-PCR offers advantages such as high reproducibility and sensitivity. To obtain the very precise differences in expressions of genes of interest in qRT-PCR, stably expressed reference genes are selected for standard correction. Different stably expressed reference genes have been reported for plants, but establishing the characterics for them stil continuous. Here, bioinformatic based prediction of conserved part of 26S rRNA was performed as the sequence is not available in public databases for one of the analysed species. Subsequently, its transferability was tested for three different medicinal species – *Pulmonaria officinalis*, *Plantago lanceolata* and *Hedera helix*. Sequence alignment resulted in a primer pair that was tested for its specifity in the real-time PCR analysis of all of species used in the study and for three plant parts – leaf, stem and flower. The amplicon of 26S rRNA was confirmed in all of analysed medicinal plant species and a its stability in tested tissues was proved. The most stable results were obtained for *Hedera helix*, but in all analysed species, the differences of expression of 26S rRNA was very low.

Keywords: housekeeping genes, sequential prediction, plant tissue stability

Introduction

Quantitative real-time PCR (qRT-PCR) is a well-established method in the analysis of plant comparative expression studies that provides effective, rapid and sensitive results [6,8,17].

One of the most important prerequisites for achieving the reliable and accurate results in qRT-PCR is connected to stable and suitable reference genes [9]. The methodological background of the use of reference genes is the correcting of the transcription efficiency cDNA amount and redeeming the purity and concentration differences of the samples to avoid the errors caused by the high dynamic state of mRNA transcription and the uncertainty of sample-operating and its downstream disposing procedures [7].

All the calculated transcription levels of the analysed genes of interest need be normalized with the transcription level of a suitable reference gene having stable expression across all treatments and conditions [10]. As the qRT-PCR was applied across plant species, many of genes started to be commonly utilized as reference genes such as glyceralde-hyde-3-phosphate dehydrogenase (GAPDH), α-tubulin (α-TUB), β-tubulin (β-TUB), actin (ACT), ubiquitin (UBQ), ribosomal RNA genes (rRNA), Acetyl CoA Carboxylase (ACCase), eukaryotic elongation factor (eEF). Many of these genes were reported as to have stable expression levels across a wide range of condition, but several studies have demonstrated considerably variation of the expression of these genes, such as actin or tubulin [12,23]. Sometimes these variations can be found across different plant species, development stages, or plant organs [4,5,22].

To date, no systematic study for the evaluation or validation of reference genes for qRT-PCR analysis in *Plantago lanceolata*, *Pulmonaria officinalis* has not been reported and only a few information was published

for individual housekeeping genes in the case of *Hedera helix*, where a total of sixteen different housekeeping genes were validated, but not a 26S rRNA [19]. Among the reported reference genes used in plant studies, those for rRNA are less likely to fluctuate under the conditions that affect the expression of mRNAs, probably due to much greater abundance of these genes that is more than >80% and the involvement of different polymerases for transcription of mRNAs and rRNAs (Lewin, 2000). 26S ribosomal RNA gene was used as reference gene in analysis of expression changes of carotenoid biosynthesis in apricot [13] and it was found that the 26S rRNA gene was stably expressed under 10% PEG treatment in herbaceous plants [18].

In this study, a sequential transferability of 26S rRNA gene for amplicon generating was analysed for selected medicinal plant as well as its expression stability in leaves, stems and flowers.

Material and Method

Plant material

Three different medicinal plant species, *Hedera helix*, *Pulmonaria officinalis* and *Plantago lanceolata* were collected in situ in the Slovak Republic, Liptov, in their mature. In every specie, leaves, stems and flowers from three different individuals were collected, sterilised in surface, cooled and transport to laboratory immediately to extract RNA.

Primers design

Primers for the expression of 26S rRNA in analysed medicinal species were designed on the base of the sequence of *Hedera helix* 26S ribosomal RNA gene (complete sequence), that is stored in the NCBI database under the accession code AF479194.1. This sequence was blasted against known sequences of *Pulmonaria officinalis* and *Plantago lanceolata*, where similarity was returned only for the whole ITS sequences of ribwort plantain with accession codes MF384885.1; KY968842.1 and AF313036.1. For lungwort, none of similar sequence was identified. All these sequences blasted has identified the conserved sequences of expressed parts for primer pair used in the study. A forward primer sequence is 5´ctcagccggaggtagggt 3´ and reverse primer sequence is 5´cactcggtcctccggatttt 3´.

RNA extraction

A total RNA was extracted by GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific) following the manufacturer instruction. The quality and quantity of extracted RNAs were checked spectrophotometrically by NanoPhotometer® P-360 (Implen).

cDNA preparation

cDNA was prepared by Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific), using specific primers designed in the study. cDNAs were synthesized after standardization to 600 ng of RNA into a reaction for every individual triplicate for all of the analysed plant parts.

qRT-PCR analysis

An optimization of annealing temperature of designed primers was performed by gradient PCR as well as an optimization of the concentration of cDNA in the reaction mixture. qRT-PCR reactions were performed in MX3005P qPCR (Agilent). The reactions were performed in triplicates for every analysed plant part by 2x Elizyme qPCR Master Mix (Elisabeth Pharmacon) with 50-fold diluted cDNA. The temperature and time conditions were as follows: 95°C 10 minutes, 35 cycles of 95°C 10 seconds and 62°C 10 seconds with fluorescence reading and melting analysis

Data processing

26S rRNA expression quantification was performed according to the Pfaffl et al. (2001) and analysed by delta delta Ct method. Standard curves for expression effectivity were prepared by 5 serial dilutions of control variant cDNA on 10-fold diluted cDNA. Melting curves of generated amplicons were determined to check the specifity.

Results and Discussion

A very few reports on reference genes in medicinal plants studied in this research were published, but some of them, such as traditional housekeeping genes - actin, GAPDH, 18S, or novel ones - PGK and Fbox

were used [2,11,19]. The stability of reference genes was reported to be variable under certain conditions and can be changed in plants in different samples, organs or developmental stages or physiological conditions [1,3,15]. In *Hedera helix*, different housekeeping genes were validated for their expression stability by Sun et al. (2016). A total of five tissues combined with seven abiotic stress conditions were analysed. The results pointed out that different reference genes should be chosen for ivy for normalization on the basis of various experimental conditions, but EXP and UBQ were the concluded as most unstable reference genes [19]. In *Plantago lanceolata*, elongation factor, GAPDH and actin were analysed for their suitability for a specific purpose of study of the resistance in the ribwort plantain against its fungal pathogen *Podosphaera plantaginis* [16].

Here, 26S rRNA expression was analysed as well as its transferability when bioinformatically predicted sequences of primers. Reference genes that correspond to the rRNAs have been reported to have the advantages for real-time PCR analysis mainly because of their nonresponsiveness to the developmental stage or external stimuli [20].

Here, the primer pair based in its designation on the sequence of ivy genomic information was proved to be transferable for both of further analysed medicinal species. In all of them, strong amplicon of appropriate length of 100 bp (that was an expected length for ivy) was generated and fully reproducible (figure 1).

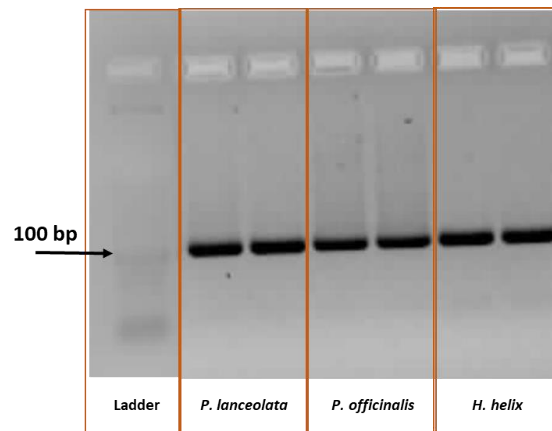

Figure 1. Amplification specifity of the 26S rRNA product in the analysed medicinal plant species in agarose electrophoresis

In qRT-PCR analysis of the 26S rRNA in the *Hedera helix*, obtained Ct values ranged from 23,17 up to the 25,32 with the average Cts for leaves with the value 24,15; for stems 24,39 and for flowers 24,97. Melting temperature for generated amlicon was with a value of 84,3 °C. Delta delta Ct analysis results (table 1) showed a very similar expression pattern among the analysed tissues of ivy with the difference in expression of only 0,07 (figure 2).

Table 1. Delta delta Ct method analysis results for the expression fold change of 26S rRNA in *Hedera helix*

Sample	$2^{-\Delta\Delta Ct}$	(SE of Mean of LOG) (+)	(SE of Mean of LOG) (-)
HH leaf	1	0,084178502	0,084178502
HH stem	0,979642	0,077397176	0,077397176
HH flower	1,049573	0,081353281	0,081353281

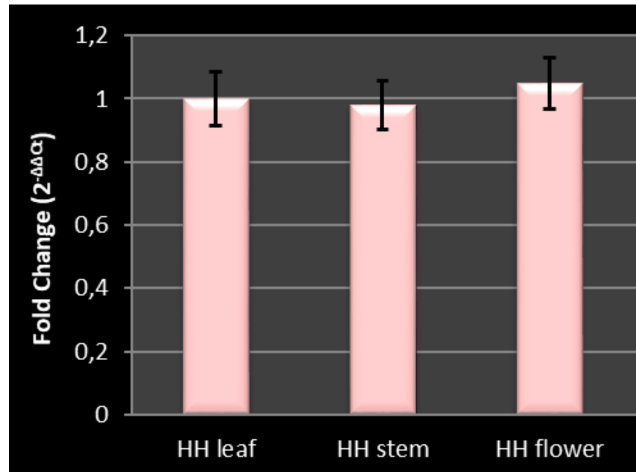

Figure 2. Graphical comparison of expression of 26S rRNA in the tissues of *Hedera helix*.

In qRT-PCR analysis of the 26S rRNA in the *Plantago lanceolata*, obtained Ct values ranged from 12.4 up to the 18.89 with the average Cts for leaves with the value 15.34; for stems 12.66 and for flowers 18.46. Melting temperature for generated amlicon was with a value of 84.5 °C. Delta delta Ct analysis results (table 2) showed again a very similar expression pattern among the analysed tissues of ribwort plantain with the difference in expression of only 0.09 (figure 3).

Table 2. Delta delta Ct method analysis results for the expression fold change of 26S rRNA in *Plantago lanceolata*

Sample	$2^{-\Delta\Delta Ct}$	(SE of Mean of LOG) (+)	(SE of Mean of LOG) (-)
PL leaf	1	0.07791194	0.07791194
PL stem	0.897198	0.074302813	0.074302813
PL flower	0.987736	0.074389036	0.074389036

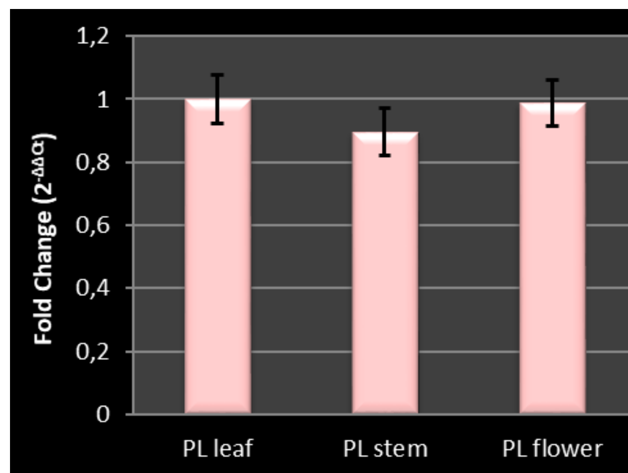

Figure 3. Graphical comparison of expression of 26S rRNA in the tissues of *Plantago lanceolata*.

In qRT-PCR analysis of the 26S rRNA in the *Pulmonaria officinalis*, obtained Ct values ranged from 19.38 up to the 20.17 with the average Cts for leaves with the value 20.04; for stems 19.52 and for flowers 20.12. Melting temperature for generated amlicon was with a value of 84.7°C. Delta delta Ct analysis results (table 3) showed the most similar expression pattern among the analysed medicinal plant species with the difference in expression of only 0.02 (figure 4).

Table 3. Delta delta Ct method analysis results for the expression fold change of 26S rRNA in *Pulmonaria officinalis*

Sample	$2^{-\Delta\Delta Ct}$	(SE of Mean of LOG) (+)	(SE of Mean of LOG) (-)
PO leaf	1	0.08154542	0.08154542
PO stem	0.929341	0.077419078	0.077419078
PO flower	0.950627	0.090918449	0.090918449

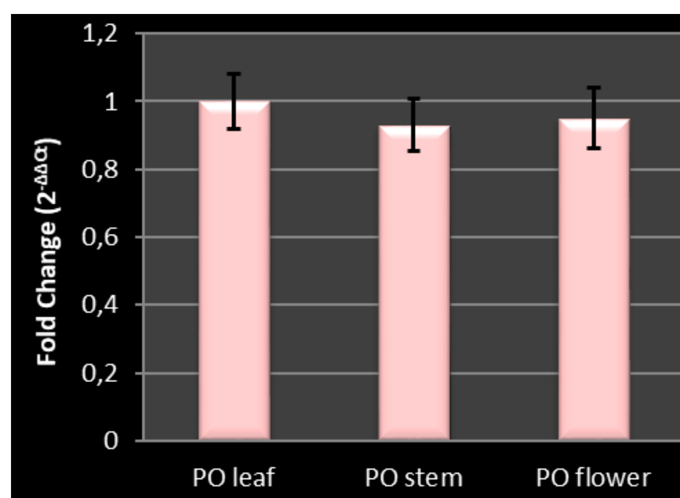

Figure 4. Graphical comparison of expression of 26S rRNA in the tissues of *Pulmonaria officinalis*.

The concept of the bioinformatics prediction of 26S rRNA as refence gene for RT- PCR analysis was applied previously by Singh et al. (2004). In their study, conserved parts of this genes were proved for the amplification in arabidopsis, tea, arnebia, caragana, rheum, picrorhiza and stevia with a positive results of its expression in young and mature leaves of this specie.

Conclusions

The results of this study showed that the bioinformatics prediction of sequence similarity is transferable into the genomic based analysis of medicinal plant species. Expression pattern of 26S rRNA have shown that using of them as a reference gene in the analysis of expression changes of the other genes of interest will provide a tissue specifity. We propose it as suitable internal control for real-time PCR analysis.

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