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Cross-species stability of reference genes in medicinal plants *Arnica montana* and *Arnica chamissonis*

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A better understanding of secondary metabolites biosynthesis requires comprehensive research at the molecular level. Although the medicinal importance of secondary metabolites extracted from *Arnica* spp. has been well documented, the very plants themselves have been poorly studied. Characterization of biosynthetic pathways and their complex regulatory mechanisms may be enhanced via transcriptomic approach. To ensure the reliability of data, RT-qPCR based gene expression studies should be preceded by the reference genes selection step. Here, ten candidate reference genes were analyzed in terms of their expression stability in developing flowers of two arnica species, *Arnica montana* and *Arnica chamissonis*. After evaluating their expression stability with four distinct algorithms (geNorm, NormFinder, BestKeeper and delta Ct method), we found that *SKIP16* and *F-box* were the most stable reference genes in *A. montana*, whereas *SAND* and *F-box* were best-performing genes in *A. chamissonis*. On the other hand, *ACT1* in combination with *F-box* could be used as internal control for cross-species investigations. This study will aid in further research on molecular mechanisms underlying the processes of flowering and secondary metabolites production in medicinal plants.

Keywords Reference genes, Arnica, Flower development, Gene expression, RT-qPCR

Many valuable secondary metabolites produced by plants belonging to the *Asteraceae* family display antimicrobial, anti-inflammatory, anti-malarial, anti-cancer, anti-hyperglycaemic, or other activities that have immense pharmacological potential[1–3] Biosynthesis of some of these bioactive compounds have already been extensively studied, for instance in *Tanacetum parthenium* (parthenolide)[4,5] in *Artemisia annua* (artemisinin)[6,7], in *Saussurea lappa* (costunolide)[8,9] or in *Stevia rebaudiana* (steviol glycosides)[10,11]. Although the medicinal properties of *Arnica montana* have been known for years[12], no significant research on its secondary metabolites biosynthetic pathways has been thus far undertaken.

Molecular studies on medicinal plants not only provide information on subsequent stages of secondary metabolites biosynthesis but also on existing complex regulatory mechanisms leading to altered transcription of biosynthesis-related genes under various conditions in different organs and growth stages[13]. Gene expression studies coupled with analytical techniques enabling qualitative and quantitative analysis of secondary metabolites facilitate the identification of critical, rate-limiting steps in the production of these compounds[14]. To obtain reliable results, gene expression studies conducted with RT-qPCR (reverse transcription quantitative real-time PCR) require data normalization against reference genes showing stable expression in the studied material. Reference genes selection is typically performed from a set of candidates which usually includes genes encoding actin (*ACT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), tubulin alpha or tubulin beta chain (*TUBa* or *TUBb*), elongation factor 1-alpha (*EF1a*) and some other less-frequently used genes which performed well in previous studies[15,16]. As the literature shows there is no such thing as universal internal control, as reference genes have been proven to display temporal and spatial differences in gene expression under various treatments[17]. Reference gene selection has already been performed and facilitated further research in several species belonging to the *Asteraceae* family. Liu et al.[18] identified stably expressed reference genes in a Chinese medicinal plant producing anti-malarial drug—*A. annua*. Liu et al.[19] tested reference genes stability in the Tibetan medicinal species *Saussurea laniceps*. Validation of reference genes in *S. rebaudiana* was carried out by Lucho et al.[20]. Well-performing internal controls were also reported for *Carthamus tinctorius*[21] and *Cichorium intybus*[22] which also are the source of some high-value bioactive compounds.

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This study aimed at identifying and validating reference genes showing stable expression in developing flowers of two arnica species—*Arnica montana* and *Arnica chamissonis*. Mountain arnica is a rare and endangered species due to threatening factors such as excessive collection for herbal use, loss of suitable habitats, climate change and genetic erosion occurring in populations at lower altitudes[23,24]. *A. chamissonis*, on the other hand, has low ecological requirements, which makes it easier to cultivate and may be considered as an herbal alternative for *A. montana*[25,26]. Since both species are rich sources of highly-valued secondary metabolites such as sesquiterpene lactones, phenolic acids, and flavonoids[27,28] our study will lay the foundation for further molecular research on various biosynthetic pathways in *Arnica* spp.

Materials and methods

Experimental material

The research material consisted of *Arnica chamissonis* and *Arnica montana* flower heads collected at different stages of development—yellow bud stage (fully developed flower buds, ray florets closed, disc florets closed), beginning of flowering (disc florets opened in two inflorescence rows, ray florets opened), and full flowering stage (half of disc florets opened), as described in Kowlaski et al.[27]. The source material for setting up the experiment comprised of seeds and seedlings deposited in the collection of herbal plants of the Department of Industrial and Medicinal Plants of the University of Life Sciences in Lublin, which originated from the Botanical Garden of the Maria Curie-Skłodowska University in Lublin (Poland). The authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

The plants' growth conditions were as described earlier in Gawlik-Dziki et al.[28]. Briefly, the plants grew on an experimental plot (51° 31′ N; 22° 45′ E) located on podzolic soil characterized by moderate content of organic matter and phosphorus, low potassium content, very low magnesium levels, and very acidic reaction. The experiment was set up on 5 m^2 plots using the randomized block method with three replications. During the plant vegetation period necessary agricultural treatments, i.e. threefold manual weeding and loosening the inter-rows, were carried out. Standard fertilization was used: nitrogen (80.0 kg ha^{-1}), phosphorus (80.0 kg ha^{-1}), and potassium (120 kg ha^{-1}) were applied every year.

The flower heads of second order were harvested from 2-year-old *A. montana* end *A. chamissonis* individuals. Plant material (four biological replicates per stage) was immediately frozen in liquid nitrogen after collection.

RNA extraction and reverse transcription

The RNA extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. The quality and quantity of RNA were assessed electrophoretically on 1.5% agarose gel and spectrophotometrically with NanoDrop2000 (Thermo Scientific™). The genomic DNA contamination removal and reverse transcription were conducted using Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Scientific™) following the manufacturer's instructions. The cDNA synthesis was carried out in a final volume of 20 µl on 3 µg of RNA. Obtained cDNA was used as a template in the RT-qPCR reactions.

RT-qPCR reactions

Ten candidate RGs (*ACT1*, *EF1a*, *eIF4e*, *F-box*, *GAPDH*, *MTP*, *PP2A*, *SAND*, *SKIP16*, *TUBb*) were chosen for expression stability analysis based on the literature review[29–32]. GeneBank sequences encoding abovementioned genes in *Helianthus annuus*, *Chrysanthemum nankingense* and *Chrysanthemum x morifolium* (the same family: *Asteraceae* and subfamily: *Asteroideae* as arnica) were used as query sequences for BLASTN search to identify respective records in the Sequence Read Archive database (SRA-NCBI) [SRA Experiment set SRX079095 for *Arnica montana* and SRX040282 for *Arnica chamissonis*]. Primers for RT-qPCR were designed with PrimerBLAST tool[33] (Supplementary Table S1.).

The RT-qPCR reactions were performed according to the methodology we used in our previous experiments[34,35]. In short, the reaction were conducted using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™). The reaction mixture of 20 µL contained 10 ng of cDNA, 1 × Master Mix, and 400 nM of each primer. The reactions were performed according to the following cycling program: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. In order to verify the specificity of PCR products dissociation curve analysis was performed after each run with continuous data collection from 60 to 95 °C. To determine amplification efficiencies for each primer pair standard curves were generated from serial dilution of pooled cDNA. All reactions were performed in three technical replicates along with no template controls (NTC) on the QuantStudio™ 3 apparatus (Applied Biosystems). Only primer pairs showing specific amplification (single peak on melting curve), reaction efficiency of 90–110%, and correlation coefficient (R^2) over 0.990 were used for further analysis.

Stability analysis of candidate reference genes

The data obtained from RT-qPCR was analyzed using a dedicated module from ThermoFisher Cloud (ThermoFisher Scientific) as we described previously[36,37]. The Cq values were corrected for reaction efficiency. Gene expression stability was determined using the following algorithms: geNorm[38], NormFinder[39], BestKeeper[40], and delta Ct method[41].The comprehensive ranking of candidate RGs was generated according to the method proposed by Velada et al.[42]. The samples were analyzed in three datasets: full dataset (combined samples from *A. chamissonis* and *A. montana*), *A. chamissonis* dataset and *A. montana* dataset. To test whether reference gene selection could affect the final results in gene expression studies, the transcript level of *FDS* gene (encoding farnesyl diphosphate synthase) was examined. The expression level of the target gene was analyzed via RT-qPCR reactions according to the procedures described above. The expression level of *FDS* in *A. chamissonis*

and *A. montana* developing flowers was normalized against the best-performing and the worst-performing candidate reference genes identified in this study.

Results

Efficiency and specificity of amplification

A set of ten potential RGs (*ACT1*, *EF1a*, *eIF4e*, *F-box*, *GAPDH*, *MTP*, *PP2A*, *SAND*, *SKIP16*, *TUBb*) was assessed in terms of their expression stability during flower development in *A. chamissonis* and *A. montana*. Primer pairs tested within this study consistently amplified single products in samples from both species, as confirmed by the presence of single peaks on the dissociation curves (Supplementary Fig. S1). Amplification efficiency ranged between 96% (*EF1a* and *ACT1*) and 109% (*eIF4e*), with all regression coefficients ≥ 0.991 (Supplementary Table S1.)

Determination of candidate RGs expression stability

Expression stability of tested RGs was assessed using 4 different methods. The geNorm algorithm ranks the candidate RGs based on the average expression stability values (M)[38]. The lower the M value the more stable the genes expression in tested samples. All tested candidate genes in all analyzed datasets had the M value lower than the 1.5 cutoff. The most stable pair of genes across both taxa was *F-box* and *MTP* (Fig. 1A). When datasets of *A. chamissonis* and *A. montana* were analyzed separately, the best-performing pair of RGs were *SKIP16* and *F-box* or *SKIP16* and *ACT1*, respectively. The highest expression variability in all analyzed datasets was displayed by *eIF4e* (Supplementary Table S2).

The NormFinder algorithm estimates expression stability based on both the overall variation of the candidate genes and the variation between sample subgroups in the sample set. The lower is calculated stability value (SV), the more stable the genes expression[39]. According to the obtained results, the *eIF4e* gene once again exhibited the least stable expression, regardless of the *A. chamissonis* and *A. montana* samples being analyzed separately or together (Supplementary Table S2). The best-performing genes in the full dataset were *EF1a* and *TUBb* (Fig. 1B). In the *A. chamissonis* dataset, the highest stability was displayed by *SAND* and *ACT1*, while in *A. montana* samples *SKIP16* and *F-box* were found to be the most stable. Similarly, when arnica species were analyzed separately the same best-scoring RGs were selected by the BestKeeper algorithm as their correlation coefficients (*r*) were most closely related to 1. However, a different pair of RGs (*TUBb* and *ACT1*) was proposed as best for RT-qPCR data normalization in the full dataset (Fig. 1C). According to the ranking generated by ΔCt method (Supplementary Table S2) *F-box* and *ACT1* were considered to be the least variable genes out of all RGs in tested research material. On the other hand, *eIF4e* was the least stable gene as it was characterized by the highest mean SD.

Determination of optimal RGs for RT-qPCR data normalization

The pairwise variation (V_n/V_{n+1}) calculated by the geNorm algorithm allows the determination of the optimal number of RGs for effective normalization of RT-qPCR data. Regardless of the dataset, V_2/V_3 values were all < 0.15 (Fig. 2), which indicates that the best pair of RGs is enough to obtain accurate results. Moreover, the comprehensive ranking, which combines the results from all used algorithms was generated (Table 1). According to the ranking, the most stable RGs during flower development in both arnica species are *ACT1* and *F-box*. In *A. chamissonis* alone, the best-performing pair of RGs was *SAND* and *F-box*, while in *A. montana* it was *SKIP16* and *F-box*. The eIF4e was shown to be the worst candidate for gene expression data normalization in all tested datasets.

Validation of candidate reference genes

To verify the reliability of the selected reference genes, the expression pattern of *FDS* gene was analyzed in developing arnica flowers. When normalized against the most stable reference genes (*ACT1* and *F-box*), either individually or in pair, *FDS* exhibited similar expression patterns (Supplementary Fig. S2). However, when the least stable candidate gene (*eIF4e*) was used as a reference, contrasting results were obtained. The most noticeable difference was shown at the full flowering stage. Normalization against best-performing reference genes revealed that *FDS* transcription was repressed during this stage of flower development. Yet, an entirely different trend was observed when the worst-performing gene was used as an internal control. The *FDS* mRNA level was overestimated, which suggested upregulation of its transcription.

Discussion

The literature shows that the expression stability of candidate reference genes might be evaluated from various perspectives, such as under abiotic or biotic stresses[43–45], under hormone treatments[19,46,47], during different developmental stages[48–50], in different genotypes/cultivars[51–53] or in organ/tissue-dependent manner[54–56]. Here, reference genes performance has been analyzed in the context of cross-species investigations and flower development.

In this study, the set of ten putative RGs was tested for its expression stability during different stages of flower development in two arnica species. According to our results, the combination of *ACT1* and *F-box* was the most suitable for gene expression investigations in both *A. chamissonis* and *A. montana*. Moreover, *F-box* remained the second-best scoring RG when arnica species were analyzed separately. The gene encoding F-box protein was also identified as very stable during the examination of floral developmental stages of *Chrysanthemum morifolium*, which, as well as arnica, belongs to the *Asteraceae* family[57]. Nevertheless, another well-performing gene in chrysanthemum, *PP2A*, showed a rather variable pattern of expression in arnica, regardless of the species

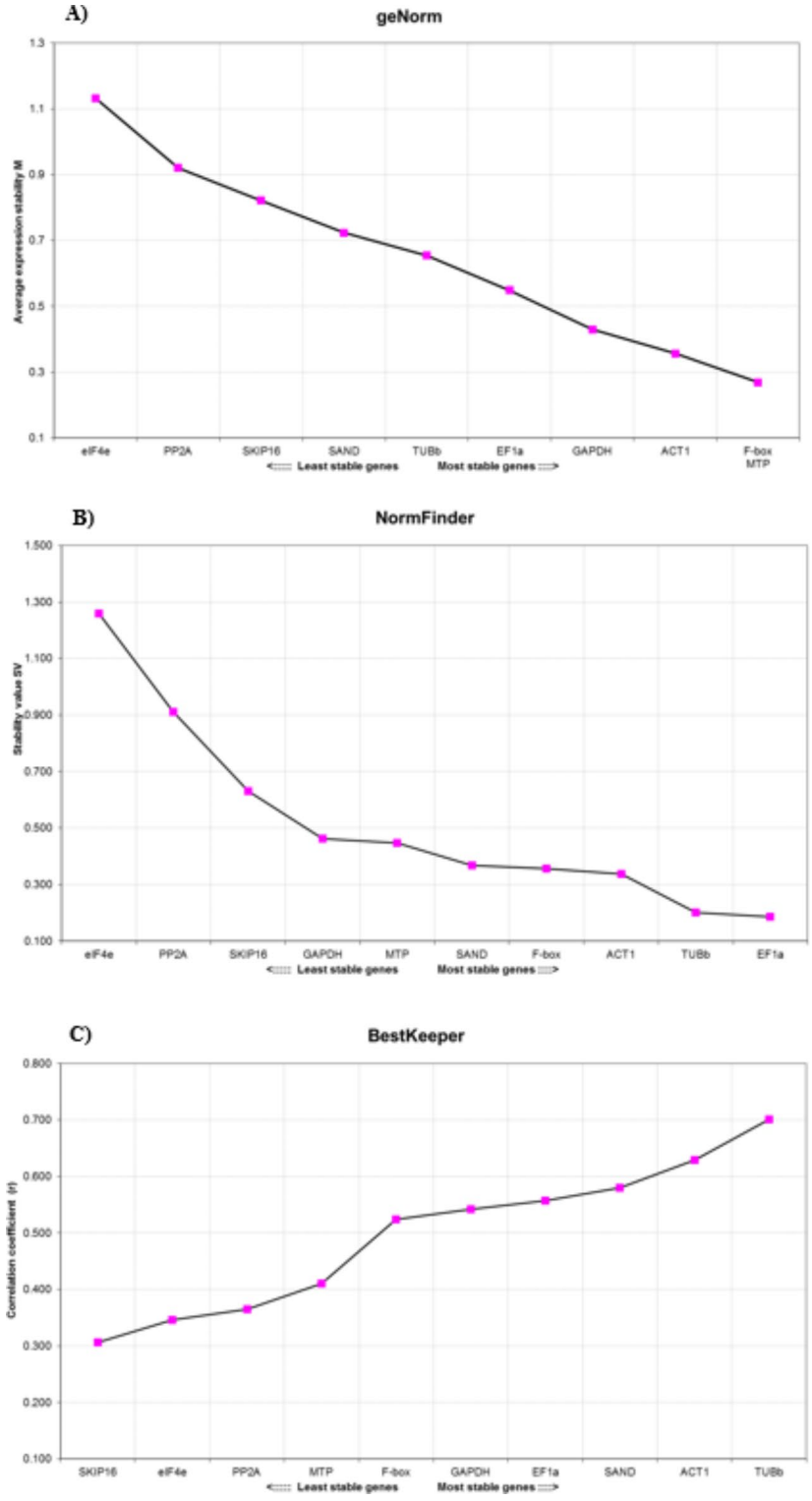

Fig. 1. (**A**) Expression stability values (M) of tested RGs determined by geNorm algorithm for *A. chamissonis* and *A. montana* samples combined together. The lower the M value the more stable the genes expression in tested samples. (**B**) Stability values (SV) of tested RGs determined by NormFinder algorithm for *A. chamissonis* and *A. montana* samples combined together. The lower the SV the more stable the genes expression in tested samples. (**C**) Correlation coefficients (*r*) of tested RGs determined by BestKeeper algorithm for *A. chamissonis* and *A. montana* samples combined together. The higher the correlation coefficient the more stable the genes expression in tested samples.

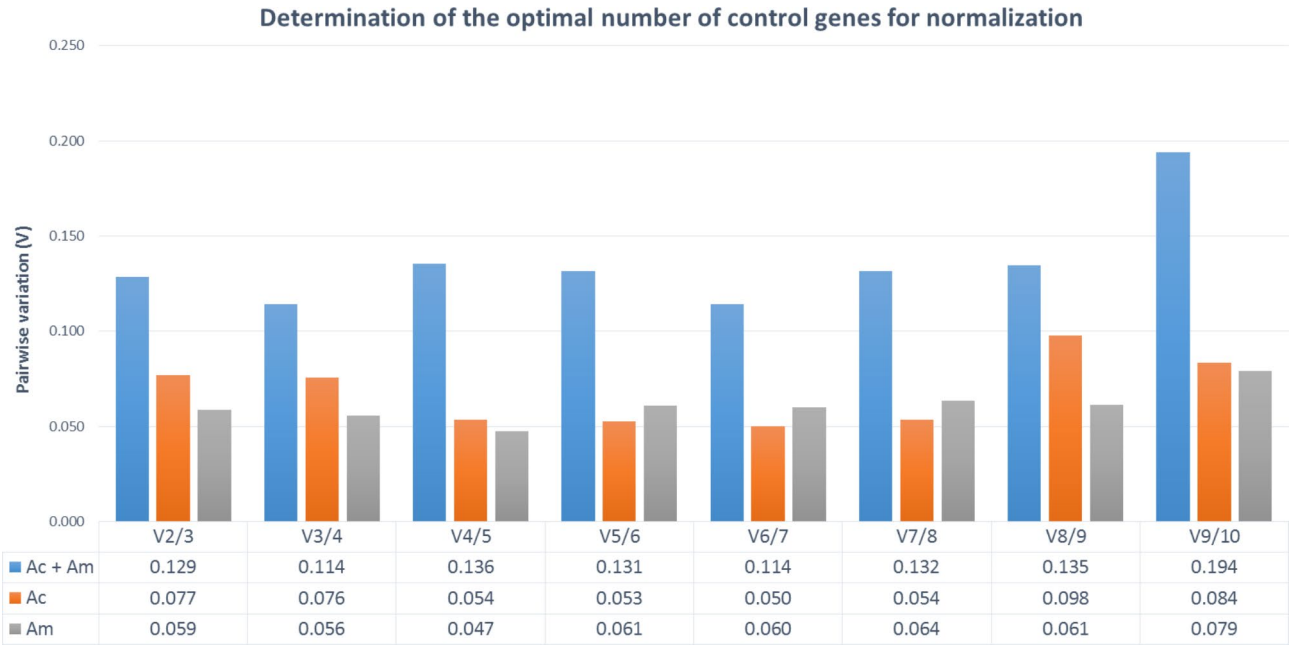

Fig. 2. Determination of optimal number of RGs calculated by geNorm algorithm. Pairwise variation (V_n/V_{n+1}) < 0.15 indicates no significant contribution made by inclusion of additional RG. Analysis was performed for: Ac + Am, *A. chamissonis* and *A. montana* samples combined together; Ac, *A. chamissonis* dataset; Am, *A. montana* dataset.

	<---------- Best ---------- Good ---------- Average ---------- >									
Dataset	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Ac + Am	*ACT1*	*F-box*	*EF1a*	*TUBb*	*MTP*	*SAND*	*GAPDH*	*SKIP16*	*PP2A*	*eIF4e*
Ac	*SAND*	*F-box*	*ACT1*	*SKIP16*	*PP2A*	*GAPDH*	*MTP*	*TUBb*	*EF1a*	*eIF4e*
Am	*SKIP16*	*F-box*	*ACT1*	*SAND*	*TUBb*	*EF1a*	*MTP*	*PP2A*	*GAPDH*	*eIF4e*

Table 1. Comprehensive rankings of RGs stability based on the results obtained from all algorithms. Ac + am, *A. Chamissonis* and *A. Montana* combined together; Ac, *A. chamissonis*; am, *A. Montana.*

being analyzed together or separately. On the other hand, with respect to cross-species stability, *PP2A* was shown to be highly stable in both *Brassica juncea* and its wild relative *Camelina sativa*[58].

Flower development was also the point of focus of the research conducted by Qi et al.[30]. The study revealed the superior expression stability of *SAND* in *Chrysanthemum lavandulifolium* samples. *ACT* and *F-box* were among the three worst-performing RGs in this species. What's more, *SAND* was recommended as the best internal control for experiments comparing expression patterns across chrysanthemum species—in both *C. lavandulifolium* and *C. morifolium.* Here, *SAND* was the best-scoring RG in *A. chamissonis*, but not in *A. montana*, where it showed moderate expression stability.

Selection of RGs that could be used in cross-species research was the objective of the study performed by Wang et al.[31]. The *EF1a* was identified as the most stable RG across examined *Chrysanthemum* taxa. However, when *C. zawadskii* and *C. nankingense* were analyzed separately, the gene ranked either second-best or sixth out of ten tested RGs. High cross-species expression stability of *EF1a* during flower development was also reported by Zheng et al.[59] in ornamental plants *Lagerstroemia indica* and *L. speciosa.* In this study, *EF1a* also showed very good expression stability in the inter-species dataset. Yet, when arnica species were analyzed separately, its performance was rather average or poor, since other candidate RGs showed higher expression stability. Here, the *ACT1* gene, which is another standard RG used in RT-qPCR experiments, was selected as the best-performing gene in terms of expression stability during flower development in both arnica species. The *ACT1* seems to represent a good choice for inter-species research, as its high expression stability across taxa was also confirmed in the abovementioned study of Wang et al.[31]. Correspondingly, *ACT* took a high position in stability rankings for the "total flower" dataset obtained from *Artemisia annua* L. wild-type and transgenic plants[60]. *A. annua* as well as arnica belongs to the *Asteraceae* family and is considered a valuable medicinal plant as it is the source of numerous pharmacologically active secondary metabolites. Moreover, actin encoding genes were found to show stable expression in the developmental stages of flowers or flower parts in *Cymbidium lowianum*[61], *Forsythia* spp[62]. *Lilium* spp[63]. and *Luculia gratissima*[64]. Contrary to this, *ACT* was found to be unsuitable for use as internal control during flower formation in *Primula forbesii*[65].

In a study conducted on flowering stages in *Iris germanica* genotypes, some traditional RGs, such as genes encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-tubulin (*TUBb*) proved to be suitable candidates for RT-qPCR data normalization[66]. On the other hand, these genes displayed average expression stability in the leaves of *Chionochloa pallens* during flowering[29]. Furthermore, *TUBb* was not recommended for the normalization of expression data obtained from *Lagerstroemia* flowers[59] and *Tagetes erecta* flower buds[67], while *GAPDH* was found to be highly unstable in *Taihangia rupestris* flowers[68]. Our study also found that *GAPDH* and *TUBb* are not the best internal controls for the elucidation of transcriptional changes occurring in arnica developing flowers.

To our best knowledge, this is the first report regarding reference genes selection in developing flowers of *Arnica* spp. The data suggests that *SAND* and *F-box* would be the most suitable reference genes for *A. chamissonis* studies, while *SKIP16* in combination with *F-box* could be used in experiments on *A. montana*. For cross-species investigations, we recommend using the pair of *ACT1* and *F-box*. Our study will benefit future research on the expression of genes in *Arnica* spp., especially in the context of secondary metabolites biosynthesis occurring during flower development.

Data availability

The datasets generated during and/or analyzed during the current study are available from the first author (Magdalena Sozoniuk) on reasonable request.

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M.S.: Conceptualization, funding acquisition, methodology, investigation, visualization, writing—original draft, review and editing, K.C.: Writing—review and editing, D.S.: Assistance in investigation, writing—review and editing, K.K.: Writing—review and editing. All authors have read and approved the final version of the manuscript for submission.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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