Sozoniuk *et al. Chem. Biol. Technol. Agric.* (2024) 11:130
https://doi.org/10.1186/s40538-024-00660-3

Chemical and Biological Technologies in Agriculture

RESEARCH

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Selection of reference genes for expression profiling in biostimulation research of soybean

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Abstract

Background Plant biostimulants constitute a promising environmentally friendly alternative for increasing crop yield and tolerance to unfavorable conditions. Among various types of such formulations, botanical extracts are gaining more recognition as products supporting plant performance. Moreover, novel tools such as cold-plasma or low-pressure microwave plasma discharge are being proposed as techniques that might improve their efficacy. Elucidation of the biostimulant's mode of action requires complex research at a molecular level. Transcriptional changes occurring after biostimulant spraying might be investigated using RT-qPCR. However, this technique requires data normalization against stable endogenous controls.

Results Here, we tested the expression stability of ten candidate genes in soybean plants exposed to various biostimulants treatment. Selection of the best-performing reference genes was conducted using four algorithms (geNorm, NormFinder, BestKeeper, and ΔCt method). According to the obtained results, *Bic-C2* (RNA-binding protein Bicaudal-C) and *CYP* (cyclophilin type peptidyl-prolyl cis–trans isomerase) showed highest expression stability, while expression of *EF1B* (elongation factor 1-beta) fluctuated the most among a tested set of candidate genes.

Conclusions Overall, we recommend using *Bic-C2* together with *CYP* for the RT-qPCR data normalization in soybean biostimulation experiments. To our best knowledge, this is the first comprehensive study of reference genes stability in plants subjected to biostimulant treatment. The results of this study will aid in further biostimulant research in crop plants, facilitating analyses performed on the transcriptional level.

Keywords Reference genes, Biostimulant, Gene expression, RT-qPCR, Soybean

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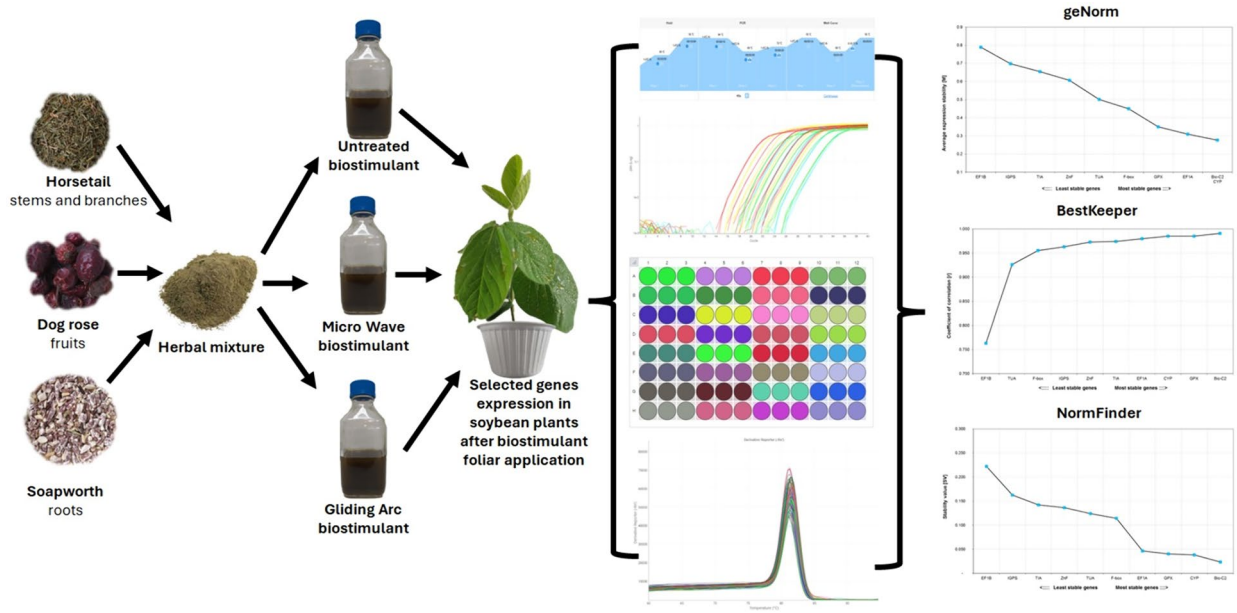
Graphical Abstract



Background

Changing climate conditions and growing world population require novel solutions to meet the demand for food and feed production without negatively affecting the environment. Various strategies are being implemented to overcome these problems—one of them is the application of biostimulants, which constitute a range of formulations based on natural products used to promote the growth and stress tolerance of crop plants [1]. The main categories of biostimulants comprise humic substances, protein hydrolysates, beneficial fungi and bacteria, chitosan and other biopolymers, seaweed extracts, and botanical extracts [2]. Compared to others, botanical extracts' mechanism of action is much less characterized. Plant-based biostimulants are rich in biologically active compounds, such as different phytohormones, antioxidants, vitamins, and other secondary metabolites, which improve overall plant performance by acting on various levels and through different pathways [3]. The characterization of these complex multilayer interactions requires more research employing omics tools (transcriptomics, proteomics, metabolomics, and phenomics) [4]. Yet, despite us not totally understanding how, botanical extracts are effective in stimulating crop growth and development under both optimal [5–7] and stress conditions [8–10].

Horsetail (*Equisetum arvense* L.), dog rose (*Rosa canina* L.), and common soapwort (*Saponaria officinalis* L.) are plants widely known for their therapeutic effects promoted by various bioactive compounds. Horsetail is distinguished by its high content of silicon within its aerial parts [11], which can be useful in two ways, including improved absorption of liquid biostimulant by treated plant and induce the metabolic response of treated plant by the microscopic disruption of its tissues by silicon particles. Moreover, horsetail extract can exhibit antifungal properties [12] and thus can serve as the contact fungicide in the biostimulant. Rosehip, as the fruit of dog rose, is a rich source of antioxidants, especially vitamin C and polyphenolic compounds [13]. Its role in biostimulants can lie in increasing the antioxidant status of treated plants and improving the stability of biostimulants. Soapwort is characterized by a high content of saponins and their glycosides [14]. These compounds are known for their potential toxicity, so their application in biostimulants should be well considered. However, they represent non-ionic biosurfactants with excellent performance [15]. In general, the presence of a surfactant agent as a detergent adjuvant is important for the optimal formulation of agrochemicals, leading to better adhesion on the surface of plants [16]. Except for this, biosurfactants may be applied in plant disease and pest control, boost plant growth through microbial interaction and enhance plant immunity [17]. Concisely, the specific properties of each of these plants predetermine their application within the complex biostimulant.

Since the use of biostimulants has been recognized as a viable method for enhancing crop resilience and yield, scientists are looking for novel ways of improving their mode of action [3, 18]. A recently proposed strategy involves cold-plasma activation of plant-based extracts, including gliding arc plasma discharge [19]. Thus far, non-thermal plasma technology has been applied in agriculture mainly for seed treatment in terms of microbial inactivation and germination improvement [20, 21]. Another reported strategy is using plasma-activated water to enhance plants' growth and tolerance to abiotic and biotic stresses [22, 23]. This is attributed to the generation of a mixture of reactive oxygen and nitrogen species during plasma discharges, some of which act as signaling molecules in cells and activate plants' defense systems [24]. Coupling plant-based biostimulants with cold-plasma activation is an innovative approach that has a high potential for improving crop yield in future [19]. Another alternative way that might influence the effectiveness of plant-derived biostimulants is the employment of microwave plasma discharge. This type of plasma treatment can operate under atmospheric pressure so that it can be simply used for the treatment of various biological materials. Along with decontamination [25] or degradation of hazardous compounds, including mycotoxins [26], microwave plasma discharge can be used to improve the extractability of bioactive compounds from plant materials [27]. The inhibition of adverse enzymatic degradation of plant materials was reported after their treatment by microwave plasma discharge [26]. The pretreatment of dried herbs by plasma discharge may potentially improve the chemical properties and stability of derived water extracts and biostimulants, respectively.

Nevertheless, it should be emphasized that the mechanisms underlying the plants' response to such novel biostimulants have not yet been elucidated. Analysis of transcriptional changes occurring after biostimulant treatment might provide insights into its mode of action. The RT-qPCR technique allows the examination of the expression profiles of various genes related, for instance, to plant redox homeostasis and defense responses, which might be involved in the process. However, this technique is sensitive to various experimental inaccuracies occurring during analysis such as differences in sample quality and quantity, RNA integrity, reverse transcription efficiency, dilution preparation, and pipetting errors. To correct for such non-biological variations, proper data normalization using reference genes (RGs) showing stable expression in tested material are required [28, 29]. Since many studies report spatiotemporal variation in the expression of commonly used reference genes, identification of reliable endogenous controls should precede analyses of genes of interest in every RT-qPCR experiment [28].

Here, we tested the expression stability of ten candidate reference genes in soybean plants sprayed with three different variants of novel plant-based biostimulants. Two variants of biostimulant were formulated using either the gliding arc plasma or low-pressure microwave plasma discharge. To our best knowledge, this is the first report on the identification of reference genes in biostimulant-treated soybean. The results of this study will aid in further biostimulant research in crop plants, facilitating analyses performed on the transcriptional level.

Materials and methods

Preparation of biostimulants

First, three different biostimulants were prepared. The biostimulants were made from the mixture of dried and milled field horsetail (*Equisetum arvense* L.) stems and branches, dog rose (*Rosa canina* L.) fruits and soapwort (*Saponaria officinalis* L.) roots in the following ratio (*w*/*w*): 95.3%: 4.6%: 0.1%, respectively. The biostimulants differed according to plasma treatment used. Untreated biostimulant (without plasma application) was prepared as follows: 25 g of the herbal mixture was mixed with 250 ml of water and then extracted at 100 °C for 30 min. Second type of biostimulant was prepared in the same manner as the control biostimulant but was subsequently treated with gliding arc (GA) atmospheric plasma discharge for 30 s with air as a working gas at a flow rate of 30 standard cubic feet per hour. In the case of the third type of the biostimulant, microwave plasma discharge [30] was applied (500 W for 30 s) on the solid herbal mixture. After the MW plasma treatment, the liquid extract was obtained under the same extraction conditions as mentioned above. Pure water served as control. The biostimulants preparation procedure is depicted in Supplementary Figure S1.

Experimental design of biostimulant application on soybean plants

The experimental material consisted of soybean plants (Abaca variety) growing separately in pots in controlled phytotron conditions (25/18 °C, photoperiod 16/8 h day/night, with photosynthetic photon flux density (PPFD) at a plant level of 500–700 µmol m^{-2} s^{-1} and 75% relative humidity). The plants were divided into four groups based on the biostimulant used: control (water only), untreated biostimulant, GA biostimulant and MW biostimulant. Each group consisted of 18 plants. The soybean seeds were pregerminated for three days on a moist filter paper. Subsequently, the seeds were sown into a sterile sowing substrate and were grown for a total of

24 days. First application of biostimulants (or water) was applied on 14th day in the form of spraying. After three days (day 17), first nine plants were harvested to collect samples for the analyses. The second spraying of the biostimulants was performed on day 21. Again after three days (day 24), the remaining nine plants were harvested to obtain samples for the analyses. Roots and aerial parts of the plants were separated in both sampling points. The experimental design is shown in Fig. 1.

Every treatment was analyzed in three biological replicates, with each sample consisting of pooled material from three randomly chosen independent plants. Collected samples of leaves and roots were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

RNA extraction and cDNA synthesis

RNA extraction, reverse transcription, and RT-qPCR reactions were performed using standard protocols which were described in our previous studies [31]. In short, collected samples were immediately homogenized in liquid nitrogen using a sterile mortar and pestle. The isolation of total RNA was performed using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. Integrity and quality of RNA samples were evaluated electrophoretically on 1.5% agarose gel and spectrophotometrically with NanoDrop2000 (Thermo Scientific™). The Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Scientific™) was used to remove the genomic DNA contamination and conduct the reaction of reverse transcription. The cDNA synthesis was carried out in a final volume of 20 µl using 3 µg of RNA. Obtained cDNA was used as a template in the following RT-qPCR reactions. The good quality of cDNA samples was confirmed via RT-qPCR reactions by analysis of amplification plots, mean Cq values, melt curves, and standard curves. Lack of genomic contamination in the samples was confirmed by NRT controls (no reverse transcriptase control).

RT-qPCR reactions and data analysis

Based on the literature review, five commonly used reference genes *CYP* (cyclophilin type peptidyl-prolyl cis–trans isomerase), *EF1A* (elongation factor 1-alpha), *EF1B* (elongation factor 1-beta), *F-box* (F-box protein), *TUA* (tubulin alpha) and five recently identified candidates showing stable expression in soybean *Bic-C2* (RNA-binding protein Bicaudal-C), *GPX* (glutathione peroxidase), *IGPS* (indole-3-glycerol-phosphate synthase), *TIA* (apoptosis-promoting RNA-binding protein TIA-1/TIAR), *ZnF* (zinc finger) were chosen for the evaluation (Table 1) [28, 32–34]. Some of the traditionally used reference genes exhibited rather poor expression stability in several reports (e.g., *GAPDH* [32, 35], *UBQ10* [36, 37]), therefore along testing the most promising conventional controls, candidates emerging from RNA-seq data [33, 34] were also included in the experimental setup.

Soybean CDS sequences and gene annotation data were retrieved from Phytozome (Phytozome genome ID: 275, annotation version: Glycine max Wm82.a2.v1) [30, 38]. Primers for RT-qPCR were designed with the PrimerBLAST tool (Supplementary Table S1) [39]. The study employed only primer pairs which showed specific amplification (confirmed with dissociation curve analysis—Supplementary Figure S2) and displayed amplification efficiency of 90–110% and correlation coefficient (R^2) over 0.990 (determined via standard curve analysis). The RT-qPCR reactions were performed on the QuantStudio™ 3 apparatus (Applied Biosystems) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™). The reactions were conducted in three technical replicates on 20 ng of template cDNA and 400 nM of each primer in 20 µl total volume, using the cycling profile recommended by the supplier.

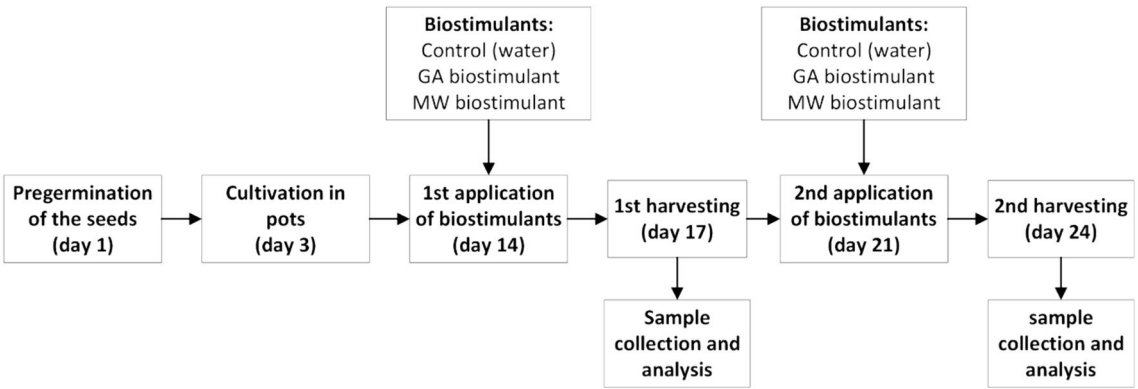


Fig. 1 Experimental design of biostimulant application on soybean plants

Table 1 Candidate reference genes details

Gene acronym	Name of the gene	Functional annotation	References
Bic-C2	RNA-binding protein Bicaudal-C	Regulation: RNA-binding, protein binding	[33]
CYP	Cyclophilin type peptidyl-prolyl cis–trans isomerase	Intra-cellular processes: protein modification, protein folding	[29, 32, 34, 36, 37, 40]
EF1A	Elongation factor 1-alpha	Information: translation, translation elongation factor activity	[29, 34, 40, 41]
EF1B	Elongation factor 1- beta	Information: translation, translation elongation factor activity	[28, 33, 36, 37, 40, 41]
F-box	F-box protein	General: protein interaction, protein binding	[32, 33, 40, 42, 43]
GPX	Glutathione peroxidase	Metabolism: redox, antioxidant activity	[33, 34]
IGPS	Indole-3-glycerol-phosphate synthase	Metabolism: nucleotide metabolism and transport	[33, 34]
TIA	Apoptosis-promoting RNA-binding protein TIA-1/TIAR	Regulation: RNA-binding, metabolism and transport	[33, 34]
TUA	Tubulin alpha	Intra-cellular processes: cell motility, structural constituent of cytoskeleton	[29, 32, 37, 41, 42]
ZnF	Zinc finger, CCCH-type	Regulation: DNA-binding	[33, 34]

Gene expression stability was determined using geNorm [44], NormFinder [45], BestKeeper (46), and ΔCt method [47]. Obtained results were subsequently compiled into the overall ranking generated as described in Velada et al. [48]. In short, each gene was assigned a weight according to its stability as assessed by above-mentioned algorithms (weight of 1 assigned to the best-performing gene, weight of 10 assigned to the worst-peforming gene). Next, the geometric means of these weights were calculated and the comprehensive ranking was obtained. Three datasets were used in the analysis—the roots samples dataset, the leaves samples dataset, and the full dataset comprising all roots and leaves samples analyzed together. For the validation of selected reference genes, the expression level of target gene *SOD* encoding superoxide dismutase [Cu/Zn] (NCBI Reference Sequence: NM_001249007.3) was analyzed under tested experimental conditions. The RT-qPCR reactions were conducted as described above using the following primers F: TCCTCTCACTGGACCAAACAA and R: TCATGACCACCTTTCCCAAGATCA. The transcript level of *SOD* was normalized against the best-performing and the worst-performing candidate reference genes according to the obtained results. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method with control samples being used as calibrator.

Results

Determination of candidate RGs expression stability

The average expression stability value (M) of reference genes was calculated by the geNorm algorithm. Candidate genes showing stable expression in the tested material have low M values, while those showing variable expression are characterized by high M values [44]. As shown in Fig. 2a, *Bic-C2* and *CYP* were the best-performing pair of genes across all tested samples in this experiment, while *EF1B* was the worst-performing gene in the full dataset. In the leaves of the soybean plants treated with various biostimulants, the highest expression stability was exhibited by *F-box* and *ZnF*, while in the roots *CYP* and *EF1A* were considered to be the most stable (Supplementary Table S2).

Analysis performed by NormFinder includes intra- and intergroup variations in the calculation of the stability values (SV), with low SV indicating low expression variability [45]. According to the obtained results, *Bic-C2* was identified as the best-scoring gene in full dataset (Fig. 2b) and ranked as second-best when roots and leaf samples were analyzed separately. In both of these datasets, the lowest variation of expression among all tested candidates was demonstrated by *F-box* (Supplementary Table S2).

The expression stability of tested genes was subsequently evaluated by the BestKeeper algorithm, which determines the correlation coefficient (r) of each candidate with the BestKeeper index (the geometric mean of all candidate genes). High values of correlation coefficient indicate high expression stability of the gene [46]. On the other hand, the ΔCt method ranks the genes based on the average standard deviation (mean SD). Both BestKeeper and ΔCt method produced identical results regarding the two most stably expressed genes in a given dataset. In full dataset, *Bic-C2* and *CYP* were identified as most stable after the biostimulants treatment. Likewise, both calculation methods indicated *EF1B* as the worst reference gene among all candidates. In the leaves dataset and roots dataset, *F-box* together with *Bic-C2* was designated as the two most stable reference genes. Nevertheless, the gene order in stability

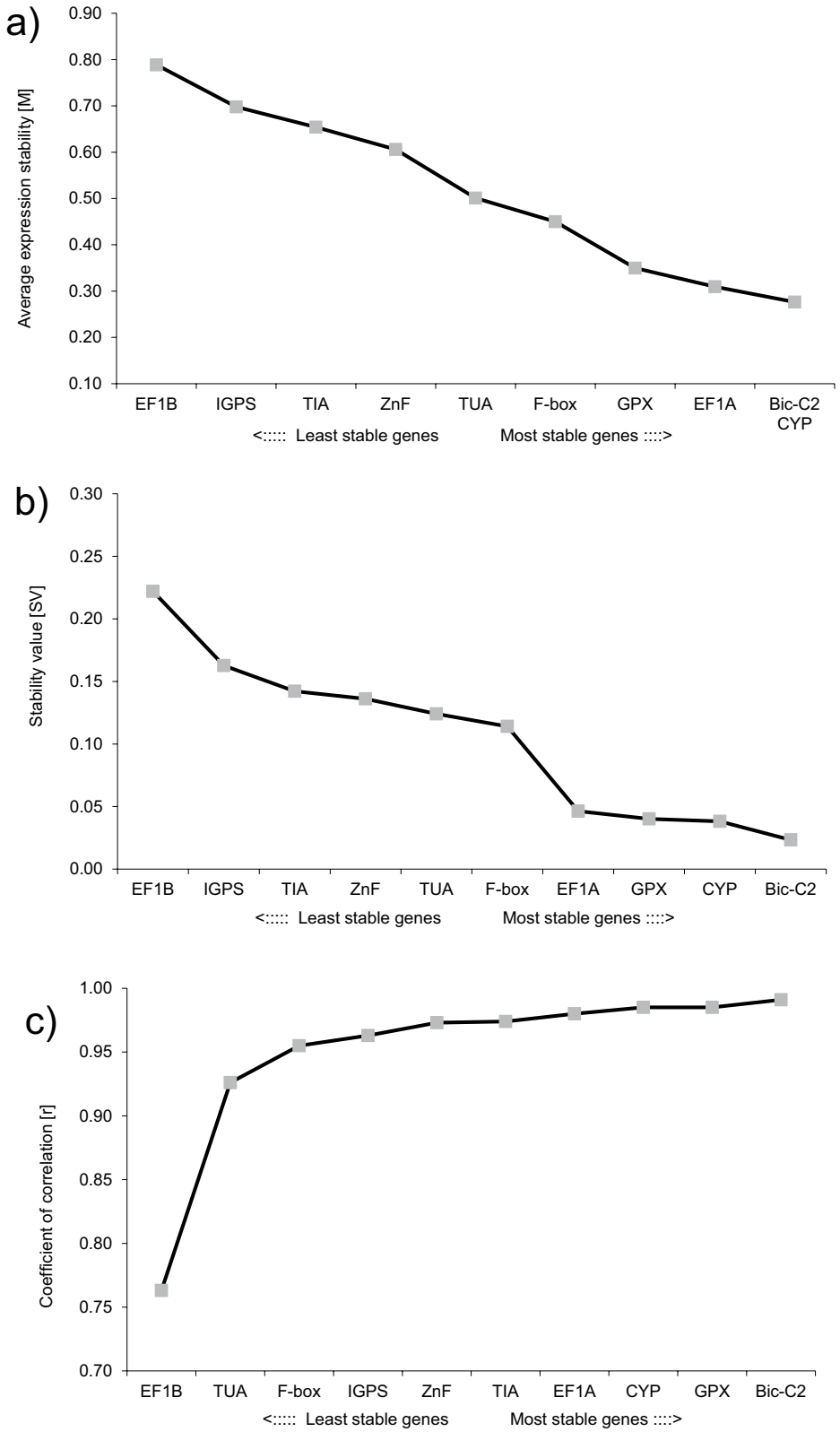


Fig. 2 Expression stability of RGs in soybean plants after biostimulant application (full dataset) evaluated by: **a** geNorm algorithm (based on expression stability values M); **b** NormFinder algorithm (based on stability values SV); **c** BestKeeper algorithm (based on correlation coefficients *r*)

rankings generated by both approaches varied in further positions (Fig. 2c, Supplementary Table S2).

To determine how many reference genes should be used for reliable normalization of RT-qPCR data, the additional analysis of pairwise variation ($V_{n/n+1}$), was performed in the geNorm algorithm. The pairwise variation value below 0.15 indicates no need for the inclusion of an additional reference [44]. Results obtained in the present study show that a pair of best-performing reference genes is sufficient for accurate normalization of the expression data, regardless of the samples being analyzed in full or separate datasets (Supplementary Figure S3.).

As a final point, the obtained results were compiled into a comprehensive ranking (Table 2). In general, *F-box* and *Bic-C2* were the most stable reference genes in the soybean leaves subjected to the biostimulants treatment. Out of all tested genes, the expression of *TUA* was the most affected by the experimental conditions used in this study. In roots, *Bic-C2* was shown to display more stable expression than *F-box*, while expression of *IGPS* fluctuated the most among a tested set of candidate genes. Nevertheless, for the experiments involving both leaves and roots samples of soybean, *Bic-C2* together with *CYP* is recommended as the best pair of controls for the normalization of RT-qPCR data.

Validation of candidate reference genes

The expression of *SOD* gene was estimated using the best- and the worst-performing reference genes identified in this study. When *Bic-C2* and *CYP* were used as internal controls, the *SOD* expression in leaves of plants sprayed with different variants of biostimulant remained stable (Fig. 3a). However, when *EF1B* was used for data normalization, obtained results suggested downregulation of *SOD* transcription. Moreover, contradictory trends were shown in the roots (Fig. 3b) of the plants treated with biostimulant activated with cold plasma. Depending on the reference genes used in the calculation, SOD expression was either upregulated or downregulated by biostimulant application. This demonstrates

Table 2 Comprehensive rankings of RGs stability based on the results obtained from all algorithms

Dataset	Best			Good		Average				
	1	2	3	4	5	6	7	8	9	10
Leaves	*F-box*	*Bic-C2*	*ZnF*	*GPX*	*EF1A*	*CYP*	*IGPS*	*TIA*	*EF1B*	*TUA*
Roots	*Bic-C2*	*F-box*	*CYP*	*EF1B*	*EF1A*	*GPX*	*TUA*	*TIA*	*ZnF*	*IGPS*
Leaves and roots	*Bic-C2*	*CYP*	*GPX*	*EF1A*	*F-box*	*TUA*	*ZnF*	*TIA*	*IGPS*	*EF1B*

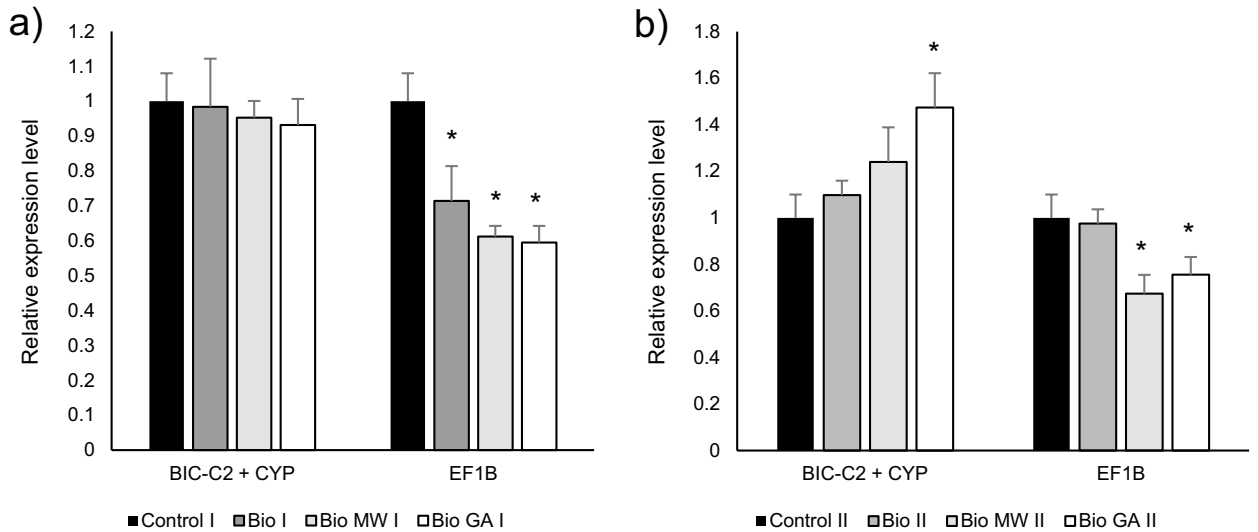


Fig. 3 The expression profiles of *SOD* gene in soybean plants after biostimulant treatment. Relative expression level in leaves after first treatment (**a**) and in roots after second treatment (**b**). Normalization was performed using a pair of the most stable reference genes (*Bic-C2* + *CYP*) in comparison to the most unstable reference gene (*EF1B*). Control: water (used as calibrator), Bio: untreated biostimulant, Bio MW: microwave plasma pre-treated biostimulant, Bio GA: gliding arc plasma pre-treated biostimulant. Data represents mean ± SD (n = 3), asterisk represents significant difference ($P < 0.05$, student's t-test)

the significance of proper reference gene selection and the influence it might have on the reliability of observed expression changes of genes of interest.

Discussion

Biostimulatory effects of plant extracts have been extensively studied in recent years as an alternative approach for promoting crop growth in sustainable agricultural production [3, 49–52]. Until now, no relevant studies have described the use of horsetail, dog rose, and soapwort in biostimulants to promote soybean plants growth and stress tolerance. Horsetail, as the main component of biostimulant in this study, is often used in organic farming for various claimed activities [53]. Its use in plant protection products was approved under Regulation of European Commission No. 1107/2009 as a basic substance since it has a preventive effect against fungal diseases due to its high silicon content. As a practical example, horsetail macerate was shown to be a promising Cu-free fungicide effective in protecting tomato plants against late blight [54]. Another study reported that horsetail extract increased the yield and improved the composition of basil essential oil, suggesting its positive effect on the quality of medicinal plants [55]. Polyphenol-rich rosehips, on the other hand, are neither commonly used nor approved for biostimulant production. However, polyphenol-based biostimulants can positively affect plant growth, especially at the root level [56], which is in coherence with using rosehip extracts as the biostimulant constituent in this study. Soapwort extract, as the material loaded with various saponins, is supposed to serve as an adjuvant in biostimulant. It occurs in this research-related biostimulant only to a minor extent, and its main purpose is to increase the efficiency of biostimulant. However, due to its properties, it may also partly act as a biocide. Application of common soapwort in biostimulants has not yet been reported in the scientific literature.

Better understanding of biostimulants' mode of action requires complex studies conducted on a molecular level [57, 58]. Investigating transcriptional changes occurring after the exogenous application of biostimulatory substances might provide insight into the complex processes leading to the beneficial effects of improved growth, yield and increased resistance to adverse environmental factors [57, 58]. The RT-qPCR is a valuable and precise tool for evaluating changes in gene expression. However, in order to obtain accurate results proper data normalization is required. Thus, the step of reference genes selection is crucial in every experiment involving this technique [59, 60].

Previous studies conducted on soybean regarding reference gene selection focused on evaluating candidate genes' stability under various abiotic and biotic stresses, in different organs, cultivars, or developmental stages [28, 32, 37, 40–42]. Although these results were obtained within the same species, they often are inconsistent or even contradictory, which might be attributed to a particular experimental setup. After testing soybean under different conditions, Wan et al. [28] reported that not a single gene displayed constant expression across all samples. Consequently, as ideal reference might not exist [61], it becomes crucial to precede each gene expression experiment with the identification of proper internal controls.

In this study, we evaluated the expression stability changes occurring in soybean plants subjected to foliar application of different variants of biostimulants. The experimental setup involved expression analysis of genes in both leaves and roots. We tested a set of ten potential reference genes—half of them represented commonly used internal controls (*CYP*, *EF1A*, *EF1B*, *F-box*, *TUA*), half comprised less-known but promising candidates (*Bic-C2, GPX, IGPS, TIA, ZnF*). Obtained data were analyzed via four different approaches (geNorm, NormFinder, BestKeeper, and ΔCt method), and the results were compiled into a comprehensive ranking of gene expression stability in leaves samples, roots samples and in the full dataset.

The results show that, regardless of the dataset, a pair of best-performing genes would be sufficient for gene expression normalization. Overall, *Bic-C2* and *CYP* outperformed all other tested candidates in terms of expression stability in whole plants after biostimulants treatment. In fact, *CYP* was previously reported as being the most stable in different soybean organs [29], which corroborates our results. At the same time, when the leaves and roots samples were analyzed separately, other gene than *CYP* was classified as better candidate for data normalization. Along *Bic-C2*, high expression stability in sample subgroups was exhibited by *F-box*. In the study by Sharma et al. [43], *F-box* also showed stable expression in both root and shoot samples of soybean exposed to macronutrient stress (irrespective of the datasets being analyzed together or separately). Likewise, *F-box* was reported to display stable expression in soybean under other abiotic stresses, such as high salinity (shoots), low temperature (shoots) and dehydration (roots and shoots) [40].

To our best knowledge, this is the first comprehensive study of reference genes stability in plants subjected to biostimulants treatment. Even though gene expression changes in plants caused by biostimulants have been reported before, typically one [62–64] or at best three traditional reference genes [65–67] were used for data normalization without previous confirmation of

their stability in the given experimental setup. Only few studies report testing a small set of three [68] or four [69] internal control candidates before analyzing genes of interest. Using unverified internal controls poses a risk of miscalculating the real expression changes of genes of interest and drawing false conclusions [70]. Therefore, the step of identification of accurate and reliable reference genes should not be omitted in gene expression studies. For instance, here *F-box* was shown to display the highest expression stability in the soybean leaves treated with biostimulants. Similarly, it was reported as the most stable gene in soybean subjected to viral stress [32]. Nevertheless, in the leaves of soybean exposed to Cd stress, it was ranked as the most unstable candidate [42]. Likewise, *ACT* was reported as highly stable in adzuki bean (*Vigna angularis*) under waterlogging stress and rust infection [71], yet it performed poorly when the plants of this species were growing under iron deficiency [72].

Many times some of the traditionally used reference genes, such as *GAPDH*, have been proven to show rather poor expression stability under given experimental conditions [32, 35, 73]. Therefore, there's a need to find new candidates for reliable reference. Using RNA-seq datasets might significantly aid in this process. Transcriptome-based identification of novel reference genes has already been conducted in some plant species, e.g., *Gossypium hirsutum* [74], *Allium tuberosum* [75], *Lactuca sativa* [76] or *Ardisia kteniophylla* [77]. Yim et al. [33] also employed such strategy in order to find better internal controls for soybean studies. One of their newly identified candidates, *Bic-C2*, outperformed all of the genes tested in this experiment. Another one, *GPX*, also showed good overall performance as it ranked third in the full dataset. Likewise, Machado et al. [34] analyzed 1298 RNA-seq soybean samples and found 452 genes displaying uniform and constant expression, which might potentially serve as reference gene candidates. Six of them were also tested in this study. While *CYP*, *EF1A* and *GPX* remained stable after biostimulants exposure across both leaves and roots, *ZnF*, *TIA* and *IGPS* exhibited rather average expression stability. Therefore, the verification of candidates emerging from RNA-seq is still needed.

Since being identified as constitutively expressed in soybean [33], *Bic-C2* has been used several times for RT-qPCR data normalization [78]. Yet, until now, its stability has not been confirmed by other authors. Based on the obtained results, we recommend *Bic-C2* to be used in pair with *CYP* as reliable internal control in biostimulant-soybean research and to be considered as a worthy candidate for studies conducted in different species.

Conclusions

In summary, in this experiment, we tested the expression stability of ten candidate genes in soybean plants treated with three novel variants of plant biostimulants. The selected candidate genes included five commonly used reference genes: *CYP*, *EF1A*, *EF1B*, *F-box*, *TUA*; and five recently identified candidates showing stable expression in soybean: *Bic-C2*, *GPX*, *IGPS*, *TIA*, *ZnF*. Comprehensive analysis conducted with four algorithms points to *Bic-C2* and *CYP* as the best-performing pair of reference genes in tested experimental material. The lowest expression stability was shown by one of the traditionally used reference genes, *EF1B*. Our results confirm that a pair of best-scoring genes will be sufficient for reliable RT-qPCR data normalization. Overall, we recommend *Bic-C2* to be used together with *CYP* as a good internal control in the research of biostimulant applications on soybean plants. Moreover, these two candidate genes could be considered for biostimulation studies conducted in other plant species. The results of this study will aid in elucidating the biostimulant's mode of action on the transcriptional level.

Abbreviations

Bic-C2	RNA-binding protein Bicaudal-C
cDNA	Complementary deoxyribonucleic acid
CDS	Coding DNA sequence
CYP	Cyclophilin type peptidyl-prolyl cis–trans isomerase
DNA	Deoxyribonucleic acid
EF1A	Elongation factor 1-alpha
EF1B	Elongation factor 1-beta
F-box	F-box protein
GA	Gliding arc atmospheric plasma discharge
GPX	Glutathione peroxidase
IGPS	Indole-3-glycerol-phosphate synthase
MW	Microwave plasma discharge
PPFD	Photosynthetic photon flux density
RGs	Reference genes
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SOD	Superoxide dismutase
SV	Stability value
TIA	Apoptosis-promoting RNA-binding protein TIA-1/TIAR
TUA	Tubulin alpha
ZnF	Zinc finger

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00660-3.

Additional file 1.

Acknowledgements

This study was supported by Polish National Agency for Academic Exchange in frame of the project "Crucial, long-term collaborations for the development of an innovative, ecological approach in biostimulants production" (BPI/PST/2021/1/00034/U/00001).

Author contributions

MS: conceptualization, methodology, formal analysis, investigation, writing—original draft; MŚ: writing—review and editing; AB: funding acquisition, project

administration, supervision; PB: funding acquisition, project administration; JB (Jan Bedrníček): investigation, writing—review and editing, visualization; FL: writing—review and editing, investigation; MJ: investigation; KP: investigation; AS (Adéla Stupková): investigation; JL: investigation; PO: investigation; JB (Jan Bárta): project administration; AS (Agnieszka Szparaga): investigation; methodology; MCPP: investigation; SK: funding acquisition, project administration, supervision. All authors read and approved the final manuscript.

Funding
This research was solely funded by the Polish National Agency for Academic Exchange, project No. BPI/PST/2021/1/00034/U/00001 "Crucial, long-term collaborations for the development of an innovative, ecological approach in biostimulants production".

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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 21 June 2024 Accepted: 25 August 2024
Published online: 03 September 2024

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