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Optimizing Gene Selection: A Mini Review on Reference Gene Normalization for qRT-PCR in *Solanaceae* Plants

LBWS Amarasena [a], Madhavi Hewadikaram [a*]
[a]NSBM Green University, Pitipana 1, Homagama, Sri Lanka

*** Corresponding author email address**: madhavi.h@nsbm.ac.lk

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Abstract

Gene expression analysis is fundamental for understanding biological processes, and quantitative real-time PCR (qRT-PCR) has become a widely used method for validating the expressions. Proper normalization across multiple samples and tissues is crucial for accurate and reliable results, requiring the selection of suitable reference genes for comparison with target genes. Finding internal reference genes in *Solanaceae* plants is crucial for precise transcript quantification using real-time PCR. Research on *Solanaceae* plants *like Solanum rostratum, Solanum melongena, Solanum tuberosum, Solanum lycopersicum, Nicotiana tabacum*, and *Capsicum* was conducted to identify reference genes for qRT-PCR normalization. Researchers can confidently assess gene expression variations in *Solanaceae* plants by establishing accurate reference genes, providing deeper insights into biological processes. This literature review provides a comprehensive overview of methodologies, challenges, and advancements in the selection of candidate reference genes for RT-PCR in *Solanaceae* plants. It has the potential to influence future research directions and methodologies, enhance scholarly discourse, and pursue excellence in molecular biology research.

Keywords - Reference genes, *Solanaceae*, qRT-PCR, Gene normalization

1 Introduction

Genomic technologies play an increasingly significant role in advancing our knowledge of complex biological mechanisms in plant science. Gene expression profiling provides new insights into regulatory gene networks, offering a deeper understanding of various biological processes. Among the reliable techniques for analyzing changes in gene expression, real-time quantitative reverse transcription PCR (qRT-PCR) is commonly employed and essential to biological research due to its higher sensitivity, specificity, superior repeatability, dynamic range, and high throughput capacity [1].

However, to ensure accuracy and dependability in qRT-PCR, it is necessary to normalize gene expression data. Normalization helps to eliminate variance caused by errors in sample measurement and sample variation that occur during experimental processes [2]. Traditionally, housekeeping genes related to basic cellular functions are used as reference genes in the normalization strategy because they are assumed to have constitutive and stable expression under various physiological conditions and experimental treatments. Nevertheless, several studies have shown that the expression of housekeeping genes is not always stable and can be influenced by developmental stage, tissue, sex, and biotic or abiotic stresses [3]. Therefore, it is essential to select suitable reference genes according to specific experimental conditions to ensure accurate results [4]. Reference genes with constant expression are required to correct and minimize errors in the qRT-PCR procedure, leading to accurate and scientifically sound data. The stability of the reference genes is crucial for the study of quantitative data [3]. Failure to choose stable reference genes could lead to misleading results and inappropriate conclusions regarding target gene expression [5].

This review will help to identify the current status of knowledge on the selection of reference genes. It will provide a comprehensive understanding of the methods and algorithms used in the selection of reference gene and their advantages and limitations. Based on the literature review, a research strategy for identifying a suitable reference gene can be developed for the specific experimental system. This may involve selecting a set of candidate genes, validating their stability across various experimental conditions, and comparing their performance in normalizing qPCR data. and potential avenues for future research.

2 Reference genes

Reference genes serve as internal reaction controls with a sequence distinct from the target gene. A housekeeping gene, expressed ubiquitously in all cells, plays a vital role in cell structure or metabolism [3]. For a gene to be considered a reliable reference, it must meet several crucial criteria. The most important aspect is that its expression level remains unaffected by experimental variables. The ideal reference

gene possesses the following qualities: stable expression across various tissues and cells, minimal impact from environmental, biological, or abiotic stresses, and expression levels comparable to the target genes. Commonly used reference genes in many plants include ACT (actin), TUA (tubulin), CYP (cyclophilin), UBI-1 (ubiquitin), and EF- α (elongation factor), as they are considered to be stably expressed [6].

In qRT-PCR analysis, reference genes are frequently employed to standardize gene expression levels to a gene that exhibits consistent expression across various research groups. They function as controls in qRT-PCR analysis, leading to more accurate interpretation of results. The most appropriate control gene for a relative qRT-PCR experiment can be selected by evaluating candidate genes and determining their expression levels across a range of experimental conditions and treatments [7]. Genes that display the most stable expression across these conditions are considered the most suitable controls.

When assessing potential reference genes for normalization in gene expression investigations, researchers commonly consider a number of standard criteria to ensure their appropriateness. These criteria include stable expression, uniform expression levels, absence of regulation, low variability, compatibility with the experiment design, and low technical variations [8].

3 Normalization of reference genes for q-RT PCR

Normalization of target gene expression measured by qRT-PCR is essential to reduce experimental bias and improve data quality. The current normalization approach involves using one or more reference genes. However, this method increases the experimental workload and relies on assumptions that can be challenging to meet and validate [9]. To address these challenges, it is preferable to use multiple reference genes that exhibit stable expression. This approach allows for more robust normalization, leading to statistically significant findings and improved detection of subtle expression changes. Normalization of target gene expression levels is crucial to account for variations in intra- and inter-kinetic RT-PCR, as well as sample-to-sample and run-to-run variations [10]. Proper normalization ensures that the expression data accurately reflects the true biological differences between samples, rather than experimental artifacts or technical variations. The steps of selecting a suitable reference gene for normalization of qRT-PCR are shown in the figure 1 below.

By employing multiple stably expressed reference genes, researchers can enhance the reliability and reproducibility of their qPCR experiments. This approach not only reduces the risk of biased results but also provides a more accurate representation of gene expression levels, facilitating meaningful comparisons and interpretations of experimental data.

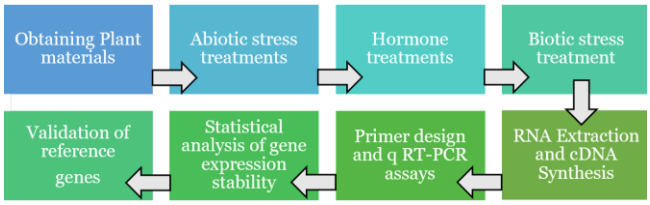

Fig. 1. Steps of selecting a reference gene for normalization of q RT PCR

4 Methods and statistical approaches

The analysis of gene expression stability is a crucial step in numerous biological research projects. To accomplish this, researchers employ a variety of techniques and statistical frameworks. Several typical examples of such methods are outlined below, along with their corresponding references:

4.1 NormFinder

This statistical algorithm ranks genes based on their expression stability, considering both intra- and inter-group variation. The stability value for each gene is calculated, and lower stability values indicate more stable expression [11].

4.2 GeNorm

Another popular method, GeNorm, determines the most stable reference genes by calculating a stability measure known as the M value for each gene. Additionally, it identifies the optimal number of reference genes required for proper normalization [11].

4.3 BestKeeper

BestKeeper utilizes pair-wise correlation analysis to identify the most stable reference genes. By calculating the coefficient of variance (CV) and standard deviation (SD) for each gene, it pinpoints the gene with the lowest variability as the most stable reference [11].

4.4 ΔCt method

This method involves comparing the cycle threshold (Ct) values of target genes with those of the reference genes. The stability of reference genes is assessed based on the standard deviation (SD) of their Ct values, with lower SD values indicating more stable gene expression [11].

These techniques enable researchers to choose appropriate reference genes for normalization, thereby improving the accuracy and reliability of gene expression analyses in biological research.

5 *Solanaceae* plants

The *Solanaceae* family, also known as nightshades, is diverse comprising approximately three to four thousand species, classified into around ninety genera. It thrives in a wide range of terrestrial habitats, from deserts to rainforests,

and includes both perennial trees and herbaceous annual species [12].

Throughout history, the *Solanaceae* family has been utilized in traditional medicine and human nourishment due to the presence of several bioactive compounds, which have been employed by various cultures worldwide [13]. Notably, some of the most economically significant crops within this family include the potato, tomato, brinjal (eggplant), and pepper [14].

The genus Solanum, a major group within the *Solanaceae* family, consists of up to 2,000 plant species that encompass both food crops and medicinal herbs. In recent decades, there has been considerable interest in the chemical and biological investigation of Solanum species. These investigations have revealed various bioactive compounds, such as steroidal saponins, steroidal alkaloids, disaccharides, flavonoids, and phenols, which have been associated with health-improving properties and their potential in combating noncommunicable diseases, major causes of global mortality [12-14]. Many species within this genus exhibit diverse pharmacological properties, including anticancer, hepatoprotective, antimalarial, anthelmintic, and other activities [15].

6 Importance of the gene expression analysis in *Solanaceae* plants

Gene expression analysis plays a crucial role in understanding the biology of *Solanaceae* plants, and qRT-PCR is a valuable technique for this purpose. qRT-PCR allows for the accurate and sensitive measurement of gene expression levels, providing insights into the regulation and functioning of genes in *Solanaceae* species. By analysing the expression patterns of specific genes, researchers can investigate various biological processes, including development, response to biotic and abiotic stresses, and synthesis of bioactive compounds. A list of main importance of gene expression analysis in *Solanaceae* plants are as follows.

Understanding Developmental Processes: Gene expression analysis helps elucidate the genetic control of developmental processes such as flowering, fruit ripening, and root development.

Stress Response Mechanisms: *Solanaceae* plants often face biotic and abiotic stresses. Analysing gene expression can identify stress-responsive genes and pathways, aiding in the development of stress-resistant varieties.

Metabolic Pathway Insights: Gene expression studies can reveal the regulation of metabolic pathways, including those involved in secondary metabolite production, which is important for flavour, colour, and health benefits.

Genetic Improvement: By understanding gene expression patterns, researchers can employ techniques like CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and RNA interference to enhance desirable traits in crops.

Comparative Genomics: Analysing gene expression across different *Solanaceae* species can provide insights into evolutionary relationships and functional conservation [16-18]. These reference genes serve as internal controls for normalizing gene expression data, enabling accurate comparisons and reliable interpretation of results. Overall, qRT-PCR-based gene expression analysis is of great importance in unravelling the molecular mechanisms underlying the diverse traits and functions of *Solanaceae* plants [19]. In addition, researchers have utilized this method to study important aspects such as fruit growth, ripening, and quality attributes, including colour, flavour, and scent. Furthermore, qRT-PCR has been instrumental in exploring the expression of genes involved in plant defence against pathogens and pests. Furthermore, it has shed light on the genes responsible for producing secondary metabolites with therapeutic and nutraceutical effects. Using qRT-PCR, significant insights into the molecular mechanisms governing these processes have been obtained, contributing to a better understanding of the complexity and diversity of *Solanaceae* plants [20].

7 Recent findings on reference gene selection for q RT-PCR in *Solanaceae* plants

Recent investigations on gene expression analysis in *Solanaceae* plants using q RT-PCR have provided valuable insights. [4] evaluated the expression stability of eight candidate reference genes in both susceptible and resistant tomato varieties under powdery mildew (PM) stress. They found that the Act gene was the most stable reference gene in both tomato varieties under PM stress. [21] conducted a simultaneous expression analysis of seven novel candidate differentially expressed genes (DEGs) in resistant and susceptible peppers using q RT-PCR, which marked the first-time investigation of these genes in peppers. Additionally, [22] researched NAC transcription factors in tomatoes using q RT-PCR. In a separate study, [23] evaluated the stability of nine commonly used reference genes for normalization through RT-qPCR analysis under different experimental conditions in eggplant. These studies contribute to a better understanding of gene expression regulation in *Solanaceae* plants and highlight the importance of using q RT-PCR for accurate and reliable gene expression analysis in this plant family. More findings are displayed in table 01.

Several plants from the *Solanaceae* family have significant medicinal and nutritional importance, but some of them have not undergone research on selecting reference genes for normalization of q RT-PCR. Angel's trumpet (*Brugmansia* genus) is utilized for medicinal purposes, although its recreational use is known to cause serious safety concerns, leading to euphoria and hallucinations. Despite being used to treat asthma and other illnesses, limited scientific data is supporting its effectiveness [24]. Belladonna (*Atropa belladonna*), also known as deadly nightshade, has historical significance as ladies used it to enlarge their pupils to enhance beauty, leading to the name "belladonna" meaning "beautiful woman" in Italian. Although only the leaves are officially recognized for medical purposes, the entire plant

is active and contains substances like atropine, hyoscyamine, and scopolamine. These substances are still used for various medical treatments, such as dilating pupils for eye exams and treating stomach and intestinal issues [19]. *Datura stramonium* (*D. stramonium*) is a well-known medicinal herb from the *Solanaceae* family. Its extracts from leaves are used to treat asthma, fever, and sinus infections, while the bark is applied externally for swellings, burns, and ulcers. This plant possesses anti-inflammatory properties and is used for various medicinal purposes, including treating dental and skin infections, alopecia, toothache, and inducing sleep [25]. Ground cherry (genus *Physalis*) is rich in vitamins A, C, and B-3 (niacin), as well as minerals like non-heme iron, calcium, and phosphorus. It is also a reliable source of vitamins B-2 (riboflavin) and B-1 (thiamine). The orange-golden hue of ground cherries is due to phytochemicals called carotenoids, which contribute to its anti-inflammatory and immune-boosting properties, beneficial for heart health, eyes, skin, and bones. Ground cherries also contain withanolides, which exhibit significant biological activities like antibacterial, anticancer, anti-inflammatory, and immunomodulatory effects, making them potentially useful in combating various types of tumour cells [14]. It is important to note that while these plants have various medicinal and nutritional benefits, further research is required to select appropriate reference genes for the normalization of qRT-PCR to improve the accuracy and reliability of gene expression analysis in these species.

Table 1
Investigations on reference gene selection for q RT-PCR in *Solanaceae* plants from 2019 to present.

Reference	Used algorithms/software	Selected reference gene
[26]	geNorm, NormFinder, BestKeeper RefFinder	*EF1α*, *ACT* and *SAND*
[27]	qPCR qTower3.0 system and the qPCRsoft software	*ALC* and *SPT*
[21]	SeqMan (v. 4.03) and MEGA7	19OrnP-PBI
[4]	geNorm, Normfinder, BestKeeper and the comparative ΔCT method.	Act

8 Challenges, limitations, and future directions for reference gene selection in *Solanaceae* plants.

Selecting suitable reference genes for gene expression analysis in *Solanaceae* plants comes with several challenges, limitations, and future directions. One major issue is the limited validation of *Solanaceae* plant-specific reference gene candidates. While numerous reference genes have been validated in model plants such as Arabidopsis or rice, it is not guaranteed that these genes will demonstrate the same stability in *Solanaceae* species. Therefore, more dedicated research and validation studies specific to *Solanaceae* plants are necessary to identify and verify reliable reference genes [28].

Another challenge arises from the diverse expression patterns of reference genes in different tissues of *Solanaceae* plants. Researchers must consider this variability and carefully select reference genes that show consistent expression across various tissues and developmental stages. Additionally, the choice of reference genes can be influenced by specific environmental factors or medical interventions under study. To ensure accurate normalization, it is crucial to assess the stability of reference genes under different experimental conditions [29].

Looking ahead, future research in reference gene selection for *Solanaceae* plants should focus on identifying and validating species-specific reference genes to improve the accuracy of gene expression analysis. Moreover, the development of standardized protocols and guidelines for reference gene validation in *Solanaceae* species would enhance the reproducibility and comparability of gene expression studies in this family of plants. By addressing these challenges and implementing robust reference gene selection strategies, researchers can enhance the reliability and utility of gene expression analysis in *Solanaceae* plants, contributing to a deeper understanding of their biological processes and potential applications in various fields.

9 Conclusion

In conclusion, the literature review underscores the significance of reference genes in ensuring accurate quantification of gene expression levels through quantitative real-time PCR (q RT-PCR). The selection of an appropriate reference gene is a critical step in the normalization process, with a profound impact on the reliability of q RT-PCR results. Several methods and algorithms, such as geNorm, NormFinder, BestKeeper, RefGenes, and genevestigator, have been devised for reference gene selection, each catering to specific experimental systems and presenting unique strengths and limitations. Moreover, the diverse uses of *Solanaceae* plants for various applications highlight the importance of stable reference gene selection to maximize their productivity.

The review also emphasizes the necessity of validating chosen reference genes across a spectrum of experimental conditions and exercising caution in interpreting q RT-PCR data. Existing literature reveals certain gaps, including the need for standardized protocols in reference gene selection and the inclusion of multiple reference genes in certain experimental setups. Overall, this literature review offers a comprehensive outlook on the present state of knowledge concerning reference gene selection for qPCR normalization. It serves as a valuable resource for researchers seeking suitable reference genes for their specific experiments and underscores the significance of continued research in this domain to enhance gene expression analysis in molecular diagnostics and medicine.

References

[1] H. Wan *et al.*, 'Identification of reference genes for reverse transcription quantitative real-time PCR normalization in pepper (Capsicum annuum L.)', *Biochem Biophys Res Commun*, vol. 416, no. 1–2, pp. 24–30, Dec. 2011, doi: 10.1016/j.bbrc.2011.10.105. https://pubmed.ncbi.nlm.nih.gov/22086175/

[2] M. Petriccione, F. Mastrobuoni, L. Zampella, and M. Scortichini, 'Reference gene selection for normalization of RT-qPCR gene expression data from Actinidia deliciosa leaves infected with Pseudomonas syringae pv. Actinidiae', *Sci Rep*, vol. 5, Nov. 2015, doi: 10.1038/srep16961. https://pubmed.ncbi.nlm.nih.gov/26581656/

[3] D. H. Pinheiro and B. D. Siegfried, 'Selection of reference genes for normalization of RT-qPCR data in gene expression studies in Anthonomus eugenii Cano (Coleoptera: Curculionidae)', *Sci Rep*, vol. 10, no. 1, Dec. 2020, doi: 10.1038/s41598-020-61739-z. https://www.nature.com/articles/s41598-020-61739-z

[4] S. Bai *et al.*, 'Selection and Evaluation of Reference Genes for Quantitative Real-Time PCR in Tomato (Solanum lycopersicum L.) Inoculated with Oidium neolycopersici', *Agronomy*, vol. 12, no. 12, Dec. 2022, doi: 10.3390/agronomy12123171. https://www.mdpi.com/2073-4395/12/12/3171

[5] E. A. Mahmoud, S. A. M. Abdelmohsen, and H. O. Elansary, 'Husk Cherry: Nutritional attributes, bioactive compounds and technological applications', *Arabian Journal of Chemistry*, vol. 14, no. 11, Nov. 2021, doi: 10.1016/j.arabjc.2021.103402. https://www.researchgate.net/publication/354124280_Husk_Cherry_Nutritional_Attributes_Bioactive_Compounds_and_Technological_Applications

[6] Li Miao *et al.*, 'Selection of reference genes for quantitative real-time PCR analysis in cucumber (Cucumis sativus L.), pumpkin (Cucurbita moschata Duch.) and cucumber–pumpkin grafted plants', *Peer J*, no. 2019, Apr. 2019. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6475253/

[7] Jing Ye *et al.*, 'Selection of suitable reference genes for qRT-PCR normalisation under different experimental conditions in Eucommia ulmoides Oliv', *Sci Rep*, no. 2018, Oct. 2018. https://pubmed.ncbi.nlm.nih.gov/30301911/

[8] W. Tingquan, 'Stability determination of candidate reference genes in cucumber plants subjected to stresses from Phytophthora melonis', *Journal of Plant Science and Phytopathology*, vol. 3, no. 1, pp. 028–035, Apr. 2019, doi: 10.29328/journal.jpsp.1001028. https://www.researchgate.net/publication/332743811_Stability_determination_of_candidate_reference_genes_in_cucumber_plants_subjected_to_stresses_from_Phytophthora_melonis

[9] L.-H. Heckmann, P. B. Sørensen, P. H. Krogh, and J. G. Sørensen, 'NORMA-Gene: A simple and robust method for qPCR normalization based on target gene data', *BMC Bioinformatics*, vol. 12, no. 1, Dec. 2011, doi: 10.1186/1471-2105-12-250. https://pubmed.ncbi.nlm.nih.gov/21693017/

[10] M. W. Pfaffl and M. Hageleit, 'Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR', 2001. https://link.springer.com/article/10.1023/A:1005658330108

[11] M. Bustamante, J. Jin, O. Casagran, T. Nolan, and J. L. Riechmann, 'Gene expression analysis by quantitative real-time PCR for floral tissues', *Methods in Molecular Biology*, vol. 1110, pp. 363–382, 2014, doi: 10.1007/978-1-4614-9408-9_21. https://pubmed.ncbi.nlm.nih.gov/24395270/

[12] C. Gebhardt, 'The historical role of species from the *Solanaceae* plant family in genetic research', Dec. 01, 2016, *Springer Verlag*. doi: 10.1007/s00122-016-2804-1. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5121179/

[13] M. Afroz *et al.*, 'Ethnobotany and Antimicrobial Peptides From Plants of the *Solanaceae* Family: An Update and Future Prospects', May 07, 2020, *Frontiers Media S.A.* doi: 10.3389/fphar.2020.00565. https://pubmed.ncbi.nlm.nih.gov/32477108/

[14] A. Ghatak *et al.*, 'Proteomics survey of *Solanaceae* family: Current status and challenges ahead', Oct. 03, 2017, *Elsevier B.V.* doi: 10.1016/j.jprot.2017.05.016. https://pubmed.ncbi.nlm.nih.gov/28528990/

[15] K. Chidambaram *et al.*, 'Medicinal Plants of Solanum Species: The Promising Sources of Phyto-Insecticidal Compounds', 2022, *Hindawi Limited*. doi: 10.1155/2022/4952221. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9519333/

[16] D. G. Aseel, S. Sobhy, M. A. Samy, E. Hamdy, S. I. Behiry, and A. Abdelkhalek, 'Comparative Analysis of the Expression Profiles of Pathogenesis-Related Genes in Tomato Systemically Infected with Tobacco Mosaic and Cucumber Mosaic Viruses', *International Journal of Plant Biology*, vol. 14, no. 2, pp. 458–473, Jun. 2023, doi: 10.3390/ijpb14020035. https://www.researchgate.net/publication/370889192_Comparative_Analysis_of_the_Expression_Profiles_of_Pathogenesis-Related_Genes_in_Tomato_Systemically_Infected_with_Tobacco_Mosaic_and_Cucumber_Mosaic_Viruses

[17] P. Fan *et al.*, 'Evolution of a plant gene cluster in *solanaceae* and emergence of metabolic diversity', *Elife*, vol. 9, pp. 1–26, Jul. 2020, doi: 10.7554/eLife.56717. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7386920/

[18] S. Jan *et al.*, 'Unleashed Treasures of *Solanaceae*: Mechanistic Insights into Phytochemicals with Therapeutic Potential for Combatting Human Diseases', Mar. 01, 2024, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/plants13050724. https://www.mdpi.com/2223-7747/13/5/724

[19] L. Zeng *et al.*, 'Development of atropa belladonna L. Plants with high-yield hyoscyamine and without its derivatives using the CRISPR/Cas9 system', *Int J Mol Sci*, vol. 22, no. 4, pp. 1–10, Feb. 2021, doi: 10.3390/ijms22041731. https://pubmed.ncbi.nlm.nih.gov/33572199/

[20] M. Y. Chung *et al.*, 'A tomato (Solanum lycopersicum) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening', *Plant Journal*, vol. 64, no. 6, pp. 936–947, Dec. 2010, doi: 10.1111/j.1365-313X.2010.04384.x. https://pubmed.ncbi.nlm.nih.gov/21143675/

[21] L. M. Bagheri, M. Nasr-Esfahani, V. Abdossi, and D. Naderi, 'Analysis of candidate genes expression associated with defense responses to root and collar rot disease caused by Phytophthora capsici in peppers Capsicum annuum', *Genomics*, vol. 112, no. 3, pp. 2309–2317, May 2020, doi: 10.1016/j.ygeno.2020.01.002. https://pubmed.ncbi.nlm.nih.gov/31923618/

[22] Na Chen, Qin Shao, Qineng Lu, and Xiaopeng Li, 'Research progress on function of NAC transcription factors in tomato (Solanum lycopersicum L.)', *research gate*, Jan. 2023. https://www.researchgate.net/publication/366988732_Research_progress_on_function_of_NAC_transcription_factors_in_tomato_Solanum_lycopersicum_L

[23] X. Zhou, J. Liu, and Y. Zhuang, 'Selection of appropriate reference genes in eggplant for quantitative gene expression studies under different experimental conditions', *Sci Hortic*, vol. 176, pp. 200–207, Sep. 2014, doi: 10.1016/j.scienta.2014.07.010. www.researchgate.net/publication/264937193_Selection_of_appropriate_reference_genes_in_eggplant_for_quantitative_gene_expression_studies_under_different_experimental_conditions

[24] A. M. Algradi, Y. Liu, B. Y. Yang, and H. X. Kuang, 'Review on the genus Brugmansia: Traditional usage, phytochemistry, pharmacology, and toxicity', Oct. 28, 2021, *Elsevier Ireland Ltd*. doi: 10.1016/j.jep.2021.113910. https://pubmed.ncbi.nlm.nih.gov/33571613/

[25] P. Soni, A. A. Siddiqui, J. Dwivedi, and V. Soni, 'Pharmacological properties of Datura stramonium L. as a potential medicinal tree: An overview', *Asian Pac J Trop Biomed*, vol. 2, no. 12, pp. 1002–1008, 2012, doi: 10.1016/S2221-1691(13)60014-3. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3621465/

[26] D. Zhao *et al.*, 'Selection of reference genes for qPCR normalization in buffalobur (Solanum rostratum Dunal)', *Sci Rep*, vol. 9, no. 1, Dec. 2019, doi: 10.1038/s41598-019-43438-6. https://www.nature.com/articles/s41598-019-43438-6

[27] C. I. Ortiz-Ramírez, M. A. Giraldo, C. Ferrándiz, and N. Pabón-Mora, 'Expression and function of the bHLH genes ALCATRAZ and SPATULA in selected *Solanaceae* species', *Plant Journal*, vol. 99, no. 4, pp. 686–702, 2019, doi: 10.1111/tpj.14352. https://pubmed.ncbi.nlm.nih.gov/31009131/

[28] I. A. Chincinska, M. Miklaszewska, and D. Sołtys-Kalina, 'Recent advances and challenges in potato improvement using CRISPR/Cas genome editing', Jan. 01, 2023, *Springer Science and Business Media Deutschland GmbH*. doi: 10.1007/s00425-022-04054-3. https://pubmed.ncbi.nlm.nih.gov/36562862/

[29] J. He *et al.*, 'Establishing Physalis as a *Solanaceae* model system enables genetic reevaluation of the inflated calyx syndrome', *Plant Cell*, vol. 35, no. 1, pp. 351–368, Jan. 2023, doi: 10.1093/plcell/koac305. https://pubmed.ncbi.nlm.nih.gov/36268892/