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Validation of Key Reference Genes in Some Improved Varieties and Landraces of Yams in Ghana

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ABSTRACT

Yam is an important staple starchy crop associated with food security and income generation. There are diverse yam species that respond differently to various manipulations under the aeroponic system. Particularly farmer-preferred varieties (landrace) have a slow growth rate in vitro and as well as when subjected to an aeroponic system. This study investigated how some nine housekeeping genes were regulated in yam accessions. Ribonucleic acid (RNA) was extracted, converted to complimentary Deoxyribonucleic acid (cDNA), and subjected to Real-Time PCR analysis. The results revealed that most reference genes used were upregulated in the improved varieties. The genes β-tubulin, Elongation factor 1-α, Malate dehydrogenase, Ubiquitin, and Tip41-Like Family Protein were upregulated in the landrace which was farmer-preferred. However, 18S Ribosomal RNA and Cycloartenol Synthase Contig7815 consistently showed low expression across all genotypes. This study has provided necessary information vital for selecting a suitable reference gene for yam gene expression analysis for researchers.

Keywords

Aeroponics system, Gene Expression, Quantitative Real-Time Polymerase Chain Reaction, Yam, Reference genes.

Introduction

Yam (*Dioscorea spp*.) belongs to the genus *Dioscorea* and is a member of the family *Dioscoreaceae*. It comprises over 600 species, but only about 10 species are normally cultivated for food [1]. However, the most economically important species cultivated are *D. rotundata* (White Guinea yam), *D. alata* (Water yam), *D. bulbifera* (Aerial yam), *D. esculenta* (Chinese yam), *D. dumentorum* (Bitter yam) and *D. trifida* (Cushcush yam) [2,3]. It is the second most important tuber crop in Africa after cassava [4]. The tuber can be used as food and/or planting material and is a good source of energy derived mostly from its carbohydrates.

Yam production in Ghana decreased from 8,520 kg in 2020 to 8,310 kg in 2021 [5]. The crop is traditionally propagated vegetatively using tubers through methods that are very slow and susceptible to disease because of the recycling of diseased planting material [6]. However, efforts have been made to facilitate the production of high-quality planting materials and high ratio propagation rates for yam. These include miniset technology, vine technology, tissue culture techniques, and aeroponic systems (AS). Aeroponics system and tissue culture rapidly multiply seed yam tubers in a clean and cost-effective manner for high ratio increased seed yam production. However, different genotypes subjected to aeroponics systems respond differently [7]. Improved yam varieties have been reported to respond better in terms of shoot length, easy canopy formation, and higher leaf chlorophyll content when established in the screenhouse than the local accessions [8]. Differences have also been observed in vine rooting, as well as mini-tubers and bulbil production. Reference genes have typically been used to understand the variation in phenotypic expression of genotypes. This study was undertaken to investigate the expression of nine reference genes in 14 improved varieties and landraces in yam. This will aid in ascertaining the level of gene expression in both local and released varieties, and their possible effect on plant growth and development.

Materials and Methods

Study Area and Genetic Material Used

The study was carried out at the CSIR-Crops Research Institute, Molecular Biology Laboratory, Fumesua-Kumasi. Fourteen (14) yam genotypes consisting of four improved varieties, nine landraces, and a check (Table 1) were used and obtained from the CSIR-Crops Research Institute (CRI)-Tissue culture aeroponics facility at Fumesua. Young tender leaves were sampled from the bottom parts of the yam plant, kept on ice immediately after harvesting, and stored at -150°C in a Haier Cryogenic Freezer before extraction.

Table 1: Genetic Materials Used for the Gene Expression.

Improved Varieties	Landraces	Check
Rotun 1	Rotun 5	Rotun 13
Rotun 2	Rotun 6	
Rotun 3	Rotun 7	
Rotun 4	Rotun 8	
	Rotun 9	
	Rotun 10	
	Rotun 12	
	Rotun 11	
	Alata 1	

RNA Extraction and Complimentary DNA Synthesis

Leaf tissue (~100 mg) was frozen in liquid nitrogen in a 2ml Eppendorf tube and crushed using pestle. Total RNA was extracted from each individual sample using NucleoSpin®filter (ThermoScientific Abgene) according to the manufacturer's instructions. The quality of RNA extracted was checked using 1 % agarose gel and the quantity also checked with a Nanodrop 2000 Spectrophotometer (Thermos Scientific, Wilmington, DE 19810 U.S.A). RNA template (1000 ng) was reverse transcripted to cDNA using QuantiTect Reverse Transcription Kit provided by Qiagen. The cDNA (100ng) was diluted to 50 µl using nuclease-free water prior to use in the qRT-PCR assays.

Verification of Amplified Products for qRT-PCR

Nine reference genes were used with each having an annealing temperature (*T*m) of 55 °C, primer lengths of 17 - 24 bp, and amplicon lengths ranging from 100 -224 bp (Table 2). Polymerase Chain Reaction (PCR) was carried out in a total volume of 15 µl, comprising 100 ng cDNA, 2X PCR Biomix Red ™ (Bioline Reagents Ltd), 10 µM forward and reverse primers, and nuclease-free sterile water. The reaction was then spun down. The PCR reactions were carried out in SEEAMP ™ DNA thermal cycler (Seegene, Inc-China). Thermal cycling conditions included an initial denaturation step at 94 °C for 4 min followed by 35 cycles of final denaturation step at 94 °C for 30 sec, primer annealing at 55°C for 1 min, and elongation at 72 °C for 1 min followed by the final step of extension at 72 °C for 10 min. The PCR products were kept at 4 °C prior to electrophoresis and later loaded on 3 % agarose gel using 1 X TAE. A 100 bp ladder (Genesis Biotech Inc.) was loaded alongside PCR products and visualized using UV transilluminator.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Quantitative (q) PCR was conducted using the GenQuant 2X SYBR qPCR Kit (Genesis Biotechnology). It was carried out in a 72-well plate with a Rotor-Gene real-time PCR (Qiagen, Germany). PCR reactions were carried out in a total volume of 10 μl, containing 20 ng cDNA, 2 X RT PCR MasterMix, 10mM primer each (forward and reverse), and nuclease-free water to a final volume of 10 μl. All reactions were performed in duplicates in 72-well reaction plates. The thermal cycling program was as follows: 95 °C for 180 sec, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 sec. The melting curves were analyzed at 60 - 95 °C with

Table 2: List of reference genes used for the study.

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')	Melting temperature (°C)	Amplified product	Reference
Actin	CTCATTGATCGGCATGGAAGC	GGGGAACATAGTTGAACCACCAC	54	137	Zhao *et al*., 2016
Elongation factor 1-α (EFI)	ACTGTTCCTGTTGGTCGTG	TCTGGGAGGGATTCGTG	54	122	Zhao *et al*., 2016
Tip41-Like Family Protein (TIP41)	TGTGCCAAATTCACCAA	CAAAACCACCTCATCATAGA	54	133	Zhao *et al*., 2016
Melate Dehydrogenase (MD)	GCCTTCCACAACCTTCAC	AGAATGGCAGCTCGTTCA	54	224	Zhao *et al*., 2016
Ubiquitin (UB)	GGGCTTTCAAGGTCGTC	TGAAGGGTTTGCTCATCC	54	100	Zhao *et al*., 2016
Beta-Glucuronidase (BG)	GCCGAGCGGATGTAAGA	TGTTGTGAGTTGCCCTGT	54	141	Zhao *et al*., 2016
β-tubulin (TB)	GCTTACTTTCTCCGTGTTCC	TGTCATAAAGAGCCTCATTGTC	54	137	Li, Jinting., *et al*. 2017
18S ribosomal RNA (R18)	CAGAACATCTAAGGGCATCACA	TAGTTGGTGGAGCGATTTGTCT	54	101	Li, Jinting., *et al*. 2017
Cycloartenol Synthase Contig7815 (CSC)	AGATGTTGAGGGAGAAGGCG	GGTCTTCCACCCAACAACAAA	54	158	Li, Jinting., *et al*. 2017

a heating rate of 0.1 °C per sec and finally a cooling step of 4 °C.

Data Collection and Analysis

Delta delta Ct values were obtained from the values generated by the qPCR machine estimated using Excel. The delta delta values were further analyzed using SPSS ver.20 to obtain the expression graph, ANOVA was performed on the delta delta CT value at a 5% significant level using Excel. The actin gene as in Table 2 was used as a calibrator for all the genes; therefore, the result was presented based on the eight genes as shown in Figure 2.

Results and Discussion

Gene Expression in the 14 Yam Genetic Materials Used

The quality of RNA extracted is essential for successful gene expression analysis. RNA purity (A260/A280) of 2.1 was observed for all 14 yam samples with the Nanodrop reading, indicating the absence or minimal presence of protein contaminants. This could also yield reproducible results with low cycle threshold (Ct) values [9].

Primer specificity study was conducted on the nine reference genes (18S ribosomal RNA, *B*-tubulin, actin, elongation factor 1α, Tip41-Like Family Protein, ubiquitin, Malate Dehydrogenase, Beta-Glucuronidase, and Cycloartenol Synthase Contig7815). These genes were tested and checked on 3% agarose gel after PCR analysis. cDNA obtained from selected yam RNA was used as a template for the PCR analysis. The agarose gel electrophoresis results revealed gene amplifying as a single band at their expected size with no primer-dimers or other nonspecific amplified products (Figure 1).

The cycler threshold (Ct) values obtained from this study were between 17.75 (18R: Rotun 7) and 27.13 (TIP41: Rotun 4). The relative expression of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method where Rotun 13 was used as a calibrator and set to 1.

The expression of the nine genes was compared between and within genes across the four improved yam varieties, the nine landraces and the check used. The higher expression means square of 617.12 was recorded within genes while between genes recorded the lower expression means square of 142.09. The probability value (P-value) was less than 0.05, indicating significant differences between the nine genes used. The expression of the eight genes across the four improved yam varieties, the nine landraces, and the check are summarized in Table 3.

Table 3: Analysis of Variance for Gene Expression Across the four improved yam varieties, the nine landraces, and the check.

Source of Variation	Df	SS	MS	Percent variation
Between Genes	8	1136.7	142.09*	18.72
Within Genes genetic material	13	8022.6	617.12*	81.28
Error	104	0.1	0.001	
Total	**125**	**9159.4**	**759.21**	

Most of the reference genes were expressed across the four improved varieties, the nine landraces and the check studied (Figure 2). However, the reference genes were down-expressed with respect to Rotun 13, Rotun 6, Rotun 7, and Rotun 8 compared with the other landraces (Rotun 5, Rotun 9, Rotun 12, Rotun 10, Rotun 8 and Alata 1). Most of the reference genes (β-tubulin, Elongation factor 1-α, Malate Dehydrogenase, Ubiquitin, and Tip41-Like Family Protein) were up-expressed for almost all the improved yam varieties, and some of the landraces (Rotun 5, Rotun 9, Rotun 12, Rotun 10, and Alata 1). All the eight reference genes used were down-expressed for Rotun 6, Rotun 7, and Rotun 8. However, Rotun 8 and Rotun 6 had the lowest expression profiles for the reference genes, while 18S Ribosomal RNA, and Cycloartenol Synthase Contig7815 consistently showed low expression across all the four improved yam varieties, the nine landraces, and the check.

Independent group t-test was designed to compare means of expression levels for each reference gene across the 14 yam genetic materials. The test was against the null hypothesis that the expression levels for the eight reference genes are the same for all 14 yam genetic materials. When reference genes were grouped as between and within genes, the calculated *p*-values for expression of all the eight genes in all the 14 yam genetic materials were less than the alpha level of 0.05. The null hypothesis was rejected in the view that differences in means were significant across and within the 14 yam genetic materials based on the expression of the eight reference genes (Table 3). Gene expression in Rotun 10, Rotun 11, and Rotun 9 were relatively favorable. β-tubulin, Tip41-Like Family Protein, and Ubiquitin, Elongation factor 1-α generally responded favorably to the study conditions. β-tubulin plays a key role in cell division, influencing mitosis and cell expansion in general [10]. Rotun 10, Rotun 11, and Rotun 1 over-

Figure 1: Cycloartenol Synthase Contig7815 gene producing a single band at 138bp on 1.5% agarose gel.

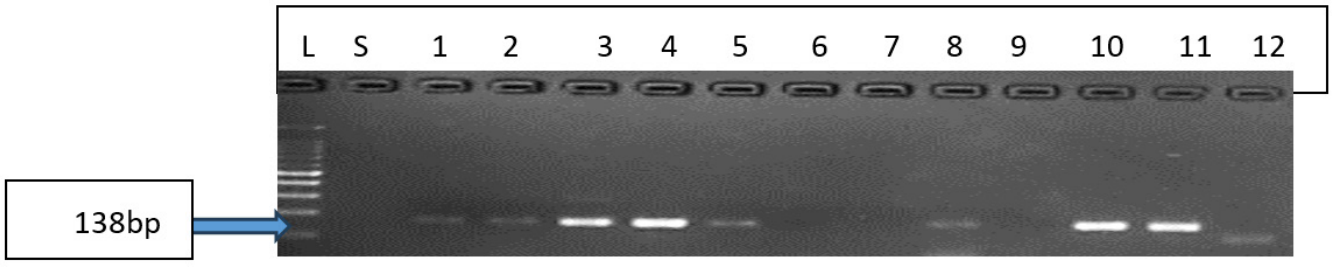
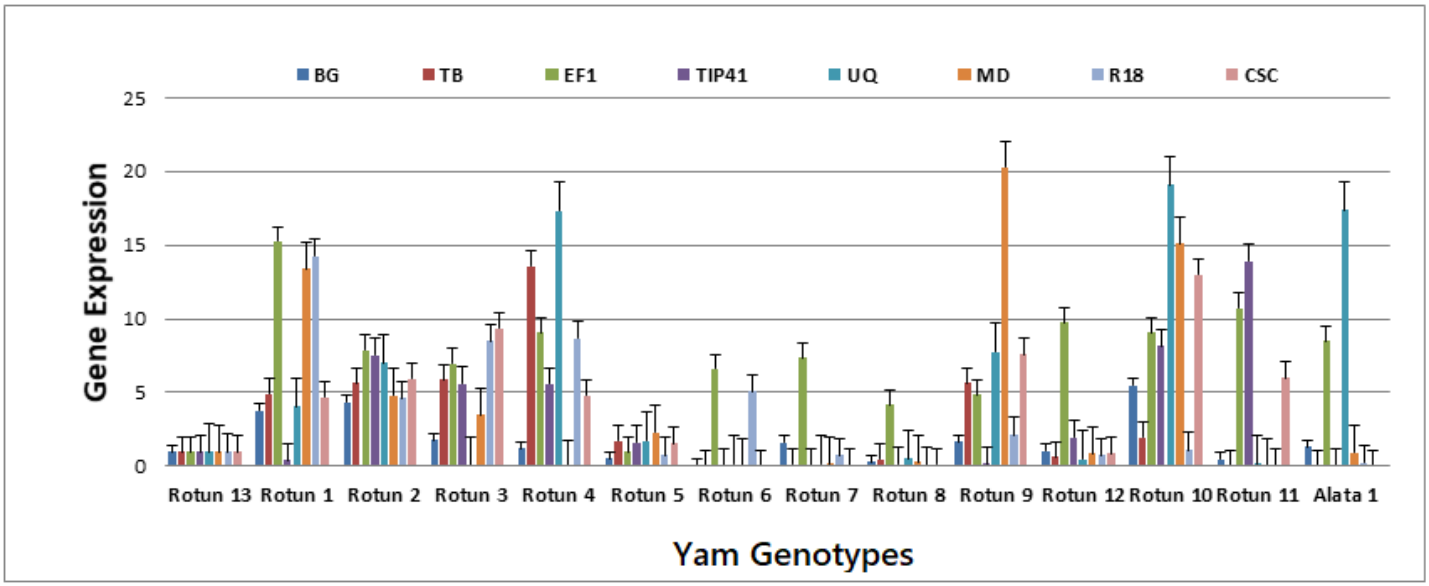


Figure 2: Expression levels of genes as shown in matching colours for each yam genetic material in the order of Beta-Glucuronidase, Beta-tubulin, Elongation factor 1-α (EF1), Tip41-Like Family Protein, Ubiquitin, Malate dehydrogenase, 18S ribosomal RNA, Cycloartenol Synthase Contig7815.



expressed Tip41-Like Family Protein, and will be more tolerant to ABA treatment. This will facilitate their growth and yield of tubers in yam production [11]. According to Belknap et al. [12], Ubiquitin is involved in plant senescence and responses to stress. This corroborates with the favorable responses to stress observed under the aeroponics setup. Rotun 9 recorded the highest level of expression for Melate dehydrogenase indicating its tolerance to the plant environmental stress to promote growth [13].

The 18S ribosomal, and Cycloartenol Synthase Contig7815 genes stimulated the genotypes poorly in this study. It is possible that the poor expression of these genes can be associated with their negative impact on the micro tuber formation, synthesis of Cycloartenol, photosynthesis, and synthesis of protein [14-16]. β-tubulin gene, Malate dehydrogenase, Ubiquitin, Elongation factor 1- alpha, and Tip41-like family protein impact the most significant differences in expression among Alata 1, Rotun 1, Rotun 10, Rotun 9 and Rotun 4. These results agree with the findings of [15] who indicated fluctuations in expression levels relative to a specific reference gene based on the genetic material. Housekeeping genes also known as reference genes are characteristically constitutively expressed genes that are essential for the maintenance of essential cellular performance and are expressed in all cells of an organism under normal and pathophysiological conditions [17]. Though some housekeeping genes are expressed at comparatively constant rates in most non-pathological conditions, the expression of other reference genes may vary depending on experimental conditions. In this study, Rotun 13 which was used to normalize results performed best in the aeroponics system in terms of the survival rate, leaf count, and chlorophyll content and also corroborates the findings of [18] on yam accessions generated from aeroponics systems. However, gene expression in the four improved yam varieties and the nine landraces varied. For instance, landraces Rotun 10, Rotun 11, and Rotun 9 had high reads for β-tubulin, Elongation factor 1-α, and Melate dehydrogenase. It could be deduced that under the aeroponics system, these genotypes were stressed and thus expressed these genes in response to their prevailing conditions.

Conclusions and Recommendations

Most of the nine reference genes used were able to express across the four improved yam varieties and the nine landraces studied. In relating gene expression to plant growth and development, the aeroponic system has to be manipulated for enhanced growth of the landrace. β-tubulin gene, Malate dehydrogenase, Elongation factor 1- alpha, and Tip41-Like Family protein varied significantly in their expression levels in specific genetic materials used and should be studied further to aid in manipulating aeroponic systems for improved growth and development of yam varieties. These will ensure clean seed yams are available in the required quantities for farmers. It is therefore, recommended that reference genes Tip41-Like Family Protein, Ubiquitin, Malate dehydrogenase, and Elongation factor 1-α were stable for the study and hence, are recommended for further gene expression studies in yam.

Reference

1. Asiedu R, Sartie A. Crops that feed the World 1. Yams. Food Sec. 2010; 2: 305-315.
2. Okorley EL, Addai EO. Factors influencing the accessibility of Yam planting materials in the Techiman District of Ghana. Ghana Journal of Development Studies. 2010; 7.
3. Weladji RB, Tchamba MN. Conflict between people and protected areas within the Bénoué Wildlife Conservation Area, North Cameroon. Oryx. 2003; 37: 72-79.
4. Bressan EA, Briner Neto T, Zucchi MI, et al. Genetic structure and diversity in the Dioscorea cayenensis/D. rotundata complex revealed by morphological and isozyme markers. Genet Mol Res. 2014; 13: 425-437.
5. Faostat FAO. Crops. Food and Agriculture Organisation of the United Nations. Available online at Google. 2022; 6: 2023.

6. Hughes JdA, Dongo L, Atiri G. Viruses infecting cultivated yams (Dioscorea alata and D. rotundata) in Nigeria. Phytopathatol. 1997; 87.
7. Balogun, Morufat, Norbert Maroya, et al. Novelty, rapidity, and quality in seed yam production: the case of Temporary Immersion Bioreactors. YIIFSWA Working Paper Series No. 6. Yam Improvement for Income and Food Security in West Africa, International Institute of Tropical Agriculture, Ibadan, Nigeria. 2014; 10.
8. Arthur M, Quain MD, Pukinka D, et al. Influence of Aeroponics, Vermiculite and Top Soil on Growth of Tissue Culture Plantlets for Seed Yam Seedling Production. Asian Journal of Agricultural Research, 2023; 17: 16-24.
9. Podevin N, Krauss A, Henry I, et al. Selection and validation of reference genes for quantitative RT-PCR expression studies of the non-model crop Musa. Mol Breed. 2012; 30: 1237-1252.
10. Li J, Han X, Wang C, et al. Validation of suitable reference genes for RT-qPCR data in Achyranthes bidentata Blume under different experimental conditions. Frontiers in Plant Science. 2017; 8: 776.
11. Punzo P, Ruggiero A, Grillo S, et al. TIP41 network analysis and mutant phenotypes predict interactions between the TOR and ABA pathways. Plant Signal Behav. 2018; 13: e1537698.
12. Belknap WR, Garbarino J.E. The role of ubiquitin in plant senescence and stress responses. Trends in Plant Science. 1996; 1: 331-335.
13. Lin HS. Genetic diversity in the foxtail millet (Setaria italica) germplasm as determined by agronomic traits and microsatellite markers. Australian Journal of Crop Science. 2012; 6: 342-349.
14. Rahikainen M, Pascual J, Alegre S, et al. PP2A Phosphatase as a Regulator of ROS Signaling in Plants. Antioxidants. 2016; 5: 8.
15. Zhao X, Zhang X, Guo X, et al. Identification and validation of reference genes for qRT-PCR studies of gene expression in Dioscorea opposita. Biomed Res Int. 2016; 3089584.
16. Hayashi R. Gene Expression and the Impact of an Antioxidant Supplement in the Cataractous Lens. In Handbook of Nutrition, Diet, and the Eye. Academic Press. 2019; 551-568.
17. Zhu J, He F, Hu S, et al. On the nature of human housekeeping genes. Trends Genet. 2008; 24: 481-484.
18. Akom M, Ennin SA, Osei K, et al. "Field Performance of Seed Yam (Dioscorea rotundata Poir) Derived from Tissue Culture and Aeroponics Seedlings". EC Agriculture. 2020; 6: 67-75.