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Validation of a reference gene for transcript analysis in cassava (*Manihot esculenta* Crantz) and its application in analysis of linamarase and α-hydroxynitrile lyase expression at different growth stages

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Relative real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a well-established method for the precise quantification of gene expression. For accurate relative real-time RT-qPCR analysis, validation of the expression of an appropriate reference gene is required. In this study, the expression of six commonly used reference genes, namely 40S ribosomal protein (40S), actin (ACT), cyclophilin C (CYCC), EF-1 alpha (EF1), TATA box binding protein (TBP) and polyubiquitin (UBI) was investigated in leaf and root samples of cassava obtained at 6, 9 and 12 months after planting (MAP). A transcript stability analysis was undertaken in two different varieties of cassava, namely Huay Bong 60 which has high cyanogenic potential (CN) and Hanatee which has low CN. The results reveal that TBP was the most stable reference gene for expression studies. This information was applied to an analysis of linamarase and α-hydroxynitrile lyase gene expression in samples from six low and six high CN cassava plants collected at 6, 9 and 12 MAP. The results indicate that at 6 MAP, the linamarase transcript from leaf of the high CN group was significantly increased, and the α-hydroxynitrile lyase transcript was significantly increased at 12 MAP.

Key words: Cassava, housekeeping gene, hydroxynitrile lyase, linamarase, real-time polymerase chain reaction (PCR).

INTRODUCTION

Accurate quantification of gene expression is important in a number of experimental situations, such as in determining the alteration in expression levels occurring at a defined biological stages and in detecting the response of genes to various stimuli (Fraga et al., 2008). Over the past decade, real-time reverse transcription quantitative PCR (RT-qPCR) has become the choice for high-throughput accurate and reproducible quantitation

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of gene expression (Vandesompele et al., 2002). Quantification of expression in RT-qPCR experiments is generally undertaken as either an absolute or as a relative quantification. An absolute quantification entails the determination of the copy number of the transcript of interest based on a calibration curve. Using a calibration curve requires the time consuming generation of a stable and reliable calibration standard which must be precisely quantified (Pfaffl, 2001). However, in most experiments, determining the absolute transcript copy number is not essential, and determining the relative changes in gene expression or a relative quantification is sufficient (Livak and Schmittgen, 2001).

For relative quantification by real-time RT-qPCR, a suitable reference gene transcript is required to normalize the target gene transcript (Pfaffl, 2001). Theoretically, the reference transcript should allow the effective normalization of different amounts of starting material (Radonić et al., 2004) and the expression levels of reference genes should be constant in all samples under investigation and under all experimental conditions evaluated (Pfaffl, 2001; Radonić et al., 2004). While there are several commonly used reference transcripts that have been reported, it is generally also accepted that no single transcript is constantly expressed in all tissues and under all experimental conditions, implying that the reference transcript should be carefully validated before each experiment for accurate quantification (Pfaffl, 2001; Radonić et al., 2004).

Cassava (*Manihot esculenta* Crantz) is an economically important human and animal food source and industrial feedstock and world cassava production is expected to increase in order to supply the industrial demand (Food and Agriculture Organization, 2012). However, the ability of cassava to produce the toxic component, hydrogen cyanide (HCN), is of significant concern. The cyanogenesis pathway has been well characterized and CN production starts when vacuoles are ruptured, releasing linamarin which is hydrolyzed by the enzyme linamarase (LNM), also known as beta-D-glucosidase (McMahon et al., 1995). The deglycosylated product, acetone cyanohydrin is then further enzymatically broken down by α-hydroxynitrile lyase (HNL) or can spontaneously decompose at pH > 5.0 to produce acetone and HCN (White et al., 1994).

The recent release of draft cassava genome sequence in “Phytozome” makes it easier to design sequences to validate reference genes and to analyze the expression of genes of interest. Although, the expression study of LNM and HNL have been identified, but the gene expression study have been done only with 4 month old plants in *in vitro* conditions (Echeverry-Solarte et al., 2013).

Since the stability of reference gene for expression study of genes affecting cyanogenic potential (CN) has not been identified elsewhere, this research therefore sought to validate potential reference transcripts across growing stages and tissues of two varieties of cassava with different endogenous CN in order to provide a transcript suitable for the accurate normalization of real-time quantitative RT-PCR data. The study utilized the most stable transcript to investigate the expression of LNM and HNL genes at 3 growing stages of cassava *in vivo*. Both root and leaf tissues were used and six plants each of low and high CN were analyzed, in order to understand more about transcription pattern at each stage and tissue systematically.

MATERIALS AND METHODS

Plant sample collection

Cassava varieties Huay Bong 60 (high CN), Hanatee (low CN), and five low and five high CN plants from a previously described F1 mapping population (Whankaew et al., 2011) were grown at the Rayong Field Crops Research Center, Department of Agriculture, Thailand. Cassava leaves and roots were collected at 6, 9, and 12 month after planting (MAP), then snap frozen in liquid nitrogen until RNA isolation. The CN in all root samples was evaluated at 6, 9 and 12 MAP using the picrate paper kit method (Bradbury et al., 1999).

RNA isolation and cDNA synthesis

Frozen tissue samples were ground into fine powder using liquid nitrogen. Fruit-mate™ for RNA purification (Takara, Japan) was applied to remove polysaccharides and polyphenols. Total RNA was then extracted using TriReagent® (Molecular Research Center, USA) according to the supplier’s instruction. The RNA was treated with DNA free kit (Ambion, USA) to remove DNA contamination. The concentration of each RNA sample was measured by a NanoDrop 1000 Spectrophotometer (Thermoscientific, USA). First-strand cDNA was synthesized from 1 µg total RNA using ImProm-II Reverse Transcription System (Promega, USA), according to the manufacturer’s instructions.

Primer design and amplification efficiency

Six reference genes including 40S ribosomal protein (40S), actin (ACT), cyclophilin C (CYCC), EF-1 alpha (EF1), TATA box binding protein (TBP) and polyubiquitin (UBI) were chosen from the cassava genome database (www.phytozome.net). Specific primers were designed using the PrimerQuest software (http://sg.idtdna.com/primerquest/Home/Index). The sequences of the target genes, LNM and HNL, sourced from previous publications (Hughes et al., 1992, 1994) were also used for primer design. The criterion for amplified products ranged from 100 to 300 bp with a Tm of 60 ± 5°C. Hairpin, self-dimer and primer dimer interactions were investigated using Oligo Analyzer (https://sg.idtdna.com/analyzer/Applications/OligoAnalyzer/).

Each primer was assessed for efficiency of amplification in series of 2 fold-serially diluted Hanatee cDNA with 3 technical replicates. Real-time PCR was carried out in 20 µl final volume containing 1 µl of 1:2 cDNA template, with final concentration of 0.2 µM of each primer and 1X of KAPA master mix (KAPA SYBR® FAST qPCR Kit, KAPA Biosystems, MA, USA). The conditions for real-time PCR was set as recommended by the manufacturer, with an initial 95°C

for 3 min, followed by a total of 40 cycles of a denaturation step at 95°C for 5 s and an annealing temperature of 60°C for 30 s. If the amplification was not efficient, 3 step cycling was tested by using an annealing temperature of 55°C for 20 s, followed by a 1 s extension at 72°C. Specific amplification was assessed by melting curve analysis. The primer efficiency (*E*) was calculated from $\%E = (10^{(-1/slope)}-1) \times 100$, according to the MIQE guidelines for qRT-PCR (Bustin et al., 2009). The efficiency of all primer between 90-105% was considered for the next step.

Determination of reference genes stability

Real-time PCR amplification of six reference gene transcripts (40S, ACT, CYCC, EF1, TBP and UBI) was evaluated using cDNA of Huay Bong 60 and Hanatee from both leaf and root samples obtained at 6, 9 and 12 MAP for three technical replicates. The most stable reference gene transcript was established using the NormFinder program (Andersen et al., 2004).

Quantification of LNM and HNL expression

LNM and HNL were chosen as genes of interest. Real-time PCR of root and leaf samples from parental Huay Bong 60 and Hanatee and five plants with low CN and five with high CN were analyzed at 6, 9 and 12 MAP with technical duplicates. The ΔCT method using the best reference gene was used for relative quantification. The expression levels of the low and high CN groups was compared using PASW Statistics 18 (SPSS Inc., 2009).

RESULTS AND DISCUSSION

Validation of reference gene transcripts

The reference gene names, primer sequences and amplicon length are given in Table 1. The efficiency of amplification of all primer pairs ranged from 95-105% and r^2>0.99, which is in accordance with established criteria for real-time RT-PCR (Bio-Rad Laboratories, 2006; Dhami and Kumarasinghe, 2014; Ma et al., 2013). In order to determine the most stable reference genes, expression levels of transcripts from these six reference genes were determined in Huay Bong 60 and Hanatee, which are significantly different in CN (Whankaew et al., 2011) at 6, 9 and 12 MAP. Stability of the reference genes according to the NormFinder software are shown in Figure 1. NormFinder calculates stability values based on variance estimations for identifying suitable normalizing genes amongst a set of candidates, with lower stability value indicating higher expression stability (Andersen et al., 2004). From the results, TBP was the most stable reference gene transcript evaluated with a stability value of 0.01 across samples. Similarly, TBP has been reported to be the most stable reference gene in several systems (Fujii et al., 2013; Nakao et al., 2015; Tratwal et al., 2014). TBP is not only the appropriate reference gene for a study of cyanogenic potential, but it can also be applied in expression studies of other traits that show differences between Huay Bong 60 and Hanatee varieties, such as starch content, yield, first branch height, etc. However, it should be noted that no reference gene is appropriate to all tissues and under all experimental conditions, and therefore the stability evaluation should be carefully validated for other organisms or different experiments for accurate quantification (Pfaffl, 2001; Radonić et al., 2004). In this study, however TBP was used to accurately determine the relative expression levels of LNM and HNL.

Expression profile of linamarase (LNM) and α-hydroxynitrile lyase (HNL)

LNM and HNL are two major genes involved in the hydrogen cyanide liberating cyanogenesis pathway (McMahon et al., 1995). The expression profiles of these genes were analyzed in six plants each of low and high CN, consisting of the parental Huay Bong 60 and Hanatee and an additional five plants with low CN and five with high CN from a previously described F1 mapping population (Whankaew et al., 2011). Expression of LNM and HNL was extremely low in root samples, with correspondingly high Ct values, and so expression analysis is only presented for leaf samples. Low expression of LMN and HNL in root samples has been previously reported by others (Santana et al., 2002; White et al., 1998).

The CN assessed in root samples and the normalized expression profiles of LMN and HNL in leaf are shown in Figure 2, and the results of statistical comparison between groups are shown in Table 2. There was no difference in the levels of CN in the low CN plants over the three time points, while the high CN plants showed significantly higher CN levels at all time points as compared to the low CN plants as well as showing significant increase in CN levels at 9 and 12 MAP as compared to 6 MAP (Table 2). LMN expression did not vary significantly in the low CN group over the three time points, and the high CN plants expression was significantly increased as compared to all other samples only at 6 MAP (Table 2). HNL expression was significantly increased at 12 MAP in both the low and high CN plants as compared to the earlier time points and at 12 MAP, HNL expression was significantly increased in the high CN plants as compared to the same time point in the low CN plants. The significantly high level of expression of the genes LNM and HNL in the cyanogenesis pathway corresponded to the high levels of CN.

Conclusion

For reliable and accurate relative quantification by real

Table 1. Primer sequences of reference genes and target genes.

Symbol	Full name	Forward primer (5' -> 3')	Reward primer (5' -> 3')	Annealing temperature (°C)	Product size (bp)
40S	40S ribosomal protein	CCTGCTGAAATTGTTGGGAAGCGT	AACACAACATCTTTGCCCGAGAGC	60	155
ACT	Actin	TCCAATTGACCGCGGCTACCTTAT	ACGCTGGATTGAAGGGAGAGTGAA	60	145
CYCC	Cyclophilin C	AGTTAGGGCTCCAAGCAGGAACAA	AACCACGCCCTCTTCTGATACGTT	60	149
EF1	EF-1 alpha	TGGTACAAGGGCCCAACTCTTCTT	TCACAACCATTCCAGGCTTCAGGA	60	172
TBP	TATA box binding protein	ATGGCAGATCAAGGAGGCTTGGAA	GCAGCGAAACGCTTAGGGTTGTAT	55 and 60	179
UBI	Polyubiquitin	AGGAGGGCCACCACTGACAAATAA	TTCCATCTAGACGCTTCCCTGCAA	60	152
HNL	α-hydroxynitrile lyase	CTGGGCTTCGTACTTCTGAG	TGAACTTCGGTCTCTGAGCC	60	127
LNM	Linamarase	GGAGACTCAGCCACAGAACC	GCACATCAACTTTACTGTCGG	55	169

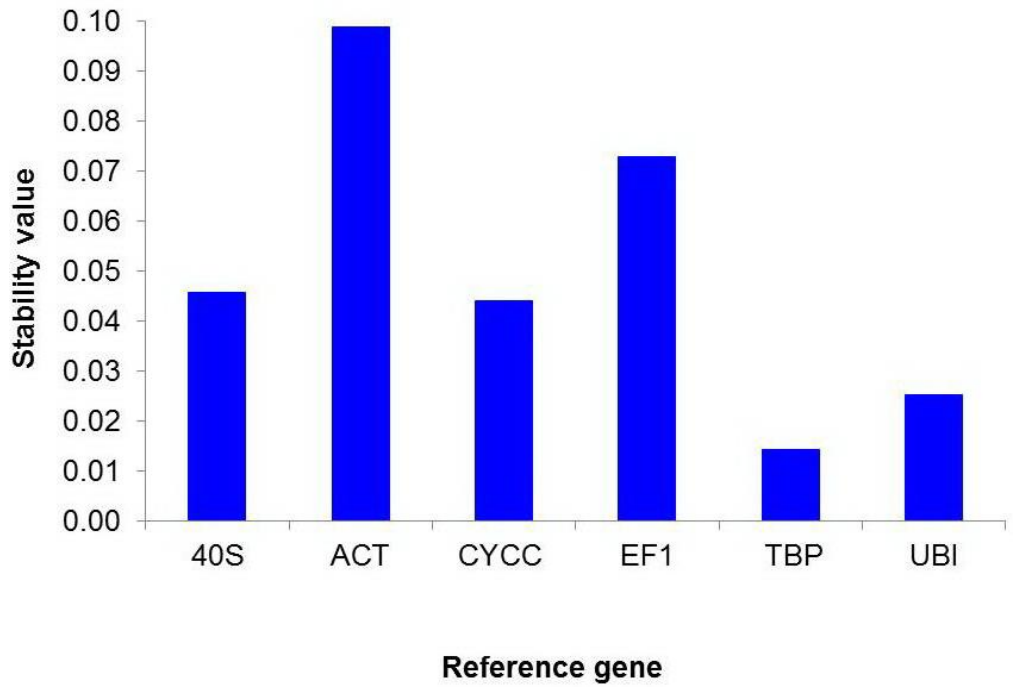

Figure 1. Stability of 6 reference genes for cyanogenic potential study for leaf and root at 6, 9, 12 MAP calculated using NormFinder. A lower stability value corresponds to a higher stability of transcript expression.

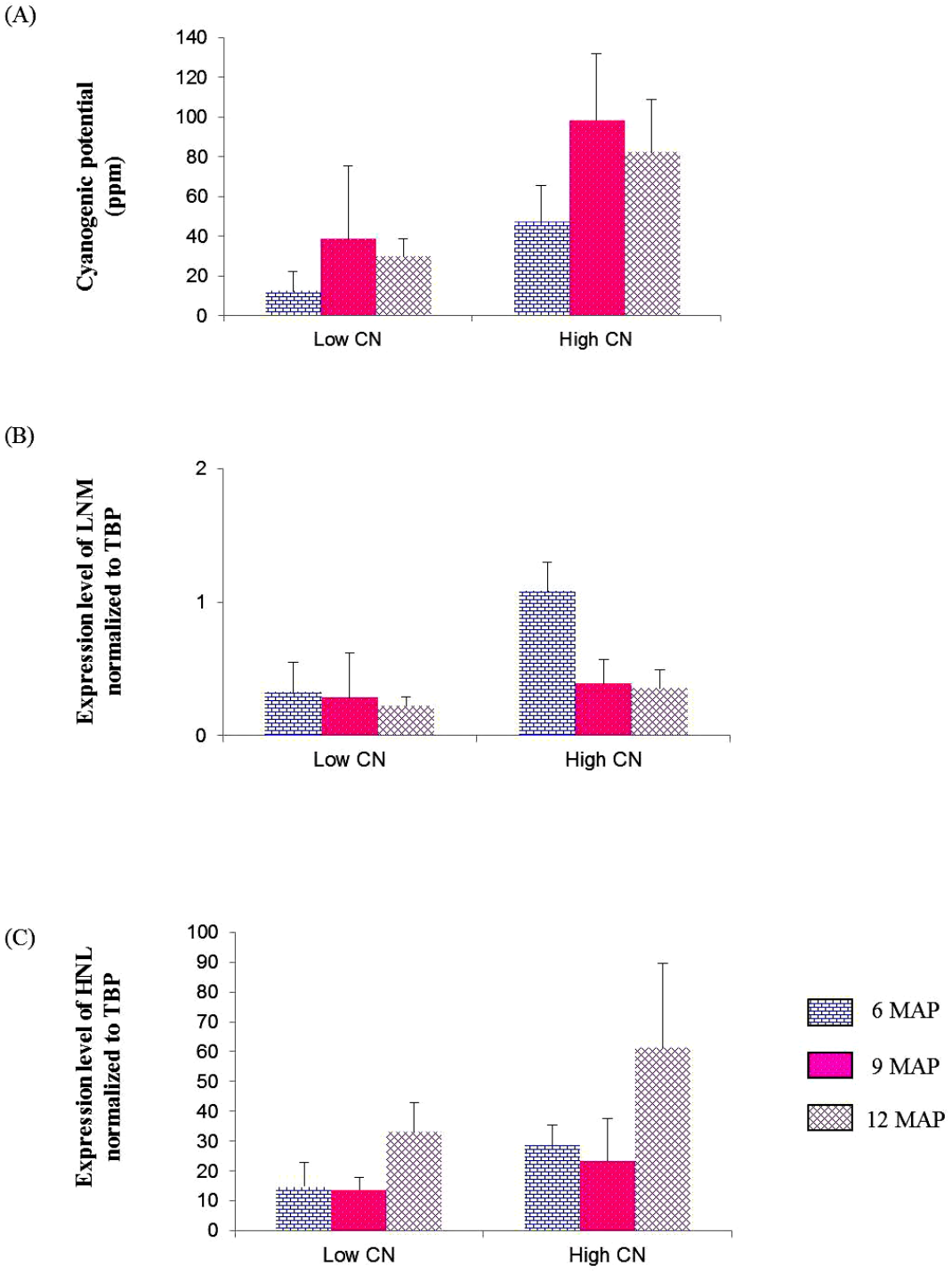

Figure 2. Expression level of LNM and HNL in cassava leaf at 6, 9 and 12 MAP. Cyanogenic potential, LNM transcript and HNL transcript of low and high groups are shown in panels A, B and C, respectively. Mean values + standard deviation are represented.

time PCR, validation of reference gene transcripts is an important first step. From the evaluation of two cassava varieties that have phenotypic differences in CN levels at 6, 9 and 12 MAP in both leaf and root, TBP was found to be the most stable gene transcript amongst the six gene transcripts evaluated. Utilizing expression of TBP to

Table 2. Tabulation of tests of significance for levels of cyanogenic potential (CN) and normalized expression of linamarase (LNM) and α-hydroxynitrile lyase (HNL). Samples were taken from cassava leaf and root at 6, 9 and 12 months after planting (MAP) of low and high CN groups. CN data is from root samples, while expression data is from leaf samples.

Parameter			Low CN group			High CN group		
			6 MAP	9 MAP	12 MAP	6 MAP	9 MAP	12 MAP
Cyanogenic potential (ppm)	Low CN group	6 MAP	-					
		9 MAP	0.072	-				
		12 MAP	0.221	0.543	-			
	High CN group	6 MAP	0.019*	0.548	0.231	-		
		9 MAP	0.000**	0.000**	0.000**	0.001**	-	
		12 MAP	0.000**	0.005**	0.001**	0.020*	0.269	-
Expression level of LNM	Low CN group	6 MAP	-					
		9 MAP	0.776	-				
		12 MAP	0.429	0.619	-			
	High CN group	6 MAP	0.000**	0.000**	0.000**	-		
		9 MAP	0.651	0.473	0.230	0.000**	-	
		12 MAP	0.806	0.596	0.298	0.000**	0.825	-
Expression level of HNL	Low CN group	6 MAP	-					
		9 MAP	0.905	-				
		12 MAP	0.046*	0.035*	-			
	High CN group	6 MAP	0.126	0.100	0.594	-		
		9 MAP	0.335	0.278	0.254	0.536	-	
		12 MAP	0.000**	0.000**	0.002**	0.001**	0.000**	-

normalize gene expression levels of LNM and HNL showed that at an early stage of post planting (6 MAP), leaves of the high CN group expressed higher levels of LNM than leaves in the low CN group, whereas HNL expression was significantly increase at the latest stage examined (12 MAP) in leaves of both low and high CN plants, although expression of HNL was significantly higher in the high CN plants than the low CH plants at 12 MAP. The results of this study will have future application on the analysis of gene expression of other important traits in cassava.

Conflict of interest

The authors declared they have no conflict of interest.

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