Menara Perkebunan 2015 83(2), 86-94

Evaluation of eleven reference genes for Reverse Transcriptase Quantitative PCR of rubber tree under water deficit

Evaluasi sebelas gen referensi untuk Reverse Transcriptase Quantitative PCR *pada tanaman karet tercekam kekeringan*

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Diterima tanggal 21 September 2015/disetujui tanggal 11 November 2015

Abstrak

Reverse Transcriptase Quantitative PCR *(RT-qPCR) merupakan teknik yang sangat ampuh untuk mendeteksi jumlah mRNA yang rendah dalam sel tanaman. Pengukuran akumulasi transkrip tersebut relatif terhadap kontrol ekspresi seperti gen-gen* housekeeping. *Keandalan teknik RT-qPCR bergantung pada pemilihan kontrol internal yang disebut pula gen referensi. Hal tersebut menjadi alasan kenapa validasi gen referensi disarankan untuk setiap set sampel cDNAs yang akan digunakan pada eksperimen RT-qPCR baru. Penelitian ini bertujuan untuk menganalisis stabilitas sebelas gen-gen* housekeeping *terpilih pada tiga organ* Hevea brasiliensis *(daun, kulit batang dan akar) tercekam kekeringan moderat selama 15 hari. RNA total diisolasi dari 18 sampel yang terdiri dari tanaman kontrol dan tercekam kekeringan pada hari ke-0 (D0), ke-5 (D5) dan ke-15 (D15). Kualitas cDNA yang disintesis divalidasi dengan amplifikasi PCR menggunakan primer* HbActin. *Kesebelas pasangan primer penyandi gen-gen* housekeeping *pada* Hevea (HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbYLS8, HbRH2b, HbRH8, HbUBC2a, HbαTub, Hb40S *dan* HbUBI) *divalidasi dengan amplifikasi PCR. Nilai* Crossing-point (Cp) *yang diukur dengan metode derivatif kedua pasca analisis RT-qPCR mengungkapkan nilai rerata Cp yang lebih tinggi secara signifikan untuk kesebelas gen* housekeeping *pada titik sampling D5 dibanding D0 dan D15. Studi ini menyarankan bahwa metode perhitungan koefisien keragaman (CV) sederhana dapat digunakan untuk menentukan peringkat gen referensi pada tanaman karet berdasarkan ekspresinya yang stabil. Lima gen* housekeeping (HbRH2b, HbRH8, HbUBC4, HbαTUB *dan* HbActin) *dapat digunakan sebagai gen referensi untuk analisis RT-qPCR pada* Hevea brasiliensis *yang tercekam kekeringan moderat. Gen* HbRH2b *memiliki ekspresi paling stabil dibanding yang lain.*

[Kata kunci : Hevea brasiliensis, *Reverse Transcriptase Quantitative PCR, akumulasi transkrip, kekeringan, kontrol internal].*

Abstract

Reverse Transcriptase Quantitative PCR (RT-qPCR) is a powerful technique in order to detect low abundance of mRNA in the plant cell. The measurement of transcript abundance is relative to the control of expression such as housekeeping genes. Therefore, the reliability of RT-qPCR depends essentially to the choice of these internal controls also called reference genes. That is the reason why a prior validation of reference genes is suggested for every set of cDNA samples used in a new RT-qPCR experiment. This study aimed to analyze the stability of eleven selected housekeeping genes in three *Hevea brasiliensis* tissues (leaf, bark and root) under15 days of moderate water deficit. Total RNA was isolated from 18 samples consisting of control and stressed-plants collected at day-0 (D0), day-5 (D5) and day-15 (D15).The quality of cDNA synthesized was examined by PCR using *HbActin* primer. The eleventh primers encoding *Hevea* housekeeping genes (*HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbYLS8, HbRH2b, HbRH8, HbUBC2a, HbαTub, Hb40S* and *HbUBI*) were validated using PCR amplification. The Crossing-point (Cp) values were measured using a second derivative method after RT-qPCR analysis revealing a significantly higher Cp mean values for 11 housekeeping genes at D5 compared to D0 and D15 sampling points. This study suggests that a simple coefficient of variation (CV) method can be used to rank *Hevea* reference genes based on its stable expression. Five housekeeping genes (*HbRH2b, HbRH8, HbUBC4, HbαTUB* and *HbActin*) can be used for RT-qPCR analysis in *Hevea brasiliensis* under moderate water deficit. The *HbRH2b* gene was the most stable among others.

[Keywords: *Hevea brasiliensis*, Reverse Transcriptase Quantitative PCR, transcript accumulation, drought, internal control].

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Introduction

Gene expression analysis has been widely used to explore biological processes related to plant developmental and environmental cues. The accumulation of messenger RNA (mRNA) is measured using a rapid, sensitive and reliable method. Currently, a fluorescence based-PCR such as Reverse Transcriptase Quantitative PCR (RT-qPCR) is a powerful technique in order to detect low abundance of mRNA in the cell (Exner, 2010; Rebouças *et al.*, 2013). The precise result expected through this method depends on several factors including the quantity and quality of samples collected, RNA, cDNA and the specificity of used primers (Udvardi *et al.*, 2008).

In most cases, the measurement of transcript abundance is relative to the control of expression such as housekeeping genes (18S rRNA, glyceraldehydes-3-phosphate (GADPH), elongation factor-1a (EF-1a), polyubiquitin (UBI), actin (Act), tubulin (TUB), etc) (Mitter *et al.*, 2009). Therefore, the reliability of RT-qPCR depends essentially to the choice of these internal controls which are called reference genes after validation against several criteria (Derveaux *et al.*, 2010). However, housekeeping or reference genes have never been identified to be stable across different tissue types or under different experimental conditions (Kozera & Rapacz, 2013). That is the reason why a prior validation of reference genes is suggested for every set of cDNAs used in a new RT-qPCR experiment (Li *et al.*, 2011). Furthermore, many RT-qPCR analyses were performed on multiple internal control genes for an accurate normalization of expression values using statistical algorithms such as geNorm and NormFinder (Vandesompele *et al.*, 2002; Andersen *et al.*, 2004). Both of these algorithms calculate the expression stability of multiple reference genes under different experimental conditions. As an alternative, the use of the coefficient of variation (CV) proposed by Hellemans *et al.* (2007) can be considered as a simple method to obtain indication of reference gene stability.

Hevea brasiliensis is the sole commercial source of natural rubber. The rubber biosynthetic pathway occurs in specialized latex cells. Latex, a cytoplasmic component of laticifers, is a colloidal suspension that contains 30 to 50% dry matter, of which 90% is rubber (Chrestin *et al.*, 1997). Latex is expelled after bark tapping (cutting of soft bark). For certain rubber clones with a low latex metabolism, application of ethephon to the bark stimulates latex flow and latex regeneration between two tappings (d'Auzac *et al.*, 1997). Tolerance of this harvesting system allows rubber tree to produce daily latex production. However, genetic aspects and environment adaptations (abiotic and biotic stress, soil compaction, etc.) are equally an important factor for sustainable natural rubber production (Jacob, 2005).

In past few years, gene expression studies using RT-qPCR rather than other techniques have been preferably carried out in rubber tree (Li *et al.*, 2011). These studies aimed to differentiate gene expression in order to understand physiological mechanisms and responses underlying the latex production during biotic and abiotic stress (Putranto *et al.*, 2015b; Putranto *et al.*, 2015a; Putranto *et al.*, 2014; Piyatrakul *et al.*, 2014; Putranto *et al.*, 2012; Duan *et al.*, 2010; Qin *et al.*, 2014; Aoki *et al*,. 2014; Xiao *et al.*, 2014; Pirrello *et al.*, 2014; Gébelin *et al.*, 2013). Study of *Hevea brasiliensis* reference genes has been initiated by Li *et al.* (2011) revealing a set of internal control usable for RT-qPCR in genotype and environmental aspects. A more specific study of the reference gene stability during latex regeneration has been carried out by Long *et al.* (2015). Even though these results were fully useful, both of these studies were focused on latex as samples undergoing anthro-pogenic harvesting stress. Information about the expression of reference gene at different tissue is not yet elucidated. Furthermore, the stability of reference genes has not yet been characterized during water deficit in *Hevea brasiliensis*.

This work is complementary to previous studies done for reference gene stability prior to RT-qPCR analysis in *Hevea brasiliensis* by Li *et al.* (2011) and Long *et al.* (2015). This study aimed to analyze the internal control stability of eleven selected housekeeping genes during 15 days of moderate water deficit in three *Hevea brasiliensis* tissues (leaf, bark and root). Firstly, the quality and quantity of total RNA was compared between control and stressed-plants. Secondly, cDNA synthesis was validated by PCR amplification using primer pair encoding *HbActin*. Thirdly, the eleventh primers encoding *Hevea* housekeeping genes were validated using PCR amplification. Fourthly, the value of Crossing-point (Cp) was measured using a second derivative method after RT-qPCR analysis for each day 0 (D0), day 5 (D5) and day 15 (D15) of water deficit. Fifth, rank stability for these reference genes during each measurement was determined using coefficient of variation (CV) method. A small number of housekeeping genes showed a stable expression during 15 days of water deficit in *Hevea brasiliensis*. The successful CV method emphasized the use of simple and yet reliable analysis to determine reference gene stability for RT-qPCR.

Material and Methods

Plant material

In vitro plantlets aged 7-months-old of clone PB260 with line CI07069 were obtained using somatic embryogenesis method developed by Lardet *et al.* (2009). The plantlets were acclimatized and grown in a greenhouse of CIRAD, Montpellier at a temperature of 27°C with 45% relative humidity. The treatment of water deficit

was carried out by subjecting dehydration to the tested plants until a moderate-water stress was reached (a value of FTSW 0.4) (Putranto *et al.*, 2012). Leaf, bark and root tissues were collected at day 0 (D0), day 5 (D5) and day 15 (D15) after dehydration treatment. The samples were conserved at -80°C until RNA extraction.

Total RNA isolation

Total RNAs were isolated using the caesium chloride cushion method adapted from Sambrook *et al.* (1989) by Duan *et al.* (2010). One gram of fresh matter was ground and transferred to a tube containing 30 mL of extraction buffer consisting of 4 M guanidiumisothiocyanate, 1% sarcosine, 1% polyvinylpyrrolidone and 1% ß-mercapto-ethanol. After homogenization, tubes were kept on ice and then centrifuged at 10,000 g at 4°C for 30 minutes. The supernatant was transferred to a new tube containing 8 mL of 5.7 M CsCl. Ultracentrifugation in a swinging bucket was carried out at 89,705 g at 20°C for 20 hours. The supernatant and caesium cushion were discarded whilst the RNA pellet was washed with 70% ethanol. After 30 minutes of air drying, the pellet was dissolved in 200 µL of sterile water. Although DNA could not cross the caesium cushion for this centrifugation condition, DNA contamination was checked by PCR amplification using primers of the *HbActin* gene including the intron sequence. The quantity of RNAs was measured using Infinite® 200 NanoQuant (Tecan, Switzerland). RNAs were conserved at -80°C.

Validation of primers

Primers used in this paper have been tested in previous experiments for its specificity by Real-Time PCR and by sequencing of PCR product (Putranto *et al.*, 2015a; Piyatrakul *et al.*, 2014; Putranto *et al.*, 2012). PCR amplification was carried out using a mix of cDNAs in order to validate the integrity of each pair of primers related to eleven *Hevea brasiliensis* reference genes (*HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbYLS8, HbRH2b, HbRH8, HbUBC2a, HbαTub, Hb40S* and *HbUBI*). The list of primers is provided in Table 1.

cDNA synthesis and Reverse Transcriptase Quantitative PCR (RT-qPCR) setup

cDNAs were synthesized from 2 µg of total RNA to the final 20 µL reaction mixture using a RevertAidTM M-MuLV Reverse Transcriptase (RT) kit according to the manufacturer's instructions (MBI, Fermentas, Canada). Full-length cDNA synthesis was checked on each cDNA sample by PCR amplification of the Actin cDNA using primers at the cDNA ends. Reverse Transcriptase quantitative PCR was finally carried out using a Light Cycler 480 (Roche, Switzerland). The RT-qPCR reaction mixtures consisted of 2 µL RT product cDNA, 0.6 µL of 5 µM of each primer, and 3 µL 2×SYBR green PCR master mix (LightCycler® 480 SYBR Green I Master, Roche Applied Sciences) in a 6-µL volume. PCR cycling conditions comprised one denaturation cycle at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 60°C for 15s, and 72°C for 20s).Real-Time PCR was done at 96-well plate.

Data analysis using coefficient of variation

The values of Crossing-point (Cp) were obtained in the end of PCR process. The determination of stability rank for each reference gene was calculated using coefficient of variation (CV). The CV is defined as the ratio of the standard deviation to the mean (Hellemans *et al.*, 2007; de Jonge *et al.*, 2007). The CV values were presented in percentage at D0, D5 and D15 sampling points.

Results and Discussion

Validation of total RNA yield cDNA and primer pairs by PCR amplification

The validation of RNA integrity is an important preliminary stage in the analysis of gene expression. The quality and quantity of RNA determines largely the quality and quantity of synthesized cDNA (Fleige & Pfaffl, 2006; Fleige *et al.*, 2006). In this work, total RNA was isolated from each 9 samples of control and stressed-plants, respectively, counting in total 18 samples (Table 2). The RNA quantification using Nanoquant showed that its concentration and purity met the requirement for transcript accumulation analysis by RT-qPCR. The purity of total RNA samples (ratio of 260/280 nm) was measured from 1.77-1.93 while the RNA concentrations ranged from 0.462-8.000 µg/µl. The yield of total RNA obtained was afterward calculated by adjusting the weight of each sampled tissue. The yield of total RNA in leaf was 10.497 and 11.590 µg/g of fresh leaf from control and stressed-plants, respectively. The yield of total RNA in bark was 7.535 and 15.036 µg/g of fresh bark from control and stressed-plants, respectively. The yield of total RNA in root was 5.758 and 9.678 µg/g of fresh root from control and stressed-plants, respectively. Statistical analysis showed no significant difference of total RNA yield between tissues or between treatments (control and stressed-plants).

The quality of cDNA synthesized using reverse transcriptase enzyme can be validated by PCR amplification using the primer of housekeeping gene such as *HbActin* (Putranto *et al.*, 2012). The total 18 cDNA samples showed a thick-positive band of *HbActin* gene (195 bp) in agarose gel electrophoresis (Figure 1). The PCR reaction was assured by the amplification of positive control, a plasmid containing *HbActin* gene. Prior to RT-qPCR analysis, eleven pairs of primer encoding *Hevea* reference genes were validated by PCR amplification. PCR results showed specific DNA

Table 1. Primer sequences of 11 housekeeping genes from *H. brasiliensis*

Tabel 1. Sekuen primer 11 gen housekeeping dari H. brasiliensis

Genes / *Gen*	Primer sequences /*Sekuen primer*		Product length / *Panjang produk* (bp)	Primer efficiency / *Efisiensi primer* (%)	Reference / *Referensi*
	Forward (5'-3')	*Reverse **(5'-3')***			
HbActin	AGTGTGATGTGGATATCAGG	GGGATGCAAGGATAGATC	195	97.50	(Putranto *et al.* 2012)
HbelF1Aa	GCGTGACTATCAGGACGACAA	CAAGACCTCCAGCAATACCCT	198	97.60	(Li *et al.* 2011)
HbUBC4	TCCTTATGAGGGCGGAGTC	CAAGAACCGCACTTGAGGAG	221	99.19	(Li *et al.* 2011)
HbUBC2b	CGACCAAGTTTTCATTTCGGGTG	AGTCTCTTCTTTGCTGGGGTTG	219	96.90	(Li *et al.* 2011)
HbUBC2a	CATTTATGCGGATGGAAGCA	CAGGGGAGTTTGGATTTGGA	197	97.90	(Li *et al.* 2011)
HbYLS8	CCTCGTCGTCATCCGATTC	CAGGCACCTCAGTGATGTC	213	96.90	(Li *et al.* 2011)
HbRH2b	GAGGTGGATTGGCTAACTGAGAAG	GTTGAACATCAAGTCCCCGAGC	215	98.90	(Piyatrakul *et al.* 2012)
HbRH8	TCACAGGGTTGGTAGATCAG	CCAAGCTCTTGCTCAATCC	209	95.80	(Li *et al.* 2011)
HbαTUB	GGTTGGTAGATCAGGCAGGTTTGG	CAACCACAAAAGTGCAATGG	207	95.00	(Duan *et al.* 2010)
Hb40S	ACAGGCTCATCACCTCCAAG	CAACCACAAAAGTGCAATGG	211	97.00	(Duan *et al.* 2010)
HbUBI	TTATCCAATGCGATCCAACC	CAAGGTCAAGGCTCACCAAT	214	91.00	(Putranto *et al.* 2012)

Table 2. Profile of total RNA isolated from three different organs of *Hevea brasiliensis* under two conditions (control and water deficit). D0 : Day 0. D5 : Day 5. D15 : Day 15. Statistical analysis was performed using ANOVA followed by Newman-Keuls test. Letter shows significancy for each yield compared.

*Tabel2. Profil total RNA dari tiga organ tanaman karet (*Hevea brasiliensis*) di bawah dua kondisi (control dan cekaman kekeringan). D0 : Hari ke-0. D5 : Hari ke-5. D15: Hari ke-15. Analisis statistik dilakukan dengan menggunakan ANOVA diikuti uji Newman-Keuls. Huruf menunjukkan angka signifikan untuk tiap rendemen yang dibandingkan.*

Treatment *Perlakuan*	Organ *Organ*	Replicate *Ulangan*	Ratio 260/280 *Nisbah 260/280*	Concentration (µg/µL) *Konsentrasi* (µg/µL)	Yield (µg/g fresh tissue) *Rendemen (µg/g organ segar)*
Control *Kontrol*	Leaf *Daun*	D0	1.89	8.000	11.590[a]
		D5	1.92	2.248	
		D15	1.87	3.667	
	Bark *Kulit batang*	D0	1.89	1.506	15.036[a]
		D5	1.85	0.462	
		D15	1.86	1.757	
	Root *Akar*	D0	1.90	1.924	9.678[a]
		D5	1.77	0.994	
		D15	1.79	1.688	
Water deficit *Cekaman kekeringan*	Leaf *Daun*	D0	1.85	2.201	10.497[a]
		D5	1.81	4.335	
		D15	1.84	8.000	
	Bark *Kulit batang*	D0	1.90	0.733	7.535[a]
		D5	1.92	1.335	
		D15	1.86	2.867	
	Root *Akar*	D0	1.87	0.926	5.758[a]
		D5	1.93	1.720	
		D15	1.92	2.143	

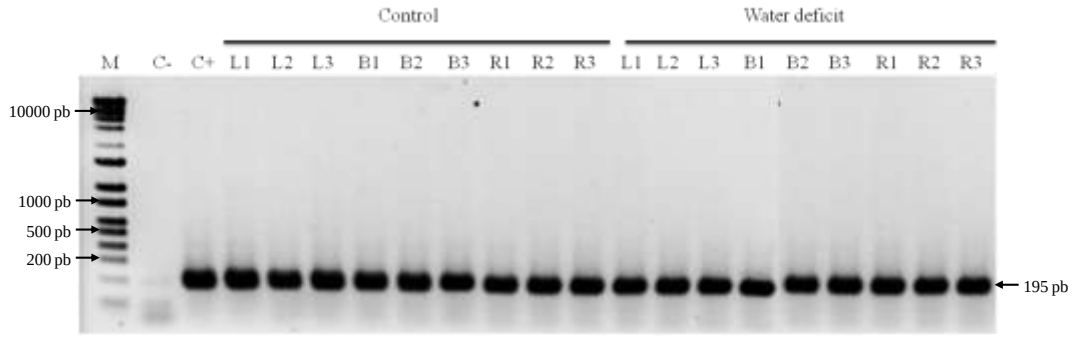

Figure 1. Profile of cDNA resulted from PCR amplification using standard primer *HbActin*. M : 2-Log DNA ladder. C- : negative control (nuclease-freewater). C+ : positive control (*HbActin* plasmid). L1-3: three leaf samples at D0, D5 and D15. B1-3: three bark samples at D0, D5 and D15. R1-3: three root samples at D0, D5 and D15. Electrophoresis migration was done at 2% agarose gel.

Gambar 1. Profil cDNA dari amplifikasi PCR menggunakan primer standar HbActin. *M : 2-Log DNA ladder. C- : kontrol negatif (air bebas-nuklease). C+ : kontrol positif (plasmid* HbActin*). L1-3: tiga sampel daun untuk hari ke-0, ke-5, dan ke-15. B1-3: tiga sampel kulit batang untuk hari ke-0, ke-5, dan ke-15. R1-3: tiga sampel akar untuk hari ke-0, ke-5, dan ke-15. Migrasi elektroforesis dilakukan pada gel agarose 2%.*

fragment for each pair of primer ranging from 192-221 bp (Figure 2). In addition, the validation of primer pairs can also be done by measuring the melting temperature (Tm) in Real-Time PCR. Single curve indicates a specific PCR product related to DNA fragment of interest (Rebouças *et al.*, 2013).

Measurement of Crossing-point (Cp) values during water deficit

The baseline and quantification of cycle reaching the threshold of fluorescence, usually called Crossing-point (Cp) were automatically determined by the LightCycler 4.05 software

(Roche) for day 0 (D0), day 5 (D5) and day 15 (D15) in control and stressed-plants. Since the Cp values occurred at the exponential phase of qPCR, they were considered as an accurate indication of efficient amplification (Luu-The *et al.*, 2005). In this experiment, the Cp values were calculated using a second derivative method. A positive peak corresponds to the beginning of the log-linear phase defined the Cp value.

The Cp values for eleven housekeeping genes encoding reference genes in *Hevea brasiliensis* have been defined for each sampling point (D0, D5 and D15) (Figure 3). The mean of Cp values at D0 for *HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbUBC2a, HbYLS8, HbRH2b, HbRH8, HbαTUB, Hb40S, HbUBI* was 18.868, 19.597, 21.578, 22.563, 21.665, 22.513, 21.006, 22.071, 22.849, 23.357 and 28.383, respectively. The mean of Cp values at D5 for *HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbUBC2a, HbYLS8, HbRH2b, HbRH8, HbαTUB, Hb40S, HbUBI* was 29.437, 22.171, 31.684, 31.304, 27.038, 27.520, 25.066, 31.335, 25.643, 27.998, and 31.717, respectively. The mean of Cp values at D15 for *HbActin, HbelF1Aa, HbUBC4, HbUBC2b, HbUBC2a, HbYLS8, HbRH2b, HbRH8, HbαTUB, Hb40S, HbUBI*was 19.557, 21.133, 24.288, 23.315, 21.786, 22.769, 22.403, 23.787, 23.382, 22.689 and 27.086, respectively.

According to Barsalobres-Cavallari *et al.* (2009) and Pfaffl *et al.* (2002), a suitable Cp value for transcript abundance analysis should range between 19-23 despite it may change depending on the degree of cDNA dilutions. Housekeeping genes performing generally constitutive expression are measured before 25th cycle. Cp value in close proximity to the 30th cycle shows low expression of housekeeping gene unsuitable to be used as a reference gene. *HbActin* had the highest level of expression with the mean Cp value as high as 18.686 and 19.557 at D0 and D15 sampling points in comparison to 22.171 for *HbelF1Aa* at D5 sampling point. The lowest level of expression was performed by *HbUBI* at three sampling points with mean Cp values of 29.383, 31.717 and 27.086 (Figure 3).

In general, the mean of Cp values for these housekeeping genes at D5 sampling point was significantly higher than D0 and D15 sampling points. For five housekeeping genes such as *HbelF1Aa*, *HbUBC4*, *HbRH2b*, *HbRH8* and *HbUBI*, the Cp values were significantly different between D0, D5 and D15 (Figure 3). These results highlighted the review of Kozera & Rapacz (2013) concerning real stable expression of reference gene has never been recorded. It underlined once again the importance of prior analysis in order to look for a stable reference gene before RT-qPCR analysis.

Stability rank of reference genes during water deficit

The coefficient of variation (CV) is used as a statistic for comparing the degree of variation between genes, even if the mean expressions are drastically different from each other. The CV equals the standard deviation divided by the mean, normally expressed as a percentage (Hellemans *et al.*, 2007; de Jonge *et al.*, 2007). The value of CV reflects the level of biological variation in the sample population. Lower CV value of a housekeeping gene is related to the low variation in a set of sample population meaning higher stability to be used as a normalization factor in RT-qPCR analysis (Wang *et al.*, 2014).

In this work, the calculated CVs for tested reference genes were ranked at D0, D5 and D15 sampling points (Table 3). The ranking results at D0 sampling point indicate that *HbRH2b*, *HbRH8* and *HbUBC4* had the most stable expression across tissues (leaf, bark and root) and conditions (control

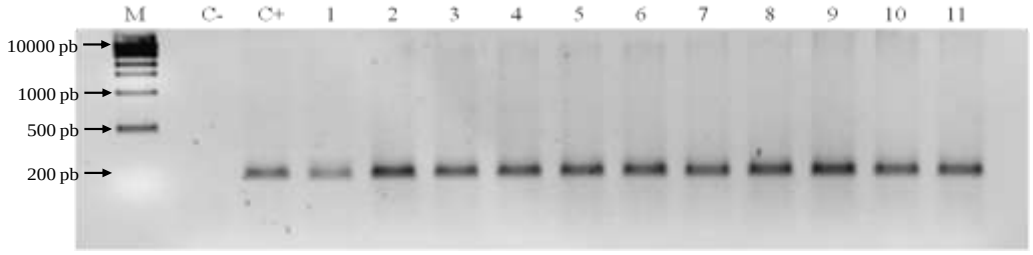

Figure 2. Profile of primers for reference gene resulted from standard PCR amplification. M : 2-Log DNA ladder. C- : negative control (nuclease-freewater). C+ : positive control (HbActin plasmid). Lane 1: *HbActin*. Lane 2: *HbelF1Aa*. Lane 3: *HbUBC4*. Lane 4: *HbUBC2b*. Lane 5: *HbUBC2a*. Lane 6: *HbYSL8*. Lane 7: *HbRH2b*. Lane 8: *HbRH8*. Lane 9: *HbαTUB*. Lane 10: *Hb40S*. Lane 11: *HbUBI*. Electrophoresis migration was done at 2% agarose gel.

Gambar 2. Profil primer gen referensi dari amplifikasi PCR standar. M : 2-Log DNA ladder. C- : kontrol negatif (air bebas-nuklease). C+ : kontrol positif (plasmid HbActin*). Lajur 1: HbActin. Lajur 2: HbelF1Aa. Lajur 3: HbUBC4. Lajur 4: HbUBC2b. Lajur 5: HbUBC2a. Lajur 6: HbYSL8. Lajur 7: HbRH2b. Lajur 8: HbRH8. Lajur 9: HbαTUB. Lajur 10: Hb40S. Lajur 11: HbUBI. Migrasi elektroforesis dilakukan pada gel agarose 2%.*

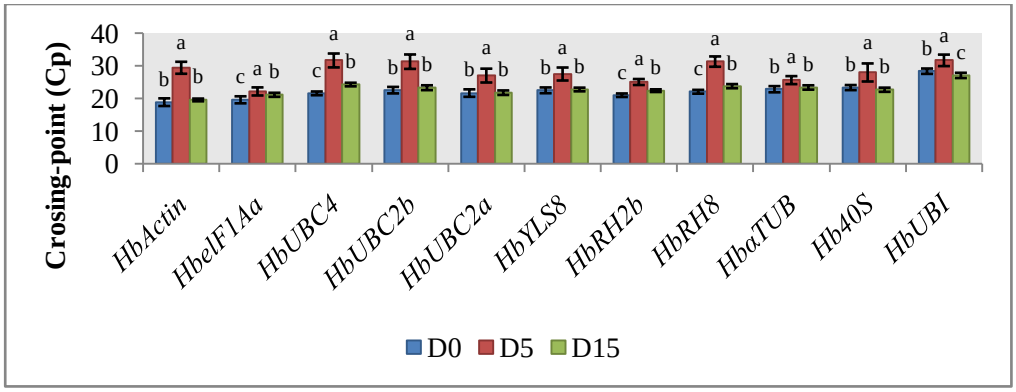

Figure 3. Profile of eleven *Hevea brasiliensis* reference genes during D0, D5 and D15 of water deficit. Reverse Transcriptase Quantitative PCR (RT-qPCR) was carried out to obtain the Crossing-point (Cp) values. The values were the mean of 6 cDNA samples (3 tissues for each treatment) at D0, D5 and D15. Vertical bars show the standard deviation for each measurement. Statistical analysis was performed using ANOVA followed by Newman-Keulstest. Letter shows significancy for each compared measurement.

Figure 3. Profil sebelas gen referensi dari Hevea brasiliensis *saat cekaman kekeringan pada hari ke-0, hari ke-5 dan hari ke-15. Reverse Transcriptase Quantitative PCR (RT-qPCR) dilakukan untuk mendapatkan nilai Crossing-point (Cp). Angka merupakan rerata dari 6 sampel cDNA(3 organ untuk setiap perlakuan) hari ke-0, ke-5 dan ke-15. Bar vertikal menunjukkan nilai deviasi standar untuk tiap pengukuran. Analisis statistik dilakukan dengan menggunakan ANOVA diikuti uji Newman-Keuls. Huruf menunjukkan angka signifikan untuk tiap pengukuran yang dibandingkan.*

Table 3. The stability rank of eleven *Hevea brasiliensis* reference genes during D0, D5 and D15 of water deficit. Coefficient of variation (CV) was calculated based on standard deviation for sample variation divided by Crossing-point (Cp) values. The reference gene with the smallest CV value is regarded as themosts table for its expression.

Table 3. Stabilitas peringkat dari sebelas gen referensi pada Hevea brasiliensis *selama cekaman kekeringan selama hari ke-0, ke-5 dan ke-15. Koefisien variansi (CV) dihitung berdasarkan deviasi standar untuk tiap variasi sampel dibagi dengan Crossing-point (Cp). Gen referensi dengan nilai CV terendah dianggap ekspresinya paling stabil.*

Rank /	D0 / Hari ke-0		D5 / Hari ke-5		D15 / Hari ke-15	
Peringkat	Gene / *Gen*	CV (%)	Gene / *Gen*	CV (%)	Gene / *Gen*	CV (%)
1	*HbRH2b*	2.55	*HbRH2b*	3.67	*HbRH2b*	1.81
2	*HbRH8*	2.63	*HbαTUB*	4.79	*HbActin*	2.11
3	*HbUBC4*	2.64	*HbRH8*	5.06	*HbUBC4*	2.26
4	*HbUBI*	2.88	*HbUBI*	5.56	*HbYLS8*	2.46
5	*Hb40S*	3.33	*HbelF1Aa*	5.61	*HbRH8*	2.70
6	*HbYLS8*	3.93	*HbActin*	6.25	*Hb40S*	2.82
7	*HbαTUB*	4.13	*HbUBC4*	6.74	*HbαTUB*	2.84
8	*HbUBC2b*	4.41	*HbUBC2b*	7.09	*HbelF1Aa*	2.94
9	*HbUBC2a*	5.30	*HbYLS8*	7.24	*HbUbi*	2.99
10	*HbelF1Aa*	5.51	*HbUBC2a*	7.87	*HbUBC2a*	3.18
11	*HbActin*	6.23	*Hb40S*	9.90	*HbUBC2b*	3.19

and stressed-plants) with CV values of 2.55, 2.63 and 2.64%, respectively. At the sampling point of D5, the top three of reference genes belonged to *HbRH2b, HbαTUB* and *HbRH8* with CV values of 3.67, 4.79 and 5.06%, respectively. At the sampling point of D15, *HbRH2b* was the most stable reference gene with a CV value of 1.81% followed by *HbActin* and *HbUBC4* ith CV values of 2.11 and2.26%, respectively. Interestingly, based on the analysis using CV, *HbRH2b* gene showed consistent and stable expressions in three sampling points with CV values ranging from 1.81 to 3.67%. According to Li *et al.* (2011), *HbRH2b* gene encodes for a DEAD box RNA helicase in *Hevea brasiliensis*. The DEAD-box helicaces are a diverse family of proteins involved in ATP-dependent RNA unwinding, needed in a variety of cellular processes including splicing, ribosome biogenesis and RNA degradation (Marchler-Bauer *et al.*, 2015).

Conclusion

In general, the results obtained in this work reconfirmed the necessity of prior analysis for reference genes before RT-qPCR due to the instability of housekeeping genes at different tissue compartments or experimental conditions. The evaluation of these reference genes should improve the possibility of having more accurate relative transcript accumulation of target genes. Despite the usage reinforcement of multiple softwares to select reference genes appropriate for each experiment, the work presented in this paper demonstrated a simple but reliable analysis using CV method to assess *Hevea* reference genes before RT-qPCR analysis. Finally, it can be concluded that five housekeeping genes (*HbRH2b*, *HbRH8*, *HbUBC4*, *HbαTUB* and *HbActin*) can be used as a set of reference genes for RT-qPCR analysis during moderate water deficit in *Hevea brasiliensis*. The *HbRH2b* gene was the most stable among other housekeeping genes.

Acknowledgement

This work was supported by the grant from the French Institute of Rubber (IFC), Michelin, Socfinco and SIPH companies. The authors thank Maryannick Rio and Christine Sanier for the experimental setup. The authors thank equally Ludovic Lardet, Florence Desailly and Gérald Oliver for the production of plant material.

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