Plant Mol Biol Rep (2012) 30:242–245
DOI 10.1007/s11105-011-0340-3

Evaluation of Reference Genes for Expression Studies in Ash (*Fraxinus* spp.)

Loren Rivera-Vega · Praveen Mamidala · Jennifer L. Koch · Mary E. Mason · Omprakash Mittapalli

Published online: 3 August 2011

Abstract Ash (*Fraxinus* spp.) is a dominant tree species in North America, in both managed and natural landscapes. However, due to the rapid invasion by the emerald ash borer (*Agrilus planipennis*), an exotic invasive insect pest, millions of North American ash trees have been killed. Real-time quantitative polymerase chain reaction (RTq-PCR) is widely used for validating transcript levels in gene expression studies for which a good reference gene is mandatory. In the current study, we evaluated the stability of ten reference genes in at least five different tissues (phloem, roots, shoots, immature leaves, and mature leaves), and two developmental stages (young and old) among three ash species including the resistant Asian Manchurian ash (*F. mandshurica*) and two susceptible North American ash species (green—*F. pennsylvanica* and white—*F. americana*). Of the examined genes, the translation elongation factor alpha (eEF1α) was observed to be most stable and thus is recommended for RTq-PCR based gene expression studies in *Fraxinus* species. To our knowledge, this is the first report on the stability of reference genes across ash species (in different tissues and during development).

Keywords *Fraxinus* spp. · Real-time quantitative polymerase chain reaction · Reference genes · GeNorm · Normfinder

Introduction

Real-time quantitative polymerase chain reaction (RTq-PCR) is a widely used technique for assessing gene expression, which allows fast and accurate quantification of even low expressed transcripts (Bustin 2002). RTq-PCR quantifies the relative expression of mRNA in real time through the detection of fluorescence after every PCR cycle avoiding post PCR processing (Ginzinger 2002). For the accurate quantification of mRNA transcripts using RTq-PCR, identification of stable reference genes is crucial to normalize the target mRNA levels (Phillips et al. 2009). Most commonly used reference genes are housekeeping genes or endogenous control genes, which are thought to be non-regulated. However, several studies revealed large variation of reference gene expression under different experimental conditions and thus to date no universal reference gene has been identified in plants or animals (Gutierrez et al. 2008). A good reference gene is considered to be one whose expression is essential for cell function and is relatively constant in tissues, development stages and under different experimental conditions (Schmittgen and Zakrajsek 2000; Guenin et al. 2009; Qi et al. 2010). The validity and accuracy of data obtained through RTq-PCR can be heavily influenced by the reference gene being used (Dheda et al. 2005). Given the variation of reference gene

L. Rivera-Vega and P. Mamidala contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11105-011-0340-3) contains supplementary material, which is available to authorized users.

L. Rivera-Vega · P. Mamidala · M. E. Mason · O. Mittapalli (✉)
Department of Entomology, Ohio Agricultural and Research Development Center, The Ohio State University, Wooster, OH 44691, USA
e-mail: mittapalli.1@osu.edu

J. L. Koch
US Forest Service, Northern Research Station, Delaware, OH 43015, USA

expression among different experimental conditions, it is essential to validate a set of reference genes (~2–3) since non-validated reference genes could greatly affect the quantification of target transcript levels in RTq-PCR experiments (Bustin and Nolan 2004; Li et al. 2010).

Ash (*Fraxinus* spp.) is a dominant tree species with widespread distribution throughout the world's temperate forests, including North America. The accidental introduction of emerald ash borer (*Agrilus planipennis* Fairmaire), an exotic invasive wood boring beetle into North America, has resulted in death of millions of ash trees including white (*F. americana* L.), green (*F. pennsylvanica* Marshall) and black ash (*F. nigra* Marshall) with significant economic and ecological impact (Kovacs et al. 2010). However, in Asia, *A. planipennis* is not considered as a major insect pest perhaps due to their co-evolutionary history with Manchurian ash (*F. mandshurica* Rupr). In an ongoing study, we have attempted to decipher the transcriptome of ash phloem in the hope to unravel candidate resistance genes to *A. planipennis* (Bai et al. 2011). This study has laid the foundation for several gene expression/functional genomics studies, for which there is an urgent need to identify/validate reference genes to be included in RTq-PCR experiments. In this study, we evaluated the stability of ten reference genes among different samples (tissues and development stages) of *Fraxinus* species including green, white and Manchurian.

Materials and Methods

Mature leaves and phloem plugs of 5 mm in diameter were collected from un-infested cultivars of green, white, and Manchurian ash in June 2010 from a common garden at the Ohio Agricultural and Research Development Center (OARDC, Wooster, OH). Two young (~5 years) and two old (~15 years) trees were sampled per species. In June 2011, samples from shoots, roots, immature and mature leaves were obtained from 4-year-old potted seedlings of the same three species at the US Forest Service (Delaware, OH) following the same protocol as the previous year. In addition to these samples, phloem plugs were also collected from girdled ash trees. Samples were obtained from above and below the girdled region from three trees per species. The post-girdled samples were obtained 21 days after girdling from a common garden at Novi, MI. Girdling was performed by peeling a 5-cm band of the bark as per Baldwin (1934). All samples were immediately placed in liquid nitrogen and kept at −80°C until further processing. Approximately 70 mg of tissue was ground to powder in liquid nitrogen for RNA extraction. Total RNA was extracted using Trizol® Reagent (Invitrogen, Carlsberg, CA) following the manufacturer's protocol. After RNA extraction, samples were treated with TURBO DNase™ (Ambion, Inc., Austin, TX) following manufacturer's protocol, in order to eliminate genomic DNA contamination and were stored at −80°C until further use.

The sequences of the potential reference genes including a cyclophilin (Cyp), elongation factor 1-beta (eEF1β), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PD), histone H3 (HIS), ribosomal protein L13 (RPL13), RNA polymerase II (RNAPII), translation elongation factor alpha (eEF1α), beta-tubulin (TUB), and ubiquitin protein ligase (E3upl) were mined from an ongoing phloem transcriptomic study of ash (Bai et al. 2011). All the sequences pertaining to the current study are available at the NCBI Sequence Read Archive under the accession number of SRA020745.3.

One microgram of RNA from each sample was used for cDNA synthesis using the SuperScript™ First-Strand synthesis kit (Invitrogen) following the manufacturer's protocol. Ten pairs of gene specific primers were designed using Beacon Designer 7 software. RTq-PCR reactions were carried out in a total volume of 10 μl containing 5 μl of 2× SybrGreen (Bio-Rad, Hercules, CA), 0.5 μl of each primer (10 μM) and 2 μl of cDNA template (40 ng/μl) and 2 μl of nuclease free water. All reactions were performed in duplicate. The cycling parameters were 95°C for 5 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s ending with a melting curve analysis (65°C to 95°C in increments of 0.5°C every 5 s) to check for nonspecific product amplification. Primer efficiencies were calculated using a standard curve that consisted of 5-fold dilutions over four points. Each point was measured in triplicate. Three standard curves were done per primer set. The primer efficiency ($10^{-1/\text{slope}}$) and R^2 presented in Table S1 are an average of these three curves. Two software programs, GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) were used for the selection of stable constitutively expressed reference genes. Cycles to threshold (C_t) values were converted according to the requirements of the software used.

Results and Discussion

Since ash phloem is the target tissue of *A. planipennis* larvae, and the adult beetle feeds on leaf tissue, we included both phloem and leaf tissue (of different ash species) from two developmental stages. Additionally, we included phloem samples from girdled trees, immature and mature leaves, shoots and roots with the goal of identifying reference genes with stable expression levels both within and across the species. Initial analysis of the ten reference genes indicated a high rate of variation; GAPDH, RNAPII, HIS and TUB were found to be the most unstable and

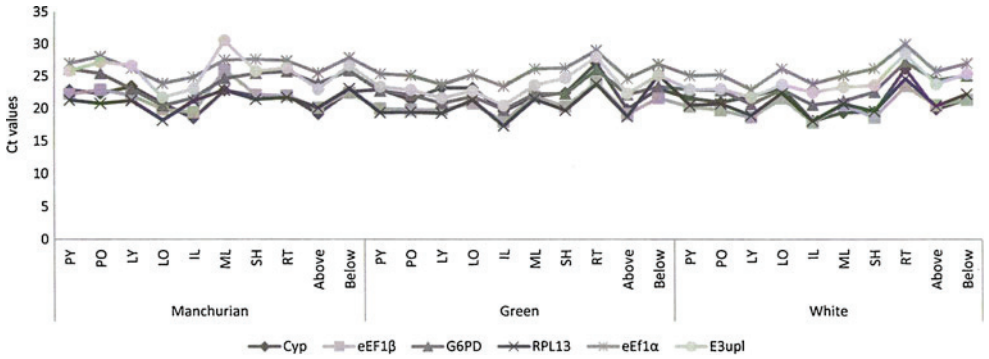

Fig. 1 Average cycles to threshold (C_t) values for the six candidate reference genes used for all the *Fraxinus* spp. samples. **PY* phloem from young trees (<5 years old), *PO* phloem from old trees (>5 years old), *LY* leaves from young trees, *LO* leaves from old trees, *IL* immature leaves, *ML* mature leaves, *SH* shoots, *RT* roots. *Above* phloem samples above girdling, *Below* phloem samples below girdling. Gene acronyms: *Cyp* cyclophilin, *eEF1β* translation elongation factor β, *G6PD* glucose-6-phosphate dehydrogenase, *RPL13* ribosomal protein L13, *eEF1α* translation elongation factor 1α, *E3upl* E3 ubiquitin protein ligase

hence, were removed from further analysis. The C_t values for the remaining six genes ranged between 17 and 33 (highest C_t value obtained for eEF1α and lowest for RPL13), the average of the C_t values is represented in Fig. 1.

GeNorm Analysis

The stability of the reference genes was calculated using GeNorm, a visual basic application (VBA) for Excel. GeNorm determines the pairwise variation of all control genes as the standard deviation of the logarithmically transformed expression ratios. It measures a gene expression stability value (*M*), which is the average pairwise variation of a gene compared to the other control genes included in the same analysis. Genes with the lowest *M* value are considered to be the most stable (Vandesompele et al. 2002). GeNorm suggests M=1.5 as a cutoff value, meaning that genes with *M* values higher than 1.5 should not be used as reference genes.

The value of M for the six reference genes showed eEF1α as a potential reference gene across all the samples assayed with E3upl and RPL13 as the least stable genes (Table 1). When all the species were considered as a pool, the most stable genes were eEF1α, eEF1β and G6PD with M values of 1.038, 1.102, and 1.222, respectively. When each species was considered as a separate subgroup, eEF1α was the most stable for all ash species. In order to discard the possibility of eEF1α and eEF1β being co-regulated, an exclusion analysis was performed wherein each gene was excluded in separate analyses. The ranking of the genes was still the same with very similar M values, indicating no co-regulation between eEF1α and eEF1β (data not shown).

Table 1 *Fraxinus* reference genes ranking according to NormFinder and geNorm software

NormFinder				GeNorm			
Overall	Manch	Green	White	Overall	Manch	Green	White
eEf1α	eEf1α	eEF1β	RPL13	eEf1α	eEf1α	eEf1α	eEf1α
(0.028)	(0.029)	(0.020)	(0.028)	(1.038)	(1.225)	(0.850)	(0.713)
eEF1β	eEF1β	eEf1α	eEf1α	eEF1β	Cyp	eEF1β	eEF1β
(0.035)	(0.041)	(0.027)	(0.031)	(1.102)	(1.252)	(0.886)	(0.741)
E3upl	E3upl	G6PD	eEF1β	G6PD	eEF1β	G6PD	RPL13
(0.042)	(0.051)	(0.027)	(0.031)	(1.222)	(1.370)	(0.906)	(0.780)
G6PD	Cyp	E3upl	E3upl	Cyp	G6PD	Cyp	E3upl
(0.044)	(0.051)	(0.032)	(0.035)	(1.231)	(1.454)	(0.976)	(0.800)
RPL13	G6PD	Cyp	G6PD	E3upl	E3upl	E3upl	Cyp
(0.058)	(0.056)	(0.047)	(0.038)	(1.327)	(1.753)	(1.002)	(0.836)
Cyp	RPL13	RPL13	Cyp	RPL13	RPL13	RPL13	G6PD
(0.068)	(0.065)	(0.071)	(0.062)	(1.472)	(1.762)	(1.589)	(0.952)

NormFinder Analysis

NormFinder is also an Excel add-in that uses a mathematical model which performs separate analysis of sample subgroups, estimates intra- and inter-expression variation and calculates a stability value (Andersen et al. 2004). Genes with a lower stability value are considered to be more stable, i.e., there is a lower variation of gene expression across the samples. NormFinder indicated eEF1α as the most stable gene for all the samples with a stability value of 0.028. When analyses were done per species, eEF1α was the most stable gene for Manchurian ash (0.029) while eEF1β and RPL13 were the most stable genes for green (0.020) and white ash (0.028), respectively. eEF1α ranked the second most stable gene for both the latter species (Table 1). The reference gene Cyp displayed the highest stability value (least stable) in the overall analysis. Despite the separate analysis, we noticed some similarities such as: (1) eEF1α was consistently among the top three genes both with and without subgroups, and (2) Cyp was consistently among the lowest three genes in all the analyses performed. This trend in the stability values obtained for eEF1α and Cyp is in agreement with other studies in plants albeit within a species (Tong et al. 2009).

Analysis of potential reference genes for the three species indicated a high variability. RTq-PCR is often used to analyze the relative expression of a gene under different conditions in the same species. However, ongoing functional studies include analysis of gene expression in different *Fraxinus* spp., thus increasing the need to find good and reliable reference genes across species. It was possible to observe that irrespective of the samples pertaining to different *Fraxinus* spp., eEF1α was observed to be highly consistent. We thus recommend eEF1α as a candidate reference gene for gene expression studies in ash. To our knowledge this is the first report on validating reference genes across different tree species.

Acknowledgements We thank Drs. Daniel Herms and Pierluigi Bonello for access to the common garden experimental plots (Novi, MI, and Bowling Green, OH) to collect relevant ash samples. The research was supported by a grant from the USDA APHIS/USDA Forest Service Accelerated Emerald Ash Borer Research Program (GRT00011769/60016270) and by State and federal funds appropriated to the Ohio Agricultural Research and Development Center, Ohio State University.

References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription–PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250

Bai X, Rivera-Vega L, Mamidala P, Bonello P, Herms DA, Mittapalli O (2011) Transcriptomic signatures of ash (*Fraxinus* spp.) phloem. PLoS ONE 6:e1636861:249–257

Baldwin HI (1934) Some physiological effects of girdling northern hardwoods. Bull Torrey Bot Club 5:249–257

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39

Bustin SA, Nolan T (2004) Pitfalls of quantitative real-time reverse transcription polymerase chain reaction. J Biomol Tech 15:155–166

Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, Rook GA, Zumla A (2005) The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem 344:41–143

Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol 30:503–512

Guenin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 60:487–493

Gutierrez L, Mauriat M, Guenin S, Pelloux J, Lefevre JF, Lovet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, Wuytswinkel OV (2008) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 6:609–618

Kovacs KF, Haight RG, McCullough DG, Mercader RG, Seigert NW, Leibhold AM (2010) Cost of potential emerald ash borer damage in U.S. communities 2009–2019. Ecol Econ 69:569–578

Li QF, Sun SSM, Yuan DY, Yu HX, Gu MT, Liu QQ (2010) Validation of candidate reference genes for the accurate normalization of real-time Quantitative RT-PCR data in rice during seed development. Plant Mol Biol Rep 28:49–57

Phillips MA, D'Auria JC, Luck K, Gershenzon J (2009) Evaluation of candidate reference genes for real-time quantitative PCR of plant samples using purified cDNA as template. Plant Mol Biol Rep 27:407–416

Qi J, Yu S, Zhang F, Zhao X, Yu Y, Zhang D (2010) Reference gene selection for real-time quantitative polymerase chain reaction of mRNA transcript levels in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*). Plant Mol Biol Rep 28:597–604

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81

Tong ZG, Gao ZH, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH 0034