Selection of Reference Genes for Expression Analysis in *Diuraphis noxia* (Hemiptera: Aphididae) Fed on Resistant and Susceptible Wheat Plants

Deepak K. Sinha & C. Michael Smith

Department of Entomology, Kansas State University, Manhattan, KS 66506-4004, USA.

The Russian wheat aphid (RWA), *Diuraphis noxia* Kurdjumov, is a major global pest of wheat and barley production that causes enormous economic damage. Few studies have been conducted to explore and decipher the molecular basis of RWA strategies to evade plant defense mechanisms. Gene expression studies of RWA in response to wheat genotypes carrying different RWA resistance genes have been initiated in our group; however, a secure and accurate understanding of RWA gene expression is dependent on identification of suitable reference genes. This study analyzed expression profiles of five potential reference genes selected and sequenced during RNA sequencing experiments. The expression of genes coding for actin and ribosomal protein L27 was comparatively less variable in RWA fed on different wheat hosts. Results of geNorm, NormFinder, and BestKeeper expression analyses support the use of actin and ribosomal protein L27 in RT-qPCR studies of RWA gene expression in studies involving RWA-wheat interactions.

The advent of next-generation sequencing technologies has resulted in a significant increase in transcriptomic data for various organisms. The generated transcriptomes have helped researchers not only to decipher expression pattern of genes and transcripts but also define the genetic architecture of many species. Validation of gene expression from such transcriptomic resources has become mandatory for reporting and reconfirming expression profiles, and reverse transcription quantitative PCR (RT-qPCR) is rapidly replacing traditional methods such as Northern blotting and Ribonuclease Protection Assays (RPA). RT-qPCR’s speed, sensitivity, efficiency and reproducibility has made it the gold standard for rapid and accurate quantification of gene expression profiles from various next-generation sequencing (NGS) datasets. Commercially available instruments and consumables have further led to universal acceptance of RT-qPCR.

Gene expression using RT-qPCR assay is based on the principle of quantifying target mRNA during the exponential phase of the PCR. During this phase, the target is doubled with each PCR cycle and the probability of PCR-bias due to limited reagents is nullified or decreased. In RT-qPCR, the amplification of product is detected on accumulation of fluorescent signal. The cycle at which the fluorescent signal exceeds background signals is referred to as the threshold cycle, or Ct (also referred to as the quantification cycle, or Cq). Analysis of Cq is used to estimate expression of the respective genes. Several factors, such as RNA quality and quantity, mastermix components used in the PCR and efficiency of PCR reaction, influence Cq values. Absolute and relative quantification methods are generally used to estimate gene expression using RT-qPCR. The absolute quantification approach necessitates generating the standard curve of known copy numbers of each target, which in turn requires standard curves for multiple targets in a study and knowledge of copy numbers of each target, thus limiting its usage. The most widely adopted approach, relative quantification, is based on estimation of gene expression normalized to the expression of a control gene known as a reference; therefore, reliable determination of reference/references is the central factor in accuracy of this method.

A gene can be used as a reference when it is highly and stably expressed in all samples under investigation and is not co-regulated with a target gene. Reference genes traditionally have been housekeeping genes believed to possess inherent stable and constitutive expression irrespective of physiological conditions in different samples or treatments under investigation. The universal stability of housekeeping gene expression was disproven in recent years, negating their use. According to the Minimum Information for Publication of Quantitative
Real-Time PCR Experiments (MIQE) guidelines\textsuperscript{16}, reference gene(s) are now selected based on their specificity in interactions between a species or cell type/s subjected to different treatments or conditions.

The Russian wheat aphid (RWA), \textit{Diuraphis noxia} Kurdjumov, originally from Central Asia, was introduced globally in the 1900s\textsuperscript{17} and has become a major destructive pest of wheat and barley and caused huge economic losses. Host-plant resistance is the most acceptable and ecologically beneficial aphid pest management strategy in wheat growing regions, yet host-plant resistance is transitory and has become a major destructive pest of wheat and barley and has been a problem traditionally has been to identify new sources of resistance such as NGS and RT-qPCR offer the possibility of understanding the molecular mechanisms of RWA virulence and engineering resistant plants with greater longevity. Our research is focused on investigating the molecular mechanisms of RWA-plant interaction with the long-term goal of finding novel and durable solutions to RWA management.

The objective of this investigation was to identify a robust RWA reference gene for use in validation of gene expression studies in RWA-wheat interactions. We identified four previously unreported RWA sequences commonly used as reference controls in other biological systems and report the most suitable reference controls for RT-qPCR assays of RWA genes expressed in aphid-wheat interactions.

**Results and Discussion**

**Analysis of RNA quality, sequencing and sequence analysis.** Bioanalyzer analysis of RNA revealed a ribosomal shift due to denaturation of 28 s rRNA (see Supplementary Fig. 1 online), the Bioanalyzer revealed fragment sizes of >230 bp. The quality of the 18 libraries (6 treatments \(\times\) 3 replications) is shown in Supplementary Fig. 2 online. Libraries were sequenced and filtered to remove low-quality sequences, providing >10 million reads for each library. Results of BLAST analysis and differential gene/transcript analysis will be reported separately.

**Gene selection and RT-qPCR.** The commonly used reference genes actin, ribosomal protein L9, ribosomal protein L27, transcription elongation factor 2 and ribosomal protein L5 were selected based on a literature survey. Actin has been used as a reference gene in many studies of model insects such as the fruit fly, \textit{Drosophila melanogaster} Meigen\textsuperscript{20}. Transcription elongation factor genes were evaluated to check their suitability as reference genes in several insects, particularly Hemipterans such as the sweetpotato whitefly, \textit{Bemisia tabaci} (\textit{Gennadius})\textsuperscript{21} and brown plant hopper, \textit{Nilaparvata lugens} (Stål)\textsuperscript{22}. Genes coding for ribosomal proteins have been reported as most reliable reference in insects such as \textit{N. lugens}\textsuperscript{23}. Many transcripts coded for these genes were identified from RNAseq data, revealing >93% homology to the respective genes in the pea aphid, \textit{Acyrthosiphon pism} (Harris) (Table 1). Representations of these genes observed in RNAseq data confirmed that there was little or no change in their expression. These genes were amplified from cDNAs, yielding amplicon sizes ranging from 220 bp to 553 bp (Table 1). These sequences have been deposited in DNA Databank of Japan with accession numbers AB914563-AB914566.

**Results and Discussion**

**Analysis of RNA quality, sequencing and sequence analysis.** Bioanalyzer analysis of RNA revealed a ribosomal shift due to denaturation of 28 s rRNA (see Supplementary Fig. 1 online), consistent with previously published examples of insect RNA\textsuperscript{18}. Multiple peaks were absent, indicating no RNA degradation. Thus, screening total RNA using NanoDrop spectrophotometry, gel electrophoresis and capillary electrophoresis produced high-quality RNA for RNAseq library preparation. The libraries analyzed using the Bioanalyzer revealed fragment sizes of >230 bp. The quality of the 18 libraries (6 treatments \(\times\) 3 replications) is shown in Supplementary Fig. 2 online. Libraries were sequenced and filtered to remove low-quality sequences, providing >10 million reads for each library. Results of BLAST analysis and differential gene/transcript analysis will be reported separately.

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**Ampliop size and melting temperature of RT-qPCR primers designed from these sequences are shown Table 2. Primer efficiency ranged from 91.50 to 102.65, with corresponding \(R^2\) values from 0.9922 to 0.9995 (Table 2). Primers were redesigned and efficiencies rechecked in cases where the efficiency or \(R^2\) values differed from the range of recommended values. Melt curve analysis of all genes showed no primer dimers or nonspecific product amplification.

**Analysis using geNorm software.** Cq values derived from RT-qPCR assay for expression stability were logarithmically transformed as

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**Table 1 | Primers designed for amplification and sequencing of selected \textit{D. noxia} genes**

| Primer | Sequence (5’–3’) | Template temperature (°C) | Amplicon size in cDNA (bp) | Efficiency (%) | Correlation coefficient \(R^2\) |
|--------|-----------------|--------------------------|---------------------------|----------------|------------------|
| Actin  | F:TGCCGTAGTGTCAGACTAC  | 55                       | 110                        | 91.99          | 0.9992           |
| RPL27  | F:AGCGTTGGTCAATTCAGTG  | 55                       | 90                         | 91.50          | 0.9995           |
| RPL9   | F:TGCAGAAGTGGGACGTCT  | 55                       | 100                        | 92.60          | 0.9993           |
| RPL5   | F:TGCAGAAGTGGGACGTCT  | 55                       | 110                        | 102.65         | 0.9922           |
| TEF2   | F:TGCAGAAGTGGGACGTCT  | 55                       | 96                         | 93.07          | 0.9993           |

**Table 2 | Primers designed for amplification and RT-qPCR of selected genes from \textit{D. noxia} feeding on different wheat genotypes**

| Primer | Sequence (5’–3’) | Tm (°C) | Amplicon size in cDNA (bp) | RT-qPCR efficiency (%) | Correlation coefficient \(R^2\) |
|--------|-----------------|---------|---------------------------|------------------------|------------------|
| RTActin| F:TGCCGTAGTGTCAGACTAC  | 55       | 110                        | 91.99          | 0.9992           |
| RTRPL27| F:AGCGTTGGTCAATTCAGTG  | 55       | 90                         | 91.50          | 0.9995           |
| RTRPL9| F:TGCAGAAGTGGGACGTCT  | 55       | 100                        | 92.60          | 0.9993           |
| RTRPL5| F:TGCAGAAGTGGGACGTCT  | 55       | 110                        | 102.65         | 0.9922           |
| TTEF2  | F:TGCAGAAGTGGGACGTCT  | 55       | 96                         | 93.07          | 0.9993           |
input for geNorm analysis. The principle behind this algorithm is that if two genes are stably expressed in a sample set then the ratio of their logarithmic transformed expression should be constant; thus, geNorm ranks genes based on their average expression stability (M), and the candidate gene possessing the lowest M value is the most stably expressed gene in that set. Figure 1 depicts the average expression stability of actin (M = 0.210) and ribosomal protein L27 (M = 0.267), the two most stable genes that should be used as references in RT-qPCR assays involving *D. noxia*-wheat interactions.

geNorm also predicts an optimal number of reference genes for accurate representation of gene expression based on calculation of pairwise variation (Vn/Vn + 1) between sequential normalization factors (NFn and NFn + 1). A cutoff value of 0.15 is considered for the ratio, below which there is no requirement of any other reference gene. Large pairwise variation represents a significant effect in gene expression due to the addition of another gene or genes, reinforcing the need for the second gene to be included to derive reliable normalization factors. Candidate genes are added based on the ranking derived using the M value. This analysis, depicted in Figure 2 for our experiments, clearly showed that adding a third gene does not increase the ratio by more than 0.15; therefore, actin and ribosomal protein L27 represent the required reference genes for accurate estimation of *D. noxia* gene expression in aphid-wheat interactions.

**Analysis by NormFinder software.** NormFinder analyzes candidate reference genes according to inter- and intra-group variation in expression. Similar results were obtained using NormFinder, which predicted stability value of 0.735 for ribosomal protein L27 followed by stability value of 0.738 for actin (Figure 3). Therefore, both geNorm and NormFinder outputs provide proof for stable ribosomal protein L27 gene that can be used as a reference in all the RT-qPCR assays that studies expression patterns of genes in...
the Russian wheat aphid feeding on different wheat varieties containing aphid resistance genes.

**BestKeeper analysis software.** BestKeeper considers the Cq values of all candidate reference genes, in order to calculate standard deviation (SD) and coefficient of variation (CV). The software excludes genes with Cq value SDs greater than 1. Results of geNorm and NormFinder analyses were further verified when Cq data were analysed using BestKeeper software, which predicts gene stability based on low CV and SD. Ribosomal protein L27 (CV = 2.48 ± 0.47%) and actin (2.85 ± 0.51%) were found to be the most stable (Table 3).

**Validation of reference genes.** Results of the current study indicate actin and ribosomal L27 to be the most appropriate reference genes in RT-qPCR assays involving RWA-wheat interactions. To validate their use, we assessed expression of tRNA-Leu, which we previously reported to be up-regulated in the gut transcriptome of RWA2 fed Dn4 plants23. Results of this experiment revealed significant (p < 0.001) over-expression of tRNA-Leu in RWA2/Dn4 interactions in comparison to other interactions (Figure 4), and very stable CV- and M values (Mean CV = 0.168, Mean M = 0.48) for the actin - ribosomal L27 combination. In contrast, use of the actin - RPL5 combination (Mean CV = 0.97, Mean M = 4.2) or the RPL27 - RPL5 combination (Mean CV = 0.952, Mean M = 3.95) - resulted in huge changes in expression, and Mean CV- and M values above acceptable stability values [homogenous (CV < 0.25; M < 0.5) and heterogeneous (CV < 0.5; M < 1)]. Thus, the results of the tRNA-Leu expression validation experiment confirm the use of actin and RPL27 for RWA expression analysis.

**Conclusions.** This study emphasizes the use of an appropriate reference gene(s) for RT-qPCR studies, reports sequences of five selected genes from the Russian wheat aphid, and identifies reference genes with stable expression in RWA feeding on wheat plants carrying different RWA resistance genes. Analyses of our data using three different statistical techniques indicated that RWA ribosomal protein L27 (RPL27) is the most stable reference gene, closely followed by actin. Ideally, both RPL27 and actin genes should be used as references for normalizing expression profiles in RT-qPCR studies. This study is an important step in our ongoing RNAseq studies to define differential expression of RWA genes in RWA-wheat interactions. Results of these experiments will be of utmost importance to RWA genome sequencing efforts as well as the huge amount of expression data derived from current studies using NGS technologies.

**Methods**

**Insect and plant material.** RWA1 (biotype 1) collected from wheat fields near Hays, KS, and RWA2 (biotype 2) individuals collected from wheat fields near Briggsdale, CO (via the USDA-ARS Plant Science Research Laboratory at Stillwater, OK), were cultured in separate housing in the greenhouse at Kansas State University on plants of susceptible wheat cultivar 'Jagger' for use in all experiments. The identity of each

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**Table 3 | Descriptive statistics based on crossing point (CP) data and expression stability of five D. noxia reference genes calculated by BestKeeper Software**

| CP factor        | Actin L27 | Ribosomal protein L9 | Ribosomal protein L5 | Transcription elongation factor 2 |
|------------------|-----------|----------------------|----------------------|-----------------------------------|
| Mean Geo CP      | 17.96     | 18.77                | 27.38                | 23.85                             | 19.28                             |
| Mean Ar CP       | 17.96     | 18.78                | 27.65                | 24.66                             | 19.33                             |
| min CP           | 17.28     | 18.07                | 23.06                | 17.66                             | 17.18                             |
| max CP           | 18.87     | 19.54                | 33.30                | 32.95                             | 21.82                             |
| CP ± SD          | 0.51      | 0.47                 | 3.72                 | 6.24                              | 0.97                              |
| CP CV (%) ± SD   | 2.85 ± 0.51 | 2.48 ± 0.47         | 13.47 ± 3.72         | 5 ± 1.95                         | 25.29 ± 6.24                       |

**Abbreviations.** CP – Crossing point, Geo – geometric, Ar – arithmetic, SD – standard deviation, CV – percentage coefficient of variation. n = 18 samples for each gene.

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**Figure 4 | Relative normalized expression of the t-RNA-Leu gene using reference gene combinations of:**

(a) actin and ribosomal protein L27 (RPL27),
(b) actin and ribosomal protein L5 (RPL5) and
(c) RPL27 and RPL5.

R1D0 = RWA1 fed wheat plants carrying Dn0 (no resistance) gene; R1D4 = RWA1 fed wheat plants carrying the Dn4 resistance gene; R1D7 = RWA1 fed wheat plants carrying the Dn7 resistance gene; R2D0 = RWA2 fed wheat plants carrying Dn0 (no resistance) gene; R2D4 = RWA2 fed wheat plants carrying the Dn4 resistance gene; R2D7 = RWA2 fed wheat plants carrying the Dn7 resistance gene.
biotype was verified independently in diagnostic plant differential greenhouse assays at Stillwater and Manhattan. Voucher specimen no. 176 (RWA2) is deposited with the Kansas State University Museum of Entomological and Prairie Arthropod Research.

RT-qPCR primer design and test of amplification efficiency. Primers of sequenced transcripts considered for RT-qPCR were designed using Primer Express Software V.3.0.1 (Life Technologies) with slight modifications of the default parameters (Table 2) to avoid hairpin, self-dimer and cross-dimer secondary structures. Primers were selected based on low penalty scores, which indicated a good match to the set parameters.

Amplification efficiency of primers was estimated by SYBR Green chemistry RT-qPCR assay using 1, 10, 10², 10³, 10⁴ and 10⁵ fold dilutions of pooled cDNAs of three technical replicates for each gene. Each PCR mixture consisted of 1 μl of cDNA, 250 mM of primer and 12.0 μl of iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) in a total volume of 20 μl. The RT-qPCR profile was 95°C for 3 min followed by 40 cycles of 95°C for 10 s, 55°C for 30 s and 72°C for 30 s. Each reaction was performed for three technical replicates in three biological replicates in 96-well optical grade PCR plates sealed with optical sealing tape (Bio-Rad Laboratories). Melting curves were generated for each sample by heating the PCR amplicon from 65°C to 95°C with a gradual increase of 0.5°C per cycle of 0.5 s.

The Microsoft Excel-based software geNorm18, NormFinder20 and BestKeeper21 was used to rank reference genes for stability of expression across all experimental samples. For geNorm and NormFinder analyses, Cq values were transformed to relative quantities using the formula 2−ΔΔCq, where ΔCq = Cq of the gene in selected sample - minimum Cq of corresponding gene in the experiment. The sample with minimum Cq or maximum expression was used as the calibrator with a set value of 1. No transformed Cq values are required for BestKeeper analysis. Other procedures were followed according to the instructions mentioned in the software manual.

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Author contributions

D.K.S. and C.M.S. scoped and designed the study; D.K.S. performed the experiments and analyzed data; D.K.S. and C.M.S. interpreted results and wrote the manuscript.

Additional information

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