Comparative Analysis of Gene Expression Level by Quantitative Real-Time PCR Has Limited Application in Objects with Different Morphology

Natalia V. Demidenko1,2*, Aleksey A. Penin1,2,3

1 Department of Genetics, Biological faculty, Mikhail Vasilyevich (MV) Lomonosov Moscow State University, Moscow, Russia, 2 Evolutionary Genomics Laboratory, Faculty of Bioengineering and Bioinformatics, Mikhail Vasilyevich (MV) Lomonosov Moscow State University, Moscow, Russia, 3 A.A. Kharkevich Institute for Information Transmission Problems, Russian Academy of Science, Moscow, Russia

Abstract

qRT-PCR is a generally acknowledged method for gene expression analysis due to its precision and reproducibility. However, it is well known that the accuracy of qRT-PCR data varies greatly depending on the experimental design and data analysis. Recently, a set of guidelines has been proposed that aims to improve the reliability of qRT-PCR. However, there are additional factors that have not been taken into consideration in these guidelines that can seriously affect the data obtained using this method. In this study, we report the influence that object morphology can have on qRT-PCR data. We have used a number of Arabidopsis thaliana mutants with altered floral morphology as models for this study. These mutants have been well characterised (including in terms of gene expression levels and patterns) by other techniques. This allows us to compare the results from the qRT-PCR with the results inferred from other methods. We demonstrate that the comparison of gene expression levels in objects that differ greatly in their morphology can lead to erroneous results.

Introduction

Over the past twenty years real-time qRT-PCR has become a powerful approach for the accurate quantification of gene expression. During the development of this technique from the first studies with ethidium bromide staining [1], several important improvements have been introduced. However, in spite of the increased accuracy of real-time qRT-PCR there are still several frequent errors in experimental procedures which can lead to the generation of biologically meaningless data.

In order to address this problem, a set of guidelines describing the minimum information necessary for the evaluation of qRT-PCR experiments was recently proposed [2]. These guidelines are now widely accepted in the biological science community; suffice it to say that the instructions for authors of several high-impact journals include the recommendation to follow these guidelines [e.g. 3].

Incorrect normalisation may lead to serious inaccuracy in data analysis. It is well-known that a normalisation strategy that relies on the use of reference genes (the genes for which expression is stable in all samples being compared) is preferable for real-time qRT-PCR experiments [e.g. 4, 5]. In some cases the degree of inaccuracy can reach a 10-fold error [6]. To avoid this problem, some approaches for validation were proposed, including geNorm, NormFinder, BestKeeper, qBase [7–10]. All of these approaches were subject to preliminary tests on human tissues, and have been applied to a wide range of other objects.

In this study we are focusing on the application of qRT-PCR to plant studies. In the case of plant studies, Brunner and coauthors [11] reported that not all of the best known reference genes are equal. Further to this, Czechowski and coauthors showed that the most frequently used reference genes are hardly appropriate for data normalisation, and proposed a number of novel reference genes [6]. To date, there are many studies in which the search and validation of reference genes are reported, but most of them are focusing on the traditionally used “housekeeping” genes, not novel candidate reference genes that have been inferred from genome-wide studies such as in [6]. This issue can be settled by obtaining ortholog sequences for novel references with the help of degenerate primers, or by searching genome/transcriptome-wide sequencing data [12] in addition to further validation. Moreover, even if reference genes have already been selected for the object, double-checking of their expression stability under experimental conditions is preferable in order to increase the accuracy of real-time qRT-PCR analysis [13].

Another group of probable source of errors is more specific, but no less dangerous, and can result in incorrect data acquisition. The qRT-PCR data generation and analysis methodology indirectly implies that the samples being compared are similar in their morphology. The extent of the applicability of qRT-PCR to comparative analysis of gene expression levels in objects which are characterised by different morphology has never been discussed. We assume that in this case the data obtained from real-time qRT-PCR
could be biologically meaningless. In order to test this, conclusions based on qRT-PCR data can be compared to those based on more direct experimental evidence such as in situ hybridisation or gene interactions predicted by mutant analysis.

To investigate the influence of object morphology on the validity of qRT-PCR data we analysed the expression of genes involved in flower development and maintenance of floral meristem. Alteration of stem cell activity in the floral meristem in mutants of *Arabidopsis thaliana* is characterised by a dramatic change in floral organ number and identity (Fig. 1).

### Results

The expression levels of genes controlling floral organ identity (*AGAMOUS* (*AG*), *APETALA2* (*AP2*), *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) and regulators of meristematic activity (*WUSCHEL* (*WUS*) and *CLAVATA1, 2* (*CLV1, 2*)) were analysed in three mutants with an altered number and identity of floral organs: *ap2-14*, *ag-1* and *clv3-2*.

The qRT-PCR analysis of the *ap2-14* mutant revealed that expression levels of *AP3* and *PI* had decreased four-fold and three-fold respectively. The expression level of *AG* had increased by slightly more than half. The expression levels of *AP2*, *CLV1*, *CLV2* and *WUS* did not change significantly (Fig. 2A).

**Figure 1.** Flowers of wild type (wt) and of three single mutants of *A. thaliana*. Scale bar = 5 mm.

doi:10.1371/journal.pone.0038161.g001
Limited Applicability of qRT-PCR

A

apetala2-14

B

agamous-1

C

clavata3-2

[Bar charts showing gene expression levels for apetala2-14, agamous-1, and clavata3-2 with asterisks indicating significant differences from control levels.]
Figure 2. The relative expression level of flower development genes. (a) ap2-14 mutant analysis, (b) ag-1 mutant analysis, (c) clv3-2 mutant analysis. Error bars represent the standard error of the mean. Asterisks indicates what values are significant to p<0.05. AP2 - APETALA2, AP3 - APETALA3, PI - PISTILLATA, AG - AGAMOUS, CLV1 - CLAVATA1, CLV2 - CLAVATA2, WUS – WUSCHEL. Dashed line indicates 1.0 expression level.
doi:10.1371/journal.pone.0038161.g002

Gene expression levels obtained from the analysis of ag-1 indicated that WUS expression was reduced by two orders of magnitude. While the expression levels of AP3 and PI had increased fourfold, and AP2 and CLV1 levels had tripled and doubled respectively. (Fig. 2B).

In the clv3-2 mutant no significant changes in gene expression levels were observed except for PI, for which the expression level was reduced two-fold (Fig. 2C).

Discussion
Over the last twenty years, many aspects of the genetic control of development in Arabidopsis thaliana have been uncovered. In particular, the mechanism underlying the determination of floral organ identity (the ABC-model) and the system for the regulation of meristematic activity of the floral meristem [14,15]. The functions and interactions of the genes involved in these processes were investigated by various methods, including the study of expression patterns by in situ hybridisation, the phenotypic analysis of mutants and transgenic plants and DNA-protein interactions.

The key gene responsible for the maintenance of meristematic activity is WUSCHEL (WUS) [16]. In wild type Arabidopsis it is characterised by a very narrow expression area. WUS is expressed in only a few cells in the shoot apical and floral meristem. The genes CLV1, CLV2 and CLV3 restrict WUS expression in the shoot apex [17]. In the flower meristem an additional gene, AGAMOUS (AG), acts to restrict WUS expression [18]. AG is crucial for the determination and development of reproductive organs (a C-class gene, in terms of the ABC model) [15]. AG expression is confined to the inner two whorls by the A-class gene AP2 [18]; in turn, AP2 translation in the third and fourth whorls is repressed by the microRNA mir172 [19]. These genetic interactions create a boundary between the perianth and the reproductive organs. The APETALA3 (AP3) and PISTILLATA (PI) genes express in the second and third whorls, conferring petal and stamen identity [15] and are involved in positive feedback interactions [20].

All of the mutants used in this study have already been characterised by several other methods. It is natural to expect that the changes in gene expression levels observed using qRT-PCR will be consistent with those inferred from other experiments. The main deviation from the expected was observed by the ag-1 mutant. This mutant is characterised by the lack of determination of the floral meristem, a phenotype that results from the impairment of AG as a negative regulator of the meristematic cell maintenance gene WUS. Thus an increase in the WUS expression level is to be expected. It has been experimentally shown that in the ag-1 mutant, a lack of negative regulation of WUS results in the proliferation and slight broadening of its expression area [21]. However, the results of the qRT-PCR contradict this; instead of increased WUS expression, a major decrease was observed. The main explanation for such a phenomenon is the inapplicability of the basic statistical method for the calculation of relative expression data – the dCt method [22]. This algorithm is based on the comparison between the ratio of reference genes to the expression levels of genes of interest, and indirectly implies that the expression pattern of these genes is similar in the samples being compared. However, in the case of ag-1, even if WUS expression is increased twofold the expression area of the reference genes is simultaneously increased by several orders of magnitude due to a strong increase in floral organ number. Such disproportionate results indicate that real-time qRT-PCR is incapable of providing accurate data for gene expression levels.

Another noticeable effect is the observed increase in expression levels of B-class genes (AP3 and PI). This is due to the expansion of their expression region – petals and stamens – that is characteristic of the ag-1 mutant phenotype. On the contrary, in the ap2-14 mutant which has reduced number of floral organs the decrease in the expression of B-class genes is observed. These variations in B-class expression are directly related to their expression patterns in both cases.

The clv3-2 mutant analysis had a similar result. WUS is negatively regulated by CLV3, thus in the case of a mutation in CLV3 an increase of WUS expression is expected. However, according to qRT-PCR analysis its expression level did not change in clv3-2 mutants. This is also associated with the mutant phenotype which is characterised by an increase in meristem activity leading to an increase in floral organ size and number. As a result, the expression area of the reference genes also increases. This change leads to the incorrect quantification of genes of interest and masks the WUS expression increase. This example also confirms the non-universality of the dCt method and non-applicability of real-time qRT-PCR for such an analysis.

All of the other results obtained were consistent with the expectations based on the mutant phenotype and present data on gene function and interaction.

The errors in this approach can seriously influence the determination of final conclusions such as the identification of gene interactions or expression area. The real-time qRT-PCR method can not lead the researcher to accurately conclude whether the expression level has increased as a result of broadening its area or because it produced more mRNA. Subsequently, it is difficult to discriminate between cadastral interactions or positive/negative regulation.

In conclusion, the present study indicates that there is a problem with the application of real-time qRT-PCR. Using the common and well-studied model Arabidopsis, particularly mutants with altered floral morphology, we have shown the influence of this factor on the accuracy and validity of qRT-PCR results. We suggest that other cases could have similar issues (e.g. interspecific gene expression studies) and lead to incorrect conclusions. One possible way to reveal that the method is the source of error is by simultaneous gene expression analyses of various genes that are involved in mutant phenotype development. Although this cannot help to reconstruct the real data, it can indicate the errors and help to avoid gathering noisy data. Alternatively, corroboration of the real-time qRT-PCR data by other methods (e.g. RNA-seq) is also suitable for obtaining the actual data.

Methods

Plant Material and Biological Samples
For the gene expression analysis, Arabidopsis thaliana plants were grown on 1:2 vermiculite:soil at 25°C, in 60% relative humidity under long day (16 hours light/8 hours dark) conditions. The mutant lines clv3-2 and ag-1 are in the Ler background thus Ler wild type plants were taken for comparison with ag-1 and clv3-2. The mutant line ap2-14 is in the Col background and Col wild type plants were used for comparison with ap2-14. Young
inflorences at the stage of the anthesis of the first flower were collected in two biological replicates. No specific permits were required for the described field studies.

**RNA Extraction and cDNA Synthesis**

Total RNA was isolated from 50 ± 5 mg of plant material using an RNaseasy Plant Kit (Qiagen, USA) with some modifications. To prevent DNA contamination, samples were treated twice with RNase-Free DNase (Qiagen, USA). The first digestion was performed according to the manufacturer’s instructions, then columns were washed with 350 μl of RW1 and the digestion was repeated. To evaluate RNA integrity, RNA was visualized on 1% SYBR-Green-stained agarose gel. Clear bands corresponding to 18 S and 23 S RNA and the absence of a smear were observed indicating minimal degradation of RNA. The concentration of isolated RNA was calculated using a Qubit (Invitrogen, USA). The concentration of total RNA was more than 100 ng/μl among all samples. Total RNA samples were stored at −80°C with the addition of RNase inhibitor RNasin (Siles, Russia) and were then adjusted to the concentration of 100 ± 5 ng/μl for reverse transcription. First strand cDNA synthesis was performed using a “First strand cDNA synthesis kit” (Siles, Russia) with a 24 T primer (0,4 nmol per reaction) in a 25 μl reaction mix according to the manufacturer’s protocol. Before each PCR run the cDNA samples were heated (65°C to 90°C, 40°C to 30°C) and then the cDNA products were diluted 10-fold prior to use in real-time PCR.

**qRT-PCR Conditions**

Quantitative real-time PCR analysis was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) using a 2.5× RT-PCR reaction mix (Syntol, Russia). Primer sequences and amplification conditions are listed in the table. To detect dsDNA synthesis EvaGreen dye was used. Each reaction was performed in a 20 μl mix containing 400 nmol of each primer and 1 μl of 1:10 diluted cDNA. qRT-PCR conditions were five mins at 95°C, then 35 cycles of 95°C at 15 s and 62°C at 60 s. Each sample was analysed in triplicate; mean Ct values were calculated. Mean Ct dispersal for technical replicates did not exceed 0,3 cycle. To reveal the absence of contamination or primer dimers a non-template control (NTC) reaction with each primer pair was run. To ensure the absence of gDNA reverse transcription negative controls were performed with each biological sample. These no-RT control reactions were run with primers to the CLF2 gene because these primers anneal within one exon. To obtain amplicon data a melting curve analysis was performed after each PCR run (Fig. S1). The list of analysed genes, primers and different parameters derived from qRT-PCR analysis is in Table S1.

**Gene Expression Analysis**

Obtained Ct values for each sample were transformed into Cq values by the standard formula: $Cq = \log(2)/\log(E)$, where $E$ is the efficiency of the amplification of each primer pair. Amplification efficiency was calculated using Minner ver. 2.2 software [23]. The relative expression levels were calculated using the ddCt method. Relative expression levels were normalised to the geometric average of the Cq values of two reference genes: AT1G34270 and AT3G25760. These genes are among the most stably expressed according to a genome-wide survey by Czechowski et al. [6].

**Supported Information**

**Figure S1** Specificity of RT-qPCR. Melting curves generated for all genes in three technical repetitions. Low-fluorescence curves indicate NTC. (TIF)

**Table S1** List of analysed genes, primers and different parameters derived from qRT-PCR analysis. (DOC)

**Acknowledgments**

The authors are grateful to Maria Logacheva for helpful comments.

**Author Contributions**

Conceived and designed the experiments: NVD AAP. Performed the experiments: NVD. Analyzed the data: NVD AAP. Contributed reagents/materials/analysis tools: AAP. Wrote the paper: NVD AAP.

**References**

1. Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Nature Biotechnology (N Y) 10: 413–7.
2. Bustin SA, Benes V, Garson JA, Hellmann J, Huggett J, et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55: 611–22.
3. Nucleic Acids Research Instructions for Authors. Available: http://www.oxfordjournals.org/our_journals/nar/for_authors/miqeRp_submission.html. Accessed: 2012 May, 5.
4. Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6: 279–84.
5. Chervoneva I, Li Y, Schulz S, Croker S, Wilson C, et al. (2010) Selection of optimal reference genes for normalization in quantitative RT-PCR. BMC Bioinformatics 11: 253.
6. Czechowski T, Stitt M, Altman T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139: 5–17.
7. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.
8. Anderson CL, Jensen JL, Ortmie 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: 5245–50.
9. Pfaffl MW, Hiebl A, Pegnoleti C, Neuhaus TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett 26: 309–15.
10. Hellemans J, Mortier G, De Paep A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Gen后备 8: R19.
11. Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4: 14.
12. Demidenko NV, Logacheva MD, Perlin AA (2011) Selection and validation of reference genes for quantitative real-time PCR in buckwheat (Fagopyrum esculentum) based on transcriptome sequence data. PLoS One 6: e19434.
13. Kadonie A, Thulke S, Mackay IM, Landi O, Siegert W, et al. (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313: 856–62.
14. Bowman JI, Smyth DR, Meyerowitz EM (1989) Genes directing floral development in Arabidopsis. Plant Cell 1: 37–52.
15. Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353: 31–7.
16. Laux T, Mayer KF, Berger J, Jurgens G (1996) The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122: 87–96.
17. Schoof H, Lenhard M, Haeker A, Mayer KF, Jurgens G, et al. (2000) The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLV3 and WUSCHEL genes. Cell 100: 635–44.
18. Liu X, Kim YJ, Muller R, Yumul RE, Liu C, et al. (2011) AGAMOUS terminates floral stem cell maintenance in Arabidopsis by directly repressing WUSCHEL through recruitment of Polycomb Group proteins. Plant Cell 23: 3654–70.
19. Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its identity by a MicroRNA and its
20. Riechmann JL, Krizek BA, Meyerowitz EM (1996) Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. Proc Natl Acad Sci U S A 93: 4793–8.

21. Lenhard M, Bohnert A, Jürgens G, Laux T (2001) Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. Cell 105: 805–14.

22. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e15.

23. Real-time PCR Miner. Available: http://www.miner.ewindup.info/Version2. Accessed: 2012 May, 5.